



European Cell Death
Organization
(ECDO)



University of Debrecen
Medical and Health
Science Center

13 th ECDO Euroconference on **Apoptosis**

BUDAPEST, HUNGARY, October 1-4, 2005

Programme and Book of Abstracts



Supported by EU within the
framework of the Marie Curie
Conferences and Training Courses



MARIE CURIE ACTIONS

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Local Organisation

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Co-Chair: Zsuzsa Szondy, M.D., D.Sc.
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Diamond Congress Ltd.

Budapest, Hungary

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Welcome

Dear Colleagues,

It is a great pleasure to welcome all the participants of the 13th Euroconference on Apoptosis in the name of the ECDO Board and the local organisers. Welcome to Budapest!

“Survival on the Danube”: this has become the calling phrase of the conference. The choice of this slogan is not without purpose, “survival” has several inferences here.

First, the city of Budapest situated at the cross-roads of ambitious nations has survived many wars and fierce battles during the centuries. Still, the spirit of the people living here, the rich history, the art and cultural attractions has made the city one of the most charming and attractive capitals of Europe to visit.

Second, ECDO and the Euroconference on Apoptosis have survived the 13 years; not only that, it prospers better than ever. We are all thankful to Michel Lanotte and Tom Cotter who started the Euroconferences, organising the first two in Paris and Kinsale and obtaining funds from the EU – with the help of the European Society of Haematology - also for the next four ones in Cuenca, Capri, Bingen and Stockholm. After these very successful conferences, ECDO had to proceed on its own resources and with the efforts of our colleagues in Israel (Ein Geddi), Switzerland (Davos) Austria (Vienna) and France (Paris), we could reach the 10th Euroconference. This was followed by the legal restructuring of ECDO and another successful EU application bringing us to the Ghent and Chania meetings and finally to Budapest. This clearly shows that apoptosis research has been very strong in Europe and I am sure it will be more and more influential during the coming years.

Third, perhaps it has never been so clear as it is now how much the pro-apoptotic and anti-apoptotic mechanisms are intertwined in cells. As it will be shown by a number of lectures and posters of the conference, the survival mechanisms in cells have recruited many of the biochemical systems which have been originally selected for other purposes in diverse metabolic and signaling pathways, in endocrine, neuronal or transcriptional regulation. We have protein families with members for either survival or death, killer proteins have turned out to facilitate proliferation as well as survival depending on cell types and tissue settings. Survival and death seem to be two fates often played out by the same molecular machinery of which more and more details have been recently revealed. This explains why cell survival was selected as the dominating topic of the apoptosis conference this year.



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In many countries “13” is considered to be an unlucky number. However, we have not been discouraged and decided to invite the apoptosis research community to enjoy a hopefully memorable 13th get-together in Budapest. This conference could have never happened without the help, advice and hard work of the Scientific Programme Committee (which met last December at the site of the Conference to plan the programme), the ECDO secretariat with Veronique Vandevoorde, the local organiser Diamond Congress and my co-chair, Zsuzsa Szondy. Many thanks to all of them.

Dear participants!

At the end of the last full day of the Conference we will take a boat together and see what it really means to survive on the Danube! Until that, enjoy the sessions and discussions, see old friends and make new ones. Have fun!

Budapest, October 1st, 2005.

László Fésüs
Chair of Conference



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Evaluation form

Also this year's Euroconference is organised within the framework of the EU Marie Curie Programme 'Europtosis'. Thanks to the financial support of the EU, a substantial number of participants received a grant to attend this meeting.

In view of this European programme, ECDO would appreciate it very much if you could fill in the **evaluation questionnaire online** upon your return. This will only take about 5 minutes of your time and the total procedure is outlined on the next page.

Thank you for your cooperation!

The ECDO board



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- 1 Choose the URL: <http://webgate.cec.eu.int/sesam/index.do>
2. In the left banner, select “**MCA Questionnaires**”
3. The “**Select questionnaire type**” page is presented below. The “Instrument”, “Project type” and “Questionnaire type” options you need to select are filled in on the example

Instrument

Project type

Questionnaire type

4. “Edit questionnaire”
5. The “**Project identification**” page is presented. The “Project ID” number you have to fill in is “**504454**”.
6. After pressing the “**Validate**” button, you will reach the “**MCA Conferences and Training Courses Assessment Questionnaire**”.
7. Please read carefully the instructions, complete the questionnaire, and submit your evaluation by pressing the “submit” button.

Project type SCF-Series of events

Project ID



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Conference Information

CONFERENCE VENUE

Danubius Thermal and Conference Hotel Helia
H-1133 Budapest, Kárpát u. 62-64., Hungary
Phone: +36 1 889-5800
Fax: + 36 1 889-5801
E-mail: helia@danubiusgroup.com
Internet: www.danubiushotels.com/helia

SCIENTIFIC CORRESPONDENCE

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REGISTRATION

To collect the conference materials you are kindly requested to register at the Conference Secretariat:
Thermal Hotel Helia – Conference Level (C floor)

Opening hours of the registration desk

September 30 (Friday)	15.00 – 19.00
October 1 (Saturday)	7.30 – 20.00
October 2 (Sunday)	8.00 – 18.30
October 3 (Monday)	8.00 – 18.00
October 4 (Tuesday)	8.00 – 12.00

Conference delegates and their companions will receive their badges, conference materials, social event tickets at the desk. Subscription for optional tours also takes place in this area. **Participants are kindly requested to wear their name badges during all events of the meeting.**



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CONFERENCE SECRETARIAT

If you need any help during the conference you can find the staff of Diamond Congress and the ECDO secretary at the registration desk. In case of emergency please call: +36 20 936 2969.

After the conference you can reach Diamond Congress at the following address:

H-1027 Budapest, Fő u. 68, Hungary

Phone: +36 1 214 7698 or 214 7701 Fax: +36 1 201 2680

E-mail: diamond@diamond-congress.hu

Internet: <http://www.diamond-congress.hu>

CONFERENCE ASSISTANTS

Conference assistants will be recognisable by their silver badge and T-shirts with a sign: HELP DESK. They will help you in all practical aspects of conference participation.

LECTURE ROOMS

The conference will take place on the **Conference Level (C floor)** of the Thermal Hotel Helia.

All lectures will be given in the conference room.

For poster presentations there will be different rooms available (Mercure, Orion A+B, Panorama, Uranus). Please look at the information boards for the local arrangement. A map of the C-level is included at the inside of the back cover.

INTERNET CONNECTION

During the conference wireless Internet access will be available close to the registration area for those participants who have their laptops with them. Additionally, a few PCs will also be provided in room Saturnus.

LUNCH AND REFRESHMENT

Organised lunches will be served at restaurant "Jupiter" at Thermal Hotel Helia for all registered participants. Refreshments are included in the registration fee for all registered participants and will be served at the lobby of the conference level.

INCOMING MESSAGES AND MESSAGE BOARD

Messages received by the desk will be posted on the message board located at the Registration Desk at C Level. Participants may also use this board to leave messages to other delegates.

SMOKING

Smoking is allowed only at the especially dedicated area of the hotel. Please smoke preferably on the terrace and outside the building.



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INSTRUCTIONS FOR SPEAKERS

We support Powerpoint 2000 or XP. If you want to use your own laptop, or you have a Macintosh, be sure that there is a VGA connector for an external monitor.

We prefer Powerpoint presentations. Your Powerpoint presentation should be on USB stick or CD.

Slide presentations are not supported.

Short orals take 15 minutes (discussion included). Hence, try to prepare a talk of 10-12 min.

You will be able to upload your presentation in the lecture room before your presentation. A technician will be of your assistance for the file upload in the conference hall.

INSTRUCTIONS FOR POSTER PRESENTERS

To allow maximal viewing of the posters, we kindly ask the presenting authors to exhibit their poster **during the entire meeting**. Poster abstracts are listed in the abstract book in alphabetical order and are presented on the poster boards during the conference in the same way. You can consult the "Poster list" in the book of abstracts to learn your poster number. Please look at the information boards in the C-lobby for the exact local arrangement of the poster panels.

Posters should be mounted at the beginning of the meeting and removed by 12 pm on 4 October (Tuesday). We cannot take the responsibility for the posters not removed till lunchtime on Tuesday 4 October.

The organisers will provide mounting materials to fix posters.

Two fixed poster sessions are scheduled during the meeting. Presenting authors of **odd poster numbers** are requested to be near their poster during the poster session on **Sunday, October 2**. Presenting authors of **even poster numbers** are requested to be near their poster during the poster session on **Monday, October 3**.

CONFERENCE WEBSITE

The Internet homepage of the Conference is kept up-to-date all the time:

<http://www.diamond-congress.hu/ecdo2005/>

<http://www.dnbr.ugent.be/ecdo/>

SOCIAL PROGRAMMES

The scientific programme of the conference will be completed with social events, allowing some time for informal discussions for the participants.

Welcome Reception

The Welcome reception will be held at the Jupiter Restaurant of Thermal Hotel Helia, the venue of the conference, at 20:00 on Saturday, October 1.

Gala Dinner on boat

The Gala dinner will be held on Monday evening, October 3 on board of 'Európa' boat cruising on the river Danube from where you will be able to admire the evening lights of the capital city. Complete dinner will be provided.

The boat leaves at 20:00 from the pier at the riverbank near Thermal Hotel Helia, so transportation will not be necessary.



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ACCOMPANYING PERSONS' PROGRAMMES

Participants are encouraged to bring their spouses to Budapest. Companions, paying the accompanying persons' registration fee, are invited to join the following events beyond the welcome reception and gala dinner:

Excursion to Gödöllő on Sunday, October 2 (half day)

This is a half day guided tour by bus to the former summer residence of Queen Elisabeth organised on Sunday. The town can be found very near to Budapest. The town's greatest treasure and draw for tourists is its 250 years old Royal Palace. Visitors can see the living quarters of Emperor Franz Joseph and Empress Elizabeth (Sissy).

Departure is from the Hotel Helia at 10:30. Planned arrival at the same place is 15.00. (lunch is not included in the programme)

Sightseeing tour with visiting the Parliament on Monday, October 3 (half day)

Budapest Sightseeing programme will be a half day guided tour by bus. The first part of the tour offers the opportunity for a visit of the impressive House of Parliament. After a walk around Hungary's largest building, interior visit of the magnificent neo-gothic Parliament (home of the Holy Hungarian Crown) with guidance through the splendid boardrooms and impressive staircases. Participants will be able to see the Hungarian Coronation Jewellery (crown, scepter, mound, mantle, and sword). Taking your passport is necessary.

After the visit at the building of Parliament, a guided sightseeing tour starts. Among several famous sights the following places will be visited: Heroes' Square, Basilica, Opera House, Buda Castle, Matthias Church, Fisherman's Bastion, Gellért Hill, Citadel, etc.

Departure is from the Hotel Helia at 9:30. Planned arrival at the same place is 14.00. (lunch is not included in the programme)

SPONSOR

Platinum Sponsor: BD Biosciences <http://www.bd.com>

EXHIBITOR

Biomedica Hungária Kft. <http://www.biomedica.hu/>

PRACTICAL HINTS

TRANSPORT TO/FROM BUDAPEST AIRPORT

Airport Minibus

Participants leaving from Budapest International Airport are advised to use the Airport Minibus shuttle service, which takes one to any address in Budapest for a fee of 2100 HUF/person (cca. 9 €). You may order your Airport Minibus at the Registration Desk or at you hotel at least one day before your departure. Telephone number: (36-1) 296-8555



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Taking a taxi

The tariffs of the taxi companies may differ to some extent, but there is one thing in common tariff ceiling. If you take a taxi, you can find the tariff chart on both dashboard and on the right rear window. The tariffs cannot be significantly more than listed below, they, however, may be lower.

	Taxi ordered by phone	Taxi from the street
Basic fee	HUF 300	HUF 300
Per kilometre tariff	HUF 180/km	HUF 240/km
Waiting tariff	HUF 45/min.	HUF 60/min.

This is the tariff chart for all rides within Budapest and the rides starting in Budapest, including Ferihegy Airport. If you take a taxi in Budapest, but your destination is outside the city, the driver may request the return price to the city border.

You get price reduction if you order your taxi by phone, so you are better off ordering a taxi from the Hotel Reception or from a restaurant, than just get in a car in front of your Hotel. Phone numbers of some taxi companies: Főtaxi 222-2222, City Taxi 211-1111, Rádió Taxi 377-7777, Taxi2000 200-0000.

INFORMATION OFFICES IN THE CITY

- Nyugati Railway Station, District XIII, phone: 302-8580
- Sütő utca 2, District V, phone: 317-9800
- Budaörs, Agip complex (M1–M7 motorway junction), phone: (06-23) 417518 (Opening hours: 15 March – 31 October: 8.00–20.00 every day)
- Király utca 93, District VII, phone: 352-1433, 352-9804 (Opening hours: 9.00–18.00 every day throughout the whole year)

TELEPHONE SERVICE

Public telephones at the conference site are available close to the elevators in several levels. They work with Hungarian prepaid Phone Cards (“telefonkártya” in Hungarian). Phone cards (HUF 500, HUF 800 and HUF 1800) can be purchased at the tobacco shops and post offices.

Phoning from/to Hungary

In Hungary, the following calling sequences are used:

From public phones, the digits 00 are used to access an international line. Most public phones work with phone cards a few with coins (10, 20, 50, or 100 HUF coins).

The **hotel room telephones** have a special procedure, see the guide in the rooms.

For numbers outside Budapest, the calling sequence starts with 06, then the area code and the number are to be dialled.

Phone numbers within Budapest have 7 digits. Be aware that in the past years, many numbers have been changed. Numbers you may still remember, starting with digit 1, were all modified, and now they start with digit 3 or 4. The information service on phone numbers in Hungary has the (local) phone number 198. Information on international phone numbers can be obtained at number 199. The country code of Hungary is 36, the area code of Budapest is 1.

Mobile phones have different “area codes”, like 20, 30, 70 etc. Therefore, e.g. the mobile phone number (20) 123 4567 can be reached from a public phone as 06-20 123 4567.



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Important phone numbers in Budapest

Emergency:	112
Medical Emergency:	104
Police:	107
Fire brigade:	105

In case of problems on the Conference site, please, contact Conference assistants!

INSURANCE

Participants are strongly advised to make their own insurance arrangements. The organisers cannot accept any liability for personal injuries sustained, or for loss or damage to property belonging to participants and accompanying persons, either during or as a result of the conference.

FOREIGN EXCHANGE AND BANKING FACILITIES

The Hungarian currency is the Hungarian Forint (HUF). Currency exchange booths are available at the airport terminals, railway stations, travel agencies, banks and various places in the city. Traveller's cheques, and convertible currency may be exchanged at these facilities. Major credit cards are usually accepted in most hotels, restaurants and certain shops in the city. Obtaining cash against ATM cards (or credit cards, but this is more expensive) is very easy in Budapest from the "Bankomats" that can be found at almost each bank office, hotel or on the street. The current rate for currency exchange is approximately 204 HUF ~ 1 USD, 246 HUF ~ 1 EUR.

TRAVEL AND TRANSPORTATION

Budapest has a reliable public transportation network: three metro lines (colour-coded: yellow, red and blue), as well as many trams, bus and trolley lines. Participants are advised to use public transportation whenever possible. Please note that you have to validate your pre-purchased tickets if you use any mean of transportation. The single ticket price is HUF 170. Tickets can be obtained also at tobacco shops, newspaper stands metro stations and at the registration desk!

CONFERENCE HOTELS

Thermal Hotel Helia****	phone: 889-5800, fax: 889-5801
Thermal Hotel Margitsziget****	phone: 889-4700, fax: 889-4988
Fortuna Hotel***	phone: 288-8100, fax: 270-0351
Hotel Ibis Centrum***	phone: 456-4100, fax: 456-4116
Hotel Ibis Váci út***	phone: 329-0200, fax: 340-8316
City Hotel Ring ***	phone: 340-5450, fax: 340-4884
Hotel Délibáb***	phone: 342-9301, fax: 342-8153
Hotel Charles ***	phone: 212-9169, fax: 202-2984



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HOW TO GET TO THE CONFERENCE VENUE (THERMAL HOTEL HELIA) FROM THE HOTELS?

Thermal Hotel Margitsziget ** (1138 Budapest, Margitsziget)**

Take the bus No. 26 till the last stop (Árpád híd – Metro) and change to metro, blue line (M3) for 1 stop till Dózsa Gy. út.

Fortuna Hotel * (1137 Budapest, Szent István Rakpart, Alsó Rakpart)**

Take trolley bus No. 79 at the corner of Szent István Park, direction to Baross tér. Thermal Hotel Helia is on the right at the 2nd stop.

Hotel Ibis Centrum * (1092 Budapest, Ráday u. 6.)**

6 stops by metro, line M3

Hotel Ibis Váci út * (1134 Budapest, Dózsa Gy. út 65.)**

10 min. walk from the venue

City Hotel Ring * (1139 Budapest, Szent István körút 22.)**

5 stops by trolley bus No.79 from Jászai Mari tér (in Budai Nagy Antal u.)

Hotel Délibáb* (1062 Budapest, Délibáb utca 35.)**

6 stops by trolley bus No. 79 from Hősök tere (Heros' Square).

Hotel Charles * (1016 Budapest, Hegyalja út 23.)**

Take the blue line (M3) at the Venue till Ferenciek tere (5 stops). Coming up from the subway take the steps on your left. The bus stop is 20m walk straight ahead, beside a church. Take bus no.8 or 112. The hotel is right at the 3rd stop (Mészáros u.).



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Training Course Programme

2nd Training Course on *Concepts and Methods in Programmed Cell Death* **“Genetic Pathways and Techniques for Detection of Cell Death”**

Budapest, Hungary, October 1, 2005

Programme

Chairs: László Fésüs and Zsuzsa Szondy

8:30-9:30	<i>Genetic pathways in C. Elegans</i> Michael Hengartner , (Zürich, Switzerland)
9:30-10:30	<i>Genetic pathways in Drosophila</i> Natalie Franc (London, UK)
10:30-11:00	Coffee
11:00-12:00	<i>Genetic pathways in mouse</i> Francesco Cecconi (Rome, Italy)
12:00-13:00	<i>Morphology</i> Walter Malorni (Rome, Italy)
13:00-14:30	Lunch
14:30-15:30	<i>Flow cytometry</i> Marie-Lise Gougeon (Paris, France)
15:30-16:30	<i>Biochemical techniques</i> Boris Zhivotovsky (Stockholm, Sweden)
16:30-16:50	Coffee
16:50-17:50	<i>Clearance</i> Ian Dransfield (Edinburgh, UK)



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Conference Programme

13th EUROCONFERENCE ON APOPTOSIS

OCTOBER 1-4, Budapest

“Survival on the Danube”

Saturday evening October 1

18:30 **Official Opening**

19:00-20:00 **Keynote lecture**

Vishva Dixit (San Francisco, USA)

The inflammasome: A dynamic caspase activating apparatus

20:00 **Welcome reception**



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October 2: Sunday morning

Session 1: Cross-talk between different cell organelles

Chair: Sten Orrenius

- 8:30-9:00 **Stuart Lipton (La Jolla, USA)**
S-nitrosylation modulates ubiquitylation: New neuroprotective treatment strategies
- 9:00-9:15 **Bernhard Gillissen (Berlin, Germany)**
Nbk induces apoptosis in a Bak independent manner via an ER pathway
- 9:15-9:30 **Helin Vakifahmetoglu (Stockholm, Sweden)**
Crosstalk between caspase-2 and p53 in DNA damage-induced apoptosis
- 9:30-10:00 **Valerian Kagan (Pittsburgh, USA)**
Cytochrome c as a phospholipid oxygenase: a new signaling role in apoptosis
- 10:00-10:30 Coffee break

Session 2: Oxidative stress

Chair: Wilfried Bursch

- 10:30-11:00 **Richard Flavell (New Haven, USA)**
Redundant and non redundant roles of effector caspases in apoptosis
- 11:00-11:30 **Boudewijn Burgering (Utrecht, The Netherlands)**
FOXO forkhead transcription factors and the control of cellular oxidative stress
- 11:30-11:45 **Christine Doblender-Gruber (Innsbruck, Austria)**
C-Raf protects against ROS-induced apoptosis
- 11:45-12:00 **Lucy Elphick (Surrey, United Kingdom)**
Oxidative neuronal cell damage results in cell cycle progression and calpain mediated cell death
- 12:00-13:30 Lunch



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October 2: Sunday afternoon

13:30-15:00 Poster session

Session 3: Genomic instability

Chair: Klaus-Michael Debatin

- 15:00-15:30 **Eileen White (New Jersey, USA)**
Hierarchy among cell death pathways avoids necrosis-driven inflammation
- 15:30-16:00 **Maria Castedo (Villejuif, France)**
Polyploidy, aneuploidy and apoptosis
- 16:00-16:15 **Simone Fulda (Ulm, Germany)**
Loss of caspase-8 expression does not correlate with MYCN amplification, aggressive disease or prognosis in neuroblastoma: a putative tumor suppressor revised?
- 16:15-16:30 **Vicente Planelles (Salt Lake City, USA)**
HIV-1 induced apoptosis is mediated by ATR, BRCA1 and GADD45, and shares its signaling with activation of the G2 checkpoint
- 16:30-17:00 Coffee break

Session 4: NF- κ B Signalling

Chair: Hans-Uwe Simon

- 17:00-17:30 **Guido Franzoso (Chicago, USA)**
The control of JNK signaling and programmed cell death by NF- κ B transcription factors
- 17:30-18:00 **Peter Vandenabeele (Ghent, Belgium)**
Caspases and NF- κ B activation
- 18:00-18:15 **Dirk Brenner (Heidelberg, Germany)**
A novel mechanism for activation or suppression of NF κ B by HPK1 determines sensitivity towards activation-induced cell death in T cells
- 18:15-18:30 **Arturo Sala (London, UK)**
The double life of ApoJ/clusterin: a stress-activated pro-survival protein, inhibitor of NF- κ B and neuroblastoma metastasis



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MARIE CURIE ACTIONS

October 3: Monday morning

Session 5: Novel mechanisms to inhibit apoptosis

Chair: Peter Vandenabeele

- 8:30-9:00 **Craig Thompson (Philadelphia, USA)**
Programmed cell death: It's not all apoptosis
- 9:00-9:30 **Dario Altieri (Massachusetts, USA)**
Survivin checkpoints
- 9:30-9:45 **Christian Widmann (Lausanne, Switzerland)**
Sequential cleavage of RasGAP by caspases: a Jekyll and Hyde control of apoptosis that can be exploited therapeutically
- 9:45-10:00 **George Kulik (Winston-Salem, USA)**
Anti-apoptotic signaling pathways in prostate cancer cells converge on BAD
- 10:00-10:30 Coffee break

Chair: Antonios Makris

- 10:30-11:00 **Nika Danial (Boston, USA)**
Integration of glycolysis and apoptosis by the BH3-only pro-apoptotic protein BAD
- 11:00-11:30 **Pascal Meier (London, UK)**
IAP-mediated regulation of caspases
- 11:30-11:45 **Morgan McKeller (Houston, USA)**
PRELI, a novel mitochondrial protein that protects B-lymphocytes from apoptosis
- 11:45-12:00 **Zsolt Sarang (Debrecen, Hungary)**
Tissue transglutaminase (TG2) acting as G protein protects hepatocytes against Fas-mediated cell death
- 12:00-13:30 Lunch



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October 3: Monday afternoon

13:30-15:00 Poster session

Session 6: Cell death in the immune system

Chair: Marie-Lise Gougeon

- 15:00-15:30 **Peter Daniel (Berlin, Germany)**
A clinical view of cell death and resistance in cancer
- 15:30-16:00 **Carlo Riccardi (Perugia, Italy)**
Molecular mechanisms of glucocorticoid-mediated control of T lymphocyte apoptosis
- 16:00-16:15 **Zsuzsa Szondy (Debrecen, Hungary)**
Retinoids might be involved in the fine tuning of the T cell selection processes
- 16:15-16:30 **David Ucker (Chicago, Illinois, USA)**
What becomes of the corpse? Molecular characterization apoptotic cell recognition determinants and immune consequences
- 16:30-17:00 Coffee break

Chair: Mauro Piacentini

- 17:00 – 18:00 ***ECDO honorary lecture:***
Peter Krammer (Heidelberg, Germany)
T cell apoptosis in AIDS
- 20:00 Gala dinner on board of 'Európa' boat



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October 4: Tuesday morning

Session 7: Autophagy and Anoikis

Chair: Wim Declercq

- 8:30-9:00 **Eeva-Liisa Eskelinen (Helsinki, Finland)**
Lysosomal membrane proteins and autophagy
- 9:00-9:30 **Maria Colombo (Mendoza, Argentina)**
Autophagy: molecular mechanisms and role in intracellular pathogens fate
- 9:30-9:45 **Julien Puyal (Lausanne, Switzerland)**
Autophagic cell death and transient focal ischemia in neonatal rats
- 9:45-10:00 **Marene Landström (Uppsala, Sweden)**
Interaction between Smad7 and beta-catenin: Importance for TGF-beta induced apoptosis
- 10:00-10:30 Coffee break

Chair: Boris Zhivotovsky

- 10:30-11:00 **Hans-Uwe Simon (Bern, Switzerland)**
An autophagy gene product as a molecular switch between autophagy and apoptosis
- 11:00-11:30 **Andrew Gilmore (Manchester, UK)**
Bax, anoikis and commitment to apoptosis
- 11:30-11:45 **Roya Khosravi-Far (Boston, MA, USA)**
Oncogene-induced regulation of proteasomal degradation pathway:
A mechanism for tumorigenicity
- 11:45-12:00 **Goran Petrovski (Debrecen, Hungary)**
Human breast cancer MCF-7 cells undergoing anoikis can shift to autophagy and get engulfed by their surviving peers and macrophages
- 12:00 Closing remarks



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MARIE CURIE ACTIONS

Lecture Abstracts: Invited speakers and short communications



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The inflammasome: A dynamic caspase activating apparatus

Vishva Dixit

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Specific adaptors regulate the activation of initiator caspases; for example, FADD and Apaf-1 engage caspases 8 and 9, respectively. The adaptors ASC, Ipaf, and RIP2 have each been proposed to regulate caspase-1 (or ICE for: interleukin-1 converting enzyme). The influence of each of these adaptors was evaluated in knockout mice. ASC was found to be essential for activation of caspase-1 in response to Toll-like receptor (TLR) stimulation and ASC-null mice were resistant to LPS-induced endotoxic shock. Additionally, caspase-1 activation was substantially attenuated in ASC-null macrophages injected with the intracellular pathogen, *Salmonella typhimurium*. Ipaf-deficient macrophages activated caspase-1 in response to TLR stimulation but not *S. typhimurium*, suggesting that IPAF provides a “private” conduit to the inflammasome for signals triggered by *Salmonella*.

S-nitrosylation modulates ubiquitylation: New neuroprotective treatment strategies

Stuart A. Lipton

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Excitotoxicity, defined as overstimulation of glutamate receptors, has been implicated in a final common pathway contributing to neuronal injury and death in a wide range of acute and chronic neurologic disorders, ranging from Parkinson's disease (PD) and Alzheimer's disease (AD) to stroke. Excitotoxic cell death is due, at least in part, to excessive activation of *N*-methyl-D-aspartate (NMDA)-type glutamate receptors, leading to excessive Ca^{2+} influx through the receptor's associated ion channel and subsequent free radical production, including nitric oxide (NO) and reactive oxygen species.

These free radicals can trigger a variety of injurious pathways, but newly discovered evidence suggests that some proteins are S-nitrosylated (transfer of NO to a critical thiol group), and this reaction can mimic the effect of genetic mutations. This posttranslational modification can contribute to protein misfolding, triggering neurodegenerative diseases. One such protein is parkin, which causes some forms of PD when mutated. We recently showed in brains from patients with sporadic PD that parkin is S-nitrosylated to increase its ubiquitin E3 ligase activity. In turn, parkin ubiquitylation of proteins such as synphilin-1 contributes to Lewy body formation, composed of aggregates of misfolded proteins that represent a hallmark of PD. Auto-ubiquitylation of parkin eventually decreases its E3 ligase activity and also abrogates its neuroprotective function.

Blockade of excessive NMDA receptor activity can in large measure protect neurons from this type of injury and death. However, inhibition of the NMDA receptor by high-affinity antagonists also blocks the receptor's normal function in synaptic transmission and leads to unacceptable side effects. For this reason, many NMDA receptor antagonists have disappointingly failed in advanced clinical trials. Our group was the first to demonstrate that gentle blockade of NMDA receptors by Memantine, via a mechanism of uncompetitive open-channel block with a rapid "off-rate," can prevent this type of damage in a clinically efficacious manner without substantial side effects. We showed that Memantine blocks excessive NMDA receptor activity without disrupting normal activity. Memantine does this by preferentially entering the receptor-associated ion channel when it is excessively open, and, most importantly, its off-rate from the channel is relatively fast so that it does not accumulate to interfere with normal synaptic transmission. Hence, Memantine is well tolerated, has been used in Europe for PD for many years, and recently passed multiple phase 3 trials for dementia, leading to its approval by the FDA for AD. Clinical studies of Memantine for additional neurologic disorders, including other dementias, neuropathic pain, depression, and glaucoma, are currently underway. We have also developed a series of second-generation drugs that display greater neuroprotective properties than Memantine. These second-generation drugs take advantage of the fact that the NMDA receptor has other modulatory sites, including critical thiol groups that are S-nitrosylated. In this case, S-nitrosylation is neuroprotective by decreasing excessive NMDA receptor activity. Targeted S-nitrosylation of the NMDA receptor can be achieved by coupling NO to Memantine, yielding second-generation "smart" drugs known as NitroMemantines.

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Nbk induces apoptosis in a Bak independent manner via an ER pathway

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BH3-only proteins have recently been recognized as essential initiators of apoptosis whereas multidomain pro-apoptotic Bax and Bak are executioners of death orders relayed by the BH3-only proteins. We have recently shown that Bax deficiency protects cells from apoptosis induction by the BH3-only protein Nbk/Bik. As these Bax-deficient cells retain, however, positivity for Bak expression this indicates that induction of cell death by the BH3-only protein Nbk is mediated specifically through a Bax-dependent pathway. Nevertheless, we could not rule out that loss of Bax protects cells just by decreasing the amount of Bax/Bak-like molecules under a critical threshold necessary for Nbk to induce apoptosis. We therefore studied the influence of Bak knock-down in the Bax-proficient cell line HCT116. Bak knock-down by siRNA does not protect these cells from Nbk induced apoptosis, whereas Bax knock-out results in complete resistance to Nbk expression. Additionally we showed, that overexpression of Bax in resistant DU145 cells markedly sensitized DU145 cells to Nbk induced apoptosis whereas Bak overexpressing DU145 cells remained resistant. Furthermore, expression of Nbk induced clustering of GFP-Bax but not of GFP-Bak fusion proteins in DU145 cells. These results indicate that Nbk acts via an entirely Bax dependent pathway.

Activation of Bax by Nbk coincides with loss of mitochondrial membrane potential. Although induction of apoptosis by Nbk involves activation of the mitochondria, Nbk does not colocalize with mitochondria but with the endoplasmic reticulum. Consequently, targeting of Bcl-2 to either of these subcellular organelles protects cells from Nbk induced apoptosis. Bcl-2 targeted to the ER inhibits dissipation of the mitochondrial membrane potential and release of cytochrome c. Therefore, in Nbk-induced apoptosis cross talk between ER and the mitochondria is crucial. Thus, Nbk induces apoptosis via an ER dependent signalling pathway. This results in downstream triggering of the mitochondria and cell death execution via the mitochondrial apoptotic machinery.

Crosstalk between caspase-2 and p53 in DNA damage-induced apoptosis

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Caspase-2 has been recognised as an apical caspase in the apoptotic cascade initiated by cell stress as well in response to DNA damage. However, the precise mechanism of caspase-2 activation is still controversial. A recent study suggested that caspase-2 activation may occur within the PIDDosome complex, constituted by PIDD, whose expression is induced by p53, the adapter protein RAIDD and pro-caspase-2. Although these data indicate a link between caspase-2 and p53, a requirement of p53 for caspase-2 activation has not been demonstrated.

Here, we investigated the possible association between p53 and caspase-2 in DNA damage-induced apoptosis. The experiments were performed using cells expressing wild-type or transcriptionally inactive p53, as well as in p53 knockdown cells.

Our results demonstrated pronounced cytochrome c release, accumulation of subG1/apoptotic cell population, oligonucleosomal DNA fragmentation and phosphatidylserine exposure in wild-type p53 cells in response to treatment with etoposide or 5 FU, while p53^{-/-} cells remained unaffected. Further experiments, in which caspase-2 mRNA and protein levels in p53 wild-type cells were lowered by siRNA, demonstrated reduced DNA fragmentation and cytochrome c release compared to cells with unaffected caspase-2 level. Combined, our data suggest that a link between p53 and caspase-2 is essential for activation of the DNA damage-induced apoptotic pathway.

¹both author contributed equally

Cytochrome c as a phospholipid oxygenase: a new signaling role in apoptosis

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Harmful or unwanted cells are eliminated through a carefully regulated biochemical death program, or apoptosis. In addition to shuttling electrons in mitochondrial inter-membrane space, cytochrome c (cyt c) – upon its release from mitochondria into the cytosol during apoptosis – cyt c plays a critical role in caspase activation. We have discovered yet another important function of cyt c in apoptosis realized via its catalytic redox interactions with a mitochondria-specific phospholipid, cardiolipin (CL). Execution of apoptotic program is often accompanied by an early mitochondrial production of reactive oxygen species whose significance has not been identified. We resolved this conundrum and established that mitochondria contain a pool of cyt c, which binds to CL and acts as a CL oxygenase activated during apoptosis. The oxidized CL is required for the release of pro-apoptotic factors. This redox mechanism of cyt c is realized earlier than its other well-recognized functions in the formation of apoptosomes and caspase activation. In the cytosol, released cyt c interacts with another anionic phospholipid, phosphatidylserine (PS), and catalyzes its oxidation in an oxygenase reaction. Peroxidized PS facilitates its externalization, recognition and clearance of apoptotic cells by macrophages. This constitutes another important redox-dependent function of cyt c in apoptosis and phagocytosis. Thus, cyt c acts as an anionic phospholipid specific oxygenase activated and required for the execution of essential stages of apoptosis.

Redundant and non redundant roles of effector caspases in apoptosis

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Although caspase 3 is known to play a prominent role in apoptosis, the function of its highly homologous family member, caspase 7, is less clear. In order to address this issue, we generated caspase 7 deficient mice. These mice are viable, appear normal, and tissues from these mice exhibit only mild deficiencies in apoptosis. Double deficiency of both these caspases, however, leads to embryonic lethality and marked apoptotic defects. These results will be discussed to highlight the key position of caspases in the molecular control of apoptosis.

FOXO forkhead transcription factors and the control of cellular oxidative stress

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Forkhead box O (FOXO) transcription factors are negatively regulated by the insulin signaling pathway through phosphoinositide 3-kinase (PI-3K) and protein kinase B (PKB, also called c-Akt). Phosphorylation by PKB leads to nuclear exclusion of FOXO, and as a result, its function as a transcriptional activator is inhibited. In *C. elegans*, an insulin-like signaling pathway controls entry into the dauer diapause, an alternative larval stage that is induced by starvation, a dauer pheromone or high temperature. Under dauer-inducing conditions, the FOXO homolog DAF-16 is activated and dauer development is initiated. In addition activation of DAF-16 can increase worm adult life-span and as such insulin-mediated control of FOXO/DAF-16 is implicated in the process of aging. Oxidative stress is thought to be a prime cause of organismal ageing and we are interested in possible cross-talks between cellular oxidative stress and the insulin/FOXO signaling pathway. An example of our efforts to understand this linkage is our recent finding of the evolutionarily conserved interaction of b-catenin with FOXO transcription factors, which is regulated by oxidative stress signaling. b-catenin binds directly to FOXO and enhances FOXO transcriptional activity in mammalian cells. In *C. elegans*, loss of the b-catenin BAR-1 reduces the activity of the FOXO ortholog DAF-16 in dauer formation and lifespan. Association of b-catenin with FOXO was enhanced in cells exposed to oxidative stress. Furthermore, BAR-1 was required for the oxidative stress-induced expression of the DAF-16 target gene *sod-3* and for resistance to oxidative damage. These results demonstrate a role for b-catenin in regulating FOXO function that is particularly important under conditions of oxidative stress.

C-Raf protects against ROS-induced apoptosis

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Oncogenic transformation relies on the deregulation of signaling pathways controlling cell proliferation and survival. Signal propagation occurs through the posttranscriptional modification of effector proteins and additional mediators among which reactive oxygen species (ROS) and Ca²⁺ feature prominent roles. Generation of ROS is observed under normal as well as deregulated activation of intracellular signaling pathways and has been shown to be essential for cell growth and transformation. Excessive ROS production has been linked to induction of cell death.

The role of C-Raf in cell survival is well established. However, the molecular mechanisms through which Raf counters apoptotic cell death are largely unknown. Previous experiments have pointed to the suppression of mitochondrial events which precede the release of apoptogenic factors. Using the IL-3 dependent promyeloid cell line 32D we demonstrate that apoptosis induction through growth factor withdrawal results in enhanced production of ROS, which is not detected in cells protected either by IL-3 or oncogenic Raf (v-Raf). Consistent with this observation, presence of antioxidants significantly delayed apoptotic cell death, also following treatment with staurosporine (STS) or the direct-acting oxidative stress-inducing agent *tert* butyl hydroperoxide (*t*-BHP), against which Raf provided efficient protection. Mitochondrial Ca²⁺ overload has been postulated to be a common endpoint for many cell death inducers. Our experiments demonstrate that apoptosis under conditions of enhanced ROS production consistently resulted in increased mitochondrial Ca²⁺ levels, which are not observed in 32D cells protected by IL-3, oncogenic Raf or antioxidants. Taken together, our data suggest that survival control by C-Raf under oxidative stress aims at maintaining mitochondrial Ca²⁺ homeostasis.

Oxidative neuronal cell damage results in cell cycle progression and calpain mediated cell death

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Acute cerebral ischaemic injury results in increased neurotransmitter release which can result in cell death via receptor and non-receptor mediated mechanisms. Glutamate induced cell death in the HT22 murine hippocampal cell line has been used as a model of non-receptor mediated glutamate toxicity and previous research has suggested that the MAP kinase family, usually associated with cell division, is in this system involved in cell death (Stanciu et al, 2000). Our investigations into the molecular mechanisms behind this cell death reveal calpains (calcium dependent proteases) to be the major protease contributing to the demise of the cell. Furthermore we show that the apoptosis associated proteases caspases, in this system contribute to the *survival* of the cell. Our investigation into the role of the stress associated MAP kinase (SAPK/JNK) resulted in abnormal nuclear morphologies which we then went onto show was the result of an upregulated cell cycle. Inhibition of not only JNK but also glycogen synthase kinase (GSK) and calpains resulted in cell cycle arrest in the G2 or pre-mitotic cell cycle phase. In conclusion we have described a model where glutamate induced ROS production damages vital cellular components resulting in the activation of the cell cycle. Upon progression through the cell cycle the damage is identified and the cells enter a caspase independent, calpain mediated cell death programme involving the kinases JNK and GSK. We further propose that the previous research into the role of MAP kinases does not in fact show a degenerative role for these enzymes but in fact shows that damaged cells upregulate the cell cycle which consequently results in the identification of cell damage and death.

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Hierarchy among cell death pathways avoids necrosis-driven inflammation

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Deficiency in pro-apoptotic BAX and BAK or BIM, or overexpression of anti-apoptotic BCL-2 renders immortal baby mouse kidney epithelial (iBMK) cells profoundly refractory to apoptosis and highly tumorigenic, but how an apoptotic defect is impacted by other common mutational events in human tumors is not known. Activation of AKT in apoptosis-deficient cells confers dramatic sensitivity to ischemia through inhibition of autophagy and activation of a necrotic pathway for cell death. In apoptotic-defective tumors, AKT stimulates tumor growth but also massive tumor necrosis and an inflammatory response that may contribute to accelerated tumor growth. This demonstrates that ischemic stress triggers apoptosis, but that a defect in apoptosis reveals an autophagy-mediated survival pathway that when inactivated by AKT, promotes death by necrosis. This stress-mediated, oncogene-activated pathway for necrotic cell death illustrates that the three modes of cell death exist in a functional hierarchy designed to avoid necrosis.

Polyploidy, aneuploidy and apoptosis

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Genomic instability is intrinsically linked to significant alterations in apoptosis control. Chromosomal and microsatellite instability can cause the inactivation of pro-apoptotic pathways. In addition, the inhibition of apoptosis itself can be permissive for the survival and ongoing division of cells that have failed to repair DNA double-strand breaks, experience telomere dysfunction or are in an abnormal polyploid state. Furthermore, DNA-repair proteins can regulate apoptosis. So, genomic instability and apoptosis are intimately linked phenomena, with important implications for the pathophysiology of cancer.

Loss of caspase-8 expression does not correlate with MYCN amplification, aggressive disease or prognosis in neuroblastoma: a putative tumor suppressor revised?

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Inactivation of caspase-8 because of aberrant gene methylation has been associated with amplification of the MYCN oncogene and aggressive disease in neuroblastoma. However, the prognostic impact of caspase-8 expression in neuroblastoma has remained obscure. We analyzed caspase-8 mRNA and protein expression in a large cohort of neuroblastoma patients (304 patients), using cDNA microarray, tissue microarray or Western blot analysis, and correlated caspase-8 expression with established markers of advanced disease and outcome. Also, we investigated the impact of MYCN on caspase-8 expression in neuroblastoma cells with transfection-enforced overexpression of MYCN or with antisense-mediated downregulation of MYCN. We found loss of caspase-8 protein expression in 75% of primary neuroblastoma samples. Surprisingly, no correlation was observed between caspase-8 expression and MYCN amplification. Similarly, ectopic expression of MYCN or antisense-mediated downregulation of MYCN had no effect on caspase-8 expression in neuroblastoma cell lines. Also, caspase-8 expression did not correlate with other parameters of high-risk disease, e.g. 1p36 aberrations, disease stage, age at diagnosis or tumor histology. Most importantly, loss of caspase-8 protein had no impact on event-free or overall survival in the overall study population or in distinct subgroups of patients. In conclusion, our findings demonstrate that loss of caspase-8 occurs in the majority of neuroblastoma and is not restricted to advanced disease stages, as previously suggested. By revealing no correlation between caspase-8 expression and MYCN amplification or other established parameters of aggressive disease, our findings in a large cohort of neuroblastoma patients challenge the previous concept of MYCN-driven inactivation of caspase-8 in advanced neuroblastoma.

HIV-1 induced apoptosis is mediated by ATR, BRCA1 and GADD45, and shares its signaling with activation of the G2 checkpoint

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The human immunodeficiency virus type-1 (HIV-1) encodes four accessory genes (*vpr*, *vpu*, *vif* and *nef*) that regulate various aspects of the host cell biology. The *vpr* gene encodes a 96-amino acid protein (Vpr) that causes cell cycle arrest in G₂ and apoptosis in the infected lymphocytes. We previously demonstrated an absolute requirement for the ATM- and Rad3-related protein, ATR, for induction of G₂ arrest by Vpr (Zimmerman *et al.*, *Mol. Cell. Biol.* 21:9286-94, 2004). ATR is a serine-threonine kinase that becomes activated in response to DNA damage, and phosphorylates eight known targets in the human proteome.

We, therefore, undertook further studies to ascertain whether phosphorylation of other ATR targets could explain the onset of Vpr-induced apoptosis. In the present study, we report that the breast cancer-associated protein-1 (BRCA1), a known target of ATR, is activated in the presence of Vpr as evidenced by phosphorylation on Ser-1423. In addition, the gene encoding the growth arrest and DNA damage-45 protein alpha (GADD45 α), a known transcriptional target of BRCA1, is upregulated by Vpr in an ATR-dependent manner. We demonstrate that RNAi-mediated silencing of either ATR or GADD45 α leads to nearly complete suppression of the pro-apoptotic effect of Vpr. Our results support a model in which Vpr-induced apoptosis is mediated via ATR phosphorylation of BRCA1, and consequent upregulation of GADD45 α .

This work establishes that the pathways leading to G₂ arrest and apoptosis by Vpr are initiated at a common signaling node (ATR), supporting the notion that both effects are not easily separable.

Importantly, key signaling aspects of the ATR pathway (such as BRCA1 phosphorylation and GAD45 α upregulation) are detected in infections of primary, peripheral CD4⁺ lymphocytes as well as thymocytes when infected by HIV-1, but not by a *vpr*(-) mutant.

The control of JNK signaling and programmed cell death by NF- κ B transcription factors

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In addition to coordinating innate and adaptive immunity and inflammation, NF- κ B/Rel transcription factors play a key role in promoting cell survival. Activation of NF- κ B antagonizes programmed cell death (PCD) induced by numerous cytotoxic stimuli, including the triggering of “death receptors” of the tumor necrosis factor-receptor 1 (TNF-R1) family. The antiapoptotic activity of NF- κ B is also crucial to lymphopoiesis, oncogenesis and chemo- and radio-resistance in cancer. Cytoprotection by NF- κ B involves upregulation of prosurvival genes. To understand the mechanisms controlling TNF-R-induced PCD and NF- κ B-dependent survival, we have employed the “death trap” screen in NF- κ B-null cells. Using this unbiased screen, we have identified Gadd45 β /Myd118 – a member of the Gadd45 family of inducible factors – as a pivotal mediator of the protective activity of NF- κ B against TNF α -induced cytotoxicity. Gadd45 β -mediated cytoprotection depends upon inhibition of the c-Jun-N-terminal kinase (JNK) cascade, and inhibition of this cascade is central to the control of PCD by NF- κ B. This Gadd45 β -mediated suppression of JNK signaling involves a direct targeting and blockade of the MAP2K, MKK7/JNKK2 – a specific and essential activator of JNK. More recently, we and others have found that NF- κ B also blunts accumulation of reactive oxygen species (ROS), which themselves are pivotal elements for induction of PCD downstream of TNF-Rs. This suppression of ROS formation in fact mediates an additional protective function that has recently been ascribed to NF- κ B. The antioxidant activity of NF- κ B depends in part upon upregulation of Ferritin heavy chain (FHC) – one of two subunits of Ferritin, the primary iron-storage protein complex of the cell – and represents another important means through which NF- κ B controls cytotoxic JNK signaling triggered by TNF α . These findings establish a basis for the NF- κ B-mediated control of ROS accumulation and JNK activation downstream of TNF-Rs. These findings might provide important new targets for antiinflammatory and anticancer therapy.

Caspases and NF- κ B activation

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Besides their proteolytic functions, caspase-1, -2 and -8 are also able to mediate NF- κ B and MAPK activation. This activity is mediated by the CARD or DED motifs in the prodomain of these caspases, which consist of 6 α helices that allow homotypic interactions. The other long prodomain caspases (caspase-9, -11, -12) do not possess this activity. Caspase-1 is considered as the prototype inflammatory caspase involved in the proteolytic activation of pro-IL-1 β . The function of caspase-2 is still unclear but may function as an intracellular stress sensor for DNA damage. We demonstrated that in the case of human and mouse caspase-1 this NF- κ B and MAPK activation occurs through recruitment of RIP2, a protein kinase implicated in innate and immune responses. In the case of caspase-2 this NF- κ B and MAPK activation depends on the recruitment of TRAF2 and RIP1, forming the stressosome complex. We will report on other interacting factors of the 700 kDa stressosome. Our results suggest that caspases by means of their prodomains indirectly activate NF- κ B-mediated anti-apoptotic mechanisms that may allow caspases to exert their non-apoptotic functions.

Using *in silico* methods for screening the human genome for new caspase recruitment domain (CARD) proteins, we have identified INCA (Inhibitory CARD) as a protein that shares 81% identity with the prodomain of caspase-1. The *inca* gene is located on chromosome 11q22, the locus where also the human caspase-12 gene is residing. The *INCA* encoding gene resides between the genes of COP/Pseudo-ICE and ICEBERG, two other CARD proteins that arose from *caspase-1* gene duplications. We have demonstrated that *INCA* mRNA is expressed in many tissues and that in the monocytic cell lines THP-1 and U937, *INCA* is specifically upregulated by IFN- γ . *INCA* physically interacts with the CARD domain of caspase-1, thus preventing the activation of this protease and the subsequent generation of IL-1 β in LPS-stimulated macrophages. Unlike COP/Pseudo-ICE and the prodomain of procaspase-1, *INCA* does not interact with RIP2 to induce NF- κ B activation.

These data suggest that the prodomain of caspases is not only involved in the recruitment of caspases in complexes, but may also initiate biological responses such as NF- κ B and MAPK activation. These pathways may contribute to non-apoptotic functions of caspases.

A novel mechanism for activation or suppression of NFκB by HPK1 determines sensitivity towards activation-induced cell death in T cells

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Apoptosis and proliferation in lymphocytes is precisely balanced throughout adult life to maintain constant levels of T and B cells. In adaptation to changing environmental stimuli antigen receptor stimulation triggers lymphocytes to undergo differentiation, proliferation or apoptosis. Stimulation of the T cell receptor (TCR) can lead to activation-induced cell death (AICD) mediated by the death receptor CD95 (Fas/Apo1) in peripheral T lymphocytes. While primary T cells are resistant towards AICD, activation of T cells is known to sensitize towards TCR-triggered apoptosis.

One general cell specific signaling mediator downstream of the TCR is the Hematopoietic Protein Kinase 1 (HPK1), a mammalian Ste20-related protein kinase. Here we describe activation of NFκB complexes in primary T-cells by HPK1. The HPK1-mediated activation of NFκB is independent of the SAPK/JNK pathway activation by HPK1 and differs from other known activators of the IKK complex.

Upon proliferation of mitogen-stimulated primary T cells, HPK1 was found to be highly upregulated in the expanding cell population. During cell expansion HPK1 was found to be cleaved by a caspase 3-like activity. This cleavage was not associated with apoptosis, but was mediated by the CD95 system and could be prevented by blocking CD95L. The resulting C-terminal cleavage product of HPK1, HPK1-C, was shown to inhibit TCR-mediated NFκB activation. Analysis of transgenic mice compared to wild type mice revealed, that stimulation of the TCR in the presence of HPK1-C leads to enhanced AICD in primary T cells. Thereby, sensitization of primary T cells to AICD can be mediated through HPK1 by suppressing the NFκB pathway.

The double life of ApoJ/clusterin: a stress-activated pro-survival protein, inhibitor of NF- κ B and neuroblastoma metastasis

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ApoJ/clusterin is an enigmatic protein that can be pro- or anti-apoptotic, depending on the cellular context and the apoptotic stimuli. ApoJ/clusterin is transcriptionally activated, in a MYB-dependent manner, by thermal injury to promote cell survival of mammalian fibroblasts. While investigating the role of ApoJ/clusterin in the invasive behaviour of neuroblastoma cells we discovered that it is an essential regulator of NF- κ B. ApoJ/clusterin knockout cells display reduced levels of the negative regulators of NF- κ B, I κ B-alpha and beta. We have observed binding of a 30kD protein to I κ Bs and enhanced endogenous NF- κ B activity in ApoJ/clusterin knockout MEFs. Importantly, forced expression of ApoJ/clusterin stabilises I κ Bs, resulting in inhibition of NF- κ B and metastatic behaviour of neuroblastoma cells in *in vitro* assays, suggesting that ApoJ/clusterin could be a metastasis suppressor gene. In agreement with this hypothesis, we have observed that neuroblastoma cells transduced with an ApoJ/clusterin-expressing retrovirus, contrary to control cells, do not invade the liver and the bone marrow of transplanted mice. Thus, we hypothesize that lack of ApoJ/clusterin expression in neuroblastoma could be associated to increased NF- κ B activity and metastatic disease. We are currently validating this hypothesis by assessing whether there is an inverse correlation between clusterin expression and nuclear translocation of NF- κ B in primary neuroblastoma samples from patients with localised or disseminated disease.

Programmed cell death: It's not all apoptosis

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Apoptosis is a well characterized form of cell death observed in metazoan organisms. It provides animals with the ability to efficiently eliminate damaged or excess cells in a manner that avoids the initiation of an immune response and preserves the metabolic resources of the host. However, apoptosis is not a universal feature of multicellular organisms. For example, plants exhibit the ability to maintain cell number homeostasis and to initiate cell death as part of a wound response without any of the core constituents of the metazoan apoptotic program. This suggests that apoptosis may represent only one of several forms of programmed cell death. Recent evidence suggests that both autophagy and necrosis can be regulated in a genetically programmed manner that allows multicellular organism with the ability to eliminate damaged or excess cells. However, the consequences to the host of these forms of cell death are distinct from those of apoptosis. Necrosis initiates an inflammatory response that may be adaptive by allowing the host to recognize damaged tissues and initiate a repair response. Autophagy initiates self-catabolism that allows for efficient elimination of cellular contents in tissues undergoing remodeling that precludes neighboring cells from phagocytosing the dying cell. These new findings suggest that the field of programmed cell death is much richer and complex than previously appreciated.

Survivin checkpoints

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Survivin is a member of the Inhibitor of Apoptosis (IAP) gene family that has attracted attention for its dual role in the regulation of mitosis and its ability to counteract apoptosis induced by a variety of stimuli. Largely undetectable in normal human tissues, survivin becomes dramatically upregulated in nearly every human cancer, and has been linked in gene profiling experiments to aggressive disease progression and unfavorable outcome in patients. For these combined properties, survivin has been vigorously pursued as a rational target for novel anticancer strategies, and several clinical trials aimed at disabling the survivin pathway in cancer patients are now underway. Recent experiments further characterized the dual role of survivin in mitotic control and regulation of tumor cell death, and how this could be best exploited for targeted anticancer therapies. These studies revealed that survivin plays an essential role in the regulation of microtubule dynamics, by enhancing microtubule stability during metaphase transition. Conversely, acute loss of survivin mediated by RNA interference (RNAi) resulted in exaggerated microtubule dynamics and profound mitotic spindle abnormalities in tumor cells. A second functional checkpoint associated with the survivin pathway was identified in the interaction between survivin and the molecular chaperone, heat shock protein-90 (Hsp90). Binding to Hsp90 was required to preserve survivin protein stability in tumor cells, whereas targeted disruption of this complex using a novel cell permeable peptidomimetic antagonist designated Shepherdin, resulted in inhibition of Hsp90 chaperone function, destabilization of survivin levels, and massive activation of apoptosis in various tumor cell types. Therefore, survivin regulates critical checkpoints in the control of microtubule function and the cellular stress response that could be exploited for rational anticancer therapy.

Sequential cleavage of RasGAP by caspases: a Jekyll and Hyde control of apoptosis that can be exploited therapeutically

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RasGAP bears two caspase cleavage sites that are used sequentially as caspase activity increases in cells. Upon mild caspase activation, RasGAP is cleaved into an N-terminal fragment called fragment N that is crucially required for the survival of stressed cells. Fragment N protects cells by activating Akt and by repressing the ability of Akt to stimulate the NFκB pathway. If caspase activity increases however, fragment N is further cleaved, annihilating its protective capacity. RasGAP functions therefore as a sensor of caspase activity to either inhibit or facilitate cell death depending on the extent of its cleavage.

The ability of RasGAP caspase cleavage fragments to regulate apoptosis can be therapeutically exploited. For example, the anti-apoptotic activity of fragment N might augment the resistance of transplanted organs to reduce procedure- and host-induced apoptosis. Consistent with this notion is our recent observation that survival of pancreatic islet cells in response to various noxious stimuli is greatly improved when cells express the N-terminal RasGAP fragment. On the other hand, the pro-apoptotic activities of the smaller RasGAP caspase cleavage fragments could be exploited to increase the sensitivity of tumors to anti-cancer drugs. We have characterized a short peptide derived from the pro-apoptotic N2 fragment of RasGAP that, when coupled to cell-permeable sequences, augments the sensitivity of tumor cells, but not normal cells, to genotoxins. It is therefore possible to increase the efficacy of anti-cancer drugs to selectively kill cancer cells using peptides derived from pro-apoptotic caspase substrate fragments.

Anti-apoptotic signaling pathways in prostate cancer cells converge on BAD

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We have identified several anti-apoptotic signaling pathways in LNCaP and C4-2 prostate cancer cells. Loss of PTEN expression results in a constitutive activation of PI3K/Akt signaling which allows these cells survive in the absence of extracellular stimuli. When PI3K activity is inhibited LNCaP and C4-2 cells undergo apoptosis; however treatment with EGF or with Epinephrine at concentrations observed during acute stress protects prostate cancer cells from apoptosis induced by PI3K inhibitors.

Thus, loss of tumor suppressor gene, activation of a receptor tyrosine kinase or hormone elevated in response to the emotional stress inhibit apoptosis in prostate cancer cells.

It was somewhat surprising that all these diverse anti-apoptotic stimuli induce BAD phosphorylation. Furthermore, we found that BAD phosphorylation is necessary for protection from apoptosis since expression of BAD with mutations in phosphorylation sites induces apoptosis that cannot be rescued by any survival signaling pathway. Subsequent analysis has shown that each anti-apoptotic agonist induces BAD phosphorylation by a distinct signaling mechanism.

Thus, EGF activates two parallel pathways: one via Ras/Raf/MEK1 cascade that lead to S112 phosphorylation by a novel BAD kinase; another pathway induces BAD phosphorylation at S136 through Rac/Pak1 signaling. Each of these two pathways is sufficient to protect prostate cells from apoptosis.

Epinephrine induced cAMP production and BAD phosphorylation at S112. Experiments with pharmacological and genetic inhibitors identified PKA as S112 kinase activated by Epinephrine.

PTEN/PI3K signaling pathway induced BAD phosphorylation at S112 and S136 in Akt-dependent fashion.

In summary BAD phosphorylation is a convergence point of several independent signaling pathways that protect prostate cancer cells from apoptosis.

Combined with recent report on increased BAD expression in prostate cancer, our data suggest that BAD phosphorylation provides an attractive target for therapies that aim to inhibit anti-apoptotic signaling in prostate cancer.

Integration of glycolysis and apoptosis by the BH3-only pro-apoptotic protein BAD

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Glycolysis and apoptosis are major pathways critical for cell survival. Recent proteomic analysis of liver mitochondria revealed BAD resides in a functional holoenzyme complex together with PKA and PP1 catalytic units regulating its activation, Wiskott-Aldrich family member WAVE-1 serving as an A Kinase Anchoring Protein (AKAP) and glucokinase (GK, hexokinase IV). Initial genetic tests utilized a *Bad* loss of function model and suggested that within this GK-containing complex, BAD serves to integrate glucose metabolism and apoptosis in that it is required to nucleate the complex and to support mitochondria-tethered GK activity and glucose-driven respiration. A non-phosphorylatable *Bad*^{3SA} knockin model provided evidence that BAD phosphorylation status does not affect complex assembly, but is required for full mitochondria-tethered GK activity. Both the *Bad*-null and *Bad*^{3SA} mice display aberrant glucose homeostasis marked by fasting hyperglycemia and abnormal glucose tolerance. We have undertaken in-vivo analysis to decipher the systemic aspects of BAD-dependent glucose metabolism. These studies revealed a central role for this BH3-only pro-apoptotic molecule in pancreatic β cell function and survival. Genetic reconstitution assays together with pharmacologic mapping of the insulin secretion defect in *Bad*-null islets indicate that BAD impacts mitochondrial proximal events to regulate glucose sensing and insulin secretion. Furthermore, BAD proved critical as a sentinel in hyperglycemia-induced β cell dysfunction and apoptosis in a diabetic model. Collectively, these findings provide a significant biologic context for the integration of glucose metabolism and apoptosis by this BH3-only molecule and present, for the first time, genetic proof for the involvement of a pro-apoptotic BCL-2 family protein in both β cell function and survival.

IAP-mediated regulation of caspases

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Apoptosis is mediated by caspases. To date 11 human, 10 mouse and 7 insect caspases have been identified. Activation of caspases is a key event in apoptotic signalling and is required to execute the cell death programme. Despite substantial progress and the identification of numerous key players of the cell death machinery, the precise mechanics of how caspases are regulated remains largely enigmatic. Certain members of the inhibitor of apoptosis (IAP) protein family play a pivotal role in regulating caspase activation and activity. IAPs, originally identified in baculoviruses, are evolutionarily conserved from insects to humans and suppress apoptosis induced by a large variety of cell death triggers.

Some of the strongest evidence for the regulation of apoptosis by IAP proteins has come from genetic studies in *Drosophila*. In *Drosophila* cell survival is ensured by DIAP1, which represents one of the last line of defences against caspase-mediated damage and cell death. Loss of DIAP1 function, either through mutation or inhibition by IAP-antagonists, triggers spontaneous activation of caspases and apoptosis. DIAP1's ability to inhibit caspases relies on its two BIR domains. While the BIR1 region binds to the effector caspases DCP-1 and drICE, the BIR2 domain directly associates with the initiator caspase Dronc. Based on the mammalian prototype IAP XIAP, the current model suggests that all caspase-regulatory IAPs neutralise effector-caspases through one and the same mechanism – namely by directly binding to their catalytically active pockets. We have tested this model by exploring the molecular mechanism of IAP-mediated regulation of caspases. We will present data indicating that IAPs are functionally non-equivalent and regulate caspases through distinct mechanisms.

PRELI, a novel mitochondrial protein that protects B-lymphocytes from apoptosis

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Apoptosis is a form of programmed cell death, in which most organelles are involved in its regulation. For instance, mitochondria are central to the release of apoptogenic proteins into the cytosol and the nucleus. Among those proteins, cytochrome c (CytC), a component of the electron transport chain, plays a key role in the triggering of apoptosis when released into the cytosol. In the cytosol, CytC forms a complex with apoptosis protease-activating factor 1 (Apaf-1) and allosterically activates caspase 9, thus setting off the lethal caspase activation cascade. The mechanisms leading to apoptosis are complex and can occur through the activation of caspase-dependent and independent pathways. Our search for mechanisms that rescue B cells from apoptosis during antigen (Ag)-dependent selection resulted in the identification of PRELI, an anti-apoptotic protein which is selectively expressed by apoptosis-prone germinal center (GC) B-lymphocytes. PRELI possesses mitochondrial localization signals within its MSF1-like domain and exhibits tandem repeats of the LEA (late embryogenesis abundant) motif, which is commonly found in stress-controlling proteins. PRELI can be detected in the inner mitochondrial space and has the capacity to sustain mitochondrial $\Delta\Psi_m$, following treatment with different apoptosis-inducing stimuli, including staurosporine, TNF- α and UV irradiation. In addition, PRELI can prevent the release of apoptogenic molecules such as apoptosis inducing factor (AIF) and CytC, inhibit caspase activity and suppress DNA fragmentation. Knock-down of PRELI expression by siRNA resulted in rapid cell death, thus confirming its capacity to inhibit apoptosis. Moreover, the relevance of the LEA motif behind PRELI's function was supported by the fact that its deletion led to the complete loss of its anti-apoptotic function. Our study thus reveals an evolutionarily conserved mechanism that sustains the integrity of mitochondria and concomitantly protects cells against both caspase-dependent and independent apoptosis.

Tissue transglutaminase (TG2) acting as G protein protects hepatocytes against Fas-mediated cell death

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TG2 is a protein cross-linking enzyme known to be expressed by hepatocytes, and to be induced during the *in vivo* hepatic apoptosis program. TG2 is also a G protein that mediates intracellular signaling by the alpha-1b-adrenergic receptor (AR) in liver cells. Fas/Fas ligand interaction plays a crucial role in various liver diseases, and administration of agonistic anti-Fas antibodies to mice causes both disseminated endothelial cell apoptosis and fulminant hepatic failure. Here we report that an intraperitoneal dose of anti-Fas antibodies, which is sublethal for wild-type mice, kills all the TG2 knock-outs within 20 hrs. While TG2^{-/-} thymocytes die in the presence of anti-Fas antibodies at the same rate as wild-types, TG2^{-/-} hepatocytes have increased sensitivity towards anti-Fas treatment both *in vivo* and *in vitro*, with no change in their cell surface expression of Fas or the levels of FLIP_L, but a decrease in the Bcl-x_L expression, and appearance of apoptotic cells with unusual morphology. The same dose of anti-Fas antibodies in wild-type mice induced mostly endothelial cell apoptosis and consequent necrosis in hepatocytes. Administration of chloroethylclonidine, an AR antagonist, to wild-type mice resulted in down-regulation of Bcl-x_L in the liver, and sensitized hepatocytes for Fas-mediated apoptosis. Accordingly, mice deficient in AR expression were also more sensitive to Fas-induced killing, their liver was more sensitive to Fas-mediated apoptosis, and expressed decreased levels of Bcl-x_L. In conclusion, our data demonstrate that the loss of TG2 sensitizes hepatocytes for Fas-mediated apoptosis, and this is related to an impaired AR signaling that regulates the levels of Bcl-x_L.

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A clinical view of cell death and resistance in cancer

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Virtually all antitumor therapies (except surgery) act via the induction of apoptotic or necrosis-like cell death and utilise to this end the endogenous death machinery. It is therefore not surprising that most therapeutic strategies fail to ultimately cure the malignant disease due to the selection of resistant clones following disruption of apoptotic programs. Multiple signaling events closely intercalate cell cycle regulation and cell death control. In cytotoxic cancer therapy, both cell cycle programs and cell death signalling cascades are targeted which ultimately converge at the level of the intrinsic mitochondrial apoptosis signaling cascade. A prominent example is the p53 signaling network which plays a key role in apoptotic cell death induced by cytotoxic therapies but also mediates arrest in the cell division cycle. Here, we provide evidence that multiple, consecutively involved p53 pathway components such as p53/Bax or p53/APAF-1 or multiple BH3-only proteins such as Nbk/Bim must be disrupted to yield a clinically relevant poor prognosis genotype. This may also explain the so far disappointing data regarding the prognostic and predictive relevance of p53 single gene analyses. Moreover, p53-induced cell cycle checkpoint signaling events may counteract cell death induction and result in rather undesirable survival of tumor cells. This is mediated at least in part via a p21^{CIP/WAF-1}-dependent cellular senescence program both *in vitro* and in a clinical context. Disruption of cell cycle arrest by pharmacological inhibition or enforced activation of p34^{cdc2} overcame arrest and the senescent state and triggered apoptotic death. Disruption of mitochondrial apoptosis by Bcl-x_L interfered, however, with cell death and enabled clonogenic survival of cells following re-entry into the cell division cycle. Thus, cellular senescence and apoptosis represent two sides of the same coin and may rather occur in a linear chain of events. Moreover, cellular senescence does not appear to be a desirable therapeutic goal in cancer, especially in apoptosis-deficient tumors where escape mechanisms facilitate clonogenic survival and clinical relapse.

Molecular mechanisms of glucocorticoid-mediated control of T lymphocyte apoptosis

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Glucocorticoids (GCs) are used as anti-inflammatory/immunosuppressive agents, in organ transplantation and in treatment of leukemia and lymphomas. Their therapeutic activity is at least in part due to the effects on cell proliferation and apoptosis. In fact, GCs can inhibit cell proliferation and induce apoptosis of normal and neoplastic lymphocytes. In particular, GCs, through interaction with glucocorticoid receptor (GR), activate non-genomic signals and regulate transcription either suppressing or inducing gene expression. Between gene whose transcription is rapidly induced by GCs, GILZ (Glucocorticoid Induced Leucine Zipper) encodes for a leucine zipper protein of 137 amino acids (aa) that displays significant homology with other molecules of the leucine zipper family. Like GCs, GILZ can either induce or counter TCR/CD3-activated apoptosis, when over-expressed in vitro, in transfected cell lines, and in vivo, in thymocytes and T lymphocytes of GILZ-transgenic mice. This apoptosis is mediated via caspases activation.

Moreover, GILZ inhibits PI3K and MAPK pathways so that its overexpression results in inhibition of NF- κ B and AP-1 activation, two transcription factors involved in cell survival and anti-proliferative activity. Studies aimed to define the possible role of GILZ in GCs-mediated effects indicate that it participates to both GCs-induced apoptosis and inhibition of cell proliferation.

In conclusion, our studies indicate that GILZ participates in the complex of genomic and non-genomic signals activated by GCs and involved in control of T lymphocyte apoptosis and development.

Retinoids might be involved in the fine tuning of the T cell selection processes

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Vitamin A deficiency has been known for a long time to be accompanied by immune deficiency and susceptibility to a wide range of infectious diseases. Suggestions have been made that the active metabolites of vitamin A, that mediate its effects on the immune system, are the retinoic acids (RAs) - all-*trans* RA and 9-*cis* RA - which are ligands for the nuclear retinoic acid receptor family. In our studies we decided to investigate whether RAs are synthesized in the thymus, and whether they can affect apoptosis of thymocytes. The effect of various synthetic retinoids was tested on the selection processes of mouse thymocytes using primary cultures of thymocytes and injecting retinoids *in vivo*. Three models of TCR-mediated death were studied: anti-CD3-mediated death of thymocytes in wild-type mice, and antigen- and bacterial superantigen-driven thymocyte death in TCR-transgenic mice expressing a receptor specific for a fragment of pigeon cytochrome c in the context of the E^k (class II MHC) molecule. The levels of various mRNAs and proteins were detected by RT-, Q-PCR and Western blot analysis respectively. The levels of endogenous retinoids were determined by HPLC, while their effect was tested using RARE lacZ mice. We have found that RAs selectively inhibited TCR-mediated death of thymocytes both *in vitro* and *in vivo*, and the inhibition was mediated via the retinoic acid receptor (RAR) alpha. Ligation of RAR alpha also promoted the differentiation of immature thymocytes and the glucocorticoid-mediated death *in vivo*. While ligation of RAR α inhibited the transcriptional activity of *nur77* and the synthesis of bim, proteins involved in negative selection, it promoted the transcriptional activity of the glucocorticoid receptor by direct interaction of the glucocorticoid receptor transactivation complex. Ligation of RAR γ , on the other hand, promoted the spontaneous cell death. We also show that retinoids are actively synthesized in the cortex of the thymus and the synthesis is correlated with the postnatal development of the thymus. Our data imply that retinoids might be involved in the fine tuning of the selection processes in the thymus.

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What becomes of the corpse?**Molecular characterization apoptotic cell recognition determinants and immune consequences**

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Physiological cell death is a process whose purpose is the elimination of functionally inappropriate cells in a manner that does not elicit inflammation. Our studies have demonstrated that the ability of apoptotic corpses to be cleared in a non-inflammatory manner is a consequence of their specific expression of determinants for recognition and modulation of pro-inflammatory responses. The acquisition of these apoptotic determinants is a gain-of-function common to all physiological cell deaths and conserved widely across species. Cells that die pathologically are recognized by a distinct mechanism and do not down-regulate inflammatory responses. The modulatory activity of the apoptotic corpse is manifest as an immediate-early inhibition of pro-inflammatory cytokine gene transcription, and is exerted directly upon binding to the phagocyte, independent of subsequent engulfment and soluble factor involvement.

Specific functional recognition of apoptotic cells is not limited to professional phagocytes. Indeed, the ability to recognize and respond to apoptotic targets appears to be ubiquitous among cells of all lineages, reinforcing the view that apoptotic recognition and modulation reflects an innate immunity that discriminates live from effete cells (without regard to self). This finding facilitates a molecular dissection of the apoptotic recognition machinery. For example, we have tested the role of the presumptive phosphatidylserine receptor (PSR) by examining apoptotic recognition by embryo fibroblasts established from mice with a targeted disruption of that locus. We find that PSR-deficient cells are fully competent to recognize apoptotic targets, definitively excluding an essential role for the PSR in apoptotic recognition and inflammatory modulation.

In contrast to their profound anti-inflammatory behavior, apoptotic cells have been suggested to be provocateurs of autoimmune responsiveness. We have begun to evaluate this issue comprehensively by testing the responsiveness of antigen-specific mouse T cells to mouse macrophages after phagocytosis of apoptotic or necrotic human cells expressing appropriate antigens. Our results to date are striking. While necrotic cell antigens are presented effectively by macrophages and stimulate T cell responsiveness, apoptotic cells do not provide an effective source of antigenic stimulation. These data suggest that apoptotic cells may potentially affect immune responsiveness just as they attenuate inflammatory responsiveness.

T cell apoptosis in AIDS

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CD95, a member of the tumor necrosis factor (TNF) receptor superfamily induces apoptosis upon receptor oligomerization. The receptor and its ligand are important for apoptosis of peripheral T cells, for downregulation of an immune response and most likely, at least in part, also for peripheral T cell tolerance. In Aids, apoptosis mediated by this system might contribute to the depletion of T helper lymphocytes. Likewise, in diseases in which liver cells are destroyed the CD95 system might play a major role.

In a search to identify the intracellular signalling pathway of CD95 several molecules coupling to oligomerized CD95 were immunoprecipitated from apoptosis-sensitive human leukemic T cell and lymphoblastoid B cell lines. The following binding molecules were only associated with aggregated and not with monomeric CD95: phosphorylated FADD (MORT1) and caspase 8. Thus, caspase 8 was identified as the most CD95 receptor proximal protease which starts the cascade of protease reactions important for CD95-mediated apoptosis. Association of FADD and caspase 8 with CD95 was not observed with C-terminally truncated non-signalling CD95. FADD and FLICE did also not associate with a CD95 cytoplasmic tail carrying the Iprcg amino acid replacement. FADD and caspase 8 form a death-inducing signalling complex (DISC) with the CD95 receptor and are, thus, the first CD95 associating proteins of a signalling cascade mediating apoptosis. The function of the DISC is discussed in detail, particularly with respect to its role in sensitivity and resistance to apoptosis.

The CD95 death system plays a role in destruction of liver tissue. In hepatitis cytotoxic T lymphocytes might use the CD95 system to kill infected hepatocytes. In M. Wilson copper overload leads to upregulation of the CD95 ligand that may finally contribute to acute liver failure. In HCC from patients treated with chemotherapeutic drugs the CD95 receptor and ligand are upregulated and may contribute to apoptosis of the tumor or, dependent on the drug sensitivity of the tumor, to the status of the tumor as an immunoprivileged site.

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Lysosomal membrane proteins and autophagy

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Autophagy is a lysosomal degradation pathway for cytoplasmic material and organelles, which is activated upon starvation. After their formation as double membrane bound vesicles with cytoplasmic contents, autophagosomes undergo a stepwise maturation process including fusion events with endosomes and lysosomes (1). Lysosome associated membrane protein type 2 (LAMP-2) is one of the ubiquitously expressed and abundant integral membrane proteins of the lysosomal and late endosomal membrane (2). In human patients, mutations in the LAMP-2 gene cause Danon disease, a fatal cardiomyopathy and myopathy characterised by accumulation of huge autophagic vacuoles in these tissues (3). We showed that deficiency of LAMP-2 in mice lead to the accumulation of late autophagic vacuoles in certain tissues including heart, muscle, pancreas, and liver. Other cell types including neurons and isolated fibroblasts did not show this phenotype (4). We concluded that in these cell types, the structurally similar lysosomal membrane protein LAMP-1 was able to compensate for the absence of LAMP-2. Accordingly, fibroblasts double deficient for both LAMP-1 and LAMP-2 showed an abnormally large accumulation of late autophagic vacuoles upon starvation (5). In a parallel study we showed that the small GTP binding protein Rab7 is necessary for the final maturation of autophagic vacuoles, probably for the final fusion step with lysosomes (6). In agreement with this, we observed that the delivery of Rab7 to autophagic vacuoles was retarded in the LAMP double deficient fibroblasts. In addition to the autophagy phenotype, the LAMP double deficient fibroblasts also show an accumulation of unesterified cholesterol in late endosomal/lysosomal compartments (5). We can abolish this cholesterol accumulation by re-expressing LAMP-2, but not by LAMP-1, suggesting LAMP-2 is more important in intracellular cholesterol traffic. At present we are aiming to understand the connection between LAMP-2 deficiency, Rab7 delivery, cholesterol traffic, and autophagy.

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Autophagy: molecular mechanisms and role in intracellular pathogens fate

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Autophagy is a highly regulated and vital cellular process that involves rearrangement of subcellular membranes to sequester cytoplasmic portions and organelles. By fusion with lysosomes, the sequestered material is degraded and the molecules reused preferentially in times of starvation or stress. Several signaling complexes and pathways are involved in the development of an autophagic response. Autophagy is stimulated by cellular signals such as starvation that inhibit the kinase Tor and by activation of PI3kinase type III. Important advances have been recently achieved to elucidate the molecules implicated in autophagy. Two protein conjugation systems are key components involved in the initial steps of the autophagic pathway: the Atg12-Atg5 conjugation and the Atg8/LC3 system. LC3 is conjugated to the lipid phosphatidylethanolamine and changes its distribution localizing to both the outer and inner autophagosomal membranes. We have presented evidence indicating that members of the Rab family of GTPases such as Rab24 and Rab7 are involved in the autophagic pathway. Rab24 localizes with LC3 upon induction of autophagy by starvation or rapamycin treatment and seems to be associated with formation of autophagosomes. Rab7 localizes on the limiting membrane of autophagic vacuoles and is involved in autophagic vacuole maturation since a functional Rab7 is required for fusion with the lysosomal compartment to degrade the incorporated material. Autophagy is not only involved in degradation but also is an essential cellular mechanism implicated in several other processes such as cell death, development and aging. Several lines of evidence indicate that certain bacteria and viruses avoid or actively subvert autophagy to promote their own replication. We have recently shown that autophagy inhibits BCG and Mycobacterium tuberculosis survival in infected macrophages representing an underappreciated innate immunity mechanism used by the host cells to eliminate intracellular pathogens. In contrast, autophagy induction favors the generation and maturation of the Coxiella burnetii-parasitophorous vacuole. Therefore, autophagy can act as a defense mechanism against invasion by certain bacteria and virus, but also can be subverted by pathogens to establish a replicative niche.

Autophagic cell death and transient focal ischemia in neonatal rats

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Autophagy, a mechanism whereby eukaryotic cells degrade parts of their own cytoplasm and organelles, is a normal physiological process active in homeostasis and atrophy. Although autophagy is a normal physiological process, autophagic cell death, or type II cell death, has been reported in neurons during normal development and in neurodegenerative pathologies Huntington's, Parkinson's and Alzheimer's diseases. The mechanisms leading to delayed cell death after hypoxic-ischemic injury are known to involve apoptosis and necrosis, but the role of autophagic cell death in the ischemic area is unclear. The aim of this study was to investigate if autophagic cell death occurs after hypoxic-ischemic injury in a transient focal ischemia model of neonatal rat. Twelve day-old Sprague-Dawley rat pups underwent permanent left middle cerebral artery occlusion (MCAO) in association with 1.5 h occlusion of the left common carotid artery. Evolution of the brain infarction was studied from 0 h to 14 days in cresyl violet-stained coronal sections. Autophagic cell death is being investigated using acid phosphatase histochemistry, immunocytochemistry and Western blots against several endocytic (EEA1, Rab5) and lysosomal (cathepsin D, LAMP1) markers and electron microscopy. The first results showed that many neurons were highly reactive to acid phosphatase histochemistry in the ischemic area from 5 h following MCAO. By using immunocytochemistry, we showed that neurons in the ischemic area were immunoreactive for EEA1 and LAMP1 from 5 h to 48 h post-ischemia. Electron microscopy revealed numerous large electron-dense membranous vacuoles or autophagosomes. In conclusion, our results suggest that neuronal death following cerebral ischemia involves an autophagic pathway.

Interaction between Smad7 and β -catenin: Importance for TGF- β -induced apoptosis

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Members of the transforming growth factor- β (TGF- β) and Wnt/wingless superfamilies regulate cell fate during development and tissue maintenance. Here we report that Smad7 interacts with β -catenin and lymphoid enhancer binding factor 1/T-cell-specific factor (LEF1/TCF), transcriptional regulators in Wnt signaling, in a TGF- β -dependent manner. Smad7 was found to be required for TGF- β 1-induced accumulation of β -catenin and LEF1 in human prostate cancer (PC-3U) cells as well as in human keratinocytes (HaCaT cells). Moreover, when the endogenous Smad7 was repressed by specific siRNA, the TGF- β -induced phosphorylation of p38, Akt Ser473, GSK-3 β Ser9 and c-Myc protein was prevented, as well as the TGF- β -induced association between β -catenin and LEF1. Notably, the observed physical association of Smad7 and β -catenin was found to be important for TGF- β -induced apoptosis, since suppression of β -catenin expression by siRNA decreased the apoptotic response by TGF- β .

An autophagy gene product as a molecular switch between autophagy and apoptosis

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Autophagy-related gene (Atg) 5 is a protein required for the formation of autophagosomes. We report here that Atg5 increases the susceptibility towards apoptotic stimuli in vitro and in vivo. Apoptosis was associated with calpain-mediated Atg5 cleavage, resulting in 24-kDa truncated Atg5, a finding, which was independent from the cell type or the apoptotic stimulus. Truncated Atg5 translocated from the cytosol to mitochondria and triggered cytochrome c release, at least partially due to antagonizing the pro-survival activities of Bcl-2 and Bcl-xL, respectively. Thus, calpain-mediated Atg5 proteolysis provokes apoptotic cell death, a finding with potential importance for clinical anticancer therapies.

Bax, anoikis and commitment to apoptosis

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Most cells require adhesion to the extracellular matrix (ECM) to provide information regarding their context within a tissue or organ. This ensures that cells are removed by apoptosis if they become displaced. Apoptosis induced by the loss of adhesion is termed anoikis. Using epithelial cells as a model, we have examined how adhesion signals regulate the spatial and temporal distribution of Bcl-2-proteins, and in particular how this regulates commitment to apoptosis. Detachment from ECM results in translocation of Bax to the outer mitochondrial membrane (OMM) within 15 minutes, but cells do not die for a number of hours. Furthermore, cells can reattach to ECM and Bax redistributes back to the cytosol, indicating that Bax translocation does not commit cells to apoptosis. Instead, other levels of Bax regulation occur on the OMM. We have investigated this using analysis of endogenous Bax and expression of Bax mutants. Using blue-native PAGE, we show that Bax translocates to the OMM where it associates with a large complex prior to cell death. Bax in this complex does not show N-terminal epitope exposure. Only at the point of cytochrome c release is N-terminal epitope exposure observed. We have analysed the role of Bax N-terminal conformation in commitment by introducing single amino acid substitutions within this region. This did not affect the regulation of Bax translocation. However, this mutant was constitutively pro-apoptotic after translocation to the OMM following detachment from ECM. Constitutively targeting this N-terminal mutant of Bax to the OMM of carcinoma cells resulted in constitutive cell death. Our data indicate that multiple regulatory steps control Bax function before and after translocation to mitochondria. The C-terminus regulates translocation, whereas the N-terminus is associated with commitment, possibly through a complex with other proteins on the OMM.

**Oncogene-induced regulation of proteasomal degradation pathway:
A mechanism for tumorigenicity**

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The roles of many of the effectors of Bcr-Abl are characterized in some detail, but little is known of the pathways that promote transformed hematopoietic cells to evade apoptosis. Here, we report a novel mechanism by which Bcr-Abl can promote survival and prevent apoptosis of cytokine-dependent hematopoietic cells. Our studies demonstrate that Bcr-Abl expression in hematopoietic cells leads to the regulation of the protein level and transcriptional activity of members of Forkhead Transcription Factor family, i.e., FOXO3a, a key regulator of cellular survival. Notably, we have observed that Bcr-Abl expression in hematopoietic cells leads to the constitutive phosphorylation of FOXO3a in an Akt-dependent manner causing the relocalization of FOXO3a in the cytoplasm. Additionally, we have demonstrated that Bcr-Abl expression also down-regulates the levels of FOXO3a protein expression through a proteasome-mediated degradation pathway. Suppression of both FOXO3a expression and activity then lead to Bcr-Abl-induced down-regulation of the components of extrinsic (TRAIL; a tumor selective inducer of apoptosis) as well as intrinsic (Bim and Hrk; members of Bcl-2 family of proteins) apoptotic machinery, causing evasion of transformed hematopoietic cells from apoptosis. Markedly, we have also observed that inhibition of the proteasomal pathway leads to enhanced FOXO3a activity and promotes apoptosis of Gleevec-sensitive as well as -resistant hematopoietic cells. Moreover, suppression of the proteasomal pathway promotes regression of leukemic phenotype in a murine model for Bcr-Abl-induced leukemia. The FOXO3a pathway, thus, will provide novel molecular targets for anti-CML therapy that acts downstream of the Bcr-Abl oncoprotein and will overcome the resistance to current therapy (Gleevec) that is caused by mutations and gene amplifications in this oncogene.

Human breast cancer MCF-7 cells undergoing anoikis can shift to autophagy and get engulfed by their surviving peers and macrophages

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Human mammary carcinoma cells (MCF-7) undergo autophagy upon starvation and tamoxifen (estrogen receptor antagonist) treatment, which climaxes by day 4 (Bursch & al. *J. Cell Sci.* **113**; 1189-98). When plated on non-adhesive substratum and in fed state, the MCF-7 cells die by anoikis peaking at day 5 (assessed by annexin/PI assay) and show autophagy peaking at day 6 (assessed by MDC/PI assay). A synergistic effect between anoikis induction and anti-estrogen treatment was noted when the cells were starved and exposed to tamoxifen on poly-HEMA coated substratum: massive autophagy occurred in these cells by day 2 (assessed by electron microscopy).

Day 5 anoikic and day 6 autophagic MCF-7 cells were phagocytosed by substratum-attached cells of the same lineage at a steady 11.2% and 11.1% rate, respectively, over the first 24 hours. The same dying cells were phagocytosed by macrophages at a steady rate of 68,1% and 41.4%, respectively. The intracytoplasmic localization of the engulfed cells after 24 hours was confirmed by confocal microscopy using different fluorescent cell labeling of the dying and substratum-attached cells. UV irradiated apoptotic MCF-7 cells were phagocytosed by substratum-attached cells of the same lineage and macrophages at a significantly lower rate over the same 24 hours.

These findings indicate that anoikis facilitates autophagy. Furthermore, anoikic and autophagic cells are steadily phagocytosed by the same type of surviving cells as well as macrophages.

Poster Abstracts



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Understanding how the Natural killer cell protease granzyme B promotes apoptosis

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Cytotoxic T lymphocytes (CTL) and Natural Killer (NK) cells can eliminate virally-infected or transformed cells either through engagement of CD95/Fas receptors on the target cell or through delivery of cytotoxic granules onto the target cell membrane. CTL/NK-derived cytotoxic granules contain a range of proteases, called granzymes, as well as other constituents that promote efficient death of target cells by mechanisms that are incompletely understood. One of the major granzymes, granzyme B, can directly process and activate members of the caspase family of cysteine proteases that play a central role in coordinating apoptosis. In addition, granzyme B can also promote processing and activation of BID, a Bcl-2 family member that can directly engage the apoptosome pathway to caspase activation through promoting mitochondrial cytochrome *c* release. However, granzyme B is also known to be capable of promoting target cell death in a caspase-independent manner, suggesting that additional granzyme B substrates exist. To search for novel granzyme B substrates we used cell-free extracts derived from a transformed human T cell line coupled with 2D-PAGE/mass spectrometry. Here we describe the results of this screen and report the identification of several novel granzyme B substrates that shed light on the mechanism of CTL/NK-mediated killing. In particular, we have identified a novel granzyme B substrate that exhibits potent pro-inflammatory properties when released from target cells during NK/CTL-mediated apoptosis. These data suggest that Granzyme B regulates a pro-inflammatory signal emitted during NK/CTL-mediated attack, possibly to amplify the immune response to viral infection or tumours.

Delayed doxorubicin-induced apoptosis in highly proliferative Jurkat cell sublines occurs by a caspase-independent mitochondrial pathway involving ROS and AIF

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Apoptosis is an evolutionary conserved and genetically regulated process controlling the homeostasis of multicellular organisms. Fas ligation, caused loss of mitochondrial membrane potential ($\Delta\Psi_m$) resulting in apoptosis by the combined action of caspases and AIF, showing cell shrinking, blebbing, nuclear condensation and fragmentation. Loss of Fas surface expression, loss of proapoptotic mechanisms or overexpression of apoptosis inhibitors lie in the molecular ethiology of cancer.

It is accepted that chemotherapeutic drugs induce tumor death through the mitochondrial pathway. Here, we studied the delayed doxorubicin-induced apoptosis in Jhp and Jws cells derived from the human T cell leukaemia Jurkat. Jhp and Jws cells showed caspase-3,8 negative expression and inactive caspase-2, -6, -7 and -9 upon doxorubicin treatment. Differently to Jurkat, drug effects were not reversed by the pan-caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk). After 48h of Jhp/Jws treatment, cells exhibited $\Delta\Psi_m$ loss, AIF nuclear translocation and AIF-peripheral chromatin condensation, confirmed by the caspase-independent induction of DNA fragmentation in high molecular weight fragments. The generation and contribution of ROS on doxorubicin toxicity is evaluated by flow citometry. Other ROS-producer drugs such as menadione or several tyrphostines in combination with the mimic SOD MnTBAP have been used to determine the relevancy of ROS in Jhp/Jws drugs-induced apoptosis. Our results indicated that the caspase-independent apoptosis induced by doxorubicin in hyperproliferative Jhp/Jws cells, followed a slower and alternative pathway involving AIF nuclear translocation, upstream regulated by ROS generation. Although present in Jurkat cells, this pathway is masked by caspase action. These data could be relevant for refractory tumours with drug resistance.

Role of hFis1 in mitochondrial division and apoptosis

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Mitochondrial fission is crucial to maintain a full complement of mitochondria in daughter cells after division and to ensure progression of the apoptotic cascade. Fragmentation is controlled by cytosolic and mitochondrial proteins, like hFis1. Mitochondria lacking hFis1 fail to divide, while its overexpression leads to mitochondrial fission, cytochrome c release and apoptosis. Its silencing confers resistance to several death stimuli. Here we show that two separate pathways operate downstream of hFis1, one that regulates the mitochondrial apoptotic pathway, the other controlling mitochondrial morphology. Overexpression of hFis1 in Bax, Bak^{-/-} (DKO) mouse embryonic fibroblasts (MEFs), which are resistant to all intrinsic apoptotic stimuli, resulted in mitochondrial fragmentation but not in organellar dysfunction and apoptosis. Mutants of hFis1 also dissociated its proapoptotic role from its effects on mitochondrial morphology. Both the cytosolic and the intermembrane space domain of hFis1 are essential for death, whereas mitochondrial fission depends solely on the cytosolic portion of the molecule. Thus, hFis1 is a bifunctional protein that can independently regulate mitochondrial fragmentation and apoptosis.

Suppression of ODC and Bax mRNA levels in bohemine treated rat prostate cancer cell lines

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Polyamines (PAs) are ubiquitous low-molecular aliphatic amines that play important roles in cell growth and differentiation. The prostate gland has the highest level of PAs in the body and they are synthesized greatly by prostate epithelium. Prostate carcinomas have high PA levels. High incidence of prostatic cancer and the limitations of its treatment caused the necessity of new therapeutic models. New synthesized plant cytokinin analogues have inhibitory effects on cyclin-dependent kinases (CDK). The aim of this study is to determine the effects of a new CDK inhibitor bohemine (BOH) on PA contents, mRNA levels of the key enzyme of PA biosynthesis ODC and pro-apoptotic gene Bax in different rat prostate cancer cell lines.

Weakly (AT-2) and highly metastatic (MAT-Lylu) cell lines (1x10⁶ cells/ml) were cultured in RPMI1640 medium, supplemented with 5% fetal calf serum, 1% penicillin-streptomycin and 15 µM BOH for 24 and 48 hours. PA contents were determined by HPLC and also mRNA levels of ODC and Bax were established by RT-PCR technique.

It was estimated that 48 hours BOH treatment inhibited the cell growth rate by 96% in both cell lines and also decreased total protein levels by 43% in AT-2, 14% in MAT-Lylu cells. It was also observed that MAT-Lylu was the most affected cell line with BOH. 48 hour BOH application reduced Put, Spd and Spm levels by 93%, 94% and 88%, respectively in MAT-Lylu cell lines. Furthermore, it was determined that BOH has an inhibitory effect on ODC and Bax mRNA expression levels in both cell lines.

In conclusion, BOH exhibited an inhibitory effect on PA levels and ODC mRNA transcription level however it is also reduced Bax mRNA levels, which is not expected. In this case it is necessary to investigate the apoptotic parameters after the application of BOH in prostate cancer cell lines.

Requirement for caspases-2, -3, -6, -8 and -9 in stress-induced apoptosis by dehydrocrotonin and its inclusion complexes with beta-cyclodextrins in HL60 cells

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Apoptosis is presently one of the most obvious targets for cancer treatment as its frequent inactivation in tumours contributes to carcinogenesis as well as resistance to chemotherapy. Low molecular weight compounds derived from plants are currently being investigated for their ability to regulate apoptosis on programmed cell death. In particular, the cytotoxic and antitumour properties of dehydrocrotonin (DHC) in systems of controlled release have been investigated. The DHC is obtained from the bark of *Croton cajucara* (Sacaca), a plant found abundantly in Brazilian Amazon. The complexation of DHC with cyclodextrins can improve its biological activities in terms of the solubility, delivery, membrane permeability and bioavailability. Current evidence suggests that there are several distinct pathways to caspase activation depending upon the stimulus that initiates the cell death program. The purpose of this work was to report the action mechanism of apoptosis induction by DHC and its inclusion complexes with β -cyclodextrins (β -CDs): β -, methyl- β - and hydroxypropyl- β -cyclodextrins (β -CD/DHC, Me- β -CD/DHC and OH-p- β -CD/DHC, respectively) on human promyelocytic HL60 leukaemia cells. HL60 cells were incubated with concentration of DHC and its inclusion complexes around to IC₅₀ values (150 and 250 μ M). In this work, we measured the caspases activation by colorimetric protease kits and the GSH level was determined by 5,5'-dithio-bis(2-nitrobenzoic acid) DTNB reaction. We have observed that the membrane mitochondrial permeability transition induction on HL60 cells by mitochondrial swelling occurred. We have also determined the mitochondrial membrane potential ($\Delta\psi_m$) by flow cytometry using a cationic lipophilic fluorochrome JC1. Our results showed that DHC and its complexes were effective in triggering the activation of caspases-2, -3, -6, -8 and -9 and decreasing the GSH level compared to control cells. DHC in a free and complexes forms induced increasing in the mitochondrial swelling indicating altered mitochondrial function. However the inclusion complexes OH-p- β -CD/DHC and Me- β -CD/DHC were more efficient in the swelling mitochondrial induction than the free DHC and the complex β -CD/DHC on HL60 cells. Moreover, the free and complexed forms showed to be effective in triggering a substantial decrease in $\Delta\psi_m$ that indicates an increase on the mitochondrial membrane depolarisation. Among the inclusion complexes, the β -CD/DHC showed be the most efficient in the caspases activation, similarly to DHC in its free form. These results suggest that DHC and its β -CDs complexes induced apoptosis partly by oxidative stress, which triggered the caspase cascade activation in HL60 cells, however with some differences in the caspases activation pathways. Based on the pattern of caspase activation and on the mitochondrial alterations, we conclude that DHC and its cyclodextrins complexes triggered apoptosis in HL60 cells through mitochondrial pathway with oxidative stress involvement.

Identification of a novel coiled coil protein similar to tropomyosin that regulates mitochondrial shape

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Tropomyosins are a family of actin binding proteins that stabilise microfilaments and are encoded by a group of highly conserved genes. There are four human tropomyosins genes: TPM1, TPM2, TPM3 and TPM4, each of them can generate multiple isoforms. In the search for regulators of mitochondrial shape in high eukaryotes, we found FLJ35417, encoding for a protein of 498 amino acids, with a putative mitochondrial targeting sequence, two transmembrane segments and coiled coil domains. It displays similarity to tropomyosin-3 and an overall 18% homology with the yeast protein Mdm33 that plays a role in the mitochondrial inner membrane fission. FLJ35417 obtained from RIKEN repository was cloned in frame with GFP or the small viral epitope V5. We are investigating whether overexpression or silencing of FLJ35417 affects mitochondrial morphology and cell viability. Overexpression of FLJ35417 promotes mitochondrial fragmentation, while its silencing enhances mitochondrial elongation. Moreover, while overexpressed FLJ35417 does not affect apoptosis, its silencing sensitizes to apoptosis by intrinsic stimuli. Given its homology with tropomyosins, that participate in cytoskeletal organization, we also addressed whether levels of FLJ35417 regulate shape of filamentous actin and α -tubulin. Precise levels of FLJ35417 are required for correct F-actin organization, while they do not affect microtubules. We conclude that FLJ35417 is a novel mitochondrially located protein that regulates shape of mitochondria and of thin filaments.

Collagen VI muscular dystrophies from animal model to human therapy: mitochondria as targets for therapeutic intervention in muscle cell death

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Collagen VI (ColVI) is an extracellular matrix protein. Inherited mutations of ColVI genes have been linked to two human muscle diseases, Bethlem Myopathy (BM) and Ullrich Congenital Muscular Dystrophy (UCMD). The pathogenic mechanisms involved in these disorders are unknown and effective therapies are missing. We have identified the pathogenic mechanism underlying muscular defects in *Col6 α 1^{-/-}* mice. Analyzing skeletal muscle fibers isolated from these mice, we showed a latent mitochondrial dysfunction due to the opening of the mitochondrial permeability transition pore (PTP), and this mitochondrial event triggers muscle fiber death. The ability of cyclosporin A (CsA), an inhibitor of the PTP, to rescue the myopathic alterations of *Col6 α 1^{-/-}* mice both *in vitro* and *in vivo* suggested a pharmacological strategy to treat patients affected by congenital ColVI deficiencies (1). We show that a PTP-dependent latent mitochondrial dysfunction is also present in myoblasts from *Col6 α 1^{-/-}* mice and from ColVI disease patients. Addition of oligomycin or rotenone to the patient's cultures caused a decrease of mitochondrial membrane potential that was never observed in cultures from healthy donors. These treatments also resulted in an increased rate of apoptosis. Both these events could be prevented by both plating cells onto purified ColVI and preincubating cells with CsA, suggesting that inappropriate PTP opening may play a key role in the pathogenesis of human ColVI myopathies. It has been shown that inactivation of GSK-3 β results in a survival signal that may prevent PTP opening (2). We have therefore tested the effects of insulin and Li⁺, which both inactivate GSK-3 β , on mitochondrial function in the same cultures. We observed that mitochondrial depolarization induced by oligomycin was normalized by pretreatment with insulin or LiCl, suggesting that GSK-3 β may play a role in the sequence of events that sensitizes the PTP to opening in ColVI diseases.

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Correlation of apoptosis and aneuploidy in the spermatozoa of infertile men

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Aim: To estimate the frequency of numerical chromosomal abnormalities in the chromosomes 13, 18, 21, X and Y of infertile men and to compare it with the sperm apoptotic index.

Materials/Methods: Semen analysis of 10 oligoteratozoospermic patients was carried out based on the WHO 1999 guidelines. Dual and triple colour FISH (Fluorescence *in situ* hybridization) was applied, according to standard manufacturer procedures (Vysis, Inc.). The probes 13 (LSI 13: Spectrum Green, 13q14), 18 (CEP 18: Spectrum Aqua D18Z1, alpha satellite DNA (18p11.1-q11.1)), 21 [LSI 21: Spectrum Orange, loci D21S259, D21S341, D21S342 (21q22.13-q22.2)], X [CEP X: Spectrum Green DXZ1, alpha satellite DNA (Xp11.1-q11.1)] and Y [CEP Y: Spectrum Orange DYZ3, alpha satellite DNA (Yp11.1-q11.1)], were used and the stained samples were observed using an Axiolab Zeiss microscope. Nick end labelling of DNA breaks in sperm nuclei have been performed with a mixture of biotinylated dUTP (Roche, stock 50 nmol) and TdT (Amersham, stock 9 units/ μ l) in TdT buffer. After the end of the reaction, the slides were incubated with Texas Red Streptavidin (1mg/ml) which revealed the apoptotic nuclei and the non apoptotic nuclei were counterstained with DAPI (1mg/ml) (Vector Laboratories). For each case 500 spermatozoa were counted.

Statistical analysis was performed with the χ^2 test.

Results: The mean frequency of aneuploidy and the percentage of apoptotic cells/patient, in infertile men, are shown in Table 1.

Subject (Total Mean)	1	2	3	4	5	6	7	8	9	10
Disomies	0.45	0.15	0.31	0.41	0.48	0.38	0.18	0,10	0,20	0.28
Nullisomies	0.86	0.54	0,70	0.94	0.97	0.88	0.21	0.35	0.26	0.61
Diploidies	0.27	0.10	0,26	0.25	0.27	0.20	0.01	0.01	0.00	0.22
Aneuploidy (%)	0.53	0.26	0.42	0.53	0.57	0.49	0.13	0.15	0.15	0.37
Apoptotic nuclei (%)	0.79	0.04	0.18	0.05	0.38	0.38	0.15	0.21	0.12	0.08

Conclusion: A correlation of aneuploidy with apoptotic nuclei was observed in 30% of the cases. Thus, infertility of these patients might be the consequence of both phenomena. In 60 % of the cases, where aneuploidy exceeded apoptosis, the cause of infertility is evident. Finally, the remaining 10% showed a negative correlation, which means that DNA fragmentation is the aetiological factor.

Synergistic cytotoxicity in leukemia cells between N-(4-hydroxyphenyl)retinamide and modulators of ceramide metabolism

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Retinoids are recognized as a family of molecules capable of cause impact on many cell functions. *In vivo* trials with animals and *in vitro* assays have shown that the synthetic retinoid, N-(4-hydroxyphenyl)retinamide (4HPR), is a promising chemopreventive and antineoplastic agent for cancer. In this sense we have previously demonstrated that *in vitro* treatments with 4HPR induce a strong apoptotic cell death in human leukemia cell lines, but not in peripheral blood lymphocytes. We have also seen that in sensitive CEM human leukemia cells, 4HPR increases intracellular ceramide by both sphingomyelin hydrolysis and *de novo* synthesis. Such ceramide accumulation is the responsible to induce an oxidative stress which in turn initiated the apoptotic mitochondrial pathway.

Related to the importance of the ceramide in the 4HPR-mediated apoptotic induction, our goal is to determine whether inhibitors of ceramide metabolism (*d, l*-threo-1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol (PPMP), *d, l*-threo-dihydrosphingosine (DHS) and *L*-threo-dihydrosphingosine (safingol) enhance retinoid effect. PPMP is an inhibitor of glucosylceramide synthase and 1-acylceramide synthase while DHS and safingol inhibit sphingosine kinase. Safingol acts also as an protein kinase C. All these pathways turn ceramide into less toxic forms so that a increased cytotoxicity is expected by treatments based on their combination with 4HPR.

When testing treatments with non cytotoxic concentrations of inhibitors (5-10 μ M of PPMP and 1-5 μ M DHS) and 4HPR (1-3 μ M), synergistic loss of viability (near 40%) has been observed at 48 hours of incubation in the following cases: 4HPR (1 μ M)+PPMP(10 μ M) and 4HPR(1 μ M)+DHS (5 μ M). The high cytotoxicity showed by 3 μ M 4HPR itself (80,47% \pm 7,84 of cell death at 48hours of incubation) hides any synergetic effect of the inhibitors. Combination of both ceramide inhibitors (10 μ M PPMP+5 μ M DHS) and 4HPR (1 μ M) results in a death ratio (>90% of cell death at 48hours) similar to that observed when applying treatments based on 3 μ M 4HPR (+10 μ M PPMP or +5 μ M DHS); furthermore, a clear synergistic increased of cell death is observed when comparing the effect of this treatment with the effect obtained by both, sum of the individual cytotoxicity or cytotoxicity of (PPMP+DHS) + 4HPR. Thus, results support the importance of the ceramide as a second messenger in the 4HPR-mediated apoptotic induction.

In order to study aspects related to the resistance of human cell lines to 4HPR, we have established 4HPR-resistant CEM lines (loss of viability < 10% at 48h of incubation with 10 μ M 4HPR) and observed the effect of some of the previously described treatments. Results indicated that these cell lines, even not being sensitive to 10 μ M PPMP, are sensitive to 4 μ M safingol and specially, to 5 μ M DHS (up to 80% of cell death at 48h). Furthermore, when adding treatment of 4HPR (1 μ M)+PPMP(10 μ M)+DHS(5 μ M), there is no difference between the effect observed on sensitive and resistant CEM lines (loss of viability of 90-98% at 48hours).

As a whole, results obtained by both 4HPR-sensitive and resistant CEM human leukemia cells, support the importance of the ceramide and its metabolism not only as a future strategy to increase the 4HPR effect (and a possible basis for a novel chemotherapy) but also because of the high cytotoxicity induced on 4HPR-resistant cells.

Role of Heat Shock Proteins(HSPs) in Tumor Necrosis Factor- α (TNF α)-induced Nuclear Factor κ B (NF- κ B) signaling pathway

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Heat shock proteins (HSPs) are a group of proteins that have been implicated in various biological functions including the response to heat shock, oxidative stress and cytokine treatment. Under normal conditions, these proteins play important roles in cell function by changing protein conformation, promoting multiprotein assembly/disassembly, facilitating protein translocation and ensuring proper folding of the nascent polypeptides during translation. It has been suggested that in accordance with their roles under normal conditions, HSPs may also play crucial role in signal transduction by promoting folding or assembly/disassembly of signaling molecules.

Of this family, we have found HSP90 protein to be upregulated in a time-dependent manner starting from 15 minutes up to 4 hours by Tumor Necrosis Factor- α (TNF α) treatment (20ng/ml) in HeLa cells. Accordingly, Electrophoretic Mobility Shift Assay (EMSA) results have shown impaired Nuclear Factor- κ B (NF- κ B) binding to radiolabeled consensus oligo in presence of the specific HSP90 inhibitor-geldanamycin (GA:0,5 μ M) These results indicate that HSP90 plays an important role in TNF α induced NF- κ B signaling pathway.

Having identified this, we want to proceed further to show protein-protein interactions of HSP90 and others by co-immunoprecipitation assays (co-IP), solving the interaction network upstream of Inhibitor of κ B Kinase (IKK) complex. As a prerequisite to co-IP assay, we have accomplished to transfect HeLa cells with Green Fluorescent Protein (GFP) with 95% efficiency via electroporation, and additionally we have optimized IP parameters, too. In near future, by co-IP experiments, we aim to search for any possible cross-talks between NF- κ B signaling pathway and other TNF α induced signaling pathways like JNK and p38 MAPK that might occur via HSP family.

A double alteration of the ADP/ATP translocator, which depends on both ROS production and caspases, triggers the mitochondrial permeability transition, dispensable during cerebellar granule cell apoptosis

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The adenine nucleotide translocator (ANT) exchanges mitochondrial ATP for cytosolic ADP, but is also a component of the mitochondrial permeability transition pore (mPTP). This poses a question as to the function of ANT in apoptosis in which ATP is needed. We investigate both ANT dependent ADP/ATP exchange and mPTP opening in homogenates from cerebellar granule cells (CGCs) undergoing apoptosis, containing initially coupled mitochondria. We show that in the first 3 hours of apoptosis (*early apoptosis*), ANT function is impaired due to production of reactive oxygen species (ROS) (there is no change in V_{max} , but the K_m for ADP increases), but no mPTP opening occurs. Over 3-8 h of apoptosis, (*late apoptosis*) the mPTP progressively opens, due to caspase action, and further ANT impairment occurs, with a decrease in V_{max} but no further change in K_m . The kinetics of ANT transport and mPTP opening are inversely correlated both in the absence or presence of inhibitors of the cell antioxidant and proteolytic systems. We conclude that *en route* to apoptosis, alteration of ANT occurs resulting in mPTP opening. This process depends on proteolysis by caspases of mPTP protein component/s other than ANT, since no change in either amount or molecular weight of ANT takes place during apoptosis as measured via Western blot. Interestingly, cell death occurs via apoptosis in the presence of cyclosporine, the mPTP inhibitor.

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Different susceptibility of solid tumor and leukemia cells to apoptosis induced by acridine derivatives

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Several classes of highly potent antitumor acridine derivatives were developed at the Gdansk University of Technology. These compounds exhibit a broad spectrum of activity against different experimental tumors. The imidazoacridinone derivative C-1311 is currently undergoing phase I clinical trials. The most active derivative of 4-methyl-1-nitroacridines C-1748 has been selected for the I clinical trials. Among triazoloacridinones, the compound C-1305 is currently undergoing pre-clinical studies.

Our previous studies showed that mentioned above acridine derivatives, at pharmacologically relevant doses (EC_{90} concentrations), induced apoptosis of different human tumor cells. Here, we compared the ability to induce apoptosis by acridine derivatives in solid tumor HT29 and human leukemia MOLT4 and HL60 cells.

DAPI staining of cytospin preparation was performed to analyze the cellular morphology. Cell cycle distribution was analyzed using flow cytometry and the sub-G1 region was used as an additional marker of apoptotic cells. Mitochondrial membrane potential was measured by flow cytometry using JC-1 fluorochrome. Caspase-3 activity and phosphatidylserine externalization was evaluated using commercially available assay kit on flow cytometry. DNA fragmentation was analyzed by 1.8% agarose gel electrophoresis.

The broad spectrum of methods allowed us to evaluate different levels of apoptotic response in tumor cells exposed to acridine derivatives. We found that exposure of MOLT4 cells to C-1311 and C-1305 induced cell cycle arrest in the G2/M phase and subsequent apoptosis. In the case of HL60 cells G2/M arrest was also evident. However, compared to MOLT4 cells their capacity to execute apoptosis after acridine treatment was somewhat weaker. We did not study the influence of C-1748 on apoptosis of leukemia cells, because our previous data showed no antitumor activity of 4-methyl-1-nitroacridines towards leukemias.

In contrast, the extend of apoptosis induced by all acridines studied was found to be much more limited in HT29 cells. Collectively, these findings identify acridine derivatives as potent inducers of cell apoptosis in the leukemia cells and to a lower extent in the colon adenocarcinoma HT29 cells.

Overexpression of cytosolic group IVA phospholipase A₂ protects cells from Ca²⁺-dependent death

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The calcium ionophore ionomycin induces apoptosis-like events in the human embryonic kidney (HEK) cell line at early times. Plasma membrane blebbing, mitochondrial depolarization, external-ization of phosphatidylserine and nuclear permeability changes can all be observed within 15 min of treatment. However, there is no activation of caspases or chromatin condensation. Expression of a fusion protein containing the enhanced green fluorescent protein (EGFP) and human cytosolic Group IVA phospholipase A₂ α (EGFP-cPLA₂ α) in these cells prevents the ionomycin-induced phosphatidylserine externalization. Cells expressing the cPLA₂ α mutant D43N, which does not bind calcium, retain their susceptibility to ionomycin-induced cell death. Both non-expressing and EGFP-D43N-cPLA₂ α -expressing HEK cells can be spared from ionomycin-induced cell death by pretreating them with exogenous arachidonic acid. Moreover, exogenous arachidonic acid decreases the mitochondrial membrane potential and, after calcium overload, mitochondrial depolarization is significantly lower in the EGFP-cPLA₂ α -expressing cells than in cells expressing normal amounts of cPLA₂ α . These results suggest that early cell death events promoted by an overload of calcium can be prevented by the presence of high levels of arachidonic acid, which induces mitochondrial depolarization.

Epithelial and Germ Cell Kinetics in the Developing Ovary

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Aim: Correlation between epithelial and germ cells proliferation and apoptosis in the developing ovary of Wistar rat embryos and neonates.

Material/Methods: Immunohistochemical localisation of antigens PCNA, Ki-67, Bax and Bcl-2 using Clone PC10 (Dako), NCL-Ki-67 (Novocastra), rabbit polyclonal (BD Pharmingen) antibodies. Gonads were examined at the foetal age of 14.5, 18.5 and 20.5 days *pc* (*post coitum*), at birth and at 1, 3, 5, 7 days *pp* (*post partum*). Labeling Index (LI) per cell type was calculated using the Image Pro Plus Software, following detection of reactive cells with Zeiss Axiolab microscope.

Results:

PCNA	14.5 dpc	18.5 dpc	20.5 dpc	22.5 dpc	1 dpp	3 dpp	5 dpp	7 dpp
Germ cells	71.19	75.66	73.26	28.57	43.58	42.17	41.71	18.41
Epithelial cells	0.70	28.90	18.03	27.66	33.69	22.88	34.25	49.22

Ki-67	14.5 dpc	18.5 dpc	20.5 dpc	22.5 dpc	1 dpp	3 dpp	5 dpp	7 dpp
Germ cells	43.67	27.09	32.11	28.62	26.83	25.99	11.88	9.42
Epithelial cells	0.87	9.74	24.57	24.96	32.12	32.53	41.92	44.28

Staining of the pro- and anti- apoptotic molecules Bax and Bcl-2 was cytoplasmic for both cell types. At 21.5 dpc a stronger reaction for Bax protein was evident in germ cells whilst the reaction for Bcl-2 was more intense at 5 dpp. An increasing positivity for both antigens was observed in follicular cells till 3 dpp. Then, the reaction remained stable for Bax but was more intense for Bcl-2.

Conclusion: Epithelial (follicular) cells, in the ovary, proliferate constantly, especially after birth. Germ cells number is declining after 18.5 dpc, following apoptosis that is more pronounced after birth. Oocytes, thereafter preserved, are protected by the anti-apoptotic action of Bcl-2 until the reproductive period.

Group VIA Phospholipase A₂-derived Lysophosphatidylcholine as a Direct *Eat Me* signal for macrophagesRebeca Pérez, María A. Balboa, and Jesús Balsinde*Eicosanoid Research Division, Institute of Molecular Biology and Genetics, Spanish Research Council and University of Valladolid School of Medicine, 47005 Valladolid, Spain*

Strong oxidants such as hydrogen peroxide are known to induce cell death by apoptosis in a variety of cells. Hydrogen peroxide-induced cell death also results in substantial hydrolysis of membrane phospholipids by calcium-independent group VIA phospholipase A₂ (iPLA₂-VIA). However, abrogation of cellular iPLA₂-VIA activity neither delays nor decreases the extent of apoptosis, suggesting that, beyond of a direct destructive role in apoptosis, iPLA₂-VIA may serve other specific roles. Here we report that phagocytosis of dying U937 cells by macrophages is blunted if the cells are depleted of iPLA₂-VIA activity by treatment with an inhibitor or an antisense oligonucleotide, and it is augmented by overexpression of iPLA₂-VIA in the dying cells. Thus the magnitude of macrophage phagocytosis correlates with the level of iPLA₂-VIA activity exhibited by the dying cells. Eliminating by antisense oligonucleotide technology the other phospholipase A₂ that U937 cells express, i. e. the cytosolic group IVA enzyme, does not attenuate phagocytosis of U937 dying cells by macrophages. Incubation of U937 cells with different fatty acids has no effect on either the extent of hydrogen peroxide-induced apoptosis or the degree of phagocytosis of the dying cells by macrophages. However, preincubation of the macrophages with lysophosphatidylcholine before exposing them to the dying cells blocks phagocytosis of the latter. Collectively, these results indicate that formation of lysophosphatidylcholine by iPLA₂-VIA in apoptotic U937 cells contributes to their efficient clearance by macrophages.

Comparing the effects of retinoids (4HPR and ATRA) on human B lymphoma cells

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Retinoids are potent anticancer drugs. There are only few reports about the effects of all-trans retinoic acid (ATRA) and its synthetic analogue, fenretinide (4HPR) on B lymphoma cells. We studied the apoptotic effect of ATRA and 4HPR in non-Hodgkin B-lymphoma cells (HT58, BL41, BL41/95). Both agents induced cell death time and dose dependently in these cell lines. The depolarization of mitochondrial membrane, as an important step of apoptosis, occurred earlier after ATRA than 4HPR treatment in HT58 and BL41/95 cells. In BL41 cells ATRA induced more intensive depolarization of mitochondrial membrane than 4HPR. Both drugs decreased the expression of Bcl-2. Reactive oxygen species (ROS) production was elevated in 4HPR treated cells but not in ATRA treated cells. Both Z-VAD-fmk and Z-IETD-fmk, caspase inhibitors, decreased the DNA-fragmentation in ATRA treated cells but not in 4HPR treated cells. At the same time Z-VAD-fmk increased necrosis in ATRA-treated HT58 cells. Endonuclease G was released from the mitochondria during 4HPR treatment, which could be an inducer for caspase-independent DNA-fragmentation. AIF remain in the mitochondria during retinoid treatment. Our results suggest that natural (ATRA) and synthetic (4HPR) retinoids induce different apoptotic pathways. ATRA induces a caspase-dependent while 4HPR a caspase-independent apoptosis in B lymphoma cells. This can be an important information for the potential use of retinoids in leukemia treatment.

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Alphavirus and apoptosis: the triggers and mechanisms behind mammalian cellular apoptosis in response to Semliki Forest virus infection

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In a multicellular organism, virally infected cells have a predilection towards undergoing apoptosis. In response to virus infection, this reaction may be viewed as an arm of the innate immune system. This can be seen as a utilitarian act if the cell succeeds in committing suicide before viral particles can be released. Its effectiveness is underlined by the existence of anti-apoptotic strategies that are employed by many viruses to combat this defence mechanism. The means by which virus infection triggers the cell apoptotic response is likely to vary between viruses, between cell types and even between cellular differentiation states. Semliki Forest virus (SFV) is in the *Alphavirus* genus of the *Togaviridae* family and is closely related to Sindbis virus. SFV has been demonstrated to induce apoptosis both in vitro and in vivo. It is likely that host cells have multiple virus detection mechanisms. PKR and RNaseL are two such molecular detection proteins present in the cytosol of host cells that may play a role in SFV detection and induction of apoptosis. Using PKR and RNaseL deficient mice and cells the roles of these two proteins in detecting SFV infection and triggering cell death are under investigation. While RNaseL deficient mouse embryo fibroblasts (MEFs) and mice show an unaltered phenotype, PKR deficient MEFs, while triggering the same caspase cascade as wild type MEFs, appear to take longer to undergo cell death after viral infection. This suggests a delay in viral detection, and an important role for PKR in the fight against SFV infection.

Cephalostatin-1 induces an ER stress-specific and apoptosome-independent apoptotic signaling pathway via ASK-1/JNK and caspase-4

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The bis-steroidal marine product Cephalostatin-1 (CPH-1) is a remarkable natural product which has been reported to induce a novel pathway of receptor-independent apoptosis that selectively utilizes Smac/DIABLO, activate caspase-9 and induces apoptosis independent of APAF-1/cytochrome C/caspase-9 apoptosome. Because CPH-1 induces an apoptosome- and receptor-independent cell death this experimental chemotherapeutic drug could be a good tool for the investigation of the ER-stress induced apoptosis.

ER stress leads to the activation of genes possessing an unfolded protein response (UPR) element, which controls the levels of molecular chaperones, such as BiP/GRP78 involved in protein folding in the ER. In fact CPH-1 was found to increase the expression of the chaperone BiP/GRP78 as well as the transcription factor CHOP/GADD153. CHOP is one of the highest inducible genes during ER-stress and known to play a role in ER-stress induced apoptosis. The expression of CHOP was activated by CPH-1 by alteration of calcium homeostasis as shown by employment of BAPTA a calcium chelator.

Another pathway in ER stress is activation of the cJUN Kinase. Recruitment of TNF receptor-associated factor 2 (TRAF2) to activated stress sensor proteins called IREs, induces the apoptosis signal-regulating kinase 1 (ASK1) / c-Jun N-terminal kinase (JNK) cascade. CPH-1 induces a strong activation of JNK as well as ASK-1. Importantly, apoptosis was abrogated in cells overexpressing a dominant negative version of ASK-1 indicating a crucial role of the ASK-1/JNK pathway in CPH-1 induced ER-stress response.

Caspase-12 and the human homolog caspase-4 are localized in the cytoplasm of the ER and function as mediators of ER-stress-induced cell death. CPH-1 leads to cleavage of the proforms of caspase-12 as well as caspase-4 and moreover inhibition of caspase-4 reduces the apoptotic efficiency of CPH-1. Pretreatment of cells with zLEVD-fmk a selective caspase-4 inhibitor clearly abrogated the apoptotic potency of CPH-1.

Taken together by use of CPH-1 which induces apoptosis independent of mitochondria induced apoptosome formation we were able to define some novel targets in ER-specific apoptotic signaling. Moreover due to this unusual signaling the requirement of this protease in CPH-1 induced cell death may be a beneficial strategy in the combat of chemoresistance.

RelA repression of RelB activity induces selective anti-apoptotic gene activation downstream of TNFR

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Tumor necrosis factor α (TNF α) is a potent pro-inflammatory cytokine that regulates immune and inflammatory responses and programmed cell death. TNF α stimulation causes nuclear translocation of several NF- κ B dimers, including RelA/p50 and RelB/p50. However, contrary to RelA, RelB entering the nucleus in response to TNF α cannot bind to DNA in mouse embryonic fibroblasts, strongly suggesting that RelB DNA binding activity is modulated by additional nuclear mechanisms. Here we demonstrate that TNF α promotes the association of RelA with RelB in the nucleus and that TNF α -induced RelA/RelB heterodimers do not bind to κ B sites. Remarkably, we show that RelA Serine 276, the phosphorylation of which is induced by TNFR ligation, is crucial for RelA/RelB complex formation and subsequent inhibition of RelB DNA binding. In absence of RelA phosphorylation on Serine 276, TNF α stimulation leads to a strong increase in the expression of endogenous NF- κ B responsive genes whose transcriptional up-regulation is mainly controlled by RelB. Since RelA has been previously shown to antagonize TNF α -induced apoptosis, we also investigated the role of RelB activity in controlling programmed cell death. We demonstrate that in absence of RelA repressive function, RelB provides an anti-apoptotic signal in response to TNF α , partly through Bcl-xL transcriptional up-regulation. Our findings demonstrate that RelA has a major regulatory role serving to dampen RelB activity in response to TNF α , and they explain, at least partly, why a significant number of RelA-deficient cells remain viable following TNF α treatment.

Keywords : NF-kappaB, RelA/RelB complexes, phosphorylation, TNFalpha, Bcl-xL, apoptosis

Inactivating/activating Bak complexes in mitochondria

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The two proapoptotic members of the Bcl-2 family, Bax and Bak, are the effectors of the mitochondrial pathway of Apoptosis. Both are activated during programmed cell death and induce permeabilization of mitochondrial outer membrane and release of apoptogenic factors (cytochrome c, Smac/Diablo) from intermembrane space to cytosol. Unlike Bax, which is translocated from cytosol to mitochondria during apoptotic mechanism, Bak is mostly localized on mitochondria in both healthy and apoptotic conditions. In the present study we investigated how Bak is associated with outer membrane. Using cell free assays and Bax-deficient cellular models we have determined in non apoptotic conditions that Bak interacts with Metaxin 2 (yeast Sam35 homolog), a peripheral protein of outer membrane involved in protein translocation. In addition we observed during apoptosis that activated-Bak does no longer interact with Metaxin 2 but requires another partner: Metaxin 1 (yeast Mas37 homolog). Metaxin 1 is another outer membrane protein with a transmembrane domain which interacts with Metaxin 2. Moreover studies using antisens strategy against Metaxin 1 demonstrated that the interaction between Bak and Metaxin 1 is essential for the Bak-induced permeabilization of outer membrane during apoptosis. VDAC 2 was identified as a partner of Bak in non-apoptotic cells (Cheng E. *et al*, 2002); here we found two other proteins which interact with Bak: Metaxin 2 like VDAC 2 interacts with Bak in non-apoptotic conditions and Metaxin 1 which is essential for apoptotic role of Bak. These results suggest that there is an active evolution in complexes containing Bak following apoptotic stimuli and these complexes play an essential role in outer membrane permeabilization capabilities of Bak.

A20 is a negative regulator of TRAIL-induced apoptosis in prostate cancer cells

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Tumor necrosis factor (TNF) apoptosis-inducing ligand (TRAIL) is a member of the TNF family of cytokines, which can induce apoptosis in a variety of tumor cells by engaging the death receptors DR4 and DR5, while sparing most normal cells. Preclinical studies in mice and non-human primates have shown the potential utility of recombinant soluble TRAIL and agonistic anti-DR5 or DR4 antibodies for cancer therapy. Moreover, it has been revealed recently that endogenously expressed TRAIL plays a vital role in immunosurveillance of developing and metastatic tumors.

Because of the tumor selectivity of TRAIL-induced apoptosis and its ubiquitous expression, it has been postulated that the apoptotic pathway induced by TRAIL is tightly regulated by several mechanisms to prevent spontaneous cell death. In this study we show that zinc finger protein A20 negatively regulates TRAIL-induced apoptosis in PC3 prostate cancer cells by inhibiting proteolytic cleavage of caspases 8, 3 and 9 and Bid cleavage. We also demonstrated that A20 interacts with DR4 and caspase-8 in Gst pull-down assay and in mammalian cells suggesting that A20 could act as a silencer of DR4. Thus, A20 appears to be a molecular switch that depending on other intracellular signals and its post-translational processing can regulate life versus death decisions of a cell in response to TRAIL.

Nitric oxide synthase upregulation in the early phase of cerebellar granule cells apoptosis

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Cerebellar granule cells (CGCs) undergo apoptosis when potassium in the culture medium is shifted from 25 to 5mM. In the first three hours of apoptosis an increase in NO production was detected, and the addition of the NOS inhibitor, L-NAME, completely quenched the NO signal as visualized by DAF-FM fluorescence. Consistently L-NAME was found to partially reduce neuronal cell death induced by K⁺ shift. In order to find out the mechanism underlying the increase in NO levels, we determined the protein levels of the three NOS isoforms, nNOS, iNOS and eNOS, by Western blot analysis. Interestingly in the first 3h after apoptosis induction, the protein level of nNOS increased by about 290% over control cells, whereas no significant expression of both iNOS and eNOS occurs neither in control nor in apoptotic cultures. It is therefore likely that the increase in the levels of cellular NO is a direct consequence of nNOS induction in apoptotic CGCs. Consistently a slight but significant increase in the mRNA of the nNOS isoform was detected. Moreover, NOS activity was found to increase up to 3 hours of apoptosis followed by a progressive decline. The increase in NOS activity well correlates with the increase in NO production, while the decrease after 8 and 15 hours of apoptosis could be due to the activation of caspases being inhibited by z-VAD. As a consequence of both NO and superoxide anion production in the early phase of CGC-apoptosis, an increase in the levels of nitrosylated proteins was detected by western blot. Taken together these results suggest that NOS upregulation takes place in the early phase of neuronal apoptosis thus allowing a sustained radical species production which is needed for the correct application of the programmed cell death.

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The Bcl-2 family members: mediators of cathepsin's-induced apoptosis

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Lysosomal proteases have been shown to play a role in several apoptotic pathways, such as oxidative stress associated with aging and the TNF- α pathway (Leist, M. and Jäätelä, M. (2001) *Nat. Rev. Mol. Cell Biol.* 2, 589-598). In some of these models lysosomal permeabilization appears to be an early event, preceding other hallmarks of apoptosis e.g. mitochondria destabilization and caspase activation. Recently, we showed that lysosomal cathepsins B, L, S, K and H cleaved and activated the proapoptotic BH3-only Bcl-2 homologue Bid (Cirman et al. (2004) *J. Biol. Chem.* 279, 3578-3587) both in vitro and in HeLa cellular model following lysosomal disruption by the lysosomotropic agent LeuLeuOMe. Using different cellular models (SH-SY5Y, HepG2, HaCaT) we can show that lysosomal disruption, followed by translocation of cysteine cathepsin translocation to the cytosol and subsequent cleavage of Bid and mitochondria destabilization (characterized by swelling and fragmentation of cristae as shown by electron microscopy), is a general mechanism not restricted to a single cell line. However, in some cellular models (MCF-7, HEK 293) Bid was not cleaved despite appearance of other apoptotic characteristics (apoptotic morphology, phosphatidyl serine exposure, increase in caspase-3-like activity, cytochrome c release into the cytosol, PARP cleavage). Therefore we looked for other possible cathepsin substrates as apoptosis mediators in this pathway. The initial studies were focused at the antiapoptotic Bcl-2 family members Bcl-2 and Bcl-XL. Both were found to be cleaved in vitro by several cathepsins with cathepsin L being the most potent enzyme. Moreover, Bcl-2 and/or Bcl-XL were also found to be degraded in several of these cell lines with cysteine cathepsins probably mediating the degradation as it could be blocked by E-64d but not by z-VAD-fmk. It can be therefore suggested that during apoptosis triggered by the lysosomal destabilization, cysteine cathepsins are the major players with the assistance of different Bcl-2 family members, depending on the cellular model. However, it is likely that more cathepsin targets exist.

Targeted Vpr-derived peptides reach mitochondria to induce apoptosis of $\alpha_v\beta_3$ -expressing endothelial cells

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Viral protein R (Vpr), an apoptogenic accessory protein encoded by HIV-1, induces mitochondrial membrane permeabilization (MMP) via a specific interaction with the permeability transition pore complex. We have designed and synthesized TEAM-VP, a peptide family composed of two functional domains, one a tumor blood vessel RGD-like 'homing' motif and the other a MMP-inducing sequence covering the minimal mitochondriotoxic domain of Vpr (Vpr67-82). When added to purified mitochondria, TEAM-VP permeabilizes mitochondrial membranes through a direct effect on the permeability transition pore (PTP). Due to its RGD sequence, TEAM-VP specifically binds to endothelial cells (HUVECs, HMVECd) and to $\alpha_v\beta_3$ -expressing tumor cells (A375M). Intracellular routing studies reveal that TEAM-VP first co-distributes with lysosomes and then exits from lysosomes to reach mitochondrial compartment. TEAM-VP triggers apoptosis of HUVECs, HMVECd and A375M with dissipation of the mitochondrial transmembrane potential ($\Delta\Psi_m$), nuclear condensation, phosphatidyl-serines exposure, caspases activation and final plasma membrane permeabilization. In contrast, TEAM-VP does not induce apoptosis of $\alpha_v\beta_3$ negative cells and peripheral blood lymphocytes. Moreover, TEAM-VP overcomes cytoprotective effect of the anti-apoptotic Bcl-2 protein. Finally, TEAM-VP cooperates *in vitro* with the topoisomerase inhibitor VP16 (a clinically used chemotherapeutic agent) to induce HUVECs death. Hence, TEAM-VP is a promising candidate for a new class of apoptogenic molecules able to selectively target mitochondria of angiogenic endothelial cells.

Human CD8⁺ are more sensitive than CD4⁺ T cell blasts to regulation by APO2L/TRAIL and by IL-2 deprivation

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The mechanisms responsible for the down-modulation of the activation of separated CD4⁺ or CD8⁺ human T-cell blasts were studied using cells obtained from healthy donors. The higher sensitivity of normal human CD4⁺ or CD8⁺ T-cell blasts to apoptosis and activation-induced cell death as compared with naïve T-cells correlated with the increased expression of Bcl-xS and Bim. In the presence of IL-2, human CD8⁺ were more sensitive than CD4⁺ T-cell blasts to regulation by APO2L/TRAIL, while both T-cell subsets were equally sensitive to Fas/CD95 regulation. This regulation was defined as inhibition of IL-2-dependent T-cell growth in the absence of cell death induction, characterized by cell cycle arrest in G2/M. CD4⁺ and CD8⁺ T-cell blasts expressed intracellular FasL and APO2L/TRAIL, which were secreted in their bioactive form to the supernatant upon PHA, CD3 or CD59 re-activation. Additionally, the inhibition of IL-2-dependent CD4⁺ or CD8⁺ T-cell blast growth upon CD3 or CD59 ligation was dependent, at least partially, on this FasL and/or APO2L/TRAIL secretion. Finally, we also showed that normal human CD8⁺ were more sensitive than CD4⁺ T-cell blasts to apoptosis induction by IL-2 deprivation, which predominated over death receptor ligation. Apoptosis by IL-2 deprivation was associated with a decrease in the expression of anti-apoptotic proteins of the Bcl-2 family, especially Mcl-1 in CD8⁺ T-cell blasts. These data define the role of APO2L/TRAIL in the regulation of human T-cell activation and point to an important role of the presence or absence of IL-2 in the type of regulation exerted.

Apoptosome independent pathways of caspase activation

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In order to identify molecules able to trigger cell death in the absence of caspase-9, we challenged cells expressing caspase-9 dominant negative with different apoptotic insults. These molecules could be interesting to identify and study alternative pathways of caspase activation and to design new antitumor drugs. Out from this screening we have discovered that PIs have the unique ability to activate effector caspases and to induce cell death through a mitochondrial Bcl-2 dependent but caspase-9 independent pathway. Stabilization of released Smac induced by blockade of the proteasome could explain the apoptosome independent cell death induced by PIs. In fact Smac/DIABLO critically supports this PI-dependent caspase activation. By using a new assay we confirm that at a single cell level both Smac and PIs can activate caspases in the absence of the apoptosome. Moreover we have observed two PI-induced kinetics of caspase activation, with caspase-9 being still required for the rapid caspase activation in response to mitochondrial depolarization, but dispensable for the slow DEVDase activation. Several studies have indicated that proteasome inhibitors (PIs) are promising anticancer agents. Our data indicate that PIs, through stabilization of released cytosolic Smac, have the peculiar ability to target the distal apoptotic checkpoint represented by the IAPs.

To identify other proapoptotic molecules able to trigger cell death in the absence of a functional apoptosome, cells mutated for caspase-9 were treated with the challenged set obtained from the Developmental Therapeutics Program of the National Cancer Institute (USA). This set contains 57 diverse compounds that are able to trigger cell death with unknown mechanisms. After this second screening we have identified two new molecules, which are able to trigger caspase activation and efficient cell death also in the presence of an inactive caspase-9. The characterization of these new compounds will be discussed.

Direct cleavage of ROCK II by granzyme B induces target cell membrane blebbing in a caspase-independent manner

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Caspase activation in target cells is a major function of granzyme B (grB) during cytotoxic lymphocyte granule-induced apoptosis. grB-mediated cell death can occur in the absence of active caspases, and the molecular targets responsible for this additional pathway remain poorly defined. Apoptotic plasma membrane blebbing is caspase independent during granule exocytosis-mediated cell death, whereas in other instances, this event is a consequence of the cleavage by caspases of the Rho effector, Rho-associated coiled coil-containing protein kinase (ROCK) I. We show here that grB directly cleaves ROCK II, a ROCK family member encoded by a separate gene and closely related to ROCK I, and this causes constitutive kinase activity and bleb formation. For the first time, two proteins of the same family are found to be specifically cleaved by either a caspase or grB, thus defining two independent pathways with similar phenotypic consequences in the cells. During granule-induced cell death, ROCK II cleavage by grB would overcome, for this apoptotic feature, the consequences of deficient caspase activation that may occur in virus-infected or malignant target cells.

Are connexins involved in the spread of apoptosis and necrosis?

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We examined the role of connexin (Cx) expression on the development of apoptosis and necrosis in HeLa cells expressing wild type Cxs (mCx30.2, Cx32 and Cx43) and their fusion constructs with GFP. Cx-GFP proteins (GFP attached to C-terminus) assembled into junctional plaques (JPs) and formed functional gap junction (GJ) channels and hemichannels. GFP-Cx proteins (GFP attached to N-terminus) formed JPs, but not functional channels. Apoptosis was induced chemically (anisomycin, etc.) or by exposing individual cells to focused UV light. Fluorescence microscopy and voltage clamp techniques were used to correlate the changes in coupling conductance, dye uptake through hemichannels and Cx expression with the progression of apoptosis and necrosis. The apoptotic reagents had no direct effect on single channel conductance, but decreased intercellular communication due to internalization of JPs. Apoptosis transformation to necrosis was faster in cells expressing wtCx43, Cx32-GFP and Cx43-GFP than in HeLa-parental cells or cells expressing GFP-Cx32 and GFP-Cx43. Dye uptake studies show that membrane permeability mediated by Cx43 hemichannels is reduced during apoptosis development. Cells exposed to UV light of relatively low or high intensities demonstrated primary apoptotic or necrotic transformations, respectively. The lag time for inducing apoptosis or necrosis was shortest in isolated cells and longest in cells that were in contact with other cells through functional GJs. The spread of necrotic transformations induced in a single cell to neighboring cells depends on GJ-mediated coupling and connexin type. Neighboring-contacting cells without JPs or cells connected through mCx30.2 junctions remained unaffected. In summary, apoptosis induced in a single cell is delayed if contacted by neighboring healthy cells and usually remains localized to that cell. However, necrosis induced in a single cell spreads very fast to neighboring cells through Cx32 and Cx43, but not mCx30.2 GJs, suggesting that the spread of necrosis can be limited by GJ channel size and permeability.

p38 α MAPK: tumor's gateway to survival and angiogenesis after photodynamic therapy

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The photochemical generation of reactive oxygen species (ROS) resulting in target cell death is at the basis of the anticancer action of photodynamic therapy (PDT), a worldwide accepted anticancer modality¹ which requires a tumor localizing- and light-absorbing drug, molecular oxygen and visible light. Recently, combination of PDT with a cyclooxygenase-2 (COX-2) specific inhibitor has been shown to increase the tumoricidal potential of PDT². Our previous studies have pointed out that activation of the p38 MAPK pathway in response to hypericin-based PDT of cancer cells stimulates an adaptive response counteracting cell death³. Consistently, we found that pharmacological inhibition or deficiency of the p38 α MAPK result in a significant increase in cancer cell susceptibility to photokilling. A key downstream target of the stress kinase p38 α cascade is COX-2, whose expression is induced in cancer cells in response to PDT². Inhibition of p38 MAPK or its genetic ablation, completely abolishes COX-2 expression and synthesis of its metabolite, PGE₂, and reduces tumor cells-mediated release of VEGF as well. In addition, we show that critical modifications in tumor cell features induced by the overexpression of COX-2 which have been reported to intensify the tumorigenic behavior, including tumor-promoted endothelial cell migration and neo-angiogenesis, are abolished in our paradigm by the selective inhibition of p38 MAPK. Thus antagonists of p38 MAPK appear to provide an additional therapeutic target upstream of COX-2, by encompassing all COX-2-dependent phenotypes. In addition to COX-2, gene expression profiles of PDT-treated cancer cells reveal that the expression of an important set of genes involved in the support of cell survival, neo-angiogenesis and inflammation depends upon the activation of p38 MAPK, further supporting the view that pharmacological targeting of this signal in the cancer cells should be explored to increase PDT tumoricidal efficacy.

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Novel pharmacological effects for the promising chemosensitizer PK11195

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The 1-(2-Chlorophenyl-N-methylpropyl)-3-isoquinolinecarboxamide (PK11195), has been recently proposed as a reliable chemosensitizer of tumour cells to a wide range of chemotherapeutic agents. PK11195, known as prototypic ligand of the Peripheral Benzodiazepine Receptor (PBR), can sensitize cells to apoptosis induction by apoptotic second messenger ceramide, anti-CD95 and the Bcl-2 inhibitor HA14.1. Although various cellular effects of PK11195 have been reported, such as the inhibition of the multiple drug resistance (MDR) pumps, activation of the p38mitogenactivated protein kinase (MAPK) pathway and activation of c-Jun NH2 terminal kinase (JNK), its precise mechanism of action still remains unclear. Experimental evidence indicates that PK11195 can reduce or abrogate the mitochondrial membrane permeabilization (MMP) inhibitory effect of Bcl-2 and related proteins. In addition, carcinoma cell lines treated with PK11195 suppress Bcl-2 and Bcl-X_L expression while the propapoptotic protein Bax is overexpressed. Here we provide evidence for novel biological effects of PK11195 unrelated to the pharmacological modulation of PBR.

In detail, employing organelle-targeted chimera of the Ca²⁺ sensitive photo protein aequorin in human tumor cell lines (HeLa) and micromolar concentrations of PK11195, we detected an increase of the ER Ca²⁺ content and consequently of the amplitude of the histamine induced mitochondrial and cytosolic Ca²⁺ transients, as well as of the cytosolic capacitative Ca²⁺ influx. In parallel, employment of organelle-targeted chimera of luciferase in the same cellular model, showed an inhibition of the mitochondrial Adenine Nucleotide Translocase (ANT) due to PK11195. Conversely, the other prototypic PBR ligand, 7-chloro-5-(4-chlorophenyl)-1,3-dihydro-1-methyl-2H-1,4-benzodiazepin-2-one (Ro5-4864) had no effect either on Ca²⁺ signalling or on mitochondrial ATP/ADP, thus, suggesting that these signalling effects of PK11195 are independent of PBR, as recently proposed also for apoptotic induction. Interestingly, the enhancement of Ca²⁺ signaling and inhibition of the mitochondrial ATP/ADP exchanger by PK11195 appear to counteract two activities of Bcl-2, enhancement of ER Ca²⁺ leakage and the ANT-dependent ADP/ATP exchange. Their interaction, and the potential usefulness of PK11195 as Bcl-2 antagonist, will be investigated.

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The role of ascorbic acid, beta-caroten, α -tocopherol, and selenium on D-galactosamine-induced liver injury of rats

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In our study, the role of the antioxidant combination on D-galactosamine-induced hepatic injury was examined in rats. Female Sprague-Dawley rats used in this study were divided into four groups. Group I: Rats injected physiologic saline water intraperitoneally. Group II: Animals given ascorbic acid (100 mg/kg/day), beta-caroten (15 mg/kg/day), α -tocopherol (100 mg/kg/day) and selenium (0.2 mg/kg/day) for 3 days via gavage method. Group III: Animals administered D-galactosamine hydrochloride (500 mg/kg) intraperitoneally a single dose. Group IV: Rats given the same antioxidant combination and injected D-galactosamine hydrochloride in same dose and time.

Histological examinations including the *in situ* terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method showed a significant increase in apoptotic hepatocytes 6 h after the administration of D-galactosamine in group III. Not only hepatic caspase-3 activity, which is responsible for apoptosis, but also hepatocyte proliferation increased significantly in this group. On the other hand, group IV which had received D-galactosamine+antioxidant combination revealed a decrease in the number of apoptotic cells, a decrease in cell proliferation and caspase-3 activity.

As a result, the antioxidants have a preventive effect on apoptosis and cell proliferation in D-galactosamine-induced liver injury of rats.

Daxx directly modulates function of chromatin-remodeling factor SMARCA4/BRG1

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Death domain-associated protein (Daxx) regulates both transcription and apoptosis. Daxx is mainly nuclear protein but its functional translocation to the cytoplasm has been also described. In the nucleus, Daxx interacts with histone deacetylases, and most likely through them represses transcription of a number of genes. Daxx repressive function is relieved upon its PML-mediated sequestration into the PML oncogenic dots (PODs). In addition (independently from PML?) Daxx was shown to enhance transactivation potential of several transcription factors. Last, but not least, Daxx is also involved in the regulation of apoptosis induced by membrane receptors (Fas, TGF β -RII), stress (UV, ROS) or during the development.

Here, we show that Daxx directly interacts with the chromatin-remodeling factor SMARCA4/BRG1. BRG1, in ATP-dependent manner, regulates assembly of the chromatin and allows an access of other regulators to the nascent DNA. BRG1 interacts with the N-terminal part of Daxx both in yeast (yeast 2-H system) and in GST-pulldown assays. Concurrent expression of Daxx with PML induces sequestration of BRG1 into PODs and PML expression also enhances BRG1 – Daxx co-immunoprecipitation. Co-expressed Daxx-enhanced BRG1-induced expression of the cell cycle regulator p21. BRG1 cooperation with Rb tumor suppressor ultimately leads to G1-arrest. We found that in SW-13 cells (BRG1-deficient adrenocortical carcinoma) Daxx affects BRG- and Rb-induced cell cycle restrictions. Currently we also analyze effect of BRG1 on apoptosis-regulating function of Daxx. Our results could re-define role of Daxx in the regulation of transcription, apoptosis and potentially also of the senescence and cell-cycle.

Upstream control of apoptosis by caspase-2 in serum-deprived primary neurons

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During development as well as in pathological situations, neurons that fail to find appropriate targets or neurotrophic factors undergo cell death. Using primary cortical neurons subjected to acute serum-deprivation, we have examined caspases activation, mitochondrial dysfunction and cell death parameters. Among a panel of metabolic, signaling and caspases inhibitors, only those able to interfere with caspase-2 like activity protect significantly primary neurons against SD-induced cell death. *In situ* detection and subcellular fractionation demonstrate a very early activation of cytosolic caspase-2, which controls Bax cleavage, relocalization and mitochondrial membrane permeabilization. Both z-VDVAD-fmk and a siRNA specific for caspase-2 abolish Bax changes, mitochondrial membranes permeabilization, as well as cytochrome c release-dependent activation of caspase-9/caspase-3, nuclear alterations, phosphatidylserine exposure, plasma membrane disruption, neurites dismantling and neuronal death. Hence, caspase-2 is an early checkpoint for apoptosis initiation in primary cortical neurons subjected to serum deprivation (Apoptosis, 2005,10, *in press*). We are currently identifying in vivo normal or pathological settings in which this process might be operative.

Augmentation of TNF-induced apoptosis in HeLa cells expressing Bcl-2

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TNF-induced apoptosis in HeLa cells depends on cytochrome c release from mitochondria and is strongly inhibited by expression of Bcl-2 and by oligomycin (Shchepina et al., 2002). Inhibitors of microtubules (colcemid, taxol), of actin cytoskeleton (cytochalasin D, latrunculin A) and of actomyosin related kinases (HA 1077, H7) do not cause apoptosis in HeLa-Bcl-2 but stimulate TNF-induced apoptosis in these cells. The cytochrome c release was observed during apoptosis, so the bypass of Bcl-2 block via direct activation of caspase 3 by caspase 8 seems not probable explanation of the effect. Bcl-2 was not completely inactivated by the inhibitors of cytoskeleton since staurosporin-induced apoptosis and apoptosis caused by energy deprivation were still inhibited in HeLa-Bcl-2. The inhibitors of cytoskeleton also released inhibition of TNF-induced apoptosis and cytochrome c release by oligomycin, indicating that direct modification of Bcl-2 was hardly responsible for abolition of its protective action. Recombinant trimeric TRAIL (TNF-Related Apoptosis Inducing Ligand) also induced Bcl-2 sensitive apoptosis in HeLa cells. The cell death and cytochrome c release was not inhibited by oligomycin indicating the difference between TNF and TRAIL signaling at the mitochondrial level. The inhibitors of cytoskeleton strongly augmented TRAIL-induced apoptosis in HeLa-Bcl-2. It is suggested that structure and activity of cytoskeleton modulate mitochondria-related steps of apoptosis caused by TNF and TRAIL.

Contribution of multiple apoptotic pathways to UVC-mediated apoptosis in fos-deficient MEFs

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Mouse embryonic fibroblasts (MEFs) deficient in the transcription factor p53 or c-Fos are hypersensitive to UV-C light. We have previously shown that UV-C light activates both the Fas (CD95, Apo-1) receptor and the mitochondrial apoptosis pathways in MEFs. In cells deficient in p53, the high level of non-repaired DNA damage forces signalling *via fasL* up-regulation and thereby enhances UV-C-induced apoptosis. In wt and c-Fos deficient cells the mechanism of apoptosis remained unclear. Here we utilized isogenic wild-type (wt) and c-Fos deficient (*fos*^{-/-}) MEFs which are both proficient for p53, in order to elucidate the pathways of UV-C induced apoptosis in p53 wt cells and the role of non-repaired DNA lesions. In wt cells apoptosis was executed via the mitochondrial pathway, as shown by the induction of the p53-regulated gene *noxa* and by activation of caspase-9. The expression of *puma*, Bax and Bcl-2 remained unaltered. In wt cells, UV-C induced a transient induction of *fasR* and *fasL*, which presumably did not contribute to apoptosis since activation of caspase-8 did not occur. It was rather blocked by c-Flip, which was down-regulated in response to UV-C in *fos*^{-/-} cells, but not in the wt. In c-Fos deficient cells, which are unable to repair UV-C damage, apoptosis is triggered via both the mitochondrial and the receptor-mediated pathway. As in wt cells, induction of *noxa* and activation of caspases-9 was observed. Additionally, c-fos deficient cells respond to UV-C with sustained up-regulation of *fasL* mRNA. Induction of FasL and FasR leads to activation of caspase-8, stabilization of Bax, down-regulation of Bcl-2 and finally to increased activation of caspase-3.

Modulation of the cell cycle in neuroblastoma by an HLH mutant domain

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Id2 (Inhibitor of DNA binding and Inhibitor of differentiation) is an helix-loop-helix (HLH) protein that heterodimerizes with basic HLH (bHLH) transcription factors preventing them from binding to DNA, so acting as a dominant negative regulator of cell transcription. Id2 has been found to reverse cellular growth inhibition by the retinoblastoma protein (pRb) through direct interaction with pRb, p107, and p130 via its HLH domain. It has been associated with the progression of human cancers, such as neuroblastomas and astrocytomas, in which Id2 is overexpressed according to tumour aggressiveness. In this study we have expressed in neuroblastoma cells an HLH dimerization domain (13I-C), isolated by phage display, containing aminoacid substitutions at residues involved in dimerization. The 13I-C mutant domain interacts with Id2 in BOSC 23 cells *in vitro* with high affinity. This result prompted us to investigate whether the expression of 13I-C mutant domain affects cell proliferation and induces differentiation in neuroblastoma cells. Stably retroviral-infected neuroblastoma cell lines expressing 13I-C mutant showed a neuronal morphology with axonal elongation and a reduced proliferation rate compared to control cells (infected with an empty vector). Immunocytochemistry demonstrated neurofilament 160 (NF160) and N-CAM expression (both markers of neuronal differentiation), which were absent in control cells. Retinoic acid treatment further increased the immunostaining. When treated with Trichostatin A, an inducer of apoptosis, these cells exhibited an higher mortality rate compared to control cells.

Taken together these results suggest that 13I-C may be useful to modulate cell cycle in neuroblastoma cells, also through their sensitization to apoptosis inducing molecules.

Role of lysosomal proteases in neutrophil apoptosis

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Like classical apoptosis, caspase-independent death programs can be triggered by death receptors or by DNA damage, both of which are controlled at the mitochondrial outer membrane and executed by proteolytic enzymes. For instance, lysosomes can also sense cellular stress and are able to define the fate of the cell through the activation of cathepsins. Therefore, we hypothesized that both caspases and cathepsins may interact in death pathways. As a cellular model, we used human blood neutrophils, since these cells are known to use cathepsins within bacterial defense mechanisms and to express caspases. Here, we demonstrate that pharmacological inhibition of cathepsins delay spontaneous apoptosis of mature neutrophils. Moreover, neutrophil death was associated with a translocation of cathepsin B and D from the azurophilic granules into the cytosol, where it targets mitochondria and triggers cytochrome c and Smac/DIABLO release. This translocation appeared to be an early event following apoptosis induction, since cathepsin inhibitors delayed caspase-8 activation and Bid/Bax cleavage. These findings were confirmed by a cell-free assay in which cathepsin B and D recombinant enzymes were able to cleave directly cytosolic caspase-8 and Bid. Taken together, these data suggest that cathepsin B and D play a crucial role in the initiation of neutrophil apoptosis.

Fenretinide induces ER-stress in Neuroectodermal tumors

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The synthetic retinoid fenretinide [N-(4 hydroxyphenyl)retinamide] induces cell death of cancer cells and acts synergistically with chemotherapeutic drugs, thus providing opportunities for novel approaches to cancer therapy. The upstream signalling events induced by fenretinide include an increase in intracellular levels of ceramide, which is subsequently metabolised to GD3. This ganglioside triggers the activation of 12-Lox (12-lipoxygenase) leading to ROS generation and apoptosis in neuroectodermal tumor cells. We have shown that fenretinide-induced death of neuroectodermal tumor cells depends from increased levels of the transcription factor Gadd153 and the Bcl-2 family member protein Bak, but the link between ROS induction and subsequent apoptosis remains unclear. Considering that Gadd153 is also up-regulated in ER-stress, we have hypothesized that the synthetic retinoid might induce cell death via ER-stress. Indeed, we show here that fenretinide-induced ROS triggers ER-stress which involved the Xbp-1 splicing and eIF2 α phosphorylation. Microarray analysis confirmed the involvement of ER-stress in fenretinide-induced apoptosis as demonstrated by up-regulation of Grp78, Calnexin and Calreticulin.

Moreover, results from microarray analysis revealed the involvement of other two ER-related genes, ERdj5 and ERp57, as genes up-regulated during fenretinide-induced ER-stress in neuroectodermal tumour cells. siRNA of either ERdj5 and ERp57 resulted in enhanced sensitivity of neuroectodermal cells to apoptosis in response to fenretinide, thus suggesting an inhibitory role for these genes in ER-stress.

Our results demonstrate that ER-stress plays a pivotal role in fenretinide-induced cell death of neuroectodermal tumour cells and indicate ERdj5 and ERp57 as new candidate targets for future chemotherapeutic drug development.

Role of Apaf1 and the Apoptosome-mediated apoptosis in PDAC (Pancreatic Ductal AdenoCarcinoma)

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Apaf1 is the molecular core of the apoptosome, a multiproteic complex mediating the mitochondrial pathway of apoptosis. The importance of this death pathway during development has been clearly demonstrated by knocking out key genes. However, a growing body of evidences indicate a possible role for the mitochondria-dependent apoptosis in different pathologies, among which we investigated Pancreatic Ductal AdenoCarcinoma (PDAC).

As well as in development, Apaf1 dosage is critical in various cancer types such as melanomas, germ line tumour, and gastrointestinal cancer. This is generally due to the inactivation of APAF1 locus by different mechanisms: methylation of promoter CpG islands or LOH, due to alterations of APAF1 regulators (e.g. p53 and E2Fs). We are studying the possible involvement of APAF1 in pancreatic ductal adenocarcinoma (PDAC). APAF1 locus abnormalities have been associated to PDAC in 60% of analyzed samples. We found that Apaf1 is variably downregulated in human specimen of PDAC and in a mouse model sensitive to PDAC, a transgenic mouse overexpressing TGF- α in exocrine pancreas (Wagner et al. 2001). Similarly, some PDAC cell lines exhibit specific low levels of APAF1. These lines showed chemoresistance to commonly used agents, such as Gemcitabine, 5-Fluorouracil, Cisplatin, and Paclitaxel. To understand if the effect was Apaf1-dependent only, we modulated APAF1 dosage in cell lines, by overexpressing Apaf1 and/or Caspase-9 cDNA. Moreover we are analyzing this feature in a mouse model in vivo (the EL-TGF- α overexpressing mice) by pancreas-specific conditional mutagenesis and overexpression.

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Human and Murine forms of Granzyme B exhibit divergent substrate preferences: implications for the mechanism of CTL/NK killing

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Cytotoxic T Lymphocytes (CTLs) destroy virally-infected and tumor cells either through engagement of the Fas receptor pathway, or through a mechanism involving the exocytosis of cytolytic granules including granzyme B and perforin. To date, several substrates for granzyme B have been identified and of these, Bid, Caspase 3 and Caspase 8 have been suggested as having important roles in the orchestration of cell death. Granzyme B can cleave the BH3-only protein Bid, which results in cytochrome c release from mitochondria with ensuing downstream caspase activation. However, granzyme B has also been shown to process and activate pro-caspases directly and this may facilitate a more direct route to death. Granzyme B has also been shown to process other important proteins such as ICAD, the cleavage of which is reported to liberate the DNase CAD resulting in DNA hydrolysis. Contradictory observations have been made by several groups with regard to the primary cellular target for granzyme B. However, a contributory factor to the confusion is that some of the studies in this area have been conducted using granzyme B of murine origin whereas other studies have used human granzyme B. This prompted us to explore whether human and murine granzyme B may actually possess distinct substrate preferences. The results of our analysis shed light on the catalytic properties of both enzymes and redefine our understanding of granzyme B-mediated killing in mouse versus man.

Src kinase phosphorylates Caspase-8 on Tyr380: a novel mechanism to suppress apoptosis and promote cancer development

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Defective apoptosis has been implicated in tumor initiation and progression. Moreover, cancer cells are often resistant to apoptosis induced by drugs and irradiation. We identified Caspase-8 as a new substrate for Src kinase. The activation of Src upon EGF-receptor stimulation, results in Caspase-8 phosphorylation and prevents Fas-induced apoptosis. We took advantage of *S. pombe* as a unique system that does not encode neither tyrosine kinases nor caspases to show that Src kinase can directly phosphorylate Caspase-8. Phosphorylation occurs on Tyr380, situated in the linker region between the large and the small subunits of human Procaspase-8, and results in delayed autoprocessing of Caspase-8 upon Fas-receptor engagement and down-regulation of Caspase-8 activity. Moreover, Src failed to protect Caspase-8-defective human cells in which a Caspase-8-Y380F mutant is expressed.

Remarkably, we observed that Tyr380 is phosphorylated in human colon cancers where Src is aberrantly activated. We are currently investigating whether and how Caspase-8 tyrosine phosphorylation plays a role in tumor progression and in cancer resistance to chemotherapy. Overall our studies provide the first evidence for a direct role of tyrosine phosphorylation in the control of caspases and reveal a new mechanism through which tyrosine kinases inhibit apoptosis and may participate in tumor progression.

The inhibition of nitric oxide synthesis protects brain against inflammation related apoptosis

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Inducible isoform of nitric oxide synthase (iNOS) plays a crucial role in oxidative stress during inflammation. Till now, little is known about the involvement of constitutive isoforms of NOS (cNOS) in brain inflammation.

The aim of this study was to analyse the expression and activity of particular isoforms of NOS and their roles in oxidative stress, neuronal degeneration and apoptosis in brain during bacterial endotoxin lipopolisaccharide (LPS)-evoked inflammation. Mice C57BL6 were injected i.p. with LPS (1mg/kg b.w.) alone or together with NOS inhibitors: 7-nitroindazol (7-NI; 25mg/kg b.w.), N^G-nitro-L-arginine (NNLA; 30mg/kg b.w.) and 1400W (5mg/kg b.w.). The studies were carried out by using RT-PCR, radio-, immuno- and immunocytochemical, fluorimetric and spectrophotometric methods. Ultrastructure analysis was performed with electron microscope.

Our results indicated that LPS exclusively enhanced iNOS expression and activity in substantia nigra. Inhibitor of iNOS and also cNOS inhibitors prevented LPS-evoked lipid peroxidation and β -NAD⁺ depletion, suggesting that cNOS were also involved in the activation of free radical dependent processes and in mitochondria alterations induced by LPS. Moreover, NOS inhibitors prevented also translocation of apoptosis inducing factor (AIF) from mitochondria to nucleus, indicating that NO is responsible for activation of AIF-dependent apoptotic cell death. Electron-microscopic analysis revealed apoptotic/autophagic alterations in many neuronal cells of substantia nigra after LPS administration. Shrunken and dark cytoplasm, deep invaginations of the nuclear membrane, swelling of mitochondria and the presence of autophagolizosomes were observed.

Our results indicated that iNOS, but also cNOS, participate in the oxidative stress leading to mitochondria damage and activation of apoptotic and/or autophagic pathways.

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Anoikis in granulosa explants: geometrical distribution pattern of dying cells

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Avian granulosa explants cultured under serum-free conditions sandwiched between their native basement membrane at the basal side and the vitelline membrane at the apical side, represent a well-characterised apoptosis model system (Mussche et al., 2000) We hypothesized that the relative survival under gonadotropin withdrawal in this model as compared to apoptotic indexes in primary granulosa cell cultures is mediated by survival pathways coupled to the preservation of cell - extracellular matrix interactions. These granulosa explants cultured non-adherently for 24 h display a complex geometrical distribution pattern of cell-free and cell-rich folds as seen on whole mounts of DAPI stained explants. In explants cultured without gonadotropic support, most apoptotic cells were clustered along the cell-free folds. The latter folds are linked to focal disruption of cellular contacts with native basement membrane, as could be documented by scanning electron microscopy.

Addition of survival factors, LH and IGF-I, not only inhibits apoptosis but also influences the extent and distribution of cell-free and cell rich folds throughout the explants.

In support of anoikis as the mechanism of apoptosis induction in the present model, we demonstrated the apoptosis-inducing effect of an antagonistic β 1-integrin antibody. Future experiments will address the contribution of metalloproteinases in the formation of the folds and thus in the remodelling of the basement membrane during apoptosis and follicular atresia.

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Structural and molecular insights in the control of LEI/L-DNase II pathway

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Caspase-independent apoptosis is mediated by different molecular actors depending on the cell type and injury. One of the effectors in caspase-independent apoptosis is LEI/L-DNase II. In its native form, LEI (Leukocyte Elastase Inhibitor), an ubiquitous cytoplasmic protein belonging to the serpin family, bears an anti-protease activity. When apoptosis is induced by certain agents (long-term culture, Na⁺/H⁺ exchanger inhibition), LEI undergoes a cleavage mediated by serine proteases like cathepsins and elastase, and is transformed into an acid endonuclease: L-DNase II. It is then translocated to the nucleus where it is responsible of DNA degradation into oligonucleosomes. Previous studies showed that LEI overexpression protects the cell if it is not transformed into L-DNase II, while an apoptotic stimulus, catalyzing its transformation, increases cell death.

In this study, we identify by bio-informatics and site-directed mutagenesis the structural elements responsible for the transformation of LEI into L-DNase II, the change of enzymatic activity (from anti-protease to endonuclease) and its nuclear translocation during apoptosis. We show that a conformational change, unmasking both endonuclease active site and a bipartite NLS, is involved in this transformation.

Bio-informatics studies have also shown the presence in both forms of the molecule of a poly(ADP-ribosylation) sequence, indicating that a poly(ADP-ribose) polymerase (PARP) might regulate L-DNase II activity by poly(ADP-ribosylation), as it is the case for other endonucleases. The interaction of LEI/L-DNase II with PARP I and its effect on endonuclease activity are evaluated. Results suggest that PARP 1 is involved in the control of this caspase-independent pathway and point the importance of this molecule in cellular management of cell death.

A20 differentially affects apoptosis in endothelial cells and smooth muscle cells

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A20 is a NF- κ B dependent stress response gene in endothelial cells (ECs) and smooth muscle cells (SMC). To the extent that it has been tested, A20 exerts a universal anti-inflammatory effect through blockade of NF- κ B activation including in EC and SMC. In contrast with its anti-inflammatory effect, its previously described antiapoptotic function is rather cell type and stimulus specific. In EC, A20 serves a broad anti-apoptotic function: A20 protects ECs from TNF and Fas-induced apoptosis by inhibiting caspase activity. In addition, A20 blunts natural killer cell-mediated apoptosis, and safeguards from complement-mediated necrosis.

In contrast, A20 sensitizes SMC to cytokine-mediated apoptosis through a novel nitric oxide (NO)-dependent mechanism. Expression of A20 in SMC significantly enhances cytokine mediated DNA fragmentation without affecting caspase activation or mitochondrial integrity. Pre-incubation of A20 expressing SMC with the nitric oxide synthase (NOS) inhibitor L-NAME totally reverts the pro-apoptotic effect of A20. In contrast, similar blockade of NOS in A20 expressing EC does not affect its anti-apoptotic function in this cell type.

The unexpected pro-apoptotic effect of A20 in SMC translates *in vivo* by the regression of established neointimal lesions following balloon angioplasty injury. Gene transfer of A20 to neointimal SMC within occlusive carotid lesions is associated with a rapid increase in apoptosis of these neointimal SMC peaking between 3 and 5 days of the gene transfer followed by a remarkable regression of the lesions. Interestingly, expression of the inducible NOS, both at the protein and mRNA levels, increases in A20 expressing neointimal SMC (day 1 after gene transfer). Increased iNOS in A20 expressing neointimal SMC *in vivo*, corroborates the involvement of NO in causing this novel pro-apoptotic effect of A20 in SMC.

Combined anti-inflammatory and anti or pro-apoptotic functions of A20 in EC and SMC respectively qualify the positive effect of A20 upon vascular remodeling and healing.

Procaspase-9 inhibition by Bir3 domain of Naip and Xiap

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Caspase-9, a key enzyme in the process of apoptosis, is normally present as proform and changes into active form following apoptosis signal. Therefore, inhibition of the proform can prevent the apoptosis prior to the commitment stage. Thus we decided to investigate the inhibition of procaspase-9 (D315, D330/A) by Bir3 domains of xiap and Naip proteins in ammonium citrate buffer. Increasing concentration of ammonium citrate led to a dramatic enhancement of the enzymatic activity presumably due to self-association of the mutant procaspase-9 molecules. Surprisingly, the enzymatic activity of the mutant protein was interfered by both Naip and Xiap Bir3 proteins. Close inspection of the status of mutant procaspase-9 in ammonium citrate buffer showed that the proform underwent a self cleavage process at a site distinct from the cleavage site of the wild type procaspase-9. Thus, we decided to determine whether the self-cleavage of the proform to the cleaved caspase-9 can be inhibited by Bir3 domain of Xiap and Naip proteins. This was indeed the case as the presence of both IAPs prevented the conversion of the proform to the cleaved form. This was unexpected, because of the general view that AXPX N-terminal sequence of the cleaved caspase-9, which is absent from the mutant procaspase-9, is required for the interaction with Xiap Bir3. It, therefore, seems that ammonium citrate facilitates the interaction of the IAPs with caspase-9 that may or may not be relevant to *in vivo* situation.

2-Methoxyestradiol-induced apoptosis in prostate cancer cells requires Smad7

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Prostate cancer is the second most common cause of death related to cancer in the Western society. 2-methoxyestradiol (2-ME), an endogenous metabolite of estradiol-17 β inhibits tumor angiogenesis while it also exerts potent cytotoxic effects on various cancer cells. 2-ME has been shown to activate the p38 mitogen-activated protein kinase (p38 MAPK) and c-Jun N-terminal kinase (JNK) pathways and to induce apoptosis in cells, while the underlying molecular mechanisms for this are unknown. Here we report that the expression of Smad7, an adaptor molecule required for activation of p38 MAPK in the transforming growth factor β (TGF- β) signaling pathway, is also required for 2-ME induced p38 activation and apoptosis in human prostate cancer cells (PC-3U). PC-3U/AS-S7 cells stably transfected with an anti-sense Smad7 construct, or PC-3U cells transiently transfected with siRNA for Smad7, were protected against 2-ME-induced apoptosis. 2-ME-induced apoptosis was found to involve p38 MAPK and JNK, since simultaneous treatments with 2-ME and a specific p38 inhibitor; SB203580, or inhibitor of JNK; L-JNK1, prevented 2-ME induced apoptosis. Interestingly, Smad7 affected the levels of β -catenin previously implicated in regulation of apoptosis, as demonstrated by anti-sense as well as siRNA techniques. Moreover, Smad7 was found to be important for the basal expression of Bim, a proapoptotic Bcl-2 family member and for 2-ME-induced expression of Bim. These results suggest that expression of Smad7 is crucial for 2-ME-induced apoptosis in human prostate cancer cells.

Phosphorylated glyoxalase I as an effector molecule of nitrosative stress-induced cell death

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Methylglyoxal (MG) is a cytotoxic byproduct of the glycolysis that is derived through enzymatic and non-enzymatic phosphate elimination from dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. MG is normally detoxified to D-Lactate by the glyoxalase (GLO) system that comprises GLO 1 and 2. The real biological function of the GLO system is still not known, but it is believed that it plays an essential role in the control of normal cellular growth.

GLO1 is overexpressed in many types of cancer and is involved in resistance of human leukaemia cells to anti-tumor agent induced apoptosis. It is also overexpressed in diabetic patients and in the brains of Alzheimer's disease patients. This de-regulated expression of GLO1 in several disease conditions suggests that MG plays a role in the development of these diseases.

We have recently discovered the phosphorylated form of GLO1, which plays an essential role in Tumor Necrosis Factor (TNF)-induced necrosis in the fibrosarcoma cell line L929. This form of cell death is characterised by increased production of oxidative stress (ROS) in the mitochondria. Phosphorylated GLO1 is not involved in the detoxification pathway of MG, but instead mediates the cytotoxic effects of MG via a pathway that leads to MG-modification of specific target molecules. The latter are formed as a consequence of oxidative stress.

Now we show that TNF-induces multiple phosphorylations on the NO-modified form of GLO1 in L929 cells. Furthermore, the NO donor S-Nitrosoglutathione (GSNO) is strongly synergistic (+ 100%) with TNF-induced cell death and induces a strong NO-modification and phosphorylation of GLO1. In addition, an inhibitor of iNOS, 1400W, reduces TNF-induced necrosis by about 50% and inhibits also the NO-modification and phosphorylation of GLO1.

These data indicate that TNF-induced necrosis is not only dependent on the induction of ROS, but also dependent on the induction of NO. Together, they give rise to nitrosative stress. In conclusion, we show that phosphorylated GLO1 is an important effector molecule in TNF-induced necrosis and that the NO donor might be an interesting adjuvant in TNF-induced tumor cell death therapy.

Interaction of pro-apoptotic TGF- β and anti-apoptotic insulin signalling pathways in the control of cell death in the postnatal mouse retina

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Running title: Interaction of TGF- β and insulin in murine retinal apoptosis

Pro-apoptotic transforming growth factor beta (TGF- β) and anti-apoptotic insulin have been shown to be involved in regulating programmed cell death of the vertebrate retina. In the present study we investigated the interaction effects of TGF- β and insulin on retinal cell death and cell survival in an organotypic culture system of early postnatal mouse retina. Addition of exogenous TGF- β resulted in a significant increase in cell death whereas exogenous insulin attenuated apoptosis and was capable of blocking the TGF- β effect. TGF- β and insulin interact at multiple levels of death/survival regulatory pathways: (a) at the level of signalling ligands and receptors, (b) at the level of signal transduction *via* Smad proteins, (c) by transcriptional up- or down-regulation of members of the anti-apoptotic Bcl-2 family, (d) by modulating the level of TIEG, a TGF- β induced immediate early gene, and (e) by activation or inactivation of effector caspases. These data reveal a complex network of interactions balancing cell survival and cell death in a developing neural tissue.

Death receptors and lipids: discerning the events that affect organelle membranes during cell death

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This work was aimed to discern the events that, following FasL ligation, impact on intracellular membranes and their lipid constituents. Despite the importance of caspases in apoptosis, their full inhibition can lead to a vacuole-mediated form of cell death that displays autophagic features. A similar vacuole-mediated form of cell death has been described following Fas stimulation in the absence of caspase 8 expression. Because knock-down of RIP, a protein kinase that is activated early after Fas stimulation, blocks vacuole proliferation and caspase-independent cell death, we have analysed the effect of genetic ablation of RIP in the FasL-induced changes in intracellular membranes of Jurkat T cells. We reasoned that if RIP were fundamental for modulating the interconnections between the membrane lipids that we find altered after FasL treatment, in particular phosphatidylcholine (PC) and cardiolipin (CL), RIP ablation would produce recognizable changes in steady-state levels of these lipids even in resting cells. The initial studies presented here show that RIP^{-/-} cells have a different profile of PC and CL, as well as an altered content of the metabolically connected phosphatidylinositol (PI), a membrane lipid most crucial for the formation of vacuoles. We discuss these results in light of the possible involvement of RIP in modulating metabolic interconnection of membrane lipids in both resting and Fas-stimulated cells that affect the proliferation of vacuoles.

CED-9 as a regulator of mitochondrial events in apoptosis

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Bcl-2 family proteins play central roles in apoptosis by regulating the release of mitochondrial intermembrane space proteins such as cytochrome c. Death-promoting members of this family, such as Bax, can promote cytochrome c release and fragmentation of the mitochondrial network whereas apoptosis-inhibitory members, such as Bcl-2 and Bcl-xL, can antagonize these events. Human Bcl-2 has been shown to be capable of acting as an inhibitor of programmed cell death in *C. elegans*, suggesting that CED-9 and Bcl-2 may share similar functions. However, it is not known whether CED-9 can act in a similar manner to Bcl-2 by regulating the release of proteins from the mitochondrial intermembrane space or mitochondrial fragmentation. Here, we have explored whether CED-9 is capable of suppressing Bax-induced cytochrome c release or mitochondrial fragmentation in mammalian cells. We have also explored whether CED-9 can serve as a negative regulator of apoptosis in the same context. We present data to argue that CED-9 may function as a regulator of mitochondrial fission/fusion dynamics but fails to regulate release of mitochondrial intermembrane space proteins.

Study of the patho-(physiological) role of mouse caspase-7

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Apoptosis is essential during different developmental stadia of a multicellular organism and depends on an intrinsic cellular suicide program. Caspases (cysteiny aspartate specific proteases) play a central role in apoptosis and inflammation (1). Caspases are produced as pro-enzymes and consist of a prodomain, a p20 and a p10 domain. After receiving the appropriate signal caspases become proteolytically active enzymes, consisting of two p20 and two p10 domains.

Caspase-7 belongs to the family of executioner caspases, together with caspase-3 and -6. It was suggested that caspase-3 and -7 are functionally redundant, because of (i) sequence identity and (ii) identical cleavage specificity of a synthetic peptide and several natural substrates. However, caspase-7 knockout (KO) mice are suggested to be lethal early during embryogenesis, in contrast with viable caspase-3 KO mice (2). Because of the lethality the characterisation of the *in vivo* role of caspase-7 KO mice was hampered. Therefore we developed a conditional caspase-7^{flox/flox} transgenic mouse. Caspase-7 activation has been shown in different models for lethal experimental hepatitis. Therefore we developed liver specific caspase-7 KO mice by crossing the caspase-7^{flox/flox} mouse with the albumin-cre mouse. In the mTNF/GaIN and anti-Fas models we didn't observe a significant difference in lethality between wildtype and liver specific caspase-7 KO mice. Caspase-7 is also highly regulated by interferon (IFN)- γ in cultured cells, such as macrophages, MEF or pancreas β -cells. We developed, by using Tat-cre protein transduction, caspase-7 deficient macrophages and MEF cells. These cells are currently evaluated for differences in apoptotic or inflammatory responses. Caspase-7 is also highly upregulated in the brain of caspase-3 KO mice in a C57/Bl6 background (3). To investigate the role of caspase-7 in the brain we are crossing the caspase-7^{flox/flox} mouse with the brain-specific nestin-cre mouse. Our experiments will shed more light on the physiological role of caspase-7.

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The secret life of caspase-14

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Caspase-14 is expressed in a limited number of epithelia, including the epidermis. This is in contrast to the other caspase family members that are ubiquitously expressed. Caspase-14 is expressed in the differentiating keratinocytes of the epidermis, the hairfollicles and sebaceous glands of the skin. In addition, caspase-14 could also be detected in the epithelial cells of the choroid plexus, the retinal pigment epithelium and the Hassall's body's of the thymus. In the skin caspase-14 activation occurs in the uppermost layers of the epidermis and correlates with epidermal cornification. Until now, no caspase-14 substrates have been identified. To unravel the possible role of caspase-14 during skin differentiation, we have generated caspase-14 deficient mice. Southern blot analysis confirmed the absence of a functional caspase-14 coding region in the knock-out mice and they were born normally with the expected Mendelian ratio. The absence of caspase-14 protein was confirmed both by western blot and immunohistochemistry. Histological sections of the back skin, brain, choroid plexus, eyes and thymus of newborn wild-type and caspase-14 deficient mice revealed no macroscopical differences. Immunohistochemical analysis of other early and late differentiation markers demonstrated a normal expression pattern of K1, K10, K14, loricrin, involucrin and filaggrin. Further analysis of different physiological parameters (such as barrier function and stratum corneum integrity) is ongoing. In addition, mouse models for 'sun burn', skin tumors or inflammation will be analyzed in order to unravel the secret life of caspase-14.

The occurrence of apoptosis during early chicken development

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We examined the distribution of apoptotic cells and the molecules ACINUS, survivin and caspase-3 during early chicken development and epithelial wound healing. Apoptotic cells were localised by *in situ* detection of digoxigenin-labelled genomic DNA according to a technique modified from the TUNEL technique. The localisation of ACINUS, survivin and caspase-3 was performed using immunohistochemical techniques.

During gastrulation, DNA fragmentation was found in nuclei throughout the embryo, according to a well-defined spatial and temporal distribution. During neurulation/somitogenesis, DNA fragmentation in nuclei could mainly be observed in the nervous system. After unilateral removal of the deep layer in the cranial part of mid-streak gastrulating embryos, we detected an increase of apoptotic cells in the epiblast above the wound.

Immunoreactivity for ACINUS, survivin and caspase-3 could be detected from gastrulation on and staining was found in the three germ layers. In the case of ACINUS and caspase-3, the highest expression could be observed in the epiblast. During somitogenesis/neurulation, strong staining could be detected in epithelial structures in the case of caspase-3. ACINUS could be localised in the neural tube, notochord, ectoderm, definitive endoderm, foregut, heart, lateral plate and intermediate mesoderm, and somites. After differentiation of the somites, we could observe stronger immunostaining in the myotome. An uniform staining for survivin was obtained. After wounding the embryos, we could not detect a difference in staining for the three molecules.

The occurrence of apoptosis during development of the chicken embryo could be a consequence of changes in cell-cell and cell-matrix adhesion, e.g. ingression of epiblast cells through the primitive streak leads to disruption of the interactions between the epiblast cells and the underlying basement membrane and to disruption of cell-cell adhesion. In conclusion, apoptosis is involved in early morphogenesis and is frequently associated with a well-defined remodelling of an embryonic tissue.

The Pollen-style system: TGase involvement in the *in planta* programmed cell death of incompatible pollen

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Sexual plant reproduction in many plants involves self-incompatibility (SI), which is one of the most important systems to prevent inbreeding through the induction of programmed cell death (PCD) in the incompatible pollen (1). SI is comprised of a self- and non self-recognition between male (pollen) and female (pistil) gametophytes, followed by inhibition of genetically identical (*self*) pollen growth. We previously demonstrated that an extracellular pollen transglutaminase (TGase), never extracellularly detected in higher plants, is an essential modulator of *in vitro* pollen growth (2). TGases are Ca²⁺-dependent enzymes considered markers of apoptosis in mammal cells, able to post-translationally modify proteins by forming bridges between protein-bound glutaminyl residues and ε-lysines or polyamines (3). The extracellular substrates and the localisation of pollen TGase inside putative focal adhesion sites suggest an *in planta* role for pollen externalised TGase in pollen tube anchorage to the stylar extracellular matrix. We analysed SI by performing *in planta* controlled crosses between pollen and compatible/incompatible styles. These studies confirmed that TGase is predominantly localised into ring-like extracellular structures surrounding the pollen tube surface, whereas in not-pollinated styles no evidence suggests the enzyme presence. Anti-TGase immunoreactive structures resembled pollen anchorage sites onto the stylar cells. In the stunted and thickened apex of incompatible pollen tubes, TGase was identified in annular structures joining the tube cell wall to the stylar surface. The analysis of TGase activity and protein synthesis in compatible and incompatible pollen-style systems is discussed with regard to the putative role of pollen TGase in the control of the SI response. One of the downstream events of SI response involves abnormal rearrangements of cytoskeleton in the incompatible pollen committed to PCD (1). Biochemical analyses indicated that tubulin polymerizing in the presence of purified pollen TGase forms aggregates morphologically different and having a reduced ability of de-polymerization in comparison to control. The label by FITC- cadaverine of TGase-treated microtubules verified that tubulin aggregates were due to the crosslinking activity of pollen TGase. In addition, pollen TGase resulted able to affect the motility of kinesin along microtubules. Preliminary immunolocalisations of pollen tubes grown *in planta* inside incompatible styles showed a punctuate pattern of actin microfilaments, possibly indicating an abnormal microfilament morphology that might resemble the formation of actin foci observed in the incompatible pollens of Papaveraceae. On the base of these data, we suggest a dual role for pollen TGase in SI, first within the early interaction between male and female gametophytes, second, when SI has been committed, in the late cytoskeleton rearrangement that takes place in the incompatible pollen tube undergoing PCD.

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Trolox is a potent modulator of arsenic-mediated cytotoxicity in vitro and in vivo

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Arsenic trioxide (As_2O_3) has shown considerable efficacy in treating acute promyelocytic leukemia (APL) with induction of programmed cell death. However, its use in other malignancies is limited by the toxicity of concentrations required to induce apoptosis in non-APL tumor cells. We reported here that Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a widely known antioxidant, enhances As_2O_3 -mediated apoptosis in APL, myeloma and breast cancer cells. We found that the combination of As_2O_3 and Trolox increased intracellular oxidative stress, as evidenced by HO-1 protein levels, JNK activation, and protein and lipid oxidation. Importantly, trolox protected non-malignant cells from As_2O_3 -mediated cytotoxicity. We further investigated whether Trolox could decrease toxicity associated with As_2O_3 monotherapy *in vivo* and whether it could decrease the tumorigenic properties of P388 lymphoma cells when administered together with As_2O_3 in mice. BDF1 mice were divided in groups and injected every other day with Trolox and As_2O_3 alone or in combination for 20 days. We found that administration of Trolox considerably reduced As_2O_3 -induced hepatomegaly, as well as serum alanine and aspartate transaminases, which are indicators of hepatocellular death. Oxidative stress markers were also upregulated in the As_2O_3 -treated group and significantly reduced in the As_2O_3 +Trolox-treated group. Additionally, we performed *in vitro* studies using P388 murine lymphoma cells and found a potentiation of As_2O_3 -mediated growth inhibition and apoptosis in this cell line. Therefore, BDF1 mice bearing P388 ascitic tumors received similar treatments for 20 days. A significant increase in life span was observed in the As_2O_3 +Trolox group compared to As_2O_3 alone. Our data suggest that trolox might prevent some of the clinical manifestations of As_2O_3 -related toxicity and increase its pro-apoptotic capacity and therapeutical potential in hematological malignancies.

Differential in vitro effect of tumor necrosis factor-alpha (TNF-alpha) on endometrial apoptosis and TNF receptor type-I (TNFR-I) expression in women with and without endometriosis

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We have demonstrated previously that spontaneous endometrial apoptosis in eutopic endometrium was significantly reduced in women with endometriosis when compared to healthy controls. This led us to suggest that abnormal apoptosis in eutopic endometrium may lead to increased viability of those shed endometrial cells during menstruation, resulting in the implantation and growth in the ectopic location, causing endometriosis. It is known that TNF-alpha is a regulator of apoptosis. The objective of the present study was to test the effect of TNF-alpha on endometrial apoptosis and expression of TNFR-I in women with and without endometriosis.

Endometrial cells from women with and without endometriosis were cultured in the presence of 0, 100, and 200 units/ml TNF-alpha. Apoptosis was measured with a cell death ELISA detection kit and TNFR-I expression was detected with RT-PCR after 24 and 48 hours of culture.

TNF-alpha stimulated apoptosis within the first 24 hours of culture in controls in a dose-dependent manner but not in endometriosis. After 48 hours of culture, TNF-alpha did not alter apoptosis in the control group but had an inhibitory effect in endometriosis. TNFR-I expression in endometrial cells was significantly stimulated by TNF-alpha in controls but was significantly inhibited in endometriosis.

From these results, we conclude that TNF-alpha exerts differential effects on endometrial apoptosis depending on the presence or absence of endometriosis. In healthy women it stimulates, and in endometriosis it inhibits, apoptosis of endometrial cells. This differential effect on endometrial cells according to their origin (with or without endometriosis) may be realized by modulation of TNFR-I mRNA expression.

Autophagic cell death of cardiomyocytes in anoxia–reoxygenation is prevented by postconditioning. K_{ATP}-channels dependent effect

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Autophagy is another type of programmed cell death, but it is not mainly described in cardiac pathology and injury. Brief episodes of ischemia in the early reperfusion period, postconditioning, reduce infarct size in experimental models in certain species. The purpose of the present work was to study autophagic cell death after anoxia and reoxygenation and to investigate the possible protective effect of postconditioning upon both types of programmed cell death, autophagic as well as apoptotic. The possible role of K_{ATP}-channels in mechanisms of autophagic cell destruction and in realization of postconditioning is also very interesting.

Primary cultures of cardiomyocytes from neonatal rats underwent 30 minutes of anoxia followed by 60 minutes of reoxygenation. Three different models of postconditioning were used: 3 cycles of 1, 3, or 5 minutes of reoxygenation followed by 1, 3, or 5 minutes of anoxia respectively. An inhibitor (glybenclamide, 100 μM) of K_{ATP}-channels was added to the culture medium immediately after anoxia, but before postconditioning. In another group without postconditioning the K_{ATP}-channel opener diazoxide (100 μM) was added immediately after anoxia. The percentage of living, necrotic, and apoptotic cells were determined by staining with Hoechst 33342 and propidium iodide. Autophagy was demonstrated by staining vacuolar structures in vivo by monodansyl cadaverine.

After anoxia and reoxygenation the amount of living, necrotic and apoptotic cells was 79 ± 1.5 %, 7.8 ± 0.9 % and 13 ± 1.5 % respectively (in unstimulated cell culture 90 ± 0.8 %, 3.3 ± 0.3 % and 5.5 ± 0.7 %, P<0.0001 for all). Postconditioning with 1 min anoxia 3 times significantly increased the amount of living and decreased number of necrotic and apoptotic cells. Postconditioning with cycles of 3 and 5 minutes had stepwise reduced the effect compared to cycles of 1 minute. The percentage of autophagic cells in control cell culture was 4.3 ± 0.3 %. This number increased after anoxia-reoxygenation to 14 ± 0.8 % (P<0.0001), and was reduced by postconditioning (5.0 ± 0.37 %, P<0.001). Glybenclamide inhibited the effect of postconditioning, whereas diazoxide alone prevented autophagy during anoxia-reoxygenation.

Thus, anoxia-reoxygenation induced autophagic, necrotic and apoptotic cell death. Postconditioning prevents autophagic cell death by a mechanism dependent from K_{ATP}-channels.

p53-dependent upregulation of Caspase-8 by Methotrexat

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Loss of Caspase-8 leads to resistance of tumor cells towards TRAIL-induced apoptosis and is associated with unfavorable prognosis for tumor therapy. Azacythidine and Interferon- γ can restore Caspase-8 expression, but show severe side effects in clinical use.

We screened a panel of Caspase-8 negative, TRAIL-resistant tumor cells which can be re-sensitized for TRAIL-induced apoptosis by recombinant expression of Caspase-8. In all cell lines, Methotrexat treatment lead to re-expression of Caspase-8 and enabled TRAIL-induced apoptosis. Methotrexat activated the Caspase-8 promoter as measured using a luciferase-coupled promoter construct. Transfection of cells with Caspase-8 siRNA disabled Methotrexat-induced sensitization for TRAIL-induced apoptosis. Methotrexat induced re-expression of Caspase-8 was mediated by p53 as it was inhibited using Pifithrin α or p53 decoy oligonucleotides.

Taken together, Methotrexat induced p53-dependent re-expression of Caspase-8 and re-sensitized Caspase-8 negative tumor cells for TRAIL-induced apoptosis. Use of Methotrexat in combination with TRAIL might thus be favorable in tumor therapy to overcome loss of Caspase-8.

Decitabine treatment sensitizes glioblastoma for TRAIL-mediated destruction in vivo

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Life expectancy of patients affected by glioblastoma multiforme (GBM) is very poor. The therapeutic use of TRAIL has been proposed to treat this disease based on its ability to kill some glioma cell lines in vitro and in vivo. Here, we showed that differently from glioma cell lines, GBM tumors were resistant to TRAIL stimulation because they expressed low levels of caspase 8 and high levels of the death receptor inhibitor PEA-15/PED. Inhibition of methyltransferases by decitabine resulted in considerable upregulation of TRAIL receptor-1 and caspase 8, downregulation of PEA-15/PED, inhibition of cell growth and sensitization of primary glioblastoma cells to TRAIL-induced apoptosis. Exogenous caspase-8 expression was the main event able to restore TRAIL-sensitivity in primary glioblastoma cells. The anti-tumor activity of decitabine and TRAIL was confirmed in vivo in a mouse model of GBM. Evaluation of tumor size, apoptosis and caspase activation in nude mouse GBM xenografts showed dramatic synergy of decitabine and TRAIL in the treatment of glioblastoma, while the single agents were scarcely effective in terms of reduction of tumor mass, apoptosis induction and caspase activation. Thus, the combination of TRAIL and demethylating agents may provide a key tool to overcome glioblastoma resistance to therapeutic treatments.

Apoptosis and Bcl-2 family members in hepatolienal period of human hematopoiesis

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Apoptosis as the essential process during embryonic development and later for the homeostasis maintenance was described even during blood cells differentiation in adult and prenatal hematopoiesis. Many authors demonstrated involvement of Bcl-2 family members for the hematopoietic precursor cell survival. In addition, Bcl-2 proteins may participate in lineage differentiation through BH4 domain of antiapoptotic members of this family. We focused on the level of apoptosis and Bcl-2 family members during hepatolienal period of hematopoiesis in which three different stages can be distinguished. The earliest stage before the 6th week of IUD is characterized by the beginning of differentiation of blood elements in the liver after its colonization by stem cells incoming from AGM system. During the second trimester blood cells differentiation in the liver culminates and is associated with stem cells release to foetal blood and the colonization of other hematopoietic organs (spleen, thymus, lymph nodes and bone marrow). In the course of the third trimester, growing bone marrow tissue takes up gradually the hematopoietic function instead of liver and the rest of the secondary organs.

We monitored the changes of apoptosis and Bcl-2, Bcl-XL and Bax proteins in the early, middle and late phases of liver hematopoiesis on paraffin sections of 14 human embryos and foetuses aged from the 5th to the 30th week of IUD. In all samples liver parenchyma cells were excluded by sequential double staining of cytokeratine 18 and Bcl-2/Bcl-XL/Bax protein or TUNEL technique used for labelling of apoptotic cells. The changes in number of CD34 positive precursors in the liver were monitored, as well. Light microscopy specimen were evaluated quantitatively using ACC image analysis system.

Our results confirmed the importance of anti-apoptotic Bcl-2 and Bcl-XL proteins in hematopoietic cells differentiation. The highest positivity of both TUNEL labelled cells and anti-apoptotic Bcl-2 proteins were observed in the middle-gestation period, thus it seems to be associated with the intensive proliferation and differentiation in hematopoietic tissue. Apoptosis was minimal in both early stages of development, when high Bcl-2 and Bcl-XL levels appear, and in the last trimester, in which all the studied Bcl-2 members were present only sporadically.

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NF κ B regulates caspase-14 expression in epidermal keratinocytes

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The expression of the caspase-14 gene is restricted to only few cell types and is strongly regulated during differentiation of epidermal keratinocytes. In silico analysis of the caspase-14 promoter region revealed several putative transcription factor binding sites including one consensus NF κ B binding site. Band shift assays showed competitive binding to the caspase-14 promoter-derived site and NF κ B consensus sequences and supershift analysis revealed that the protein complex, that targets the caspase-14 promoter sequence, was composed of the NF κ B subunits p50 and RelB. The functionality of this predicted binding site was demonstrated by promoter-reporter analysis using mutated constructs. The involvement of NF κ B in caspase-14 regulation was further corroborated by the findings (1) that the activity of the caspase-14 promoter was reduced by the NF κ B-inhibitor CAPE and (2) that caspase-14 expression was suppressed by dominant negative IKK2, overexpression of I κ B α proteins, as well as by siRNA-mediated p50 knockdown in epidermal skin equivalents. Our results establish NF κ B as an important regulator of a differentiation-associated gene in the epidermis.

Tomato phytocomplex but not lycopene alone is able to induce apoptosis in HL60 human leukemia cells

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Clinical studies demonstrate that diets rich in antioxidant molecules are associated with a diminished risk of cancer. Moreover the data from recent literature suggest that the carotenoids, in particular the lycopene, could also be used as anticancer molecules. Chemically, lycopene is an acyclic carotene and is the major carotenoid found in tomatoes. Many *in vivo* and *in vitro* studies demonstrate the efficacy of lycopene and its oxidized products in prevention of cancer and in the ability of killing cancer cells. These interesting results encouraged us to investigate whether lycopene alone or in combination with other components from tomato extract (phyto-complex) is responsible for induction of apoptosis in tumor cells. In our experiment we used pure Lycopene and Lycopene phyto-complex (commercialized at 6% in title of pure lycopene and used to produce dietary and cosmetic products) at different concentrations ranging among 1 and 25 microM; as control we used cells treated with the vehicle used to solubilize both substances. We monitored: cell viability, ROS production and mitochondrial changes and the induction of apoptosis as subdiploid DNA content and phosphatidylserine exposure. We used HL60 leukemia cell culture, considered the best cell model to evaluate the markers of apoptosis. After 24 h of lycopene and lycopene phyto-complex addition to the cell medium, we found the classical hypodiploid peak, accompanied by scrambling of phosphatidylserine, decrease of mitochondrial transmembrane potential and increase in ROS production only in cells treated with lycopene phyto-complex. Our experiment clearly demonstrate that lycopene alone had no effects on HL60 cells and that the phyto-complex containing 6 % lycopene was responsible of the induction of apoptosis in leukemia cells. We speculated that the phytomixture could contain other carotenoids or antioxidant molecule or oxidized products derived from Lycopene itself that could act synergistically in the induction of cell death in human leukemia cells.

The TRAF3 binding site of human molluscipox virus FLIP molecule MC159 is critical for its capacity to inhibit Fas-induced apoptosis

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Viruses have evolved diverse strategies to prevent the death of infected cells. Members of the viral Flice/caspase-8 inhibitory protein (v-FLIP) family prevent induction of apoptosis by death receptors through inhibition of both the processing and activation of pro-caspases-8 and -10 at the level of the death receptor-associated signaling complex (DISC). We have addressed the molecular function of the v-FLIP member MC159 of the human molluscum contagiosum virus. MC159 FLIP powerfully inhibited both caspase-dependent (apoptotic) and caspase-independent cell death induced by Fas. The full anti-apoptotic capacity of MC159 depended on its C-terminal domain, which bound TRAF3 and was necessary for optimal TRAF2 binding, and mediated the recruitment of both TRAFs into the Fas DISC. The intact TRAF3 binding site was required for the complete inhibition of FasL-induced caspase-8 processing, mitochondrial membrane potential loss and Fas internalization. Our findings provide evidence that a MC159 FLIP/ TRAF2/TRAF3 complex regulates an as yet unrecognized aspect of Fas-mediated apoptosis, and identify MC159 FLIP as a molecule that targets multiple features of Fas-induced cell death.

Studies of chemoresistance in human B lymphoma-derived cell lines: role of the apoptosome

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Resistance to apoptosis is one of the cardinal features of cancer cells, and contributes to their chemoresistance. Our recent studies have shown that chemoresistance is a common feature of Burkitt's lymphoma (BL)-derived B cell lines, and have demonstrated, for the first time, the sequestration of apoptotic protease-activating factor-1 (Apaf-1) in the plasma membrane of the prototypic BL cell line, Raji (Sun et al., Blood, 2005). Administration of lipid raft-disrupting agents with subsequent liberation of constitutive Apaf-1 from the plasma membrane, or enforced expression of exogenous Apaf-1 in the cytosolic compartment, rendered BL cell lines sensitive to apoptotic stimuli, including etoposide and staurosporine. However, apoptosis induction was delayed in this model. More recent studies have shown that BL cell lines express elevated levels of cIAP2 (cellular inhibitor of apoptosis protein-2), an endogenous caspase inhibitor. Furthermore, introduction of Smac peptides, with the aim to alleviate IAP inhibition of caspases, was found to potentiate apoptosis induction by staurosporine and lactacystin (a proteasome inhibitor) in Apaf-1-overexpressing BL cells, but failed to do so in mock-transfectants. Similarly, immunodepletion of cIAP2 sensitized only Apaf-1-overexpressing BL cells to cytochrome c-dependent caspase activation, and was ineffective in wild type cells. In sum, these studies demonstrate a key role for apoptosome activation in BL-derived cell lines, and indicate that other apoptosis-regulating molecules acting downstream of mitochondria may contribute to chemoresistance in these cells. These findings are of relevance for the treatment of human cancer, and suggest, among other things, that the use of small-molecule Smac mimetics, or proteasome inhibitors, for the eradication of cancer cells is likely to require cytosolic expression of Apaf-1, and a functional apoptosome.

The permeability transition in mitochondria from cyclophilin D-null mice

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Mammalian mitochondria possess a high conductance inner membrane channel, the Permeability Transition Pore (PTP), which plays a critical role in cell death. Sustained PTP opening induces collapse of the mitochondrial membrane potential ($\Delta\psi_m$) and depletion of pyridine nucleotides, and may cause rupture of the outer membrane and release of proapoptotic proteins. An important discovery is that the PTP is inhibited by Cyclosporin A (CsA) with high affinity (1). This immunosuppressant induces PTP inhibition in the same range of concentrations that inhibits its putative receptor, the peptidyl prolyl *cis-trans* isomerase Cyclophilin D (Cyp-D) (2). In order to unambiguously define the role of Cyp-D in PTP modulation we have generated a mouse model in which the expression of *Ppif*, the gene encoding for Cyp-D, has been eliminated by knock-out strategies (3); and we have studied the properties of the PTP in mitochondria of *Ppif*^{-/-} mice. Mitochondria from *Ppif*^{-/-} mice had no Cyp-D, and displayed a striking desensitization of the PTP to Ca²⁺, in that pore opening required about twice the Ca²⁺ load necessary to open the pore in strain-matched, wild-type mitochondria. Mitochondria lacking Cyp-D were insensitive to CsA, which increased the Ca²⁺ retention capacity only in mitochondria from wild-type mice. The PTP response to ubiquinone 0, depolarization, pH, adenine nucleotides and thiol oxidants was similar in mitochondria from wild-type and *Ppif*^{-/-} mice. These experiments demonstrate that (i) the PTP can form and open in the absence of Cyp-D; (ii) that Cyp-D represents the target for PTP inhibition by CsA; and (iii) that Cyp-D modulates the sensitivity of the PTP to Ca²⁺, but not its regulation by the proton electrochemical gradient, adenine nucleotides and oxidative stress. These results have major implications for our current understanding of the PTP and of its modulation *in vitro* and *in vivo*.

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Effects of Calsenilin/DREAM/KChIP on Ca²⁺ homeostasis

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The specificity of the calcium signals is guaranteed by the coordinated activity of different transporters, i.e. channels, pumps and exchangers, and by Ca²⁺-binding proteins. Recently a new member of the Neuronal Calcium Sensor (NCS) protein family, Calsenilin/DREAM/KChIP3, has been cloned. This Ca²⁺ binding protein has different functions, probably depending on its intracellular localization and/or interactions with different partners. Calsenilin has been described as a protein which interacts with Presenilin, a protein localized in ER membrane; DREAM acts as a Ca²⁺-regulated transcriptional repressor and KChIP3 as a modulator of A-type potassium channels. In particular, DREAM (DRE-antagonist modulator) represses the transcription of the human prodynorphin, *c-fos* and other mammalian genes by binding to the DRE sequence in the absence of calcium. Following the increase of Ca²⁺ levels it dissociates from DRE, derepressing the transcription. Moreover, Calsenilin overexpression induces apoptosis in rat neuroblastoma cells and increases susceptibility to apoptotic triggers, possibly by altering endoplasmic reticulum calcium signaling and activating caspase and calpain activities.

The possible influence of DREAM/Calsenilin/KChIP3 on Ca²⁺ homeostasis has been investigated in this work. Since DREAM is mainly present in neuronal cells we generated stable SH-SY5Y transfectants expressing EFmDREAM and have explored the effects of its overexpression using the Ca²⁺-sensitive photoprotein aequorin targeted to the cytoplasm and to the lumen of the ER. Ca²⁺ concentrations were measured in resting cells and in cells stimulated with the agonist ATP. The results show that overexpression of the EFmDREAM significantly reduces the increase in cytoplasmic calcium both in a medium containing 1mM Ca²⁺ and in the absence of extracellular Ca²⁺. The steady state concentration of Ca²⁺ in the lumen of the ER is also significantly lower in the cells overexpressing EFmDREAM as compared to the control cells.

Cell death pathways in development and diseases of the nervous system: the role of the apoptosome and Faf1

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In the attempt to unravel the role of the apoptosome in the onset and progression of neurodegenerations we have previously found that Apaf1^{-/-} NPCs (ETNA^{-/-} cells) resist apoptosis induced by amyloid- β A β peptide. This strongly suggests that the apoptosome pathway is a key route of cell death in Alzheimer's disease (AD). This kind of analysis, however, implies the use of micromolar levels of A β peptide which are in contrast with the low nanomolar levels of natural A β found in the brain and cerebrospinal fluid. Therefore, a different approach to define the mechanism of A β peptide toxicity is to use cell culture models which produce low levels of A β peptide. For this purpose we produced ETNA cells stably or inducibly (TET-On system) overexpressing a mutant form of APP which is associated with familial AD: APP^{swe} (Swedish mutation: KM670/671NL). This mutation determines a much higher production of A β when compared to the APP^{wt}.

We analyzed ETNA-APP^{swe} cells and we found that they produce a large amount of APP and of A β ₁₋₄₂. Moreover, we checked the level of tau phosphorylation and we found that it is increased in cells induced to produce APP^{swe}. We are now analyzing the level of apoptosis in ETNA-APP^{swe}.

Moreover, we are studying the involvement of the novel gene Faf1 (Fas Associated Factor 1) in the regulation of neuronal cell death. Faf1 knock-out mice show early embryonic lethality and the heterozygotes may show phenotype in testis leading to male sterility. Intriguingly, we have noticed a specific expression of Faf1 during embryogenesis in telencephalic hemispheres, which is dynamic in embryonic stages and progressively becomes to be localized to restricted regions of the brain. The concomitant functional study of Faf1 in ETNA^{+/+} cells is aimed to define its role in neuronal cell death both in development and neurodegeneration.

Induction of apoptosis is one of the mechanisms involved in the antigenotoxic activity of some natural chemopreventive agents

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The possibility of modulating the cellular responses to genotoxic agents by manipulating dietary factors has opened a new frontier for cancer control. Changes in cell ploidy, non-random chromosome aberration, and DNA damage are frequently associated with chemical toxicity and carcinogenesis. Preventing the manifestation of these events may prevent the induction of cancer. Apoptosis plays an essential role as a protective mechanism against carcinogenesis by eliminating genetically damaged cells, initiated cells, or cells that have progressed to malignancy. The induction of apoptosis thus is a highly desirable chemopreventive strategy for cancer control.

A number of natural compounds with inhibitory effects on tumorigenesis have been identified from our diet. Among these, isothiocyanates (ITCs) and anthocyanins have gained much attention because of their potential anticarcinogenic properties in culture models as well as in animal models. In this study we evaluated the ability of the ITC sulforaphane (present in Cruciferous vegetables) and the anthocyanin cyanidin 3-O- β -glucopyranoside (Cy-g) (present in red oranges) to protect cultured human lymphocytes from genotoxicity induced by three different agents: ethyl methanesulfonate (EMS), H₂O₂, and mitomycin C (MMC). In order to understand the mechanisms of action of sulforaphane and Cy-g, the cultures were treated before, during, and after treatment with the mutagens; in addition, the induction of apoptosis was evaluated. Sulforaphane reduced the genotoxicity induced by EMS, H₂O₂ and MMC in at least one of the treatment protocols; it had no effect on H₂O₂ genotoxicity in the post-treatment protocol and it increased MMC genotoxicity in the pre-treatment protocol. Cy-g was able to reduce EMS and H₂O₂ genotoxicity using all three treatment protocols, but it had no significant effect on genotoxicity of MMC in any of the protocols. Apoptosis was produced in the cultures treated with sulforaphane or Cy-g and increased under conditions where the two natural agents produced anti-genotoxic effects. This suggests that sulforaphane or Cy-g mediated-apoptosis may remove highly damaged cells induced by genotoxic agents. Taken together, our findings indicate that sulforaphane and Cy-g possess anti-genotoxic activity in vitro and that further studies are warranted to characterize this property in vivo.

DNase1L2 degrades DNA in terminally differentiated keratinocytes of human skin

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Degradation of nuclear DNA is an important step in terminal differentiation of epidermal keratinocytes (KC). Whereas KC nuclei are degraded along with other organelles during differentiation in normal skin, incomplete breakdown of nuclei results in parakeratosis, a prominent sign of skin diseases such as psoriasis. Here, we investigated the potential involvement of DNase1L2, a member of the DNase1 family of DNases, in this process. Tissue screening revealed that DNase1L2 is expressed almost exclusively in skin where it is primarily present in the outermost KC layers. By contrast, DNase1L2 was barely detectable in parakeratotic skin lesions. Expression of DNase1L2 mRNA and protein was weak in proliferating KC, but increased strongly during cell differentiation *in vitro*. To test the hydrolytic activity of DNase1L2 towards chromosomal DNA, cultured KC were permeabilized and incubated with recombinant DNase1L2. Indeed, nuclear DNA was efficiently degraded under conditions similar to those found in the superficial layers of the epidermis. Extraction and immuno-affinity enrichment showed that enzymatically active DNase1L2 is present in the cornified layer of human skin. In summary, our results establish DNase1L2 as the first skin-associated DNase and suggest a function in the breakdown of nuclear DNA during KC differentiation.

Initiator caspase-8 and -10 have overlapping and unique substrate specificities

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While the key role of caspase-8 in death receptor-mediated apoptosis is well established, the function of its closest homologue, caspase-10, is largely unknown. There is no doubt that caspase-10 can be recruited to death receptor signaling complexes, but it remains controversial whether caspase-10 can fully substitute for caspase-8 and whether it exerts a specific or redundant role. The different phenotype of patients with caspase-10 mutations suffering from autoimmune lymphoproliferative syndrome-type II and patients with caspase-8 mutations, might suggest that both initiator caspases serve a specific function. A key to the understanding of caspase function is the analysis of its downstream targets. For instance, cleavage of RIP kinase-1 by caspase-8 shuts down NF- κ B controlled cell survival pathways. Furthermore, caspase-8 initiates the mitochondrial apoptotic pathway by cleaving Bid to its proapoptotic form tBid. However, specific substrates of caspase-10 are as yet completely unknown. Therefore we have compared caspase-8 and caspase-10 regarding their substrate cleavage. We found several proteins that are differentially cleaved by caspase-10 and -8. The functional consequences of these differences in substrate cleavage for the propagation of the apoptotic signal and the interference with the mitochondrial pathway will be discussed.

Time-course dependent transcriptional regulation of T cell apoptosis upon IL-2 withdrawal

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Apoptosis of mature T lymphocytes is an essential process for maintaining immune system homeostasis. In particular, the cytokine IL-2 plays a critical role in this process since T cells deprived of IL-2 undergo apoptosis. However, the details of the molecular signaling pathways leading to T cell apoptosis are poorly understood. To dissect the transcriptional program in cell death, we used cDNA microarrays containing more than 15,000 murine genes to study the gene expression profile in T lymphocytes at different time points of IL-2 withdrawal (3h, 6h and 18h). Comparison of the gene expression profiles revealed that 98% of the genes were unaffected by cytokine starvation. Interestingly, the apoptotic program rather seems to activate gene expression in the early phase of cell death. At 6 hours of IL-2 deprivation, 60% of the differentially expressed genes were transcriptionally induced. On the contrary, transcription was strongly repressed in later stages of apoptosis since 80% of differentially regulated genes were downregulated at 18 hours of IL-2 starvation. SOM clustering of the 365 differentially expressed transcripts revealed specific temporal expression patterns supporting the idea that IL-2 deprivation triggers a tightly regulated transcriptional program to induce T lymphocyte death. In addition, to validate microarray results, changes in gene expression following IL-2 deprivation were confirmed for selected genes by real-time RT-PCR. Several of the genes that underwent transcriptional changes following IL-2 starvation have already been implicated in apoptosis regulation such as AIF, bnip3, cdc25a, egr-1 or c-myc. However, we have also identified a broad array of novel genes whose function in apoptosis has not been described yet. Taken together, these results provide novel insights into the temporal regulation of gene expression during T lymphocyte death.

Effects of Bim in the mitochondrial apoptotic pathway

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Members of the Bcl-2 family which can be divided into anti- and pro-apoptotic proteins, are crucial regulators of programmed cell death. The pro-apoptotic subgroup of so-called BH3-only proteins share homology in only one of the four conserved regions termed Bcl-2 homology (BH) domains 1 to 4. These BH3-only proteins are thought to continuously sense the cellular integrity at various subcellular levels. Activation of these proteins by a variety of stimuli leads to activation of the pro-apoptotic multidomain proteins Bax and Bak and, consequently, to induction of apoptosis by release of pro-apoptotic factors from the mitochondrial intermembrane space into the cytosol. Bim, a BH3-only protein, is expressed in three main isoforms, BimS, BimL and BimEL. BimS is believed to be a stronger killer than BimL or BimEL. To investigate the function of BimL and BimS, we used a regulable adenoviral expression vector based on the Tet-Off system. In our experiments, the adenoviral expression of both BimL and BimS revealed an identical apoptotic potency.

To study the role of Bak and Bax in Bim-induced apoptosis, we overexpressed BimL and BimS as well as Nbk (another BH3-only protein) in Bax-negative DU145 cancer cells. These cells were resistant to apoptosis induced by all three proteins tested. Therefore, we stably overexpressed Bax or Bak in DU145 cells. We demonstrated that DU145 cells overexpressing Bax are sensitized for BimL- and BimS-induced apoptosis. This is in accordance with the data obtained for Nbk, that acts in an entirely Bax-dependent fashion (Gillissen et al. EMBO J 2003, 22, 3580). However, Bax-negative, Bak-overexpressing DU145 cells were sensitive to both BimL- and BimS-induced apoptosis. This is in contrast to apoptosis induced by Nbk which was not affected by Bak-overexpression. These results indicate, that in contrast to Nbk, BimL and BimS exert their apoptotic function via a Bak- and Bax-dependent pathway.

Glutathione efflux through an SLCO/OATP-like transporter occurs in two stages during FasL-induced apoptosis, and is necessary for the changes in cell ionic homeostasis and cell shrinkage

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Apoptosis is characterized by the activation of specific biochemical pathways, from which intracellular GSH depletion and changes in the intracellular ionic homeostasis are important hallmarks. To date, neither the mechanisms involved in the reduction of the intracellular GSH concentration, $[GSH]_i$, nor its connection to the progression of apoptosis have been clearly elucidated. In this work, we studied the mechanism by which apoptosis induces the reduction of $[GSH]_i$ and its relationship with the progression of cell death. We focused particularly, on its correlation with cell shrinkage and changes in the ionic homeostasis of Jurkat cells exposed to FasL. A reduction in the total $[GSH]_i$ in response to FasL-induced apoptosis was shown to occur in two stages. The first stage of $[GSH]_i$ loss was modulated by the activator caspases 8 and 9, and was correlated with a slight decrease in cell size, increased intracellular Na^+ concentration, and plasma membrane depolarization. The second stage was coupled to a secondary intracellular Na^+ and K^+ loss and enhanced cell shrinkage. This stage was modulated by the activity of the execution caspases 3 and 7. Significant levels of mRNA were detected in Jurkat cells, for the members of the GSH-efflux pumps multidrug resistance proteins (ABCC/MRP), and organic anion transport polypeptide proteins (SLCO/OATP). FasL-induced GSH efflux was trans-stimulated by the organic substrates MK571, probenecid, taurocholic acid, estrone and bromosulfophthalein, and was inhibited by high concentrations of extracellular GSH supporting the role of the SLCO transporters in GSH efflux. High extracellular GSH medium also prevented the changes in ionic homeostasis, cell shrinkage and membrane depolarization. We propose for the first time that $[GSH]_i$ efflux during FasL-induced apoptosis, occurs in two stages, and that it is mediated by an SLCO-like transport mechanism. The reduction in $[GSH]_i$ is necessary for the changes in intracellular ionic homeostasis and cell shrinkage to occur during FasL-induced apoptosis.

OPA1, an inner mitochondrial membrane dynamin related protein, controls apoptotic remodelling of cristae and cytochrome c mobilization independently from fusion of the mitochondrial reticulum

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Complete release of cytochrome *c* from mitochondria during apoptosis is granted by remodelling of the cristae structure and by fragmentation of the organelle. While the latter has been ascribed to gain and loss of function of certain “mitochondria-shaping” proteins including dynamin related protein-1 and mitofusin-1, molecular mechanism of the former remain uncertain. Here we show a role for the inner mitochondrial membrane dynamin-related protein OPA1, independently of mitochondrial fusion. Active OPA1 protects from apoptosis at the mitochondrial level by preventing cytochrome *c* release and dysfunction following intrinsic stimuli, including the “BH3-only” molecule BID. OPA does not interfere with activation of multidomain proapoptotics that mediate release of cytochrome *c* release across the outer mitochondria membrane, but it regulates cristae remodelling and mobilization of cytochrome *c* stores from the cristae induced by BID. This regulation does not require mitofusin-1, essential for mitochondrial fusion by OPA1, but depends on the inner membrane rhomboid protease PARL.

Involvement of DNA-double-strand breaks in activation of caspases

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DNA-damage and DNA-repair mechanisms may play a critical role in sensitivity and resistance of tumor cells after cytotoxic drug treatment. In leukemias and solid tumors cytotoxic drugs have been shown to induce apoptosis and to activate apoptosis pathways. Deficiencies in these pathways may lead to resistance towards chemotherapy. However, the role of DNA-damage and DNA-repair in activation of apoptosis pathways are not well understood. Here, we investigate whether DNA-damage and DNA-repair may play role in apoptosis sensitivity and apoptosis resistance.

DNA-double-strand breaks (DBS) can be caused by radiation or cytotoxic drugs interacting with the DNA. If left unrepaired, DNA-double-strand breaks may cause cell death. The catalytic subunit of DNA-dependent protein kinase (DNA-PK) and ligase IV is required for non-homologous end-joining (NHEJ) pathway that repairs DNA-double-strand breaks induced by anticancer drugs such as doxorubicin or cisplatin. We found that DNA-PK +/+ and Ligase IV +/+ cell lines, intact in DNA-repair and NHEJ, were resistant to doxorubicin treatment and were defective in activation of caspases-3, caspase-9, caspase-8 and PARP cleavage after doxorubicin treatment in comparison to DNA-PK -/- and Ligase IV -/- cells lines which show a defect in DNA-repair and NHEJ. Inhibition of DNA-repair enzymes with DNA-repair enzyme inhibitors such as Wortmannin restored doxorubicin-induced apoptosis, activation of caspases, PARP cleavage and DNA-damage in repair intact DNA-PK +/+ cell line as well as in apoptosis resistant and anticancer drug resistant cell lines, which express higher levels of DNA-repair enzymes in comparison to parental sensitive cell lines. We conclude that DNA-damage and DNA-double-strand breaks are involved in activation of caspases by doxorubicin. In addition, apoptosis resistance and chemoresistance, at least in part, depend on loss of DNA-damage and higher rate of DNA-repair which may be modulated by different inhibitors of DNA-repair enzymes.

The mitochondrial effects of small organic ligands of BCL-2 at the BH3 domain. Sensitization of BCL-2 overexpressing cells to apoptosis without mitochondrial toxicity by a pyrimidine-2,4,6-trione derivative

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BCL-2 belongs to a family of proteins that regulate apoptosis, or programmed cell death. This family includes both antiapoptotic proteins such as BCL-2 and BCL-XL and proapoptotic proteins such as BID, BAD, BAK and BAX. A computer screening has identified HA14-1, a small organic ligand that is able to displace peptides modeled on the BCL-2 binding region of BAK, a proapoptotic member of the family. HA14-1 was able to cause cell death that was preceded by activation of caspase 9 and 3, and caused mitochondrial depolarization *in situ* (1). We have investigated the mitochondrial effects of BH3I-2', Chelerythrine and HA14-1. All compounds displayed a biphasic effect on mitochondrial respiration with uncoupling at low concentrations and respiratory inhibition at higher concentrations. At concentrations lower than required for uncoupling all compounds sensitized the PTP to opening both in isolated mitochondria and intact cells. BCL-2 overexpression did not sensitize but rather protected cells from the cytotoxic effects of BH3I-2', Chelerythrine and HA14-1. In order to assess whether the BCL-2-binding and PTP-inducing effects could be separated from the effects on respiration, we have tested a set of HA14-1 analogs from the Hoffmann-La Roche chemical library. We have identified a pyrimidine-2,4,6-trione derivative (EM20-25) as a molecule devoid of effects on respiration that is able to induce PTP opening, to disrupt the BCL-2/BAX interactions *in situ* and to activate caspase-9 in BCL-2-overexpressing cells. EM20-25 neutralized the antiapoptotic activity of overexpressed BCL-2 towards staurosporine, and sensitized BCL-2-expressing cells from leukemic patients to the killing effects of staurosporine, chlorambucil and fludarabine. These results provide a proof of principle that the potentially toxic effects of BCL-2 ligands on mitochondrial respiration are not essential for their antiapoptotic activity, and represent a step forward in the development of tumor-selective drugs acting on BCL-2.

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Combined supplementation of folic acid and vitamin E diminishes diabetes-induced dysmorphogenesis in rat embryos

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Diabetic embryopathy and growth disturbances are 3-5 fold more common in infants of diabetic mothers than in offspring of non-diabetic pregnancy. The mechanisms causing these disturbances are likely to be multifactorial.

It has been suggested that oxidative stress and apoptosis plays an important roles during organogenesis. These notion may also be involved in the induction of embryonic dysmorphogenesis in diabetic pregnancy. We aimed to investigate the effect of administration of folic acid alone or combined with vitamin E on embryonic malformations and different markers of apoptosis, such as NFK β activity, Bcl-2, Bax, cytochrome c protein levels and p-53 protein and mRNA expression.

Diabetes was induced by a single injection of streptozotocin. Pregnant, non-diabetic and diabetic, rats were treated with daily injections of folic acid or treated with both folic acid and 5% vitamin E in the diet. Untreated rats from both groups served as controls. Embryos were collected on gestational day 10 or 11 and were evaluated with regard to malformations and growth retardation. Some embryos were used for NFK β assay, western blot and cDNA synthesis.

We found increased malformations and growth retardation in a diabetic milieu compared to normal environment. Supplementation of folic acid alone and combined with vitamin E normalized these parameters

In addition, we found decreased NFK β activity and Bcl-2 protein, increased expression of Bax, cytochrome c, p53 protein and mRNA in embryos of diabetic rats compared to normal rats. Administration of folic acid to the diabetic rats increased NFK β activity and Bcl-2 protein. Combined administration of folic acid and vitamin E normalized Bcl-2 expression and cytochrome c protein in the diabetic environment.

Combined folic acid and vitamin E supplementation to pregnant diabetic rats diminished diabetes-induced dysmorphogenesis and had some beneficial effects on embryonic apoptotic rate.

Genetic study of DNA damage checkpoint signaling in *C.elegans* reveals *cep-1/53* dependent and independent components needed for DNA damage induced apoptosis

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DNA damage checkpoint pathways are needed to detect DNA damage and transduce a signal that elicits cell cycle arrest, DNA repair, and/or programmed cell death. Defects in DNA damage checkpoint signaling, have a dramatic impact on genome stability and are implicated in tumorigenesis, as well as in some genetic instability disorders like, for instance, Li-Fraumeni syndrome and Ataxia Telangiectasia. Although the link between these genetic instability disorders and a failure in the proper DNA lesion processing is clearly established, the signaling network that communicates between DNA perturbation and the cell death machinery remains ill defined. Furthermore, DNA damage induced apoptosis can not be studied in yeast. Previously we defined a core pathway needed for DNA damage induced apoptosis that includes conserved upstream checkpoint genes, needed for sensing of DNA damage 1) 3), as well as the *C. elegans* homolog of p53 which is specifically needed for DNA damage induced apoptosis 2). Furthermore, we uncovered a novel mechanism of *cep-1/p53* regulation through *gld-1/quaking* mediated translational repression 4).

To define further components of DNA damage signaling we screened through a collection of radiation sensitive Rad mutants (reduced survival of the progeny of irradiated worms) and found mutants specifically defective in DNA damage induced apoptosis, mutants specifically defective in the S-phase checkpoint and mutants defective in DNA damage induced cell cycle arrest and apoptosis. The mutants defective in DNA damage induced apoptosis fall into two classes. The first class is likely to act in the *cep-1/p53* pathway and is defective in the *cep-1* dependent induction of *egl-1* while the second class seems to affect a pathway parallel to *cep-1/p53* as DNA damage induced *egl-1* transcription is normal. Interestingly, both classes of mutation classes genetically interact. We already pinned down one locus corresponding to the second class of mutations. Based on complementation analysis we are confident that most loci define new genes. The separation of function revealed by mutant phenotypes could reveal new features of the signaling network between apoptosis activation and DNA damage checkpoints. We are currently establishing the genetic relations between these mutants and started to map 6 checkpoint defective mutants. We will give an update of our results.

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Caspase 3 and 9 expression during retinoic or irradiation induced apoptosis in early eye development

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Programmed cell death is a key physiological process in embryonic development. During eye development, two cell-death periods can be distinguished: an early phase and a later phase. Similarly apoptosis is often part of abnormal development. Teratogen agents, such as retinoic acid and irradiation, induce an episode of apoptosis and subsequently give rise to cranio-facial defects including eye malformations. Within the apoptosis pathway, caspases play a central role. The aim of this research was to study the expression of caspase-3 and caspase-9 during apoptosis induced by retinoic acid and irradiation in early eye development, and determinate similarities and differences of these two models. Pregnant C57Bl/6j mice received 80mg/kg of all-trans retinoic acid or 2 grays irradiation on gestation day 9 (G9). Embryos were obtained (3 hours, 6 hours, 12 hours and 24 hours after exposition). Coronal cut was performed, and stained using anti caspase-3, anticaspase-9 immunohistochemistry and TUNEL. In apoptosis induced by retinoic acid, caspases 3 and 9 were located in cells at the ventral part of the optic stalk, and close to the lens placode. In irradiation-induced apoptosis, caspase 9 was found in the optic vesicle cells, while caspase 3 was located in both optic vesicle and lens placode cells. TUNEL positive cells are located at the same site as caspase 3 positive cells.

The spatial pattern of the expression of both caspases 3 and 9 after in-vivo exposition to retinoic acid or irradiation suggests that the apoptosis mechanisms are correlated to both cell type and stimulus nature in the developing eye.

The inverse effect of cathepsin B on TNF- α mediated hepatocyte apoptosis and proliferation

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Cathepsin B is a cysteine proteinase, considered to have an important role on the apoptotic cell death mechanism, which is activated by D-galactosamine and tumor necrosis factor- α (D-GalN/TNF- α). Benzyloxycarbonyl-L-Phenyl-Alanine fluoromethyl ketone (Z-FA.FMK) is a cathepsin B inhibitor used in researches on cathepsin B. The aim of this study was to investigate the role of cathepsin B on the cellular mechanism of apoptotic cell death and cell proliferation induced by D-GalN/TNF- α combination in mice, immunohistochemically. In this study, after 1 hour administration of 8 mg/kg Z-FA.FMK by intravenous injection, the liver damage was induced by a single intraperitoneal injection of D-GalN (700 mg/kg) and TNF- α (15 μ g/kg). Apoptotic hepatocytes were determined by *in situ* terminaldeoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay and immunohistochemical method for casapase-3. The proliferative hepatocytes was established by immunohistochemical staining against proliferating cell nuclear antigen (PCNA). In the group given D-GalN/TNF- α , a significant increase in the number of both TUNEL and caspase-3 positive hepatocytes and a decrease in the number of PCNA positive hepatocytes were observed. In contrast, in the group given D-GalN/TNF- α and Z-FA.FMK a significant decrease in the number of both TUNEL and caspase-3 positive hepatocytes and a significant increase in the number of PCNA positive hepatocytes were determined. As a result, microscopic evaluations indicate that cathepsin B contributes to TNF- α mediated hepatocyte apoptosis and inhibits hepatocyte proliferation in D-GalN/TNF- α induced liver injury model.

Molecular determinants of the apoptosis induced by interferon alpha in human myeloma cell lines

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Interferon- α (IFN α) has been used for more than 20 years for the therapy of multiple myeloma (MM), a still incurable disease. While some patients benefit from this treatment, others exhibit no significance response. Congruent with these observations, IFN α induces apoptosis in some, but not all, myeloma cell lines. Thus, understanding the mechanism(s) of IFN-induced apoptosis could be useful in establishing criteria for eligible patients for IFN therapy. It has been reported that IFN could exert its apoptotic effects by inducing the synthesis and secretion of Apo2L/TRAIL. We have analysed the sensitivity to IFN α of five different MM cell lines and found no correlation between the sensitivity to IFN α and Apo2L/TRAIL. Pre-treatment of sensitive cell lines with IFN α for 48 h induced apoptosis that was not associated with synthesis or secretion of TRAIL. Some cell lines became less sensitive to Apo2L/TRAIL after IFN α pre-treatment. IFN induced apoptosis was not prevented by co-incubation with the blocking anti-Apo2L/TRAIL antibody 5C2. The pan-caspase inhibitor Z-VAD-fmk and the caspase-8 selective inhibitor Z-IETD-fmk completely abolished apoptosis induced by Apo2L/TRAIL, but only reduced slightly the toxicity of IFN α . On the other hand, PI3-kinase inhibitors prevented IFN α apoptosis with no significant effect on TRAIL apoptosis. Rapamicyn blocked IFN α induced apoptosis in one of two sensitive MM cell lines. IFN α treatment induced proapoptotic conformational change of Bak protein but not of Bax. Levels of Mcl-1 protein did not significantly change upon IFN α incubation. We propose that IFN α uses the intrinsic pathway to induce apoptosis and that one or more BH3-only proteins, inducible by IFN, could be the key mediator(s).

Influence of autocrine factor deficit on energy metabolism and survival of CTLL-2 cells under oxidative stress

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Last time a lot of data show that survival of mammalian cells is under the control of growth factors and autocrine survival factors (AF). We studied the influence of AF deficit on survival, intracellular ATP content and transmembrane potential of mitochondria of IL-2-dependent CTLL-2 cells under oxidative stress. Autocrine factor deficit in cell culture was formed by primary cultivation of CTLL-2 cells at high density (1×10^6 cells in a pellet) for 12–14 hrs and following transfer of the cells with fresh medium into low density culture (1×10^5 cells/ml).

The cells cultivated under deficit of AF have been shown to be more susceptible to oxidative injury in comparison with CTLL-2 cells cultivated without deficit of AF (control) and their death came at smaller concentrations of H_2O_2 than in control cell case. Cell surface blebbing of experimental cells occurred at smaller concentrations of H_2O_2 than control cells too. The ATP content in CTLL-2 cells was depressed under AF deficit conditions even without any stress and treatment of the cells by hydrogen peroxide resulted in additional large decrease of it. Autocrine factor deficit in cell culture strongly affected on transmembrane potential of mitochondria of CTLL-2 cells under oxidative stress. It caused the appearance of the cells with depolarization of mitochondria and portion of such cells increased with time.

These data indicate deficit of AF in cell culture causes the decrease of intracellular ATP content which intensifies oxidative injury to CTLL-2 cells and accelerates cell death.

Induction of the intrinsic apoptotic pathway by inhibition of protein kinase CK2 in LNCaP prostate cancer cells

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Protein kinase CK2 is a ubiquitous serine/threonine kinase participating in a variety of cellular processes like transcription, proliferation, cell cycle regulation and apoptosis. CK2 is composed of two regulatory β -subunits and two α and/or α' -subunits. Its activity is upregulated in highly proliferating cells and so it is not surprising that in many tumours CK2 protein and activity is elevated.

Prostate cancer is the most common cancer among elder men in Western countries. The identification of signalling pathways that play a dominant role at certain stages of the disease is a prerequisite for a successful therapy. Components of the signalling pathways are mostly regulated by phosphorylation and dephosphorylation. Among these kinases is protein kinase CK2. We knew from recent work that inhibiting CK2 induces apoptosis in LNCaP prostate cancer cells. We now analyzed the mechanisms leading to programmed cell death. By treatment of the cells with emodin apoptosis was induced over a period of 24 hours as observed by PARP cleavage. DNA-fragmentation was shown after 48 hours by TUNEL-staining. Apoptosis induction went along with the stabilization of the growth suppressor protein p53.

Since we found no indication for a cleavage of Bid and on the other hand a release of cytochrome c from mitochondria during the apoptotic response we conclude that inhibition of CK2 by emodin leads to the induction of the intrinsic pathway of apoptosis in prostate cancer cells.

siRNA experiments revealed that repression of CK α seems not to be sufficient for induction of apoptosis. Experiments inactivating CK2 α' subunit as well as both catalytic subunits by siRNA are in progress.

Apico-basolateral polarity is a major determinant of the outcome of Fas/CD95 apoptotic signalling in hepatocytes

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Maintenance of epithelial cell shape and polarity determines many vital cell functions, including the appropriate response to external stimuli. Hepatocytes are epithelial cells of complex polarity : each cell contains several small apical membrane domains delimited by tight junctions. In vivo, the apical domains of adjacent cells align to form specialised structures called bile canaliculi. Primary or immortalised hepatocytes maintained in standard monolayer cultures are flat and largely lose both the apico-basolateral polarisation and their differentiated phenotype. In sharp contrast to their sensitivity to death receptor stimulation in vivo, cultured murine hepatocytes are resistant to Fas/CD95 engagement in culture.

In order to examine the impact of cell polarity on the sensitivity to apoptosis signalling, we have set up a three-dimensional culture system in which murine hepatocytes formed highly polarised organoids, with apical domains surrounding one or several lumens. The hepatocyte organoids were functional, as judged by the high level of albumin secretion and the accumulation of bilirubin.

Stimulation of the Fas/CD95 death receptor, which is highly hepatotoxic in vivo, was a strong inducer of apoptosis in the polarised organoids. This was in sharp contrast to the monolayer hepatocyte cultures, which were protected from death by an exacerbated NF- κ B signalling following engagement of the death receptors. Thus, hepatocytes in polarised, functional organoids modulate intracellular signal transduction pathways that determine their sensitivity to apoptotic stimulation. Experiments are in progress to define the molecular basis of altered NF- κ B signalling in polarised versus de-differentiated hepatocytes.

Interestingly, our results are in contrast to the reported behaviour of mammary epithelial cells, which become resistant to a range of apoptotic stimuli when organised into polarised acini-like structures. This difference recapitulates the in vivo sensitivity of the two types of epithelial cells to Fas death receptor stimulation, strengthening the argument of the physiological relevance of our data.

Implication of PTEN in neuronal apoptosis induced by oxygen and glucose deprivation (OGD)

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The tumor suppressor PTEN (phosphatase and tensin homologue deleted on chromosome 10) is a lipid and protein phosphatase. Its pro-apoptotic function has been mostly studied in several tumors and is associated to its capacity antagonizing PI3K/Akt signalling. Increasing evidence points out the importance of PTEN in maintenance of CNS development. However, little is known about its role in regulating neuronal apoptosis under pathological conditions. The aim of this study was to characterize the role of PTEN in neuronal apoptosis after the onset of oxygen and glucose deprivation (OGD), which mimics many aspects of cerebral ischemia. OGD was carried out for 4 h in cultures of embryonic cortical neurons and primary hippocampal cells from rat. Staining with Hoechst 33258 revealed a significant increase in apoptotic damage 16 h after re-oxygenation. In addition, we examined the expression and phosphorylation status of PTEN. Western blots demonstrated that the level of total PTEN was not changed from 2 h to 24 h after re-oxygenation. Interestingly, a significant decrease in the level of P-PTEN was observed in a parallel experiment, accompanied by a decrease in the levels of P-Akt and P-Erk_{1/2}, suggesting an increase in PTEN activity after OGD. Moreover, knockdown of PTEN by using a specific antisense significantly protected hippocampal cells from apoptotic damage induced by OGD. In addition, PTEN knockdown resulted in enhanced levels of P-Akt and P-Erk_{1/2}. Our findings suggested that PTEN was implicated in neuronal apoptosis induced by OGD. We conclude that knockdown of PTEN can enhance survival signalling pathways thereby providing an anti-apoptotic effect in neurons. PTEN could become a potential target for the therapy of stroke.

Translational upregulation of Apaf-1 via Internal Ribosome Entry site is required for UV-induced apoptosis

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Components of the cellular translation machinery, including the initiation factor eIF4G, are targets of caspase-mediated cleavage during apoptosis that correlates with the inhibition of protein synthesis that accompanies apoptosis. Paradoxically, however, protein synthesis is required for apoptosis to occur in many experimental settings. Previous studies showed that two proteins that regulate apoptosis by controlling caspase activity, XIAP and Apaf-1, are translated by a unique, cap-independent mechanism. This cap-independent translation is mediated by an IRES (Internal Ribosome Entry Site) elements that are found in the 5' UTR of XIAP and Apaf-1 and are used preferentially under conditions in which normal cap-dependent translation is repressed, such as during cell cycle, apoptosis or a broad range of cellular stresses.

The present study investigated the ability of UV irradiation, as an apoptotic trigger, to modulate activity of XIAP and Apaf-1 IRES and the mechanisms behind the IRES activation. We show that UV irradiation leads to the inhibition of proteins synthesis and cell death. In contrast, the cell survival is greatly enhanced by pre-treatment of cells with protein-synthesis inhibitor cycloheximide suggesting that protein synthesis is required for apoptosis to occur. We further show that the IRES-mediated translation of Apaf-1, but not XIAP, is enhanced by UV irradiation and that this increase in Apaf-1 translation is necessary for apoptosis. The enhanced Apaf-1 IRES translation is independent of caspase-activation but instead depends on PERK kinase. These data suggests that progression of UV-induced apoptosis requires IRES-mediated translation of Apaf-1 that ensures continuous levels of Apaf-1 despite the overall suppression of protein synthesis.

High effectiveness of platinum(IV) complex with adamantylamine in overcoming resistance to cisplatin and suppressing proliferation of ovarian cancer cells in vitro

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[(OC-6-43)-bis(acetato)(1-adamantylamine)amminedichloroplatinum(IV)], coded as LA-12, is a novel octahedral platinum(IV) complex containing a bulky hydrophobic ligand - adamantylamine. The use of bulky hydrophobic amines as a non-leaving ligands, may increase penetrability of the compound to the cancer cells. Therefore, the effects of LA-12 on sensitive(A2780), with acquired resistance(A2780cis) and with intrinsic cisplatin resistance(SK-OV-3), ovarian cancer cell lines were investigated and compared to those of cisplatin. IC₅₀ and IC₉₀ concentrations of LA-12 were 6-(A2780) or 18-fold(A2780cis) lower than those for cisplatin (MTT assay). Equitoxic concentrations (IC₅₀ or IC₉₀) of both compounds caused a significant and similar time- and dose-dependent inhibition of cell proliferation and an increase in the number of floating cells which corresponded to the decrease of total cell viability. A different type and dynamics of cell cycle perturbation after cisplatin and LA-12 treatment were detected. Exposure to LA-12 resulted in transient accumulation of A2780 and A2780cis cells in S phase, while cisplatin caused G₂/M arrest in sensitive and S phase arrest in resistant cells. A relatively low rate of apoptosis after exposure to IC₅₀ or IC₉₀ of both complexes was observed, markedly higher in resistant A2780cis cells. Western blot analysis indicated a concentration-dependent p53 level increase in both lines(higher after cisplatin treatment). PARP cleavage was observed only in A2780cis cells. In conclusion, LA-12 was found to be significantly more efficient than cisplatin, and it was able to overcome the acquired cisplatin resistance(showing resistance factor 2.84). In spite of the low rate of apoptosis, LA-12 caused increase of p53 level and cell cycle perturbations in the ovarian cancer cell lines studied.

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Identification and characterization of caspase-14 substrates

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Caspase-14 is the most recently discovered member of the caspase-family. Although caspases are normally ubiquitously expressed, the expression profile of caspase-14 appeared to be restricted to the differentiating keratinocytes in skin, the Hassall's bodies in the thymus and keratinizing cells of the forestomach in mice. Active cleaved caspase-14 has been shown in skin, but so far no *in vivo* substrates of caspase-14 have been reported.

By means of different proteomic approaches we want to identify caspase-14 substrates, in order to unravel the physiological role of caspase-14 during terminal keratinocyte differentiation. Preferentially we made use of the non-gel COFRADIC technology (combined fractional diagonal chromatography) (Gevaert et al., 2003). Epidermal lysates derived from wild-type mice and caspase-14 knock-out mice are compared differentially. After a tryptic digest the epidermal proteins isolated from caspase-14 wild-type skin are labelled with light isotopes (O¹⁶) and the epidermal extract derived from caspase-14 knock-out skin is labelled with heavy isotopes (O¹⁸). After this step both preparations are mixed and the N-terminal peptides of the highly complex peptide mixture are isolated by diagonal chromatography and analysed by LC-MS/MS. Caspase-14 substrates, only present in wild-type samples, will appear as single O¹⁶ peaks. This technology enabled us to identify some potential caspase-14 substrates that need to be confirmed in *in vitro* assays using recombinant caspase-14. Discovery of substrates of caspase-14 will give us insight into the role of caspase-14 in terminal keratinocyte differentiation.

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Isolation and characterization of novel Bcl-xL interacting proteins

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The proto-oncogene Bcl-xL belongs to a class of genes that favours neoplastic transformation not by conferring a proliferative advantage to the transforming cell but rather by drastically limiting its potential to undergo programmed cell death (apoptosis). During neoplastic transformation the capability to escape apoptosis given by the over-expression of the Bcl-xL gene confers resistance to a broad range of potentially apoptotic stimuli, such as the intrinsic apoptotic effect of proliferative oncogene activation, hypoxia and matrix detachment. Bcl-xL over-expression has been also correlated with chemo-resistance. Protein-protein interaction among members of the Bcl-2 protein family is an essential control point in apoptosis, and has been shown to be a promising level of intervention for cancer therapy. To better understand the biochemical implications of Bcl-xL overexpression in tumour development, we have recently started a project aimed at the identification of new Bcl-xL interacting proteins. We performed an extensive screen of membrane proteins interacting with Bcl-xL using the Tandem Affinity Purification (TAP) technology from detergent solubilised total cell membranes. This strategy led to the identification of several novel proteins. We focused our effort on the characterization of one of those, annotated in the database as JM4 and belonging to the PRA1 (Prenylated Rab Acceptor) protein family. The ability of JM4 to interact with Bcl-xL was confirmed by co-immunoprecipitation using a myc-tagged JM4 protein. Fusion of JM4 with GFP shows a marked perinuclear staining, with partial colocalisation with the Golgi and the Rab7-positive late-endosomal compartment. Transient transfection of the JM4 cDNA in HeLa cells results in a time dependent induction of apoptotic cell death, which is prevented by the co-transfection with the Bcl-xL cDNA or treatment with the caspase inhibitor zVAD. Strikingly knock-down of JM4 expression by RNA interference reproducibly decreases the sensitivity of HeLa cells to cell death induced by glucose starvation and the chemotherapeutic drug etoposide. We are currently investigating the possibility that JM4 acts as a GDI displacement factor for Rab7 and can influence susceptibility to cell death by modulating the balance of recycling vs degradation of membrane receptors and/or transporters.

Abrogation of caspase-independent cell death (CICD) by TPCK or TLCK (unlike to UCF101) is not mediated by Omi/HtrA2

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Serine protease inhibitors (UCF101, TLCK, TPCK, AEBSF) can hamper several forms of caspase-independent cell death (CICD). UCF101 is familiar as to be specific for HtrA serine proteases. The less selective inhibitors (TPCK, TLCK) were usually so far also assumed to inhibit the mitochondrial Omi/HtrA2 protease known to play role in CICD. Contradicting to this assumption we found using bacterially expressed recombinant Omi/HtrA2 protease (peT Omi-134-458) that TPCK and TLCK did not inhibit Omi/HtrA2 even at much higher concentration (cc) than required them to abrogate CICD.

Previously we have shown that staurosporine (1 μ M) can induce CICD in the presence of pan-caspase inhibitor, Z-VAD(OMe)-FMK (50 μ M) in HL-60 leukemia cells in forms of apoptosis or necrosis after 8 hrs exposure (Cell Death Differ 11: 1357, 2004). In this study we show that UCF101 (30 μ M) abrogated both forms of CICD in parallel. However, the effects of the unselective serine protease inhibitors were different: TPCK abrogated CICD-apoptosis at 3 μ M while CICD-necrosis only at >30 μ M cc; TLCK abrogated CICD-necrosis at 100 μ M but not CICD-apoptosis. In the case of UCF101 the required cc to abrogate cell death was comparable to the cc required to inhibit the activity of the recombinant Omi/HtrA2 protease (20 μ M) in a 15 min. inhibition test, ex vivo. In contrast with UCF101, the unselective inhibitors did not inhibit recombinant Omi/HtrA2 protease even at one order higher cc that required them for abrogation of cell death (TPCK > 400 μ M, TLCK > 800 μ M) in a 2 hrs ex vivo test.

Our results indicate that TPCK or TLCK serine protease inhibitors suspend the CICD pathway by targeting a (serine) protease that is distinct from Omi/HtrA2.

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Members of the MAGUK family are substrates for the executioner caspases and cathepsins, but not for serine protease Omi

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MAGUKs (membrane associated guanylate kinases) are a large family of proteins involved in sequestering protein complexes at the plasma membrane, signal transduction pathways and formation and maintenance of different cell-cell contacts. Members of this family occur in all multicellular organisms and have specific domain organization – several PDZ domains, a SH3 or WW domain and GUK (guanylate kinase) domain. Since a dying cell during apoptosis isolates herself from the neighboring cells, thus disrupting cell-cell contacts, and MAGUKs with their protein-protein interaction domains function as scaffolding proteins at cell-cell contacts, it is believed that they are cleaved by caspases during apoptosis and/or other proteases that are involved in apoptosis.

We and some other groups have shown, that some members of MAGUK family are cleaved by the executioner caspases during apoptosis. In in vitro experiments we have shown that MAGI-1, MAGI-2, MAGI-3, DLG1, PSD-95, PSD-93, SAP-102, ZO-1, ZO-2 and ZO-3 are cleaved by caspases-3, -6 and -7 and degraded by several cathepsins (lysosomal proteases). Serine protease Omi did not cleave any of the MAGUKs tested. By inducing the apoptosis with UV and staurosporine in different epithelial cell lines (HaCaT, MDCK, CaCo-2,...), we showed that caspases can also cleave MAGI-1, DLG1, ZO-1 and ZO-3 in different cell models. Cleavage of MAGUKs could be prevented by 20 µM z-VAD-fmk, a pan-caspase inhibitor. Using a selective lysosomal disrupting agent LeuLeuOMe, which induces apoptosis by translocating the lysosomal proteases to the cytosol, where they can cleave proapoptotic Bcl-2 homologue Bid, we have further shown that DLG1, ZO-1 and ZO-3 are cleaved/degraded in HaCaT and CaCo-2 cells. This cleavage/degradation could be prevented by 20 µM E-64d, an inhibitor of papain-like cysteine proteases, but not by 20 µM z-VAD-fmk. Immunohistological stainings in HaCaT, MDCK and CaCo-2 cells showed that DLG1, ZO-1 and ZO-3 proteins are localized to cell membrane and that after induction of apoptosis with various apoptotic agents localization of DLG1, ZO-1 and ZO-3 is disrupted, which correlates with the loss of cell-cell contacts. The same was shown in HaCaT cells transfected with fusion constructs of MAGI-1 and MAGI-3 with EGFP, but not in cells transfected with the dominant negative mutant of MAGI-1 (D761A).

Role of the alternative NF- κ B pathway in the control of apoptosis

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RelB belongs to the family of NF- κ B transcription factors that are key regulators of inflammatory and immune responses, but also controls cell growth, apoptosis and tumor development. The activity of NF- κ B is activated by at least two different signaling pathways with different kinetics: the canonical pathway that relies mostly on the IKK β - and IKK γ -dependent I κ B α degradation which results in the rapid but transient activation of mainly RelA containing dimers, and the alternative pathway which proceeds via the IKK α -induced p100 processing, thus leading to a late but sustained activation of both RelB/p52 and RelB/p50 dimers. It is well characterized that activation of RelA antagonizes apoptosis through the activation of pro-survival genes. In contrast, how RelB contributes to the control of apoptosis is unknown. We have screened high density microarrays using as probe total RNA extracted from wild type and RelB-deficient mouse embryonic fibroblasts either untreated or stimulated by lymphotoxin β , an inducer of the alternative NF- κ B pathway in these cells. Semi-quantitative RT-PCR verified expression changes in 20 genes and revealed three distinct classes of genes: genes that are inhibited by RelB, genes that are activated by RelB and genes whose constitutive level is regulated by RelB. These genes include cytokines and chemokines, but also genes that are involved in the control of cell cycle and apoptosis. Future work will be aimed at studying RelB in these processes as well as its potential in the development of hematopoietic malignancies.

Keywords : NF-kappaB, RelB, Lymphotoxin β , gene expression regulation, apoptosis

H1 histamine antagonists induce phospholipase C dependent programmed cell death through modulation of Ca⁺² homeostasis in human malignant melanoma cells but not in normal melanocytes

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Histamine is a well known biogenic amine. Recently, it has been proved that histamine has an important role as a mediator in normal and malignant cell proliferation. Previously we found that the H1 histamine antagonist diphenhydramine induces apoptosis in human acute T- lymphocytic leukaemia cells (Jangi et al, 2004). Human melanoma cells have been reported to contain high activity of histidine decarboxylase, the unique enzyme responsible for histamine synthesis and RNA expression. In the current study we tested the effect of diphenhydramine and other specific H1 histamine antagonists like terfenadine (TEF) and astemizol on human malignant melanoma cells. Here, we show that *in vitro* treatment with H1 histamine antagonists rapidly induced apoptosis in several different human malignant melanoma cell lines, namely A375, HS294T, HT144 and MJOI. Apoptosis was determined by DNA fragmentation assays and phosphatidylserine exposure detected by Annexin V binding to the surface of the cells. Importantly, H1 antagonist treatments did not adversely affect the viability of human melanocytes and murine fibroblasts at the same doses and time points. Treatment of melanoma cells with TEF induced an increase in the concentration of intracellular Ca⁺² resulting from depletion of the endoplasmic reticulum Ca⁺² stores, followed by caspase-2, -3, -6, -8 and caspase-9 activation. General caspase inhibitor (z-VAD-FMH) and selective inhibitors of caspase-2 (z-VDVAD-FMK) protected melanoma cells from TEF-induced apoptosis. Conversely, a caspase-8 inhibitor (z-IETD-FMK) had non-significant effect, which argued against an important role for caspase-8 and was consistent with the finding that death receptors were not involved in TEF-induced apoptosis. Furthermore, we found that mitochondria plays an important role in the apoptotic process, which was characterized by the dissipation of mitochondrial transmembrane potential, caspase-9 activation and the release of cytochrome c from mitochondria into the cytosolic compartment. Interestingly, we found that phospholipase c inhibitor neomycin prevent elevation of cytosolic Ca⁺² levels and protect melanoma cells completely from apoptosis-inducing effect of TEF. In addition, by using ultrastructural analysis by electron microscope, we found typical characters of autophagic cell death, like multiple vacuoles formation and autophagosomes. We conclude that H1 histamine antagonist kill human malignant melanoma cells by several types of programmed cell death by triggering the mitochondrial pathway of apoptosis at least in part through the generation of reactive oxygen species. We conclude that H1 histamine antagonists kill human malignant melanoma cells by different types of programmed cell death and the death way process is triggered by caspase-2 activation and it is phospholipase C dependent.

Cell death induced by the viral protein Apoptin is modulated by Bcl-2 family members and Apaf-1 dependent

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Apoptin, a chicken anemia virus-derived protein, selectively induces apoptosis in transformed but not in normal cells, thus making it a promising candidate of a novel anticancer therapeutic. The mechanism of apoptin-induced apoptosis is largely unknown. Contrary to previous assumptions, we observed that Bcl-2 and Bcl-x_L inhibited apoptin-induced cell death in several tumor cell lines. Furthermore, Bax expression sensitized cells to apoptin-induced cell death, whereas deficiency of Bax conferred resistance. Cell death induction by apoptin was associated with cytochrome c release from mitochondria as well as with caspase-3 and -7 activation. zVAD-fmk, a broad spectrum caspase inhibitor, was highly protective against apoptin-induced cell death. Apoptosis induced by apoptin required Apaf-1, as immortalized Apaf-1-deficient fibroblasts as well as tumor cells devoid of Apaf-1 by siRNA were strongly protected. Thus, our data indicate that apoptin-induced apoptosis is not only Bcl-2- and caspase-dependent, but also engages an Apaf-1 apoptosome-mediated mitochondrial death pathway.

Signalling pathway involved in the apoptotic effect of dopamine in the GH3 pituitary cell line

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At the end of the lactation, massive apoptosis of lactotroph cells occurs in pituitary gland. The factor(s) responsible for this phenomenon is (are) still largely unknown. Dopamine (DA) is the main regulator of lactotrophs by inhibiting, via its D2 receptor, the synthesis and release of prolactine as well as the cell proliferation. Moreover, DA is known to induce apoptosis in various cell types via its transporter (DAT). The aim of the present study was to test the pro-apoptotic effect of DA in a model of lactotroph cells, the GH3 cell line.

To this end, different features of apoptosis were investigated. Thus, we show that DA induces in these cells 1) loss of the mitochondrial potential, 2) translocation of Bax from cytosol to mitochondria, 3) cytochrome c release from mitochondria to cytosol 4) caspase 3 activation and 5) nuclear fragmentation, in a time and dose dependent manner. We then tried to determine the mechanism involved in DA-induced apoptosis. Until now, the expression of DAT in pituitary was controversy, however our results show that DAT is expressed in GH3 cells and is involved in pro-apoptotic effect of DA since apoptosis is significantly reversed in presence of mazindol (a DAT inhibitor). In neurons, it is well established that DA triggers apoptosis by ROS formation and oxidative stress. In GH3 cells, direct measurement of intracellular ROS show that DA increases rapidly their formation. Moreover, co-incubation of DA with NAC (an anti-oxidant) completely inhibits the apoptotic effect of DA. Neither JNK nor P38 are involved in this process, however we find that MPT is likely the target of the ROS formed by DA in these cells.

These data provide first evidence that DA is able to trigger apoptosis in pituitary cells by a mechanism involving DAT and oxidative stress. These findings could be particularly relevant to understand the plasticity of lactotrophs during the post-natal life.

Alteration of spleen Pan T cells, CD4 cells, MHC Class II molecule expressed cells and macrophages after chronic methanol intoxication

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Background & Rationale:

Severe exposure to methyl alcohol leads to liver damage, blindness, and finally death. The adverse effects of methanol toxicity receive attention only when severe signs and symptoms of mass methanol intoxication manifests in or after death. Its action on the immune system remains unexplored.

Aim & Objectives:

The present study has been carried out to explore the effects of chronic methanol intoxication on the immune system.

Material & Methods:

Male wistar albino rats (200-220gm) were exposed to methanol (2.37gm/Kg body weight in saline/day IP) for 30 days and control, immunized control animals that were killed on 31st day. Splenic cell suspension was prepared and were isolated using Midi- MACs system using protocols suggested by the manufacturers (Miltenyi Biotec, Glagbach, Germany). The results were analyzed by one way analysis of variance (ANOVA) followed by Tukey's multiple comparison.

Results & Conclusions:

In chronic methanol exposed as well as in immunized chronic methanol exposed groups there was a significant decrease ($p < 0.05$) in the cell count of pan T cell, CD4 and macrophages, when compared to the control group as well as with the immunized control group. However chronic methanol exposure was not found to alter the MHC class II molecule expressions. To conclude, chronic exposure of methanol at the aforescribed dosages was found to affect spleen pan T cells, CD4 cells, and macrophages with out affecting the MHC II molecule expression (Financial assistance by UGC, New Delhi is gratefully acknowledged).

Induction of apoptosis and autophagy in ALCAM/CD166 deficient breast cancer MCF-7 cells

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An adhesion molecule ALCAM/CD166 (Activated Leukocyte Cell Adhesion Molecule) is expressed in different kinds of normal and neoplastic tissues. Our studies on tissue distribution of ALCAM/CD166 suggest that this protein is suppressor of breast cancer cells invasion. Apoptosis and autophagy are two forms of programmed cell death that play important roles in the removal of unneeded and neoplastic cells. While these two forms of programmed cell death are morphologically distinct, our studies indicate that apoptotic and autophagic cell death utilize some common regulatory mechanisms. Using laser scanning cytometry and confocal microscopy as well as western blot technique we have shown that presence of ALCAM/CD166 is associated with programmed cell death regulation. Overexpression of *bcl2* gene (major apoptosis inhibitor) in: estrogen-dependent breast cancer cell line MCF-7 (MCF-7 *bcl2/neo*) but also in metastatic breast cancer cell line MDA MB 231 (MDA MB 231 *bcl2/neo*), and reference breast cell line HBL-100 (HBL-100 *bcl2/neo*), significantly increased ALCAM expression accompanied by decrease of MMP2 (Matrix Metalloproteinase 2) degradation protease activity. Appearance of ALCAM protein was surprisingly denoted in HBL-100 *bcl2/neo* in contrast to almost uncovered level in HBL100. *ALCAM* gene silencing in MCF-7 cell line [MCF-7 *ALCAM* (-)] significantly decreased concentration of BCL-2 and increased levels of apoptosis (89kDa PARP, active kaspase7) as well as autophagy (MAP1 LC3, Beklin 1) markers. Those data suggest that high expression of ALCAM/CD166 promotes breast cancer cells survival by restrain cell death pathways. These studies focus on meaning of adhesion molecule in apoptosis and autophagy and are an attempt of explaining how this knowledge can be transformed into effective treatments of breast cancer.

Identification of autoimmunity susceptibility genes by ENU mutagenesis

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The genetic factors involved in autoimmunity remain unresolved. We have used ENU mutagenesis in an attempt to identify autoimmunity susceptibility genes in mice by screening for anti-nuclear antibodies (ANA). ANA are a defining feature of Systemic Lupus Erythematosus and have also been observed in some cases of Autoimmune Lymphoproliferative Syndrome. Peripheral T cells from this pool of mutants with ANA were then assessed for proliferation to CD3 and CD28 antibodies, antigen-induced cell death (AICD), and cytokine withdrawal death defects. Five strains were identified with a variety of defects in these assays. Notably, one strain was identified with ANA, decreased AICD, and increased cell death upon cytokine withdrawal. Further analysis of this strain revealed normal cell death upon gamma-irradiation but poor cell death upon Staurosporine and Etoposide treatment. Mapping crosses to identify the specific genetic mutation along with relevant cellular and biochemical experiments are underway for all five strains.

Cisplatin effects on apoptosis and cytoskeleton derangement in LLC-PK are mediated by peroxynitrite formation

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Cisplatin-induced renal injury is associated with apoptosis induction and cell detachment by cytoskeletal alterations in pig renal tubular epithelial cells (LLC-PK). Generally, it is supposed that cisplatin coordinates to DNA and that this complex not only inhibits replication and transcription of DNA, but also leads to apoptosis. During apoptotic processes in renal cells, it was also seen that the cytoskeleton collapses because of the cleavage of cell-adhesion molecules which leads to loss of contact with neighboring cells, proteolysis, disassembly and reorganization of actin-filaments. It is also well known that oxidative and nitrosative stress to the cell alters many cellular programmes. In cisplatin-treated organisms, the renal content of total nitrate/nitrite was found to be increased supposing that nitric oxide production is enhanced. It is known that nitric oxide reacts with the radical superoxide anion under stress conditions to form peroxynitrite which could nitrify other proteins on tyrosine residues and alternate thus their function in the cell. Taken account all the above, we hypothesizes that peroxynitrite might be involved in cisplatin-induced renal cell apoptosis and cytoskeleton derangement.

Functional interactions between apoptotic nuclease DFF40/CAD and histone H1

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The major apoptotic nuclease, DNA fragmentation factor (DFF40/CAD), is primarily responsible for internucleosomal DNA cleavage during the terminal stages of programmed cell death. Previously we have demonstrated that several chromatin proteins, including HMGB1/2, histone H1 and topoisomerase II, greatly enhances naked DNA cleavage by this nuclease *in vitro* (histone H1 stimulates DFF40/CAD cleavage of DNA ~20-fold). Here we investigate the mechanism of stimulation of DNA cleavage by histone H1. Addition of histone H1 either during or after caspase-3 treatment of DFF causes the same stimulatory effect on DNA cleavage, indicating that histone H1 affects DFF40/CAD enzyme activity, but not caspase-3-dependent activation of the nuclease. We have found that each of the six somatic cell histone H1 isoforms, which differ in primary sequence, equally activate DFF40/CAD. Using a series of truncation mutants of recombinant mouse histone H1-0, we demonstrate that the H1-0 C-terminal domain (CTD) is responsible for activation of DFF40/CAD. We show further that the intact histone H1-0 CTD and certain synthetic CTD fragments bind to DFF40/CAD. These interactions enhance the ability of DFF40/CAD to bind to DNA. We have concluded that the interactions between the histone H1 CTD and DFF40/CAD target and activate linker DNA cleavage during the terminal stages of apoptosis.

Effects of leptin on rat testes development and maturation

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Physiological role of leptin in human regarding with the insulin resistance in hypothalamic-pituitary function becomes clear at present. It has been claimed that plasma leptin levels which increase rapidly (just) before puberty in male and female play an important role in the onset of puberty. Our aim was to investigate the effects of leptin on seminiferous tubule development and maturation and onset of puberty

This study was performed on 40 male newborn rats, as 20 for controls and 20 for the study group. In the study group 0.25 µg/ml of leptin was given subcutaneously twice a day for 14 days starting from the first day of birth. The rats were decapitated on the 15, 25 35 and 45 days, 5 rats from both the study group and controls in each time and testes were removed. Light microscopic follow-up was applied after the tissues were fixed in bovine's solution. In order to evaluate the presence of apoptosis TUNEL method was performed by labelling DNA strand breaks with the in-situ cell death detection kit. No significant difference was detected between the leptin-administered group and controls.

Key Words : Testes, TUNEL, Puberty

Effects induced by chemical hypoxia in primary cultures of astrocytes isolated from A₃ adenosine receptor knock-out and wild type mice

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Adenosine acts as a neuromodulator in the brain through the activation of four distinct G-protein-coupled (A₁, A_{2A}, A_{2B} and A₃) adenosine receptors (AR). Under stress conditions adenosine levels get substantially elevated. The A₃AR has been found to mediate both protection and cell death depending on the degree of receptor activation and type of toxic insult. We studied the role of A₃AR by comparing the effects of hypoxia-like conditions in primary astrocytes from wild type and A₃AR knock-out mice. Chemical hypoxia was induced by exposure to cobalt chloride (CoCl₂), which caused a dose-dependent ATP depletion. Occurrence of apoptosis was indicated by phosphatidylserine exposure on the plasma membrane and the appearance of condensed nuclei with DNA fragmentation. Necrotic cells were also detected. We observed increased expression of genes regulated by Hypoxia-Inducible Factor-1 α (HIF-1 α), such as pro-apoptotic factor Nip3 and inducible nitric oxide synthase (iNOS). Pre-incubation of astrocytes with bongkreikic acid reduced ATP depletion significantly, indicating an action of Nip3 on mitochondria permeability transition pore opening. Caspase activation contributed modestly to apoptosis, as shown by low levels of cleaved PARP and lack of protective effect of the inhibitor Z-VAD-FMK. Co²⁺ increased ERK 1/2 phosphorylation, but inhibition of MEK1 did not prevent cytotoxicity. Pre-treatment with antioxidants did not reduce the damage, suggesting that oxidative stress is not likely to trigger cobalt toxicity. ATP depletion, chromatin rearrangements and membrane permeability alterations did not differ between the wild type and A₃AR knock-out astrocytes. In conclusion, chemical hypoxia induces several features also associated with the deleterious effects of low oxygen *in vivo*, e.g. cell death by apoptosis and necrosis, HIF-1 α stabilization and increased expression of Nip3 and iNOS. Since we observed no differences in cobalt-induced toxicity in A₃AR knock-out astrocytes we propose that A₃AR does not play a major role in susceptibility of astrocytes to hypoxia.

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Betulinic acid as new activator of NF- κ B: Molecular mechanisms and implications for cancer therapy

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Recent evidence demonstrates that the anticancer activity of Betulinic acid (BetA) can be markedly increased by combination protocols, for example with chemotherapy, ionizing radiation or TRAIL. Since Nuclear factor-kappaB (NF- κ B), a key regulator of stress-induced transcriptional activation, has been implicated in mediating apoptosis resistance, we investigated the role of NF- κ B in BetA-induced apoptosis. Here, we provide for the first time evidence that BetA activates NF- κ B in a variety of tumor cell lines. NF- κ B DNA-binding complexes induced by BetA consisted of p50 and p65 subunits. Nuclear translocation of p65 was also confirmed by immunofluorescence microscopy. BetA-induced NF- κ B activation involved increased IKK activity and phosphorylation of I κ B- α at serine 32/36 followed by degradation of I κ B- α . Reporter assays revealed that NF- κ B activated by BetA is transcriptionally active. Interestingly, inhibition of BetA-induced NF- κ B activation by different chemical inhibitors (proteasome inhibitor, antioxidant, IKK inhibitor) attenuated BetA-induced apoptosis. Importantly, specific NF- κ B inhibition by transient or stable expression of I κ B- α superrepressor inhibited BetA-induced apoptosis in SH-EP neuroblastoma cells, while transient expression of I κ B- α superrepressor had no influence on BetA-induced apoptosis in two other cell lines. Thus, our findings that activation of NF- κ B by BetA promotes BetA-induced apoptosis in a cell type specific fashion indicate that NF- κ B inhibitors in combination with BetA would have no therapeutic benefit or could even be contra-productive in certain tumors, which has important implications for the design of BetA-based combination protocols.

Investigating the role of the pro-apoptotic BH3-only protein BID in the hematopoietic compartment and in death receptor mediated hepatitis

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BH3-only members of the Bcl-2 family initiate programmed cell death (apoptosis) by acting as molecular sensors for various intracellular stress signals. Bid is a BH3-only protein reported to connect the death receptor (e.g. CD95/Fas, TNFR1/2) mediated apoptotic pathway to the stress induced apoptotic pathway. Activation of death receptors leads to caspase-8 mediated processing of Bid to its potently pro-apoptotic p15 fragment, tBid, which translocates to and initiates the disruption of the mitochondrion in a Bax/Bak dependent manner. However, whether and in what compartments Bid is essential for death receptor mediated apoptosis *in vivo* is still debated.

Here we report the generation of a *bid* knockout mouse on a pure C57BL/6 genetic background. *Bid*^{-/-} mice appear normal, have normal body weight and organ size and are fertile. *In vitro* death assays on various lymphocyte subsets show no significant resistance for *bid*^{-/-} over wildtype control cells to various stress stimuli, including γ -irradiation, treatment with etoposide, dexamethasone, PMA, ionomycin or recombinant Fas ligand. Consistent with previous results, our *bid*^{-/-} mice are resistant to Fas receptor mediated fatal hepatitis after *i.v.* injection of anti-Fas antibody or, as shown for the first time, recombinant Fas ligand. Interestingly, however, *bid*^{-/-} mice are not resistant to massive T-cell activation induced hepatitis (Con A injection), which is Fas and also TNFR mediated. We are currently generating *bid*^{-/-}*relA*^{-/-} mice to clarify the *in vivo* role of Bid in TNFR1-mediated death as embryos deficient in the NF- κ B subunit *relA* die around E14.5 due to extensive, TNFR1-signalling dependent hepatocyte apoptosis. This will indicate whether, *in vivo*, Bid is a more general player of death receptor induced apoptosis in relevant tissues or whether it is restricted to Fas signalling.

Effects of exendin-4 on pancreatic beta cells regeneration and apoptosis in neonatal STZ-diabetic rats

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Diabetes mellitus are characterized by an insufficient extent of beta (β) cell replication needed to compensate for the loss or dysfunction of β cells occurring in diabetes. There some attempts to cure diabetes by evaluating the regenerative potential in experimentally induced diabetic animal models. One of the experimental models useful to study the regeneration of β cell is the neonatal rat with diabetes induced by streptozotocin (STZ). STZ is selectively cytotoxic to β cells by induction of lesion in DNA, and induces β cell death. Exendin-4 has been shown to promote β cell proliferation and islet neogenesis. In this study, an experimental model was designated to study the apoptotic cell death and regeneration potential of the β cells in exendin-4 treated neonatal STZ (nSTZ) diabetic rats. On the second day birth 100 mg/kg STZ was given i.p to two groups of the newborn rats. First group was diabetic. To the second group starting from third day, 3 μ g/kg/day exendin-4 was given for 5 days. Third group was non-diabetic. The pancreas tissue samples were taken under the ether anesthesia at 7th day then they neutral formalin fixed paraffin embedded. The tissue sections were immunostained with insulin, PCNA and caspase-3 antibodies.

While the expanded caspase-3 expression was seen in nSTZ-diabetic group, the decrease of caspase-3 expression is seen in exendin-4 treated group. PCNA and insulin co-expressed islet β cells were more numerous in exendin-4 treated rats compared to nSTZ diabetic rats. Moreover, in extra pancreatic β cells number insulin and PCNA co-expression were close to the treated and untreated diabetic groups.

Our results suggested that exentin-4 treatment decreases caspase-3 expression and induces beta cell proliferation in neonatal STZ diabetic rats.

INCA, a novel human caspase recruitment domain protein that inhibits interleukin-1 β generation

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Caspase-1 contributes through two independent pathways to the inflammatory response. On the one hand it mediates the maturation of pro-interleukin (IL)-1 β and pro-IL18 through its proteolytic activity while on the other the N-terminal caspase recruitment domain (CARD) is necessary and sufficient for the activation of NF- κ B and p38 MAPK. The latter is dependent on the interaction with RIP2, a CARD-containing serine/threonine kinase involved in multiple NF- κ B activating pathways. Using *in silico* methods for screening the human genome for new CARD proteins, we have identified INCA (Inhibitory CARD) as a protein that shares 81% sequence identity with the prodomain of caspase-1. The *INCA* gene is located on human chromosome 11q22 between the genes of COP/Pseudo-ICE and ICEBERG, two other CARD-only proteins that arose from caspase-1 gene duplications. These gene duplications must have occurred recently, because the mouse and invertebrate genomes do not encode such CARD-only proteins. Here we show that INCA mRNA is expressed in many tissues and is, like procaspase-1, specifically upregulated by interferon- γ in the monocytic cell lines THP-1 and U937. Like COP/Pseudo-ICE and ICEBERG, INCA is able to interact physically with procaspase-1 thereby preventing the maturation and release of pro-IL-1 β from LPS stimulated macrophages. However, unlike COP/Pseudo-ICE and procaspase-1, INCA and ICEBERG no longer interact with RIP2 nor induce NF- κ B activity. Hence, these four homologous CARD-proteins can be divided in two groups, the NF- κ B activators (procaspase-1 CARD and COP/Pseudo-ICE) and the non-activators (INCA and ICEBERG), in order to identify critical amino acids on the surface of the procaspase-1 CARD domain for the interaction with RIP2 and the subsequent activation of NF- κ B.

NF- κ B suppresses mitochondrial permeability transition pore opening and apoptosis of ventricular myocytes during hypoxic injury

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In this report we provide evidence for the operation of the cellular factor NF κ B as a key regulator of the mitochondrial function and the cell death of ventricular myocytes during hypoxia. In contrast to normoxic control cells, ventricular myocytes subjected to hypoxia displayed a 9.1 fold increase ($p < 0.05$) in apoptosis as determined by Hoechst 33258 nuclear staining and vital dyes. Mitochondrial defects consistent with permeability transition pore (PTP) opening, loss of mitochondrial $\Delta\Psi_m$, and Smac release were observed in cells subjected to hypoxia. This was accompanied by a concomitant increase in the post-mitochondrial caspase 9 and caspase 3 activity in hypoxic myocytes. Adenovirus mediated delivery of wild type IKK β (IKK β wt) resulted in a significant increase in NF κ B dependent DNA and gene transcription in ventricular myocytes. Interestingly, cells rendered defective for NF κ B activation with a kinase defective IKK β_{K-M} (IKK β mt) or a non-phosphorylatable form of I κ B α were sensitized cells to mitochondrial perturbations and hypoxic injury. Hypoxia-induced, mitochondrial defect and cell death were suppressed in cells expressing IKK β wt but in not cells expressing the kinase defective IKK β . To our knowledge, the data provide the first direct evidence that IKK β -mediated NF κ B activation suppresses hypoxia-induced cell death of ventricular myocytes through a mechanism that impinges upon the mitochondrial death pathway.

Preincubation of HT-29 cells with 5-LOX inhibitor (MK-886) induces changes in cell cycle and increases of apoptosis after photodynamic therapy with hypericin

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In spite of many specifics, high doses of photodynamic therapy (PDT) can damage surrounding tissue, induce light and drug hypersensitivity, respectively. Progressive anticancer drug is PDT active hypericin – naturally occurred in *Hypericum sp.* Modulation of arachidonic acid metabolism (concretely lipoxygenase pathway) is related with regulation of cell proliferation and type of cell death.

Pre-treatment of HT-29 cells (colorectal adenocarcinoma) for 48h with low toxic concentrations of 5-LOX inhibitor MK-886 induced apoptotic form of cell death as primary in cell population after photodynamic therapy with hypericin.

With the assistance of MTT assay were selected concentration of both anticancer agents (MK-886 and hypericin), which were characteristic with minimal antiproliferative and cytotoxic effect. Subsequently were analysed cytokinetical parameters after their mutual combination (MTT, total cell number, floating cells quantification, viability, cell cycle progression and BrdU incorporation – flow cytometry). Apoptosis was studied morphologically – fluorescence microscopy, changes of PARP expression – western blotting, mitochondrial membrane depolarisation and ROS production – flow cytometry.

Pre-treatment of cells with MK-886 (2.5 μ M) for 48h induced only 0.5% of apoptosis. After photodynamic therapy with hypericin (0.1 μ M) population of apoptotic cells was enhanced to 19%. However, in combination of pretreatment of cells with MK-886 and photodynamic therapy with hypericin increased percentage of apoptotic population to 31% (2.5 μ M MK-886/0.1 μ M hypericin), it presented enhancing more than 60%. Increasing of apoptosis was determined also by changes in mitochondrial potential and distinct PARP (116kDa) cleavage on characteristic 89kDa fragment.

Increasing of MK-886 concentration induced massive changes in cell cycle progression. Pre-treatment of HT-29 cells with MK-886 alone (15 μ M; - 48h) caused accumulation of cells in S-phase (58% in S-phase and 36% in G0/G1 phase). However, after followed hypericin fototoxic action comes to displacement of cells into G0/G1 phase of cell cycle (more than 90% in G0/G1). Reduction of proliferating part of polulation was confirmed by BrdU incorporation.

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Key words: photocytotoxicity, hypericin, MK-886, HT-29, cell cycle, apoptosis

Extensive glycosylation of DR6 regulates its localization at the cell surface

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DR6 (death receptor-6) is a death domain containing receptor of the TNFR (tumor necrosis factor-receptor) superfamily that can participate in the regulation of proliferation and differentiation of T- and B-lymphocytes. The deduced 655-amino acid protein contains N-terminal signaling sequence followed by four cysteine-rich domains (CRD), and a 120-amino acid linker region. The cytoplasmic part encompasses 135-amino acid death domain (DD) and a 150-residue tail [1]. Overexpression of DR6 in HeLa or HEK/293 cells leads to apoptosis, and/or to activation of NF- κ B and stress kinases of JNK/SAPK family. DR6-knockout (DR6^{-/-}) mice have expanded T-cell populations, increased secretion of IL-2 and IL-4 in response to mitogens and grafted DR6^{-/-} HSC induce more severe graft-versus-host disease [2, 3]. DR6^{-/-} B cells show increased proliferation in response to anti-IgM, anti-CD40 or LPS[4]. DR6 ligand was not, in contrast to majority of other TNFR receptors, yet identified.

We found that DR6 is expressed in at least 2 forms of apparent molecular weights approx. 90 kDa and 110 kDa, although its predicted (and even published) molecular weight is only 68 kDa. Subsequent analysis revealed that both N- and O- glycosylation of amino acids in the extracellular part are mainly responsible for the observed differences in apparent molecular weights. Chemical inhibitors and site-directed mutagenesis confirmed that six Asn are N-glycosylated and that there is at least one O-glycosylation site. DR6(p110) form is both N- and O-glycosylated, whereas DR6(p90) form is only N-glycosylated and seems to be an intermediate product of DR6 processing. Inhibition of DR6 glycosylation leads to compromised localization of DR6 at the plasma membrane. TNF α can induce expression of both p110 and p90 forms in prostate tumor cell line LnCAP. Translation of DR6 can be initiated at either Met-1 or Met-25 but eventual alternative translation initiation of DR6 has no effect on its processing or its expression at the cell surface.

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Iron deprivation induces cell death in human Raji cells independently of mitochondrial pathway

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Iron deprivation induces cell death in some tumour cells while other cells are resistant. The aim of the study was to contribute to our understanding of mechanisms involved in cell death induction by iron deprivation in human Raji cells (Burkitt lymphoma). In order to discriminate between changes coupled with cell death induction by iron deprivation and changes resulting just from iron deprivation but unrelated to cell death induction, original Raji cells, sensitive to cell death induced by iron deprivation, and a cells of derived subline Raji/50FeC, resistant to cell death induced by iron deprivation, were employed. Raji/50FeC cells were derived from the original Raji cell line by adaptation in medium with decreasing level of iron source in the form of ferric citrate. Iron deprivation was achieved by cultivation in defined medium without iron source added. First, we tested the activation of executioner caspases (3, 6, 7) and the cleavage of their substrates (PARP, lamin). We detected the activation of caspase-3 and also the cleavage of its substrate PARP in sensitive cells under iron deprivation but not in resistant cells. However, we did not detect the activation of caspase-6 and caspase-7 under iron deprivation. Concerning upstream caspase-9, we detected only a slight increase of caspase-9 activity in sensitive cells under iron deprivation. However, we did not detect any release of cytochrome c from mitochondria and we did not also detect Bax translocation from cytosol to mitochondria in sensitive cells under iron deprivation. We did not demonstrate any change in mitochondrial localization of AIF and endonuclease G due to iron deprivation. It seems that the slight activation of caspase-9 does not represent here a part of signaling pathway leading to the cell death under iron deprivation. We can conclude that the cell death induced by iron deprivation in sensitive Raji cells involves caspase-3 activation but not cytochrome c release from mitochondria.

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The different apoptotic response of gastrocnemius and musculus soleus muscle fibers against to strenuous exercise in rats

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Recently apoptosis, regulated program of the cell death, has gained the interest of many exercise scientists because in addition to necrotic cell death, evidence indicates that apoptotic cell death also occurs with exercise. The exact mechanism of apoptosis during exercise is unclear and various hypotheses are proposed. One hypothesis depends on the triggering of apoptosis by mitochondria (internal pathway); the other depends on the glucocorticoids and cytokines effects (external pathway). To contribute and to clarify on this subject this study was designed to determine predominant apoptotic mechanism in strenuous exercise model (at a speed of 25 m·min⁻¹ and a slope of 5°) in rat (L. Wistar albino), comparing gastrocnemius (low level mitochondria) and soleus (high level mitochondria) muscle fibers. Determination of single stranded DNA (ssDNA), caspase 8, 9 and 3 activities, cytochrome c, lipid peroxidation (thiobarbituric acid reactive substance, TBARS) and reduced/oxidized glutathione ratio (GSH/GSSG) in muscle cytosolic and mitochondrial fractions and plasma IL-6 and TNF- α levels were used as a markers of internal and external apoptotic process in a time dependent manner. The experiments were carried out with forty-nine male, 24 weeks old, L. Wistar rats. After a week of acclimation period, all rats were randomly assigned to one of the following seven groups: rested controls (Group I, n=7), immediately after exercise (Group II, n=7), 3 hours after exercise (Group III, n=7), 6 hours after exercise (Group IV, n=7), 12 hours after exercise (Group V, n=7), 24 hours after exercise (Group VI, n=7), 48 hours after exercise (Group VII, n=7). The ratio of ssDNA of soleus muscle fibers in all groups were shown immediately rises after exercise compared with the control group whereas gastrocnemius muscle fibers ssDNA ratios were shown rises gradually in a time dependent manner. The highest ratio was found in Group II (0h) of soleus muscle fibers and Group IV (6h) of gastrocnemius muscle fiber. All data of groups in gastrocnemius and soleus muscle fibers were significantly different from the control group and each of other (p<0.05). Mitochondrial TBARS levels were found significantly higher and the ratios of GSH/GSSG were significantly lower after exercise compared with the control in soleus muscle than the data of gastrocnemius muscle (p<0.05). In a time dependent manner cytosolic caspase 9, caspase 3 activities and cytochrome c levels were predominant in soleus muscle while caspase 8 activity was predominant in gastrocnemius muscle statistically (p<0.05). The data of plasma TNF- α and IL-6 were shown the same time pattern with caspase 8 in gastrocnemius muscle (p<0.05). In summary, this study provided evidence that apoptosis is triggered by strenuous exercise. In addition to this, in soleus muscle mitochondrial pathway and oxidative stress, in gastrocnemius muscle external pathway and cytokine increases are predominant in triggering of apoptosis.

Investigating the role of BH3-only genes in resistance of glioblastoma cells against hypoxia-induced apoptosis

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The oxygenation status of solid tumors is negatively correlated with their resistance against antineoplastic treatment. The molecular mechanisms underlying this correlation are complex and might include enhanced resistance to stimuli triggering the mitochondrial pathway of apoptosis. Glioblastoma multiforme (GBM) is amongst the most hypoxic and therapy-resistant tumors known. The aim of this study was 1) to analyze the variations of different glioblastoma cell lines in resistance to cell death induced by oxygen withdrawal (< 0.1 % O₂), hypoglycemia and acidosis, 2) to investigate the functional integrity of hypoxia-induced apoptotic signaling pathways in these cell lines and 3) to correlate alterations in apoptotic signaling with resistance to mitochondrial dysfunction and apoptosis in GBM. Analysis of basal and hypoxia-induced expression of BH3-only proteins BNIP3, NIX, Noxa and PUMA in seventeen glioblastoma cell lines revealed that loss of expression/lack of induction of BH3-only proteins occurred in a significant number of the investigated cell lines. Subsequent FACS analysis of mitochondrial dysfunction (TMRM fluorescence) and quantification of cell death (propidium iodide uptake) after oxygen withdrawal, hypoglycemia and acidosis in the presence or absence of small-molecule Bcl-2 antagonists revealed pronounced differences in the sensitivity of individual cell lines to these stress stimuli. Our data indicate a strong correlation between loss of expression/lack of induction of individual BH3-only proteins and enhanced resistance to apoptosis in GBM cells. Inactivation of hypoxia-triggered mitochondrial death pathways and ensuing apoptosis deficiency might contribute to therapy resistance in GBM.

Anticancer drugs target survivin via a PI3K- dependent but Akt-independent signaling pathway in immature neutrophils

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Myelosuppression is the most common unwanted side effect associated with the administration of anticancer drugs and infections remain a common cause of death in chemotherapy treated patients. Several mechanisms of the cytotoxicity of these drugs have been proposed and may synergistically operate in a given cell. Survivin expression has been associated with cancer, but recent reports suggest that this molecule is also expressed in several immature and mature hematopoietic cells. Here we provide evidence that specific survivin depletion leads to apoptosome formation in immature neutrophils. Moreover, treatment of these cells with anticancer drugs reduced endogenous survivin levels causing apoptosis. The anticancer drugs did not directly target survivin, instead they blocked the activity of phosphatidylinositol-3-OH kinase (PI3K), which regulated survivin expression and apoptosis in immature neutrophils. Strikingly, and in contrast to other cells, this pathway did not involve the serine/threonine kinases Akt. Moreover, in combination with anticancer drug therapy, rapamycin did not induce increased myelosuppression in an experimental lymphoma mouse model. These data suggest that drugs, which block either Akt or signaling molecules located distal to Akt may preferentially induce apoptosis of cancer cells since they exhibit no cytotoxicity for immature neutrophils.

The mechanism of TNF- α and sodium butyrate interaction during differentiation and apoptosis of colon epithelial cells

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Previously, we have demonstrated the interaction between TNF- α and butyrate (NaBt) using human colon adenocarcinoma cell line HT-29. To validate our findings more generally we have investigated this interaction in another human colon adenocarcinoma cell line – Caco-2, and in normal human foetal colon cell line – FHC. TNF- α suppressed differentiation and potentiated cell death induced by NaBt in both CaCo-2 and FHC cell lines. Considering this possibility for affecting the butyrate effects on the intestinal epithelium, our results imply the role of TNF- α in colon epithelial cell injury and/or carcinogenesis during prolonged chronic inflammation.

Following up this matter, we have focused on mechanism of the NaBt and TNF- α interaction using FHC and HT-29 cells. The possible role of arachidonic acid (AA) metabolism was investigated using specific inhibitors of cyclooxygenase, lipoxygenase and phospholipase A₂ (PLA₂). Apoptosis induced by NaBt and TNF- α co-treatment was elevated by all inhibitors and the level of differentiation was not changed by any inhibitors used in both cell lines. The expression of PLA₂ was suppressed during combined treatment compared with NaBt alone treatment. NaBt induced cell level of reactive oxygen species (ROS) detected by flow cytometry was not changed by TNF- α . In FHC cells, the NF- κ B expression was decreased by TNF- α , PPAR- γ expression by NaBt and expression of both factors by combined treatment. No alteration of the transcription factor expression was observed in HT-29 cells. On the other hand, NF- κ B activity was significantly enhanced by TNF- α alone compared with untreated control. After 4 hours of NaBt and TNF- α co-treatment, additional significant increase of NF- κ B activity was observed in comparison with TNF- α alone, but this effect disappeared after 24 hours. PPAR- γ activity was significantly induced by NaBt alone, and while TNF- α alone had no effect on PPAR- γ activity, it significantly enhanced the NaBt-mediated PPAR- γ activity induction after 4 or 8 hours. Our findings did not prove the role of ROS but indicated presumable role of AA metabolism and NF- κ B and PPAR- γ transcription factors in mechanisms of detected NaBt and TNF- α interaction.

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Natural polyphenolic Curcumin overcomes resistance to apoptosis and induces mitotic catastrophe in Bcr-Abl expressing cells

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Bcr-Abl expression is the main genetic feature of Chronic Myeloid Leukemia (CML), which consist ~ 20% cases of all types of leukemias in adults. Clinically, CML could be divided into three phases, the chronic phase (low Bcr-Abl level), accelerated phase, which leads to blast crisis (high Bcr-Abl level), characterized by drug and apoptosis resistance. It was suggested that the expression of Bcr-Abl plays a significant role in the achievement of drug resistance. We investigated the influence of curcumin on low and high Bcr-Abl expressing cells. Curcumin (diferuloylmethane) a natural, non-toxic, yellow pigment of turmeric (*Curcuma longa*) is one of the most studied chemopreventive agents. Curcumin is described as an anti-oxidative, anti-inflammatory, anti-cancer agent, capable to trigger apoptosis in many cancers. Our research shows that 20 μ M curcumin diminished proliferation of Bcr-Abl expressing cells (measured by BrdU incorporation assay) as well as viability (measured by MTT assay). Curcumin arrested cell cycle at G2/M phase. As the cell cycle analysis by flow cytometry do not allow distinguishing cells in G2 from cells in M phase and the DNA content in both cases is equal, we used MPM-2 assay to measure the mitotic index. We observed that curcumin treatment increase the number of mitotic cells in comparison with untreated cells. Moreover, curcumin treatment changed the level of cell cycle regulatory proteins like cyclin D2 (downregulation) and p21 (upregulation). Microscope analysis of Hoechst stained nucleus morphology after 6 and 18h incubation with curcumin displayed aberrant mitosis in ~ 20% cases of low and high Bcr-Abl expressing cells. Morphological symptoms typical for novel type of cell death – mitotic catastrophe, including mitotic spindle desorganization: monopolar spindles, multipolar spindles, micronucleation and cytokinesis disturbances were observed. On the other hand we excluded classical apoptosis pathway as a primary mechanism responsible for all changes observed in mitotic cells. Our results show that mitotic catastrophe is followed by caspase activation (measured by procaspase cleavage, and caspase 3 activity assay) and apoptotic DNA fragmentation (estimated by the cell cycle analysis by flow cytometry and by the level of phosphorylation of H2AX histone). In conclusion, treatment overcoming apoptosis resistance caused by Bcr-Abl, particularly the mitotic catastrophe, seem to be very promising strategy in cancer treatment. Curcumin is one of the potential mitotic catastrophe inducers and the molecular mechanism is currently under our investigation.

Apoptotic and necrotic cells internalised differently by a macrophage cell line without activation of NF- κ B transcription factor

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In the present study we further characterized two distinct internalisation mechanisms (Krysko et al. 2003) used by macrophages to engulf apoptotic and necrotic cells. To address this issue we used an *in vitro* phagocytosis assay with a mouse macrophage cell line (Mf4/4) and as target murine L929sAhFas cells, which were induced to die in a necrotic or apoptotic way by TNFR1 or Fas stimulation, respectively. Scanning electron microscopy proved a striking difference in the internalisation mechanisms of apoptotic and necrotic cells used by macrophages. Apoptotic cells are taken up by complete engulfment of apoptotic bodies as single entities forming a tight fitting phagosome, resembling the “zipper”-like mechanism of internalisation. Primary and secondary necrotic cells are internalised by a macropinocytotic mechanism with formation of multiple ruffles by the ingesting macrophages. To exclude macropinocytosis as the mechanism of uptake for apoptotic cells, we applied a marker of macropinocytosis - horseradish peroxidase (HRP) and demonstrated at ultrastructural level that HRP was excluded from the phagosomes containing apoptotic bodies while the tracer was present inside the macropinosomes of macrophages exposed to primary and secondary necrotic material. Addition of wortmannin (50 nM and 100 nM), an inhibitor of phosphatidylinositol 3`-kinase (PI3K), reduced (by 50%) the uptake of apoptotic bodies by macrophages, while the engulfment of necrotic cells remained unaffected, suggesting a differential involvement of PI3K. Induction of a pro-inflammatory response in macrophages by necrotic cells is still controversial and therefore we investigated whether following the uptake of apoptotic and necrotic cells the activity of NF- κ B transcription factor (a key regulator of genes involved in immune and inflammatory reactions) would be modulated. To this end we generated a lentiviral vector containing a NF- κ B-dependent luciferase reporter gene and transduced Mf4/4 with this vector. This approach revealed that neither apoptotic nor necrotic cells induce NF- κ B activation in macrophages upon their uptake. In conclusion the present study demonstrates (1) the existence of different mechanisms of internalisation of apoptotic and necrotic cells used by macrophages and (2) that uptake of neither apoptotic nor necrotic L929 cells modulate the NF- κ B activity in macrophages.

NF κ B-dependent enhancement of UVB-induced apoptosis is associated with lack of I κ B reappearance

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Activation of the transcription factor nuclear factor- κ B (NF κ B) by interleukin-1 is generally associated with the induction of antiapoptotic pathways. Accordingly, NF κ B inhibits death ligand-induced apoptosis (CD95L, TRAIL) via upregulation of antiapoptotic inhibitor of apoptosis proteins (IAPs) and and FLICE-inhibitory protein (FLIP) and thus has been regarded to act universally in an antiapoptotic fashion. However, apoptosis induced by ultraviolet-B radiation (UVB) is enhanced in a NF κ B-dependent manner, indicating that NF κ B can act also in a proapoptotic fashion. We could show that NF κ B activation in the presence of UVB represses the genes of antiapoptotic proteins (IAP, FLIP). Simultaneously, tumor necrosis factor α (TNF) is released which activates the TNF receptor-1 (TNF-R1). Triggering of TNF-R1 can transduce either pro- or antiapoptotic signals depending on whether the proapoptotic adapter protein FADD or the antiapoptotic adapter proteins TRAF-1, -2, -6 are recruited to TRADD. We have demonstrated that NF κ B activation in the presence of UVB represses the TRAF genes. Therefore, the balance at the TNF-R1 is shifted towards promotion of the proapoptotic pathway which in concert with the downregulation of IAPs and FLIP finally results in the enhancement of UVB-induced apoptosis.

Activation of IL-1R by binding of IL-1 results in kinase-dependent phosphorylation of the cellular inhibitor of NF κ B inhibitor of κ B (I κ B), followed by ubiquitination and proteasomal degradation allows NF κ B to translocate into the nucleus and bind to responsive promoter elements. Once activated NF κ B induces transcription of its inhibitor resulting in reappearance of I κ B in the cytoplasmic fraction of the cell within 2h. I κ B had been described to then translocate back into the nucleus arranging termination of NF κ B mediated transcription.

In the case of costimulation of cells with IL-1 + UVB instead reappearance of I κ B occurs to be completely inhibited over hours. Lack of reappearance was shown to be in part due to reduced transcriptional output but predominantly resulted from immediate proteasomal degradation of resynthesized I κ B in the cytoplasm. Lack of NF κ B inhibitor I κ B leads in turn to prolonged nuclear persistence of NF κ B without alteration in NF κ B subunit composition. As a consequence NF κ B can long-ranging exert its transcriptional effects, meaning accelerated TNF α production and enduring repression of antiapoptotic genes. As a result both effects cooperate to mediated enhancement of UVB-induced apoptosis.

Early stages of cold stress-induced programmed cell death in tobacco cell line BY-2

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Programmed cell death (PCD) is an active process, which occurs as the tool of normal plant development or in response to different environmental stimuli. PCD could be induced in BY-2 cell line by application of various abiotic stressors, e.g. heavy metals (Fojtová *et al.* 2002, Kuthanová *et al.* 2004) or low temperature. Similar features of this PCD process (collapse of cytoplasmic strands, increasing vacuolization, nuclear fragmentation and DNA destruction) were observed both after treatment with Cd (Kuthanová *et al.* 2004) and when exposing cells to low temperature (4°C). However the appearance of the DNA fragmentation was substantially delayed in case of the cold treatment.

To study the timing of early stages, PCD was induced in exponentially growing BY-2 cell culture (3-days old) by treatment in 4°C for 1, 3, 7, 12 and 24h. The cells were subsequently frozen in liquid nitrogen. This enzymatically non-destructive procedure enabled to evaluate the progress of DNA fragmentation as executive phase of PCD at the same conditions for both stresses-cultivation at 26°C. Both DNA ladder and TUNEL reaction clearly demonstrated that PCD was induced in about 40% of cells as early as after 1 hour of the treatment in 4°C. About half of this amount was observed after Cd-treatment and no DNA fragmentation was observed in control cells, which were also all killed by the freezing directly without treatment. Similarly to Cd-treatment, early stages of cold-induced PCD were reversible after transforming cells in the permissive temperature.

We could conclude, that the slow progression of executive phase of PCD during cultivation at 4°C is not caused by slower induction phase but probably by lower activity of degrading enzymes at this temperature. Different frequency of TUNEL-positive cells after both stresses (Cd-15% and 4°C-30%) may indicate different induction mechanism of the PCD. In this context the role of rapid cold induced changes of cytoskeleton in the PCD induction is discussed. To test a potential role of caspases-like proteases in these early stages of PCD a construct, consisting of CFP and YFP connected with caspase cleavage sequence (DEVD, YVAD) for Fluorescence Resonance Energy Transfer analysis (FRET) was prepared.

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Thapsigargin and apoptotic hepatocyte injury

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Hepatocyte death is a cardinal feature of almost every liver disease and apoptosis is one of its modes characterized by specific biochemical and morphological features. Evidences suggest that an increase in cytosolic calcium (Ca^{2+}_c) and a modulation of nitric oxide (NO) are associated with apoptotic signaling, but the cross talk of these signals is still ambiguous. We sought to investigate the events of endoplasmic reticulum (ER) calcium depletion by thapsigargin (TG) with the consequent transient increase in Ca^{2+}_c in relation to NO production and apoptotic and/or necrotic markers during 24h of hepatocyte culture. Hepatocytes were isolated from rats and then treated in culture with complete medium or TG (1 or 5 μ M) for 24h. NOS expression was determined by NO production estimated as NO_2^- . Hepatocytes were evaluated for MTT test, urea synthesis and ALT leakage. Caspase-3 activity was detected by a colorimetric caspase-3 assay kit and cytosolic cytochrome-c by a sandwich ELISA. Hepatocytes were investigated by light and fluorescent microscopy to evaluate morphological features of apoptosis and to detect Annexin-V/apoptosis positive cells. Both concentrations of TG produced significant increase in caspase-3 activity while cytochrome-c and MTT transformation were only slightly elevated 24h after TG incubation. Medium nitrite levels were reduced after the higher TG concentration with concomitantly increased ALT leakage. The decreased NO production was accompanied by an increase in the urea synthesis. Histologically, an increased number of apoptotic events especially at low TG concentration was confirmed. Higher TG concentration did not produce corresponding increase in apoptotic morphological markers. In summary, increasing the basal Ca^{2+}_c by TG treatment that accompanies the depletion of ER calcium alter apoptotic/necrotic parameters in different ways, depending on the concentration of TG used. Lower concentration of TG produced clear apoptotic feature in hepatocytes during 24h incubation and did not reduce NO production. On the other hand, higher concentration of TG produced apoptosis and necrosis as well, evidenced by the increased ALT leakage, and moreover highly significantly decreased medium NO. Nevertheless, higher TG concentration was accompanied by a decrease of cell volume of viable cells, an indication of initial stages of apoptosis. We may conclude that the extent of the increase in Ca^{2+}_c and the modulation of NO production by hepatocytes contribute to hepatocyte apoptotic and/or necrotic events.

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Resveratrol protects against 4-Hydroxynonenal-induced apoptosis: a role for JNK and c-Jun/AP-1

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In the present study we have studied the effect of resveratrol in signal transduction mechanisms leading to apoptosis in 3T3 fibroblasts when exposed to one of the major end product of oxidized fatty acid metabolism, 4-hydroxynonenal (HNE). Mitogen-activated protein kinases (MAP kinases) and various caspases have been proposed to mediate stress-induced apoptosis in many cell types. HNE induced early activation of JNK and p38 proteins but downregulated the basal activity of ERK 1/2. We were also able to demonstrate the release of cytochrome c from mitochondria, caspase-9 and caspase-3 activation as shown by active caspase fragments and fluorometric caspase assays. Treatment of fibroblasts with specific inhibitors of MAPKs and caspases provided further mechanistic evidence for JNK, caspase-9 and caspase-3 involvement but neither p38 nor ERK 1/2 in mediating HNE-induced apoptosis. Studies undertaken by MAPK and caspase inhibitors as well as resveratrol indicated that resveratrol was as effective as JNK inhibitor in preventing JNK and caspase activation hence apoptosis. Increase in c-Jun and phospho-c-Jun and decrease in c-Fos protein levels took place within 1 h of HNE treatment which was accompanied by an increase in the DNA binding of AP-1. This effect could be overcome by pretreatment of cells with resveratrol as shown by immunoblots and gel shift assays. Overexpression of dominant negative c-Jun and JNK1 prevented HNE-induced apoptosis, which indicates a role for JNK-c-Jun/AP-1 pathway in HNE-induced apoptosis.

In light of the JNK-dependent induction of c-Jun transcription and AP-1 upregulation induced by HNE and the protective role of resveratrol, these data may show a critical potential role for JNK in the cellular response against toxic products of lipid peroxidation. In this respect resveratrol acting through MAP kinase pathways and specifically on JNK could have a role other than acting as an antioxidant.

The apoptosis/autophagy paradox. Autophagic vacuolization before apoptotic death

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Autophagic cell death is morphologically characterized by an accumulation of autophagic vacuoles. Here, we show that inactivation of LAMP2 by RNA interference or by homologous recombination leads to autophagic vacuolization in nutrient-depleted cells. Cells that lack LAMP2 expression demonstrated an enhanced accumulation of vacuoles carrying the marker LC3, yet a decreased co-localization of LC3 and lysosomes, suggesting that the fusion between autophagic vacuoles and lysosomes was inhibited. While a fraction of mitochondria from starved LAMP2-expressing cells co-localized with lysosomal markers, within autophagolysosomes, no such co-localization was found upon removal of LAMP2 from the experimental system. Of note, LAMP1 depletion had much less effects and did not aggravate the phenotype induced by LAMP2-specific small interfering RNA. Serum and amino acid-starved LAMP2-negative cells exhibited an accumulation of autophagic vacuoles and then succumbed to cell death with hallmarks of apoptosis such as loss of the mitochondrial transmembrane potential, caspase activation, and chromatin condensation. While caspase inhibition retarded cell death, it had no protective effect on mitochondria. Stabilization of mitochondria by overexpression of Bcl-2 or the mitochondrion targeted cytomegalovirus protein vMIA, however, blocked all signs of apoptosis. Neither caspase inhibition nor mitochondrial stabilization did antagonize of autophagic vacuolization in LAMP2-deficient cells. Altogether, these data indicate that accumulation of autophagic vacuoles can precede apoptotic cell death. These findings argue against the clear-cut distinction between type 1 (apoptotic) and type 2 (autophagic) cell death.

Gene expression analysis of genistein treated human prostate cancer cells PC-3 using cDNA microarrays and proteins encoded sequences

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cDNA microarrays was developed to investigate the relative expression of the corresponding total RNA in prostate cancer cell line PC-3 (AR-) treated with therapeutically doses of Genistein 50uM compared with untreated PC-3 prostate cancer cells. For generating fluorescently labeled cDNA samples to be used in microarray screening we used the SuperScriptTM Indirect cDNA Labeling System (InvitrogenTM). Hybridization procedure was performed overnight using a humidified Corning chamber at 45^o C in water bath. The image analysis was performed using GenePix Pro 41121 and ImaGene and for Data analysis we used GeneSight, GeneSpring and MATLAB (MatArray and maanova) software for expression analysis.

The gene expression analysis revealed that Genistein at therapeutically and physiologically doses as well caused a differential expression of: "6-phosphofructo-2-kinase/fructose-2, 6-biphosphate 3", "ATPase, Ca⁺⁺ transporting, ubiquitous", "BTG family, member 2", "H2A histone family, member L", "H3 histone, family 3A" "UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 2", "calcium channel, voltage-dependent, L type, alpha 1C subunit", "calmodulin 3 (phosphorylase kinase, delta)", "caspase 5, apoptosis-related cysteine protease", "chaperonin containing TCP1, subunit 7 (eta)", "general transcription factor IIH, polypeptide 4, 52kDa", "phosphatidylinositol transfer protein, membrane-associated", "polymerase (RNA) II (DNA directed) polypeptide A, 220kDa", "potassium voltage-gated channel, KQT-like subfamily, member 2", "protein tyrosine phosphatase, non-receptor type 3", "protein tyrosine phosphatase, non-receptor type 7", "ras homolog gene family, member N", "transcription elongation factor B (SIII), polypeptide 1 (15kDa, elongin C)", "transcription factor B2, mitochondrial", "tumor necrosis factor, alpha-induced protein 2", "Cdc42 guanine nucleotide exchange factor (GEF) 9, DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 38 acrosomal vesicle protein 1, protocadherin 1 (cadherin-like 1), putative nucleolar RNA helicase, regulator of G-protein signaling 3, ubiquitin specific protease 11, vesicle-associated membrane protein 2 (synaptobrevin 2).

We conclude that Genistein can preferentially cause a decrease of some gene involved in apoptosis pathways and an increase in others in human prostate cancer PC-3 cell line. We made a computational analysis for the identified genes involved in apoptosis induced by Genistein into PC-3 cells in order to predict the protein encoded sequences, transcripts and metabolites. As a conclusion we predicted some pathways involved in the apoptosis induced of Genistein into the prostate cancer cells PC-3 which should be further investigated, as well as molecular markers.

Keywords: prostate cancer PC-3 cells, Genistein, apoptosis, gene expression analysis, cDNA microarrays

The involvement of insulin and insulin signaling pathway in the skin cell death

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Maintaining skin integrity and proper wound healing is essential for life. This is achieved by continuous skin cell proliferation, differentiation and programmed cell death.

In our previous studies we have demonstrated that insulin and insulin-like growth factor 1 (IGF-1) are regulators of skin proliferation and differentiation. In the current work we have investigated the effects of these hormones on skin apoptosis. We have focused on physiological apoptosis of skin epidermal keratinocytes, which is part of the normal turnover of skin, and compared it to UVB-induced pathological skin cell death.

We found that insulin accelerated the physiological cell death process, but had no effects on the UVB-induced pathological cell death. IGF-1, on the other hand, was not involved in regulation of the physiological cell death process, but facilitated cell death under pathological conditions.

In order to identify specific pathways involved in these effects, we have studied genetically manipulated skin cells. We used primary keratinocytes manipulated to under- and over-express insulin receptor (IR) or the insulin receptor substrates 1 and 2 (IRS-1 and IRS-2 respectively).

Surprisingly, while manipulating IR and IRS-2 expression had no significant effects on skin cell death, IRS-1 played a key role in pathological cell death of keratinocytes. Over-expression of IRS-1 resulted in a marked increase in the apoptotic cells population after UVB irradiation. Unexpectedly, lack of IRS-1 expression had no significant effect, probably due to redundancy between the members of the IRS family.

In conclusion, these data provide evidence that insulin and IGF-1 participate in programmed cell death in skin cells. Pathological (UVB-induced) apoptotic signal in keratinocytes is mediated by IRS-1 pathway.

Unveiling the mechanism of insulin and IGF-1 involvement in skin turnover is of major importance for our understanding of the wound healing process and hopefully will lead to new insights on the pathogenesis of skin diseases.

Molecular Characterization of FAT10 in response to UV-induced DNA damage

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Ubiquitin-like modifier (UBL) proteins are involved in various cellular regulatory roles. Among this family of proteins, one of its members, FAT10 (diubiquitin), has recently generated much interest. The expression of FAT10 was reported to be upregulated in various gastrointestinal and gynaecological cancers. However, the molecular characteristics of FAT10 remain largely unclear. In this study, the effect of FAT10 on colon cancer cells in response to UV-induced DNA damage was investigated. We show that overexpression of wild type FAT10 did not affect cell cycle and apoptosis profiles when compared to endogenous levels of FAT10, irrespective of UV irradiation. The functions of UBLs may be mediated by specific conserved residues such as glycine-glycine (diGly) residues at the carboxyl termini. In this regard, FAT10 was further characterized by examining the effect of mutation at the conserved diGly and lysine residues. In response to UV, FAT10 diGly mutants showed significant increased levels of apoptosis, suggesting that the cells were sensitized to apoptosis. These data suggest that the diGly residues in FAT10 may be functionally important in mediating apoptosis in response to UV-induced DNA damage.

Involvement of MAPKs and NF- κ B in diosgenin-induced differentiation in HEL cells with release of apoptotic bodies but not functional platelets

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Recent reports demonstrated that NF- κ B activation participated in megakaryocytic differentiation. A growing number of studies demonstrated the key role of the MAPK pathway during megakaryocytic differentiation. After differentiation, the fate of mature megakaryocytes is to produce platelets. Platelet shedding results from cytoplasmic fragmentation, which leads to the formation of denuded megakaryocytes constituted of the megakaryocyte nucleus, its envelope, and a ring of cytoplasm. These senescent megakaryocytes have been identified as apoptotic cells. Furthermore, platelet formation has recently been shown to require precise caspase activation. These data emphasized the involvement of apoptotic-related phenomena in thrombopoiesis.

The treatment of HEL cells with 10 μ M diosgenin caused an initial activation of ERK1/2 within 5 min (5-fold over untreated cells), which was sustained up to 12h. Furthermore, diosgenin induced a rapid and sustained de-activation of p38. With diosgenin stimulation, we found an early slight activation of NF- κ B at 24h. Then, diosgenin progressively inhibited NF- κ B translocation after 48-96h of treatment leading to a complete inhibition at 192h of treatment. During diosgenin treatment, two distinct bursts of caspase-3 activation were observed. At 48h stimulation, we observed a rapid and strong increase in active caspase-3. Then, active caspase-3 levels rapidly decreased at 96h but remained higher than controls. Afterwards, caspase-3 activity increased again and once more reached high levels by the end of the treatment. In addition, the intensity of the PARP cleaved 85 Kd fragment followed the kinetics of caspase-3 activation. Diosgenin treatment also led to the fragmentation of differentiated cells. Cellular fragmentation started at 96h post-stimulation and at the end of the treatment (at 192h), most of the cells were fragmenting or already fragmented. Moreover, the fragments shedded from diosgenin-differentiated cells were platelet-sized particles but not fonctionnal platelets as they were unable to aggregate under ADP stimulation and as electron microscopy revealed that these particles contained no granulations.

In conclusion, diosgenin induced the megakaryocytic differentiation of HEL cells through a combined activation of the ERK signaling pathway and inhibition of the p38 MAPK pathway. Afterwards, differentiated cells showed a marked inhibition of NF- κ B nuclear translocation and an activation of caspase-3 together with PARP cleavage. The apoptotic cell death of diosgenin differentiated cells then led to the release of apoptotic bodies but not functional platelets.

Cross-talk between endoplasmic reticulum and mitochondria during apoptosis involves VDAC-dependent calcium channeling and PTPC opening

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The mitochondrial Permeability Transition Pore Complex (PTPC) is a polyprotein complex that has been implicated in the mitochondrial membrane permeabilization (MMP), a process leading to apoptosis. Calcium, released from endoplasmic reticulum (ER) by the inositol 1,4,5 triphosphate channel receptor (IP3R), was shown to induce MMP, a phenomenon thought to be facilitated by a local coupling between closely apposed regions of the ER and mitochondria. Here, we investigated the role of PTPC in the ER stress-induced apoptosis signaling pathway. Using a cell-free system of purified organelles, we demonstrated that Ruthenium Red (Ca^{2+} uniporter inhibitor), DIDS and NADH (two VDAC inhibitors) reduced Ca^{2+} influx into mitochondria, indicating that Ca^{2+} could penetrate across the outer mitochondrial membrane, at least, by VDAC and subsequently enter the matrix via the uniporter. In HeLa cells, pro-apoptotic agents that specifically act on the ER, such as tunicamycin, thapsigargin, and brefeldin A, elicited the opening of the PTPC, which was prevented by Bcl-2 or vMIA, two potent MMP inhibitors. The opening of the PTPC was also inhibited by Ruthenium Red, suggesting that ER stress-induced apoptosis is dependent upon mitochondrial calcium influx. Finally, we show that the IP3R-1 receptor co-immunoprecipitated with ANT¹ and VDAC, two major components of the PTPC. Nevertheless, no direct interaction between purified VDAC and IP3R-1 was observed *in vitro*, suggesting either the requirement for unknown co-interactors in ER/mitochondria complexes or a specific membrane environment. Altogether these results suggest that IP3R1 and VDAC are functionally involved in the cross-talk between ER and mitochondria facilitating Ca^{2+} entry into mitochondria and leading to the PTPC-dependent irreversible phase of apoptosis.

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Regulatory mechanisms involved in morpho- physiological changes of testis in the seasonally breeder bat *Corynorhinus mexicanus*

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Temporal asynchrony of male reproductive functions occurs in vespertilionid bat *Corynorhinus mexicanus*. Spermatogenesis occurs on summer, whereas spermatozoa are stored into epididymis, accessory sex glands remain active and copulation take place during fall-winter when testes are quiescent; therefore is a good model to study factors that modulate morpho- physiological changes of testis in seasonally breeder mammals. Adult bats were captured monthly at Central Mexico during a year. We evaluate annual variation in weather and photoperiod of that region. Fecal testosterone (T) and androstenedione (A) were determined by Enzyme Immunoassay. Testes were dissected, measured, and weighed from two specimens. Specific activity of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and gamma-glutamyl transpeptidase (γ -GT) was determined in raw homogenates of left gonads spectrophotometrically. Right testes were immersed in formaline, then processed for light and fluorescence microscopy. Spermatogenesis was assessed in tissue sections stained with H-E; apoptosis in sections processed by TUNEL. 5 stages were distinguished in testis of *C. mexicanus* throughout year. I-Inactivity (late October-April); dry-cold weather, short photoperiod (10.5hL/13.5hD) season, bats passes through daily torpor. Testicular size Minimum, maximum cell number and low degeneration index in interstitial compartment. Germinal epithelium reduced to spermatogonia and Sertoli cells. Highest CAT and GPX protect against H₂O₂ a potent cellular peroxidant. II-Recrudescence (May-early June); highest temperature (16°C), photoperiod (13hL/11hD) season; T, and A peak, spermatogonial renewal occurs. SOD increasing generate H₂O₂ by superoxide anion dismutation, then, degenerative index in both testicular compartments augment. III-Increase (late June-July); rainfall augmented drastically. Submaximum androgens, spermatocitogenesis begins. Higher SOD, lowest CAT and GPX. Maximum degeneration index in both compartments. IV-Maximum development (August); testes mass increase 40 times in comparison to reproductive inactivity. Minimum androgens and interstitial cell number; in tubular compartment, lumen was clearly evident, and maximum cell number observed; spermiogenesis occurs. Degenerative index diminished in both compartments. SOD decrease, and GPX increase to protect against other H₂O₂ sources. V-Involution (September-early October); increase in GPX continues, highest γ -GT a Sertoli cell marker that might act with GPX via renovation of glutathione.

Protein Kinase C ζ associates with death inducing signaling complex and regulates Fas ligand-induced apoptosis

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Previous studies have shown that Protein Kinase C (PKC) stimulation may interfere with Fas signaling pathway and Fas ligand (FasL)-induced apoptosis. In this study, we investigated in Jurkat cells, a FasL-sensitive human T-cell model, whether PKC ζ targets apical events of Fas signaling. We describe for the first time that in Jurkat cells, both PKC ζ and Prostate apoptosis response-4 (Par-4), one of the major endogenous PKC ζ regulators, are components of the death inducing signaling complex (DISC). Using PKC ζ overexpressing cells or si-RNA depletion, we demonstrate that PKC ζ interferes neither with Fas expression nor Fas clustering in raft microdomains, but negatively regulates FasL-induced apoptosis by interfering with DISC formation and subsequent caspase-8 processing.

Live cell analysis of lysosomal and mitochondrial injury and cell death during Fas receptor-mediated apoptosis

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Lysosomal damage followed by a subsequent leakage of cathepsins contributes in the regulation of mitochondria-dependent apoptosis. However, the exact nature of injury of organelles and kinetics of lysosomal and mitochondrial membrane permeabilization remain elusive. In this study, we used live cell confocal microscopy to measure the kinetics of the leakage of cathepsin B-green fluorescent protein in relation to mitochondrial membrane potential during Fas receptor-mediated apoptosis. These experiments revealed that cathepsin B-green fluorescent protein appeared into the cytosol several hours before mitochondrial depolarization. The latter was rapidly followed by morphological signs of apoptosis suggesting that mitochondria commanded cells to die. LysoTrackerRed-uptake assay with flow cytometry and confocal microscopy showed that the cells maintained lysosomal pH gradient until very late stages of Fas-induced apoptosis. Cathepsin B inhibitor, CA-074-OMe, reduced mitochondrial dysfunction and cell death. Also, a general cysteine protease inhibitor, E-64d, was able to decrease cell death, whereas cathepsin D inhibitor pepstatin A, failed to do so. Taken together, our results suggest that the cells retain the proton gradient across lysosomal membrane during cathepsin B release and that the mechanism of Fas receptor-induced mitochondrial permeabilization and cell death involve the activity of cathepsin B.

c-Jun/AP-1 regulates Secretogranin II, a new class of protein that mediates neuronal differentiation and protection from nitric oxide-induced apoptosis

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The AP-1 transcription factor family comprises many homo- and heterodimeric complexes (composed of c-Jun, c-Fos, ATF-2, etc) that regulate different sets of target genes. However, identification of AP-1 target genes involved in cell death and survival is still at an early stage. Secretogranin II (SgII), an abundant component of neuroendocrine secretory granules was found to be an AP-1 target gene in a microarray analysis aiming to identify potential neuro-protective genes that counteract nitric oxide (NO)-induced apoptosis in human SH-Sy5y neuroblastoma cells. NO transcriptionally activates *sgII* through a conserved cyclic AMP response element (CRE) motif in its promoter and promotes SgII protein secretion. Basal and NO-inducible expression of *sgII* mRNA as well as SgII protein synthesis/export were severely compromised in neuroblastoma cells stably transformed with dominant-negative c-Jun. Cells expressing dominant-negative c-Jun, which were sensitized to NO-induced apoptosis and failed to undergo nerve growth factor (NGF)-dependent neuronal differentiation, were stably transformed with *sgII* mRNA. Neuronal differentiation and resistance to NO were restored in independent clones re-expressing *sgII*. Knockdown of *sgII* with RNA interference in cells expressing functional c-Jun abolished neuronal differentiation and rendered the cells more sensitive to NO-induced apoptosis. We conclude basal and NO-inducible transcription of the *sgII* gene requires a transcription factor in the AP-1 family. Importantly, SgII represents a new class of AP-1-regulated protein that counteracts NO toxicity and mediates neuronal differentiation of neuroblastoma cells.

Diabetes increases mitochondrial production of ROS and activates apoptotic cell death pathway after transient cerebral ischemia

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Diabetes and hyperglycemia aggravate brain damage in experimental ischemic animals and clinical stroke patients. Diabetes and hyperglycemia accelerate maturation of the neuronal damage, increases infarct volume and includes post-ischemic seizures. The mechanism by which hyperglycemia/diabetes worsens ischemic brain damage is not fully understood. The objective of this study is to determine whether hyperglycemia induced either by streptozotocin-injection or glucose loading activate mitochondria-initiated apoptotic cell death pathway after transient cerebral ischemia. Ischemic models included a 5 min global ischemia followed by 6 hrs, 1- 3- and 7- days of recovery in 4-week diabetic rats, a 30 min focal ischemia followed by 6 hrs, 1- and 3-days of recovery in 4-week diabetic rats and a 15 min global ischemia followed by 30 min, 1-, 3-, and 6-hrs of reperfusion in glucose-infused hyperglycemic rats. Normoglycemic, non-diabetic rats were used as control. The results showed that 1) Hyperglycemia/diabetes increased neuronal death in the brain and shorten the damage maturation period. 2) Hyperglycemia/diabetes enhanced production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). 3) Hyperglycemia/diabetes caused early mitochondrial swelling as examined by electron microscopic studies, release of cytochrome c, activation of caspase-3, cleavage of PARP and fragmentation of DNA. 4) Hyperglycemia/diabetes activated caspase-3 via the mitochondria-initiated cytochrome c – caspase-9 pathway but not through receptor binding mediated Fas – caspase-8 pathway. 5) Although neuronal death was characterized by ladder DNA fragmentation, TUNEL positive, chromatin condensation and nuclear shrinkage after ischemia in hyperglycemic/diabetic brain, there was no apoptotic body identified in these animals. It is concluded that activation of apoptotic cell death pathway may contribute to hyperglycemia/diabetes-exaggerated brain damage and that such aggravated damage has both apoptotic and necrotic components

Keywords: apoptosis, reactive oxygen species, cerebral ischemia, caspase, mitochondria, diabetes, hyperglycemia

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Protection of betulin against cadmium-induced apoptosis in HepG2 cells involves Fas downregulation, blockage of ROS generation, and cell cycle arrestSeon-Hee Oh[†] and Sung-Chul Lim^{†‡}

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The protective effects of betulin (BT) against cadmium (Cd)-induced cytotoxicity have been reported. However, the mechanisms responsible for these protective effects are unclear. Therefore, this study examined the mechanisms responsible for the protection of BT against Cd-induced cytotoxicity in human hepatoma cell lines, HepG2 and Hep3B cells. Pretreatment with 0.5 and 5 µg/ml of BT resulted in the almost complete inhibition of Cd-induced cytotoxicity in the HepG2 cells. These effects appeared to be related to the inhibition of apoptosis, as determined by PI staining and DNA fragmentation analysis. The anti-apoptosis exerted by BT involved the recovery of the Cd-induced reactive oxygen species (ROS) levels to the basal control levels, the abrogation of the Cd-induced Fas upregulation, and the subsequent blocking of caspase-8-dependent Bid activation. Cd induced the accumulation of the p53 and p21 proteins, which arrested the cell cycle at the G₀/G₁ phase, and a corresponding peak in the sub-G₁ cells at the late stage. The BT pretreatment did not affect the p21 and p53 expression levels, when compared with those of the treated cells with Cd alone. BT was also observed to induce the arrest of the transient S-phase at an early stage and the arrest of the G₀/G₁ arrest at a relatively late stage, without affecting the sub-G₁ apoptotic peak.

In the Hep3B cells, the BT pretreatment partially inhibited the Cd-induced apoptosis via the blockage of caspase-8 and Bid activation. However, it did not cause the blockage of caspase-9 or -3 activation, nor did it activate Bax.

Overall, it was found that Cd can induce apoptosis via the Fas-dependent and -independent apoptosis pathways. However, the observed protective effects of BT were sensitive to Fas-mediated apoptosis, and may be regulated via the blockage of Cd-induced ROS generation and the arrest of the cell cycle.

Effects of heterologous expression of PICK1, a PKC α -specific anchoring protein, on cell growth and programmed cell death

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The various isozymes of protein kinase C (PKC) translocate to distinct subcellular compartments upon stimulation. The translocation is postulated to be mediated, at least in part, by isozyme-specific anchoring (targeting) proteins. We have shown previously that PICK1 played a role as a targeting subunit of PKC α which anchored the kinase to mitochondrial and modulated activity of the kinase. Interaction of PICK1 and PKC α is mediated through the carboxylate-binding loop of the PDZ domain in PICK1 and the extreme C terminus of PKC α . To investigate the cellular significance of targeting of PKC α to mitochondria by PICK1, cell clones that stably expressed PICK1 were selected. Heterologous expression of PICK1 did not appear to affect growth rate or survival rate of cells under normal growth conditions. Upon drug treatment, cells overexpressing PICK1 exhibited a higher survival rate than the parental cells as examined by trypan blue exclusion and appearance of apoptotic nuclei. Activation of caspases 9 and 3 were significantly suppressed in PICK1-overexpressing cells indicating that PICK1 interfered with the mitochondria-dependent death pathway. An inhibitor that selectively inhibited conventional PKC abolished the drug resistance of PICK1-overexpressing cells whereas it had no apparent effect on the survival rate of parental cells. These results indicated that PICK1-mediated drug resistance required the activity of conventional PKC. It has been shown in a number of studies that over-expression of protein kinase C α rendered cells more resistant to apoptosis induced by various stresses, however there is no direct evidence, thus far, demonstrating a physical link of PKC α to mitochondria in association with its anti-apoptotic effect. Our results suggested that PICK1 anchored PKC α to mitochondria, phosphorylated particular substrate(s) and thus rendered cells more resistant to apoptosis.

Hypoxia induces p53-dependent transactivation and Fas-dependent apoptosis

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p53 triggers cell cycle arrest or apoptosis in response to different stress stimuli, e.g. DNA damage, activated oncogenes and hypoxia. We have analyzed p53-dependent gene and protein expression in response to hypoxia using HCT116 colon carcinoma cells carrying wild type p53 and the p53 null isogenic line HCT116. Hypoxia induced p53 protein levels and p53-dependent apoptosis in these cells. RNA from hypoxia-treated cells was hybridized to a cDNA microarray containing 20,000 transcripts. This revealed that only a limited number of genes are regulated by p53 in response hypoxia. Most classical p53 target genes are not upregulated. However, we found that the known p53 target genes Fas/CD95 and MDM2 were induced in response to hypoxia in a p53-dependent manner, along with several novel p53 target genes that have been implicated in control of cell growth, including ANXA1, DDIT3/GADD153 (CHOP), SEL1L, SMURF1 and SMURF2. Fas/CD95 was also induced in hypoxia-treated normal human diploid fibroblasts. Disruption of Fas signalling using either anti-Fas blocking antibody or a caspase 8 inhibitor abrogated p53-induced apoptosis in response to hypoxia. We conclude that hypoxia triggers a p53-dependent gene expression pattern distinct from that induced by other stress agents and that Fas is a critical regulator of p53-dependent apoptosis upon hypoxia.

Key words: p53, hypoxia, apoptosis, microarray analysis, p53 target genes,

Effects of highly active antiretroviral therapy (HAART) regimens containing the protease inhibitor (PI) nelfinavir on apoptosis of peripheral blood mononuclear cells

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AIM: Negative mitochondrial effects of nucleoside analogues have been firmly established. However, controversies regarding the effects of PI on mitochondria remain: while some authors have claimed that PIs have beneficial effects through inhibition of mitochondrially-driven apoptosis, other have reported an increase of apoptotic events. We evaluated the effects of HAART regimen containing the PI nelfinavir on peripheral blood mononuclear cells (PBMCs) expression of Bcl-2 (antiapoptotic protein) and cleaved, active caspase-9 (pro-apoptotic protein).

MATERIAL AND METHODS: Present cross sectional study included 16 HIV negative patients (HIV-), 20 HIV positive patients naïve for antiretrovirals (HIV+) and 19 HIV positive patient receiving nelfinavir plus AZT+3TC or ddl+d4T (HIV+PI) as first line antiretroviral treatment at least during the previous 12 months. PBMCs were isolated and Bcl-2 and caspase-9 analyzed by Western blot. Bcl-2 expression was normalized by mitochondrial content using VDAC quantification, while caspase-9 expression was normalized by beta-actin. A pro-apoptotic index calculating the ratio caspase-9/Bcl-2 was also estimated

RESULTS: Bcl-2 expression was lower in HIV+ individuals than in HIV- (0.99 ± 0.57 vs 1.60 ± 0.92 respectively, $p=0.08$), while caspase-9 expression was higher (0.49 ± 0.40 vs 0.14 ± 0.08 , $p<0.01$). Apoptotic index was very significantly increased in HIV+ respect to HIV- individuals (0.50 ± 0.33 vs 0.06 ± 0.04 , $p<0.001$). HIV+PI patients exhibited a trend to normalization for Bcl-2 expression (1.25 ± 0.88 , $p=0.34$ respect to HIV+) as well as for caspase-9 expression (0.45 ± 0.58 ; $p=0.27$ respect to HIV+), while apoptotic index significantly improved (decreased) (0.31 ± 0.25 , $p<0.05$ respect to HIV+). No differences were found between the subgroup of HIV+PI according to concomitant PI drugs either AZT+3TC or ddl+d4T.

CONCLUSIONS: HAART regimens containing the PI nelfinavir seems to have some beneficial effects to counteract the increased apoptosis present in HIV positive untreated people (Grant: Marató TV3-020210).

Role of Bcl-2 superfamily proteins and caspases in the initiation phase of Doxorubicin-induced apoptosis in human leukaemia cells

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While the main molecular events in the execution phase of apoptosis induced by doxorubicin (Dox) are well delineated, a comparable description of the initiation phase is not available. In particular, it is unclear the degree of essentiality of caspases to speed up the apoptotic process and the role of particular BH3-only proteins. We have evaluated the relative roles of caspases and Bcl-2 superfamily proteins in two leukaemia cell lines (Jurkat, U937) differing in their dependence of caspases for rapid apoptosis execution. We have analysed the participation of multidomain proteins, pro- (Bax, Bak) and anti-apoptotic (Bcl-2, Bcl-x_L, Mcl-1), of BH3-only proteins (Bim, Bik, Bid, PUMA) and caspases in the destabilization of mitochondria leading to $\Delta\Psi_m$ loss and cytochrome c and AIF release. Dox treatment induced caspase-independent conformational changes of Bax and Bak in U937 cells and of Bak in Bax-negative, Jurkat cells. Overexpression of Bcl-2 or Bcl-x_L prevented these conformational changes in both cell lines. Down-regulation of Bak expression by siRNA silencing inhibited Dox-induced apoptosis in Jurkat but not in U937 cells. Mcl-1 levels diminished upon Dox-treatment in both cell lines. Cotreatment with Z-VAD-fmk prevented apoptosis and Mcl-1 reduction in Jurkat but not in U937 cells. Attenuation of Mcl-1 expression by siRNA increased sensitivity to apoptosis in both cell lines. Co-immunoprecipitation studies indicate that Bax and Bak became associated during apoptosis in U937 cells. Bim protein could not be found associated to either Bax or Bak. These results suggest that apoptosis induction is mediated by down-regulation of Mcl-1 and the Bax and/or Bak conformational change, followed by release of cytochrome c and AIF to cytosol. In Jurkat cells, not expressing Bax, caspases are needed to cause a rapid mitochondrial disruption through an amplification loop, whereas in U937, expressing Bax and Bak, the degree of mitochondrial disruption is sufficient to trigger apoptosis.

Nanoparticles illuminate death path

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Quantum dots (QDs) are luminescent nanoparticles with unique optical properties that make them advantageous for multicolor and long-term live cell imaging. Moreover, QDs are excellent candidate agents for *in vivo* diagnostic imaging. QDs must be inert and non-toxic to fulfill the requirements of high-quality fluorescent probes and diagnostic agents. When coated with proteins or biocompatible polymers, QDs are not deleterious for cells and organisms. However, they are retained in the cells and accumulated in the body for a long period of time during which the coatings may be degraded. We explored the toxicity of unmodified cadmium telluride QDs that could represent the degradation products of well protected, polymer or protein-coated nanoparticles. Results from these studies show that unmodified QDs induce plasma membrane, mitochondrial and nuclear damage leading to cell death. Cell death in human epithelial breast cancer cell line (MCF-7) induced by QDs is not classical necrosis or apoptosis. Reactive oxygen species (ROS) are important players in mediating cellular damage and the powerful antioxidant NAC prevents QD-induced ROS production and cytotoxicity. Results from these studies show that QD-induced cytotoxicity can be reduced or even eliminated without covalent binding of protective agents to QD surface. Moreover, the study provides evidence for molecular mechanisms and a role of several subcellular compartments in QD-induced cell death. A thorough understanding of the toxicity of QDs is crucial for their application in biology and medicine.

Keywords: nanoparticles, fluorescence, quantum dots, oxidative stress

The roles of lipids in the process of Bax activation

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Upon activation, Bax undergoes several conformational rearrangements. It translocates to mitochondria, oligomerizes, and inserts in the bilayer in a form that cannot be detached by alkali treatment. This process can be mimicked in vitro with recombinant proteins and liposomes made from synthetic or purified lipids. The composition of these liposomes has been shown to influence their degree of permeabilisation by Bax. These observations were proposed to reveal the nature of the pore through which apoptogenic factors exit mitochondria. We are trying to understand how the properties of the liposomes influence the process of Bax activation itself.

Protective effect of (-) epicatechin on the biomarkers of oxidative stress in erythrocytes from normal and hypertensive subjects

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Hypertension is a major health problem worldwide. It is one of the most pervasive disorders in India and its prevalence is as high as 80 per 1000 urban population and 40 per 1000 rural population. Recent evidence suggests that reactive oxygen species (ROS) radical may play a role in the development of organ damage associated with cardiovascular disease and hypertension. (-) Epicatechin, a member of tea catechins belonging to flavonoids group, is known to be a potent anti-oxidant. The present study was undertaken to evaluate the effect of (-) epicatechin on the biomarkers of oxidative stress: malondialdehyde, protein carbonyls content, membrane sulfhydryl groups and reduced glutathione content in erythrocytes from hypertensive subjects. The effect of (-) epicatechin was also compared with a known anti-oxidant L-ascorbic acid. The erythrocyte malondialdehyde (MDA) and protein carbonyls (PC) were found to be significantly increased while membrane sulfhydryl (SH) groups and intracellular glutathione (GSH) content were decreased in hypertensive subjects. Invitro incubation with (-) epicatechin caused a significant decrease in both MDA and PC content of erythrocytes while GSH and SH groups of erythrocyte gets increased an effect which was more pronounced in erythrocytes from hypertensive subjects. Similar results were obtained with L-ascorbic acid. The increase in MDA and PC contents and decrease in GSH and SH groups in hypertensive erythrocytes is an indicator of oxidative stress condition. Increase in the GSH level and protection of MDA, PC and SH group oxidation by (-) epicatechin suggests that high dietary intake of foods rich in catechins may help to reduce oxidative stress and concomitant free radical damage in hypertensive subjects.

Fragmentation of mitochondrial network could be induced by reactive oxygen species produced by respiratory chain

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Mitochondria in living cell display a variety of shapes, ranging from small and spherical to extended tubular networks. This reflected structural dynamics of mitochondria, which includes fusion, fission, and branching. Fragmentation of mitochondria usually accompanied apoptosis and could play some significant but unresolved role in apoptotic signalling. In our experiments we have investigated the effect of inhibitors of mitochondrial functions on mitochondrial shape and location in the cell. HeLa cells were transfected with mito-EYFP (Clontech) and stable transfected clone was selected. We have shown that piericidin (an inhibitor of complex I) and myxothiazol (an inhibitor of complex III) of respiratory chain induce fragmentation of mitochondrial reticulum, which was completed in several hours. Oligomycin (an inhibitor of F₀F₁-ATP synthase) was ineffective indicating that cessation of ATP synthesis was not critical for fragmentation of mitochondria. Uncouplers (FCCP, DNP) induced more rapid fragmentation accompanied by swelling of mitochondria. It was suggested that fragmentation of mitochondria induced by the inhibitors of respiration was mediated by increased production of reactive oxygen species in the apical segment of the respiratory chain. This hypothesis was supported by the observation on rapid (less than 1h) fragmentation of mitochondria induced by H₂O₂ (0.1-0.2 mM). When cells were preincubated with 10-(6'-ubiquinolyl)decyltriphenyl-phosphonium (MitoQ, a mitochondria-targeted antioxidant) at 20 nM for 7 days mitochondria became resistant to fragmentation induced by inhibitors of respiration and by H₂O₂ but remained sensitive to uncoupler-induced fragmentation. Moreover, prolonged incubation of cells with MitoQ caused significant improvement of mitochondrial reticulum. The structural changes (elongation of the mitochondrial reticulum) were correlated with increase of the size of electrically connected areas of the mitochondrial network. It was shown that local photodynamic damage of rhodamine-loaded tubular mitochondria using laser focused by confocal microscope resulted in depolarisation (rhodamine release) of the large area and 30-50% of total mitochondrial population. In control HeLa cells the area of depolarisation was much smaller and no more than 10% of mitochondria were depolarised. The presented data indicated that mitochondrial ROS production could modulate mitochondrial dynamics (structural and functional) both under the stressful conditions and in traditional cell culture.

Mitochondrial morphology

Changes in apoptotic endonucleases and morphology in the rat thyroid gland after ¹³¹I-induced injury

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Incorporated ¹³¹I irradiation induced significant morphological and functional changes in the rat thyroid gland. The central zone (CZ) of thyroid appeared to be more sensitive to irradiation than the peripheral zone (PZ). Recently two DNA-degrading enzymes, endonuclease G (EndoG) and DNase I, were shown to be important in different tissue injuries induced by chemical or physical pro-apoptotic stimuli. In the present study, we hypothesized that the previously observed predominant damage of the CZ during ¹³¹I-induced injury is caused by the difference in the endonuclease profiles of the thyroid CZ and PZ. Materials and methods. Female Wistar rats (n=13) were injected with 5.5 MBq ¹³¹I. Rats were studied one week, 4 weeks or 10 weeks after injection. Control animals (n=7) received NaI. Quantitative computing immunohistochemistry was used for measurements of EndoG and DNase I, PARP p85. Relative volumes of epithelium, stroma and colloid, cell density (CD) and apoptotic index (AI) were quantified. Results. One week after ¹³¹I administration, the quantity of EndoG increased simultaneously in the CZ and PZ (2.5- and 2.1-fold, respectively), and DNase I was only 1.3 fold higher in both zones. The EndoG/DNase I ratio was 6.7 in the CZ and 9.3 in the PZ. Considerable accumulation of EndoG (10-fold both in the CZ and PZ) and DNase I (11-fold and 23-fold in CZ and PZ, respectively) were found 4 weeks after ¹³¹I administration. The EndoG/DNase I ratio was 4.2 in the CZ and 2.78 in the PZ. Numerous apoptotic bodies and PARP p85-positive cells were observed, especially in the CZ. Nuclear translocation of EndoG and DNase I was found in apoptotic cells. The AI was significantly increased to 60-fold compared to the control. The PZ was characterized by complete restoration of epithelial and colloidal components of parenchyma to the control values. Ten weeks after injection, a partial deprivation of DNase I was observed. The EndoG/DNase I ratios in the CZ and PZ were 3.5 and 12.7, respectively. Cell death in the PZ was low, while in the CZ it was increased 30-fold above the control level. The CD was decreased by 50% and 11% in the CZ and PZ, respectively. Conclusion. Thyroid cell injury strongly depends on the amount of endonucleases, and these enzymes play a critical role in thyroid follicular cell death induced by ¹³¹I administration.

Curcumin and vincristine induce mitotic catastrophe in apoptosis-resistant HL-60-derived HCW-2 cells

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The term mitotic catastrophe has recently become widely used to describe a form of death affecting many cancer cells. Mitotic catastrophe is manifested by unsegregated chromosomes and multimicronucleation of nonviable cells, which due to severe DNA or mitotic spindle damage are not able to bypass mitosis. We show here that cells of HL-60-derived HCW-2 line highly resistant to apoptosis treated with curcumin, a known apoptosis inducer, undergo mitotic catastrophe indistinguishable from that induced by vincristine, an agent causing mitotic spindle depolarization. Both treatments decrease cell proliferation and arrested cells in G₂/M phase of cell cycle. They also decreased cell survival and induced morphological changes characterized by cell enlargement, chromatin condensation and micronucleation. Cell synchronization with thymidine block increased the number of cells arrested in G₂/M and the number of "catastrophic" cells after curcumin treatment. However, prolonged cell incubation with curcumin or vincristine led to cytochrome c release from mitochondria, caspase 3 activation and oligonucleosomal DNA fragmentation suggesting that the process of mitotic catastrophe may eventually be followed by apoptotic cell death.

mTOR acts through Mdm2 to signal cell survival *in vivo*

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Coordination of cell death and survival is crucial during both embryogenesis and adulthood, and alterations to this balance can result in degeneration or cancer. Growth factor receptors such as Met can activate PI3K, a major intracellular mediator of growth and survival. PI3K can antagonise p53-triggered cell death but the underlying mechanisms are not fully understood. Using genetic and pharmacological approaches, we found that PI3K can act through mTOR to regulate p53 activity both *in vitro* and *in vivo*. mTOR inhibits p53 by increasing protein levels of Mdm2, the negative regulator of p53. This pathway makes selective use of mTOR effectors: p70 ribosomal S6 kinase (p70^{S6k})/S6 ribosomal protein pathway is required for Mdm2 up-regulation, whereas 4E-binding protein 1 (4E-BP1) is not. Unexpectedly, although it is required for nuclear translocation of Mdm2, Akt is dispensable for Met-triggered activation of mTOR and up-regulation of Mdm2 protein. Inhibition of either mTOR or Mdm2 signalling is sufficient to block cell survival induced by HGF/Met *in vitro* and to induce p53-dependent apoptosis *in vivo*. Our studies identify a novel mechanism for control of cell survival *in vivo*, involving positive modulation of Mdm2 activity by mTOR. This new role, in addition to the known effects of mTOR on growth, reinforces its potential as a drug target in cancer.

PPAR gamma dependent programming of macrophage capacity for phagocytosis of apoptotic cells

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Macrophages acquire their capacity for efficient phagocytosis of apoptotic cells during their differentiation from monocytes. The peroxisome proliferators activated receptor- γ (PPAR γ) is highly up-regulated during this maturation program. We have observed that when human monocytes were differentiated to macrophages in the presence of an antagonist of PPAR γ (GW9662), the capacity of the mature macrophages to engulf apoptotic human neutrophils was significantly decreased. The effect of the antagonist was dose dependent; it had to be present during the differentiation process and also could inhibit the previously observed augmentation of phagocytosis by glucocorticoids. Rosiglitazone, an agonist of PPAR γ , did not show any effect in these cellular assays. Blocking ligand activation of PPAR γ had no influence on the expression of PPAR γ itself or typical differentiation markers such as CD206 and CD16. However, it could down-regulate the mRNA level of CD36, ABCA1, phosphatidylserine receptor and transglutaminase 2 which have been previously implicated in the phagocytosis process. These results suggest that during programming of monocytes to macrophages ligands of PPAR γ are formed and drive expression of genes responsible for effective clearance of apoptotic cells.

H₂O₂ mediated apoptotic internucleosomal nDNA fragmentation, antioxidant response and protease dynamics in wheat (*Triticum aestivum* L.)

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Hydrogen peroxide induced antioxidant defense response, apoptotic internucleosomal nDNA degradation, and protease dynamics were studied during early ontogenesis in different organs of etiolated wheat seedlings. An organ specific dual anti/pro-apoptotic affect was observed. Application of 100mM H₂O₂ induced the internucleosomal nDNA fragmentation just after 48 hours of treatment. In coleoptile H₂O₂ induced apoptosis 24 hours earlier compared to the onset of apoptosis in control. Down regulation of ascorbate and catalase levels were accompanied with induction of apoptotic internucleosomal nDNA fragmentation. Moreover increased proteases level overlapped with the course of apoptosis induction. Dynamics of both antioxidants provided evidence that they play a key role in modulating the H₂O₂ induced apoptosis. Contrary to this, exogenous application H₂O₂ showed an anti-apoptotic affect in roots as it completely abolishing the apoptotic internucleosomal nDNA fragmentation: a hallmark of apoptosis observed during normal ontogenesis in roots of control seedlings. This effect was coupled with higher levels of ascorbate and catalase, which further supported the H₂O₂ induced anti apoptotic effect in wheat roots via better antioxidant defense. Details of new H₂O₂ mediated anti-apoptotic effects and dynamics of antioxidants and proteases with special reference to apoptosis are discussed.

Stress- or vector-induced heat shock protein accumulation in cells before irradiation can attenuate DNA breakage and p53-dependent apoptosis

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The aim of our study was to examine whether the heat shock transcription factor 1 (HSF1)-mediated stress response and excess heat shock proteins (Hsps) contribute to cell resistance to radiation-induced apoptosis. Two cognate cultures of mouse embryo fibroblasts with *HSF1*-gene knockout (HSF1 *-/-* cells) and with normal wild-type HSF1 expression (HSF1 wt cells) were preconditioned by heating (43 C, 30 min) without or with Quercetin (an inhibitor of HSF1) and then exposed to gamma-rays. Some cell samples were infected with special virus-based vectors to overexpress the constitutively active (mutant) form of HSF1 or individual Hsps.

Without any pretreatments, both the cultures were equally radiosensitive exhibiting apoptotic death of 40-60% cells after irradiation at doses 4-6 Gy; herein, enhanced p53 expression, caspase-3 activation and characteristic cleavage of poly ADP-ribose polymerase (PARP) indicated triggering of the p53-dependent apoptotic pathway. The heat preconditioning transiently up-regulated the Hsp level in HSF1 wt cells and significantly reduced their post-radiation apoptosis; these effects could be abolished by Quercetin or simulated (without preheating) by the HSF1 overexpression. In contrast, no attenuation of apoptosis was found in heat-preconditioned HSF1 *-/-* cells unable to trigger the HSF1-mediated Hsp induction after heating. However, when the constitutively active HSF1 was overexpressed in HSF1 *-/-* cells, the latter accumulated Hsps and became more resistant to radiation-induced apoptosis like heat-preconditioned HSF1 wt cells. The vector-induced overexpression of Hsp70 or Hsp27 also reduced apoptosis in the irradiated cells of either culture.

Studying of mechanisms of the Hsp-mediated radioprotection has revealed that Hsp70 and Hsp27, being accumulated in the target cells, are able to attenuate single- and double-strand DNA breakage resulting from irradiation. This attenuation of the DNA breakage was shown to lead to the impaired p53 expression and, as a consequence, to weaker stimulation of the p53-dependent apoptotic pathway that was manifested in the suppressed PARP cleavage by caspase-3 and the lower intensity of apoptosis. It is yet unclear how Hsps can protect DNA in irradiated cells; one of potential mechanisms is that excess chaperones promote accelerated synthesis and nuclear import of proteins essential for early repair of the DNA breaks. The other possibility is that the HSF1 activation or Hsp27 accumulation indirectly yields up-regulation of the intracellular pool of glutathione known as a radioprotector.

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In situ detection of starvation-induced autophagy

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Autophagy is a regulated bulk degradation process inside cells. In addition to maintaining cellular homeostasis, there is growing evidence for the participation of autophagy in many different human pathologies. However, transmission electron microscopy is currently the only reliable method for monitoring autophagy in situ. Because TEM is labor intensive, we questioned whether useful marker proteins can be found for unambiguous detection of autophagy in tissue via routinely used colorimetric, immunohistochemical or fluorescent techniques. Starved HepG2 hepatocytes and nutrient deprived liver tissue were used as a model for the initiation of autophagy. Our findings indicate that starvation-induced autophagy in HepG2 cells is neither associated with differential mRNA gene expression nor with changes in the expression level of known autophagy-related proteins (beclin 1, ubiquitin, cathepsin D, DAP kinase). On the contrary, both transcription and translation were inhibited (reduced de novo protein synthesis, decline in total protein and RNA content, dephosphorylation of p70 S6 kinase), suggesting that the identification of autophagy-specific biomarkers for tissue is highly compromised. Microtubule-associated protein 1 (MAP) light chain 3 (LC3), which is an attractive marker of autophagosomes, revealed a relatively low expression level in tissue and cultured cells. Nonetheless, liver tissue from nutrient deprived transgenic GFP-LC3 mice showed a significant upregulation of LC3 immunopositive dot-like structures as compared with nonstarved control tissue. Our findings suggest that TEM remains an indispensable technique for evaluation of autophagy in situ. However, LC3 can be used as a marker for autophagosome formation in situ but only when this protein is overexpressed.

Mitofusin-2, mutated in Charcot-Marie-Tooth type IIa, links endoplasmic reticulum to mitochondria

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Endoplasmic reticulum (ER) and mitochondria are in close juxtaposition to support their intercommunication essential in Ca²⁺ homeostasis and apoptosis. Molecular mechanisms controlling ER-mitochondria interactions are unknown. Mitofusin-2 (MFN2), a dynamin-related protein of the outer mitochondrial membrane mutated in Charcot-Marie-Tooth type IIa, specifically controls morphology not only of mitochondria, but also of ER. In cells lacking *Mfn2*, ER-mitochondria interactions are largely reduced. GTPase and RAS-binding domains of MFN2 proved essential to regulate ER morphology and interaction with mitochondria, consistent with an increase in phosphorylation of downstream RAS targets in *Mfn2*^{-/-} cells. MFN2 is the first mitochondrial protein that regulates ER-mitochondria interaction.

Extending myocardial viability during heart preservation with cyclosporine A

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Hypothermic preservation (PRES) of donor hearts is limited to 12-14 hours for complete functional recovery following reperfusion. In a canine heterotopic heart transplant model 50-60% functional recovery returned following 18 hours of PRES with University of Wisconsin solution (UW). Concomitant with attenuated functional recovery, there were significant increases in apoptotic cells and caspase 3 at 2 and 6 hours of reperfusion with a concomitant decrease in lamin B₁ with no necrotic cells. ATP and CP concentrations were reduced during PRES. ATP increased but remained below control during 6 hours of reperfusion while CP returned to levels above control. These results suggested that reduction of apoptosis may prolong myocardial viability during PRES and reperfusion. Donor hearts were subjected to 18 and 24 hours of PRES (2-4° C) with and without cyclosporine A (CsA) treatment (apoptosis blocker). CsA was given to the donor animal (10 mg/kg), in the PRES solution (10⁻⁵ mol/l) slowly infused during the PRES period (1 ml/min) and to the recipient animal (2.5 mg/kg). After 18 hours of PRES with CsA, function returned to 100% within 1 hour and stayed at this level throughout a 6 hour recovery period. There were no apoptotic myocytes nor caspase 3 activity after 18 hours PRES with CsA treatment and lamin B₁ remained at 100% in the nuclei. 24 hours PRES in UW resulted in no functional recovery. However, after CsA treatment, functional recovery returned to 100% after 4 hours of reperfusion. ATP and CP concentrations were surprisingly the same with or without CsA treatment at 18 hours and lower with 24 hours but returned during reperfusion to 18 PRES levels. The mechanism of action may be associated with the mitochondrial permeability transition (MPT) pore via cyclophilin D binding. In conclusion, by blocking apoptosis with CsA, myocardial viability during PRES was maintained with 100% functional recovery during reperfusion.

Peroxynitrite induces senescence and apoptosis of red blood cells through the activation of aspartyl and cysteinyl proteases

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Changes in the oxidative status of erythrocytes can reduce cell lifetime, oxygen transport and delivery capacity to peripheral tissues and have been associated with a plethora of human diseases. Among reactive oxygen and nitrogen species of importance in red blood cell homeostasis, superoxide and nitric oxide radicals play a key role. In the present work, we evaluated subcellular effects induced by peroxynitrite, the product of the fast reaction between superoxide and nitric oxide. Peroxynitrite induced: i) oxidation of oxyhemoglobin to methemoglobin, ii) cytoskeleton rearrangement, iii) ultrastructural alterations, iv) altered expression of band-3 and decreased expression of glycophorin A. With respect to control cells, this occurred in a significantly higher percentage of human red blood cells (about 40%). The presence of antioxidants inhibited these modifications. Furthermore, besides these senescence-associated changes, other important modifications, absent in control RBC and usually associated to apoptotic cell death, were detected in a small but significant subset of peroxynitrite-exposed RBC (about 7%). Active protease cathepsin E and μ -calpain increased, activation of caspase 2 and caspase 3 was detected and phosphatidylserine externalization, an early marker of apoptosis, was observed. Conversely, inhibition of cathepsin E, μ -calpain as well as of caspase 2 and 3 by specific inhibitors resulted in a significant impairment of erythrocyte "apoptosis". Altogether these results indicate that peroxynitrite, a milestone of redox-mediated damage in human pathology, can hijack human red blood cells towards senescence and apoptosis by a mechanism involving both cysteinyl and aspartyl proteases.

The study of the apoptogenic effect of pyrimidines derivatives on murine leukemia cells

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Eight pyrimidine derivatives with thio- and hydrazine- groups were investigated for their ability to inhibit the growth of tumor cells and apoptogenic activity. Their action was compared with the action of 5-fluoropyrimidines, including the widely used in chemotherapy 5-fluorouracil. 2-thiouracil and 6-hydrazinouracil did not influence the growth of murine leukemia cells. 2,4-dithiouracil, 2-thio-4-hydrazinouracil, 2-hydrazinouracil and 2-thio-5-fluorouracil decreased cell proliferation (IC_{50} were respectively), but even at highest studied concentration (1000 μ M) had no cytostatic action. Only high concentrations of 2,4-dihydrazinouracil and 2-chloro-4-hydrazinouracil had strong cytotoxic action. The most potent inhibitor was 5-fluorouracil with an IC_{50} of 1 μ M and IC_{90} of 7 μ M, which means that its active concentrations are near 100 times lower than that's of other compounds. For detection of the cell fraction undergoing apoptosis a morphological analysis was made using fluorescent dye propidium iodide. Only 5-fluoropyrimidines and 2,4-dihydrazinouracil caused the appearance of apoptotic cells with typical fragmented condensed nuclei, ghosts and apoptotic bodies. In contrast dead cells treated with 2-chloro-4-hydrazinouracil did not have apoptotic morphology. Combined treatment of cells with 5-fluoropyrimidines and thymidine led to decreasing of the amount of apoptotic cells compared to the treatment with fluoropyrimidine alone, but the combination with leucovorin resulted in increased amount of apoptotic cells. The pattern of modulation of apoptosis induction by 5-fluorodeoxyuridine points to the domination of DNA-directed apoptogenic action based on the inhibition of thymidylate synthetase. In contrast to this, the induction of apoptosis by other fluoropyrimidines proved to be connected to both mechanisms of its inhibitory effects: DNA-directed and their RNA-directed action.

Transposon tagging in yeast to identify genes involved in resistance to Bax lethality

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Previous work, using EMS mutagenesis, identified yeast proteins that positively or negatively modulate the lethal effects of Bax, indicating that Bax lethality in yeast is not the result of generalized cytotoxicity. To identify cellular components that participate in suppressing the effects of Bax, the yeast strain EGY48 was transformed by homologous recombination with a transposon-mutagenized yeast library. The pool of mutant cells was super-transformed with a Bax-expressing plasmid and selected on restrictive medium. Isolated colonies, which were reproducibly resistant to Bax, were further analysed. The integration sites from three of the isolated strains were cloned and identified as, a subtelomeric helicase ORF, previously associated with telomerase independent telomere maintenance, the AFG3 protein, which is a member of the conserved AAA mitochondrial proteases, and a mitochondrial HSP protein.

In a reverse approach, aiming to identify positive effectors of Bax lethality in yeast, an EMS-derived mutant strain exhibiting resistance to Bax and defects in mitochondrial import, was used. Most of the isolated library clones that restored Bax sensitivity also restored the mitochondrial import process. The identified genes include the thioredoxin peroxidase TSA2 and the Old Yellow Enzyme 2, a conserved mitochondrial NADPH/FMN-dependent oxidoreductase.

Violacein and violacein-loaded poly (D, L-lactide-co-glycolide) nanoparticles induce cell differentiation and trigger apoptosis via a mitochondrial pathway in human leukemic cells

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Violacein, a pigment isolated from *Chromobacterium violaceum*, a bacterium found on Amazonian region, has been reported to have multiple biological activities including in vitro antitumoral effects. Its activity on promyelocytic leukaemia (HL60) cells is of special interest because the compound effectively induces cell death in these cells. Advances in nanobiotechnology have resulted in evolution of several novel colloidal carrier systems such as liposome, polymeric micelles, nanoparticles and nanoemulsions to achieve these multiple objectives. Polymeric nanoparticles made from natural or synthetic polymers have drawn major attention due to higher stability and opportunity for further surface nanoengineering. Our results show that Violacein-loaded poly (D,L-lactide-co-glycolide) (PLGA) nanoparticles has a similar inhibitory effect evaluated by trypan blue assay on leukemic HL60 cells when compared to free form. However the cytotoxic effects evaluated by phosphatase activity and MTT reduction assays were lower on encapsulated form than on free violacein. Based on morphological and biochemical changes, violacein and violacein entrapped in nanoparticles were found to induce terminal differentiation (assessed by nitro blue tetrazolium reduction assay) in HL60 cells. Thus, both formulations inhibit HL60 cell growth in vitro partly by inducing cytotoxic effects and cell differentiation. Flow cytometric analysis of HL60 cells after treatment for 12 h showed that violacein-loaded PLGA induced apoptosis, with maximum cell death at a concentration of 2 μ M. Violacein and violacein/PLGA induced opposite changes in the mitochondrial swelling indicating altered mitochondrial function. The mitochondrial activity was also checked by flow cytometry studies, labelling cells with the probe JC1, that displayed a basal hypopolarised status of the mitochondrial in treated cells. We postulate that the effects of nanoparticles (violacein-loaded or violacein plus empty nanoparticles) in inhibit the swelling mitochondrial occurrence is caused by Pluronic membrane sealing capability as a hypothesis described by several authors with different cells. Pluronic used in the nanospheres preparation inhibits mitochondrial swelling however it was not able to blockade the $\Delta\psi$ loss in the treated cells. In summary, based on the changes in the $\Delta\psi$ and in the 9-caspase activation, we conclude that violacein (free form and in nanoparticles) triggered apoptosis in HL60 cells probably through cytochrome c release and apoptosome formation. On theoretic grounds, selective eradication of transformed cells by use of mitochondrion-specific agents should be effective. One strategy, yet to be developed would aim at exploiting differences in the composition or regulation of the permeability transition pore complex.

Inhibition of constitutive activation of NF- κ B in cutaneous T cell lymphoma cells induces apoptosis and enhances sensitivity to death-inducing agents acting through the mitochondrial pathway

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Advanced stages of cutaneous T cell lymphoma (CTCL) are associated with a poor response to death-inducing chemotherapeutic agents, including mitochondrial drugs [Michel *et al*, 2003]. Since it has been shown that constitutive activation of the Nuclear factor- κ B (NF- κ B) pathway is involved in the resistance of tumor cells to apoptosis in several human malignancies of the hematopoietic lineage, we investigated the status of NF- κ B in CTCL cell lines and in circulating tumor cells from patients with Sézary syndrome (SS) and its role in resistance to mitochondrial drug-induced apoptosis. The NF- κ B status was determined using EMSA, immunoblotting and confocal microscopic analysis in CTCL cell lines (HUT-78, MyIA and SeAx) and in peripheral blood mononuclear cells from 30 patients with Sezary syndrome showing a high ratio of tumor cells. The effects of bortezomib (PS-341), an inhibitor of the proteasome which has shown to exhibit potent anti-tumor properties, were examined on the NF- κ B status and tumoral cell viability.

Results demonstrate the presence of active NF- κ B complexes (p50/p65) in the nucleus by confocal microscopy and interaction with κ B elements by EMSA. Selective inhibition of NF- κ B was achieved in the SeAx cell line by transduction of a super-repressor form of I κ B α , with subsequent induction of tumor cell death. Similarly, PS-341 completely reverses the constitutive nuclear translocation of NF- κ B, in a dose- and time-dependent fashion, in both CTCL cell-lines and tumor cells from SS patients. The NF- κ B inhibition by bortezomib lead to induction of apoptosis, as determined by annexin V / propidium iodide staining and mitochondrial transmembrane potential alterations, and by immunoblot analysis of procaspase 3 and poly(ADP-ribose)polymerase cleavage. Immunoblotting analysis also revealed that bortezomib-induced apoptosis of CTCL cells was associated with an up regulation of the pro-apoptotic Bax member of the bcl-2 family, while expression of contra-apoptotic molecules such as Bcl-x(L) or Bcl-2 was not altered.

Our results demonstrate that constitutive activation of the NF- κ B pathway plays a key role in the survival of CTCL and in the resistance of this latter malignancy to apoptosis induced by mitochondrial drugs. They also emphasize that NF- κ B activation is reverse by the proteasome inhibitor bortezomib *in vitro*. This set of data warrants further studies investigating *in vivo* the effects of bortezomib in CTCL patients showing resistance to classical systemic treatments.

Hypoxia induces protection against etoposide-induced apoptosis in HepG2 cells : role of p53, HIF-1 and AP-1 transcription factors

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If there is an important number of data describing the importance of mutations in genes involved in the apoptotic process on one hand, and the role of hypoxia on the other hand, in the development of tumors and in their resistance to the various anti-cancer therapies, few elements are currently available as for the link which could exist between these two processes. The goal of our work was thus to study the relationship existing between both processes regarding cancer cell survival.

In this study, physiological hypoxia was shown to inhibit apoptosis induced in HepG2 cells by etoposide, a topoisomerase II inhibitor. Indeed, hypoxia reduced DNA fragmentation, caspase activation and PARP cleavage induced by etoposide. Etoposide is known to induce apoptosis through p53 activation. However, hypoxia did not influence this activation, indicating that it is not through p53 inactivation that hypoxia protected cells against etoposide-induced apoptosis. Two transcription factors are activated by hypoxia, HIF-1 and AP-1, that could play a protective role under hypoxia. This hypothesis was tested experimentally. The results show that etoposide did not influence HIF-1 activation by hypoxia and the use of anti-HIF-1 α siRNA did not worsen etoposide-induced apoptosis under hypoxia, thus excluding a protective role of HIF-1. On the other hand, etoposide decreased AP-1 activity under hypoxia and SB600125, an inhibitor of JUN kinases, inhibited the hypoxia-induced protection against etoposide-induced apoptosis. These results suggest that AP-1 could play an anti-apoptotic role under hypoxic conditions. Furthermore, data from DNA microarrays indicate that the expression of several pro- and anti-apoptotic genes was modified.

These results are very interesting because it is a clear demonstration that hypoxia has a direct protective effect on apoptotic cell death. This observation is an important data in order to understand how tumor growth can occur in challenging environmental conditions and how cancer cell can survive chemotherapeutic agents.

Mutated p53 in colon adenocarcinoma HT29 cell line predominate cell death signalling in photodynamic therapy with hypericin

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Photodynamic therapy (PDT) is a new rapidly developing approach in cancer therapy based on administration of nontoxic or weakly toxic photosensitive compound and its activation with light of appropriate wavelength. Although PDT is of use in clinical practice, new promising photosensitive compounds with advantageous attributes are discovered continuously. The effect PDT in treatment of cancer is a consequence of multiple factors such as spectral specification and power input of light source, light dose, character and concentration of photosensitive compound or incubation time. PDT also requires the presence of oxygen for its photodynamic action.

Here, we report by analysis of cytokinetical parameters and flow cytometric analysis of cell cycle response of colon adenocarcinoma cell line HT29 cells to photocytotoxic effect of hypericin as a function of two variables - hypericin's concentration and light dose.

Contribution of both factors to overall cytotoxicity and cell cycle arrest has a cumulative character. Despite of hypericin's ability to induce apoptosis with high efficiency and absence of anti-apoptotic Bcl-2 expression, necrosis seems to be a dominant mode of cell death in HT29 exposed to different PDT doses. It is likely that mutation of p53 in HT29 cells plays a crucial role in cell death signalling in HT29.

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Loss of p21 and 14-3-3sigma enhances apoptosis induction by p14ARF

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The human INK4a gene locus encodes two structurally unrelated proteins termed p16INK4a and p14ARF (p19ARF in the mouse) latter of which is transcribed in an alternative reading frame. Both p16INK4a and p14ARF have been widely implicated in tumor suppression. However, in particular p14ARF has been shown to play a unique role in both the induction of apoptosis and the regulation of cell cycle and senescence. In contrast to the initial notion that the activity of p14ARF strictly depends on a functional p53/mdm-2 rheostat, we recently demonstrated that p14ARF is capable of inducing an arrest in the G2, but not the G1-phase of the cell cycle in cells lacking functional p53, p21, or both by inhibiting the p34cdc2 (cdk1) kinase. To further extend these studies, we investigated the capacity of p14ARF to induce cell cycle arrest and apoptosis in cells deficient for p21, 14-3-3sigma, or both as compared to cells wildtype for p21 and 14-3-3 σ , respectively. Here, we show that loss of both p21 and 14-3-3sigma does not block the induction of G2-arrest upon expression of p14ARF. However, the combined loss of p21 and 14-3-3sigma completely abolished the ability of p14ARF to inhibit DNA-synthesis. Similar to p21-deficient cells, expression of p14ARF downmodulated both cdk1 protein expression and kinase activity in cells deficient for either 14-3-3sigma or both p21 and 14-3-3sigma. Noteworthy, triggering of the mitochondrial apoptosis pathways with subsequent caspase-activation and the occurrence of apoptotic DNA-fragmentation was substantially augmented in cells deficient for both p21 and 14-3-3sigma. These data demonstrate that both p21 and 14-3-3sigma are dispensable for the induction of G2-arrest upon expression of p14ARF. However, S-phase control by p14ARF critically depends on the presence of both p21 and 14-3-3sigma. As the apoptotic response to expression of p14ARF is strongly enhanced by loss of p21 and 14-3-3sigma our data implicate that cell cycle checkpoint control by these proteins critically determines apoptosis sensitivity.

BID knock-down upregulates Hrk and induces autophagy in breast cancer MCF-7 cells exposed to camptothecin

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The BH-only death factors share exclusively the short BH3 domain with the other Bcl-2 family subclasses. With the exception of Bid which might also bind to Bax, they are thought to act by binding to and neutralizing the Bcl-2 like survival factors. Human Hrk is a member of this BH3 subfamily of proapoptotic proteins. Hrk is activated by the mechanism of transcriptional upregulation and was found to be preferentially expressed in spleen bone marrow and neurons.

This study focused on the role of BID and Hrk during apoptosis and autophagy in breast cancer MCF-7 cells exposed to the cytostatic drug, camptothecin (inhibitor of DNA topoisomerase I). Confocal microscopy, MicroImage[®] System, Western blot and immunoelectron microscopy were used to examine apoptosis, autophagy, BID and Hrk expression within tumor cells. The RNAi technique was used for silencing *BID* and *Hrk* expression and to investigate their role in apoptosis and autophagy.

Exposure of breast cancer cells to CPT induced apoptosis, which was demonstrated by an increase in the content of the 89 kDa PARP degradation fragment (product of caspase 7 activity). In cells with knock-down of *BID* (*BID* (-)) the 89 kDa PARP fragment was not detected, indicating effective suppression of apoptosis. Surprisingly knock down of *BID* gene increased the Hrk level. This effect was not observed in *BID* (+) cells exposed to CPT. The expression of Hrk increased markedly within 6 h of CPT treatment in *BID*(-) cells and was completely blocked by actinomycin D and cycloheximide. It indicates that Hrk is probably synthesized *de novo* by Bid-deficient MCF-7 cells.

The electron immunocytochemical analysis confirmed enhanced expression of proapoptotic protein Hrk and revealed its subcellular colocalization with antiapoptotic protein BCL-2 in CPT-treated *BID*(-) MCF-7 cells.

Electron micrographs of CPT-treated cells revealed that apart of classical features of apoptosis like: cell shrinkage, condensation and margination of chromatin, a typical feature of autophagy existed in the same cell. Among them the most characteristic were: giant autophagolysosomes containing visible organelle and vacuolisation of the cytoplasm without disrupted nuclear envelope and plasma membrane. It suggested that another form of cell death might interfere with apoptosis. Suppression of CPT-induced apoptosis in *BID*(-) was accompanied by the intensification of autophagy, measured by analysis of Beclin1 and MAP I LC3 protein level. Hrk knock-down, which is strongly expressed in *BID*(-), cells did not affect MAP I LC3 content.

In conclusion, our study indicate that BID may serve as a molecular switch between apoptosis and autophagy in breast cancer cells.

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Histone deacetylase inhibitors strongly sensitise neuroblastoma cells to TRAIL induced apoptosis by caspases dependent changes in the ratio of pro- and anti-apoptotic proteins

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Childhood neuroblastoma (NB) is a clinically and biologically heterogeneous neoplasm which behaviour can be explained by differential regulation of apoptosis. Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) selectively induces apoptosis in most tumour cells, but not in normal tissues. The non-invasive S-type caspase-8 positive NB cell lines are weakly sensitive to TRAIL, whereas the invasive N-type caspase-8 negative NB cell lines are resistant. Histone deacetylase inhibitors (HDACIs) are a new class of anti-cancer agent inducing apoptosis or cell cycle arrest in tumour cells with very low toxicity toward normal cells. HDACIs were recently shown to increase apoptosis induced by TRAIL in several tumour cells weakly sensitive to TRAIL.

We show that the HDAC inhibitors Sodium Butyrate (NaB), suberoylanilide hydroxamic acid (SAHA) and Trichostatin A (TSA) induced apoptosis in S-type and N-type NB cell lines. HDACIs induced cell death by activating the caspases cascade, Bid and the mitochondrial pathway. In addition, sub-toxic doses of HDACIs strongly sensitise the caspase-8 positive S-type NB cell lines to TRAIL induced apoptosis in a caspases dependent manner. Combined treatments increased the activation of caspases, Bid and Bim_{EL}, and the inactivation of the anti-apoptotic proteins XIAP, Bcl-x, RIP, Flip and survivin. It also enhanced the loss of $\Delta\Psi_m$ and the release of cytochrome c and AIF from the mitochondria into the cytosol. HDACIs trigger the mitochondrial pathway and sensitise NB cells to TRAIL by accelerating the kinetics of the apoptotic cascade and by increasing the ratio between pro and anti-apoptotic proteins.

HDACIs are therefore interesting new anti-tumour agents for targeting heterogeneous tumours such as neuroblastoma as these agents display a strong toxicity toward the most aggressive N-type NB cells and highly sensitise the S-type NB cells to TRAIL induced apoptosis.

Proteasome inhibitors as agents of pharmacological preconditioning

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We have shown earlier, that proteasome inhibitors trigger the apoptotic programme in cardiomyocytes and proteasomal activity is being decreased in anoxia and is restored in reoxygenation. And apoptotic cell death is being executed specifically during reoxygenation. At the same time phenomenon of preconditioning is well-known (prevention of cell injury at ischemia-reperfusion by previous short ischemic cycles) and it can be reproduced by training of cells with different damaging agents mimicking ischemia. We hypothesized that proteasome inhibitors might serve as preconditioning agents.

To check this supposition we have used primary cultures of cardiomyocytes from neonatal rats. These cells underwent 30 minutes of anoxia followed by 60 minutes of reoxygenation. Proteasome inhibitors clasto-lactacystin β -lactone and MG132 were added to cardiomyocyte culture before anoxia. Clasto-lactacystin β -lactone is a more specific and powerful proteasome inhibitor than MG132. The percentage of living, necrotic, and apoptotic cells was determined by staining with Hoechst 33342 and propidium iodide.

After anoxia-reoxygenation the amount of living, necrotic and apoptotic cells was 79.1 ± 1.31 %, 7.7 ± 1.11 % and 11.9 ± 1.04 % correspondingly. Proteasome inhibitors significantly affect the ratio of living, necrotic and apoptotic cardiomyocytes in dose-dependent manner. Clasto-lactacystin β -lactone (5 μ M) and MG132 (10 μ M) induce the development of apoptotic cell death but at the same time they do not significantly effect the quantity of necrotic cardiomyocytes. In lower concentrations (2.5 μ M and 5 μ M correspondingly) the inhibitors during incubation for 90 minutes do not cause significant augmentation of cell death. During anoxia-reoxygenation clasto-lactacystin β -lactone significantly decreased the amount of necrotic (1.9-fold, $P=0.015$) and apoptotic (1.8-fold, $P<0.001$) cardiomyocytes (4.0 ± 0.42 % and 6.57 ± 0.40 % correspondingly). The quantity of living cardiomyocytes, to the contrary, increased up to 85.9 ± 0.47 % compared to anoxia-reoxygenation (in control - 89.8 ± 0.90 %). MG132 analogically affected the ratio of cells after anoxia-reoxygenation: the amount of necrotic cells was decreased to 4.5 ± 0.32 % ($P=0.03$) and of apoptotic - 6.2 ± 0.51 % ($P<0.001$).

Thus, inhibition of proteasome activity before modeling anoxia-reoxygenation in cardiomyocyte's culture prevents the cell death through necrosis and apoptosis. In general, proteasome inhibitors may be used as novel pharmacological agents for reproducing preconditioning. We suppose that mechanisms of this effect are due both to prevention of restore of proteasome activity in reoxygenation and to moderate damaging action of proteasome inhibitors.

Selective release of SMAC/Diablo from the mitochondria in response to combined treatment with TRAIL/APO2L and proteasome inhibitors

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Tumor Necrosis Factor (TNF)-Related Apoptosis-Inducing Ligand (TRAIL/APO2L) is currently among the most intensively studied potential anti-tumor agents. Proteasome inhibitors overcome resistance to TRAIL/APO2L-induced apoptosis in many tumor cell types. However, the molecular mechanism of the synergistic interaction is not fully understood. Proteasome inhibitors (Epoxomicin; 1 μ M and MG-132; 3 μ M) sensitized the completely TRAIL-resistant RKO and partially TRAIL-sensitive HT29 colon carcinoma cell lines to TRAIL/APO2L-induced apoptosis. DNA fragmentation determined by flow-cytometry was inhibited by both the general caspase inhibitor Z-VAD-FMK and the caspase-8 inhibitor IETD-FMK. Epoxomicin induced 50% increase in cell surface TRAIL-R2/DR5 expression while expression of anti-apoptotic proteins including Bcl2, BclxL, survivin or XIAP was unchanged. In RKO cells, DNA fragmentation, mitochondrial membrane depolarization and increased caspase-3-like enzyme activity was exclusively induced only by combined treatment with epoxomicin and TRAIL/APO2L. The combination treatment induced the release of cytochrome-c, OMI/HtrA2 and SMAC/Diablo from the mitochondria, however epoxomicin alone induced the release of cytochrome-c and OMI/HtrA2 only, while TRAIL/APO2L treatment alone did not cause significant release of any of these mitochondrial pro-apoptotic proteins. These results are consistent with a model where the full activation of caspase-3 by caspase-8 in response to TRAIL/APO2L or by the apoptosome/caspase-9 in response to epoxomicin is dependent on the selective release of SMAC/Diablo in response to the combined treatment with both agents.

The role of mitochondria in the pathogenesis of liver damage induced by TNF- α and Fas receptors

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We studied the effects of Cyclosporin A (CsA) administration (i) on the properties of the permeability transition pore (PTP) in mitochondria subsequently isolated from the liver; and (ii) on the susceptibility to hepatotoxicity induced by activation of TNF or Fas receptors in mice. We first studied the time-course and dose-dependence of PTP inhibition in liver mitochondria *ex vivo* according to a recent protocol established in our laboratory for rats. Next, we verified that treatment of naïve mice with 20 $\mu\text{g} \times \text{kg}^{-1}$ LPS (which causes TNF- α release) plus 700 $\text{mg} \times \text{kg}^{-1}$ D-GalN (which sensitizes the liver to the proapoptotic effects of TNF- α through a transcriptional block) caused the expected liver damage as judged from hematoxylin-eosin-stained, paraffin-embedded liver sections and from activation of caspase 3. All these effects of LPS plus D-GalN treatment were prevented by pretreatment with CsA for 1 hour, indicating that CsA prevents the hepatotoxic effects of TNF- α by blocking the mitochondrial proapoptotic pathway through inhibition of the PTP. We then studied whether the PTP plays a role in the Fas receptor-dependent death pathway. To induce liver damage we used the well-characterized J-o2 antibody, which causes Fas receptor cross-linking and activates caspase 8 and caspase 3, mimicking treatment with Fas ligand. A study of the dose-dependence of treatment revealed that caspase 8 could be activated already at 10 $\mu\text{g} \times \text{kg}^{-1}$ of J-o2 antibody, while caspase 3 activation required at least 200 $\mu\text{g} \times \text{kg}^{-1}$. At variance from the case of LPS plus D-GalN, CsA did not confer major protection from hepatic damage, suggesting that activation of caspase 3 by cross linking of the Fas receptor may not require recruitment of the PTP.

Induction of apoptosis by N-(4-Hydroxyphenyl)retinamide in human cutaneous T-cell lymphoma cells

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CTCL represents a heterogeneous group of skin homing T cell lymphomas that shows considerable variation in histology, phenotype and prognosis. CTCL are clonal neoplasm characterized by progressive accumulation of cells that resemble activated/memory CD4+ T cells. These malignant lymphocytes are resistant to dying with most chemotherapeutic agents. Existing treatments for CTCL (topical agents, chemotherapy, photopheresis, IF α ...) have cosmetic benefits but no impact on survival of patients. N-(4-hydroxyphenyl)retinamide is a low-toxicity synthetic-derivative of retinoic acid, which has shown great potential as quimiopreventive and antineoplastic agent. In previous works we have demonstrated that 4HPR activates the mitochondrial apoptotic-pathway on several lymphoblastic leukaemia cell lines. In this work, we examined the effects of 4HPR on viability, cell cycle, apoptosis induction, ROS overproduction, clonogenic proliferation, release of proapoptotic factors and caspase-3 activation, and expression of survivin (member of IAP family) and other apoptosis-related proteins, in MJ, Hut78 and HH established CTCL cell lines.

Our results showed that *in vitro* treatments with 4HPR increased the number of hypodiploid cells and annexin V binding in a time-dependent manner, and triggered cytochrome *c* release and caspase-3 activation after 12 hours of incubation. Moreover, early ROS increase was correlated with 4HPR-induced cell death. 48h 4HPR treatment had also a clear effect on cell cycle progression. Also, a decreased potential to form colonies on methylcellulose was shown on remaining lymphocytes after incubation with 4HPR. 4HPR decreased survivin levels, but had no effect on expression of Bcl-2.

We conclude that 4HPR at clinically relevant concentrations has proapoptotic effects and could be important as a new treatment for CTCL, alternative to conventional drugs.

Full-length p73 α represses drug-induced apoptosis in SCLC cells

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The p73 gene, a member of the p53-family, encodes several isoforms through the use of alternative promoters and alternative splicing. At the N-terminus, two different promoters give rise to full length and Δ N isoforms, the latter lacking transactivation domain. At the C-terminus, seven isoforms generated through alternative splicing have been cloned. Previous studies have demonstrated that Δ Np73 exerts a dominant-negative effect on p73 by blocking their transactivation activity and hence their ability to elicit cell cycle arrest and induce apoptosis. Considerable efforts are made to identify the functional diversity of the C-terminal p73 variants. We have examined the effect of p73 and found that p73 α inhibited drug-induced apoptosis in small cell lung carcinoma cells, whereas p73 β promoted it. The inhibition of cell death occurs upstream of caspase activation and mitochondrial events, such as cytochrome c release, suggesting that p73 α acts in the initiation phase of apoptosis and has a rather broad-range inhibitory effect preventing the caspase and mitochondria-dependent cell death pathways. Moreover, we found p73 α to inhibit bax activation and PUMA-induced apoptosis. This isoform were also able to inhibit apoptosis induced by PUMA over expression, but failed to inhibit apoptosis induced by bax over expression. These findings adding to the idea of the inhibition of apoptotic cell death by p73 α occurring in an early event of the apoptotic mechanism. The observed inhibitory effect of p73 α is dependent on the carboxyl-terminus region containing the SAM domain and requires the DNA binding domain. Furthermore, we discovered that p73 α is able to inhibit the pro-apoptotic effect of p73 β and, inversely, that p73 β could counteract the anti-apoptotic function of p73 α , demonstrating the existence of an equilibrium between these two p73 isoforms. In addition to the previously described antagonistic effect of Δ Np73 on the p73 isoforms, our finding of the existence of pro- and anti-apoptotic members within the p73 variants, reveal the complexity of the outcome of p73 gene expression. Furthermore, besides to its upregulation in certain tumor types, the ability of p73 α to inhibit drug-induced cell death uncovers potential oncogenic activities for this protein.

Identification and biochemical characterisation of a serine protease-mediated cell death pathway activated in human acute myeloid leukaemic HL-60 cells

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An increasing number of studies demonstrate that the general protein kinase inhibitor staurosporine can activate features of cell death under caspase-inhibiting circumstances. The proteolytic mechanisms required for cell death under these circumstances have not been elucidated. Here we set out to determine what proteolytic networks were responsible for sts-induced human acute myeloid leukaemic HL-60 cell death under circumstances of caspase-inhibition. We found that HL-60 cells exposed to staurosporine (sts) and treated with the general caspase inhibitor Z-VAD.fmk still died in an apoptosis-like fashion, exhibiting cytochrome c release, nuclear shrinkage and fragmentation, DNA laddering and apoptotic-associated morphology. Measurement of DEVD-dependant caspase activity demonstrated a Z-VAD.fmk-sensitive increase in sts-treated HL-60 cells. This data suggests a simultaneous activation of at least two death systems within the same cell, one regulated via caspase-dependant mechanisms and other being a caspase-independent system. To determine the components of the latter, HL-60 cells were co-treated with sts and various inhibitors of non-caspase proteases. It was found that only inhibitors of chymotrypsin-like serine proteases (TPCK; (N-tosyl-L-phenylalanine chloromethyl ketone), DCI (3,4-dichloroisocoumarin) and FFCK (5(6)-carboxyfluoresceinyl-L-phenylanyl-chloromethyl ketone)) protected the cells from nuclear shrinkage and fragmentation induced by staurosporine. TPCK also abolished apoptosis-associated morphology, oligonucleosomal DNA fragmentation, and partially prevented caspase-3 processing and activity; however, it did not prevent sts-induced cytochrome c release. Activation of chymotrypsin-like proteolytic activity, as detected through cleavage of the substrate AAF.AMC, was observed in apoptotic and apoptotic/Z-VAD.fmk treated cells. FFCK, a fluorescent tag of active serine proteases was used in conjunction with anti-fluorescein antibodies in a western blot analysis to demonstrate activation of a 16kDa protein species under conditions of apoptosis. In summary we have identified a novel serine-protease mediated route to cell death that can take place in parallel to caspase activation in HL-60 cells.

Cell death – induction in normal haematopoietic and leukaemic cells by oxazaphosphorines

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A great deal of attention has recently become focused on elucidating the cell response to chemotherapy. Oxazaphosphorines are a class of alkylating agents with major anti-neoplastic activity in many cell types. Mafosfamide cyclohexylamine salt (D-17272), 4-hydroperoxy-cyclophosphamide (D-18864) and cyclophosphamide (CP) have been commonly used during chemotherapy. Glufosfamide (β -D-glucosyl-isophosphoramidate mustard, D-19575), is a newly synthesized drug, currently undergoing experimental and clinical trials.

Cell death is a highly regulated process. Apoptosis, necrosis, autophagy and mitotic catastrophe are accepted to be major cell death pathways. Cell death and cell cycle progression are believed to be closely linked and regulated and a failure of these processes determines profound dysregulation of cellular homeostasis.

Available information about possible effects on cell death and cell cycle progression induced in normal haematopoietic and leukaemic cells by the oxazaphosphorines is scarce. Thus, the study was undertaken to show and compare the extent to which the chemotherapeutic agents D-19575, D-18864, D-17272 and CP can affect induction of cell death and changes in the cell cycle in the mouse haematopoietic system and leukaemic cell lines.

The experiments were carried out *in vivo* on the mouse bone marrow and peripheral blood cells and *in vitro* on human promyelocytic HL-60 and histiocytic U-937 leukaemic cells. Temporary morphological and functional changes were studied after treatment of mice with the alkylating drugs and exposure of leukaemic cells to these agents. The research was conducted using light, fluorescence and confocal microscopy as well as flow and laser cytometry methods.

The different patterns of alterations in cell morphology, mitochondria and lysosome function, DNA degradation and cell cycle progression in normal haematopoietic and leukaemic cells exposed to the oxazaphosphorines, were determined. A mode of cell death and changes in the cell cycle induced in the mouse haematopoietic system and leukaemic cell lines were dependent on the compound applied and its dose, the time intervals after the drug application and the type of cells being stimulated to die.

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Hepatocellular carcinoma in hereditary tyrosinemia type I: Breaking the balance between life and death

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Hereditary Tyrosinemia type 1 (HT1) is an autosomal recessive disorder caused by a deficiency of fumarylacetoacetate hydrolase (FAH), the last enzyme in the breakdown cascade of tyrosine. The lack of FAH leads to the upstream accumulation of three major toxic metabolites: fumarylacetoacetate (FAA), maleylacetoacetate (MAA) and succinylacetone (SA). The abnormal presence of high amounts of FAA combined to its apoptogenic, cytostatic and mutagenic properties are thought to be responsible for the progressive liver and kidney damage distinctive of HT1. Moreover, among all human pathologies, HT1 presents the highest susceptibility for developing hepatocellular carcinoma (HCC). Despite the recent use of 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexadione (NTBC) as an effective treatment to stop the progression of liver and kidney damage, HCC development remains frequent in the HT1 patients, as well as in murine models of the disease.

These observations suggest a double and paradoxical role of FAA in the clinical manifestations of the disease: in one side, FAA leads to the apoptotic death of hepatocytes, and, on the other, it elicits a survival signal which is at the basis of the carcinogenesis phenomenon.

The knockout murine model of HT1 (*fah* ^{-/-}) was used to analyze *in vivo* the potential implication of specific survival signaling pathways in the establishment of the observed cell death resistance. The study was performed on adult, 4-months-old, mice treated with the NTBC from fetal stage to avoid the neonatal death and, withdrawn from NTBC treatment for different periods (from 1 to 5 weeks). Our results have shown an activation of the Akt/PKB survival cascade in *fah* ^{-/-} mice as soon as the NTBC treatment was discontinued. This activation causes the inhibition of caspase 9-dependent apoptotic features, of the pro-apoptotic protein Bad, the pro-apoptotic kinase GSK3 β and the transcription factor FKHR. Furthermore, this survival state is reinforced by inhibition of the intrinsic apoptosis pathway at two levels: upstream of the mitochondrion through the induction of anti-apoptotic BCL-X and BCL-2 proteins and at the level of the apoptosome through the action of Hsp27, Hsp70 and Hsp90.

Thus, we have demonstrated that the chronic stress induced by liver disease in HT1 activates the survival signal of the Akt/PKB kinase and inhibits the intrinsic apoptosis pathway to provide cell death resistance *in vivo*.

Dual involvement of PKC delta in osteoblastic cell apoptosis induced by syndecan-2

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We recently showed that overexpression of syndecan-2, an heparan sulfate transmembrane proteoglycan, induces apoptosis in osteoblastic cells. Protein Kinase Cs (PKCs) are known to be associated with syndecan functions, lying up- or downstream the proteoglycans in the signaling cascade events. PKCs are diversely involved in the apoptotic or survival signaling. However, there are increasing evidences that PKC δ , a novel PKC isoform, is implicated as a proapoptotic kinase. In this study, we examined the involvement of PKC δ in the proapoptotic signaling induced by overexpression of syndecan-2 in osteoblastic MG63 cells.

We observed by immunofluorescence microscopy and immunoprecipitation assays that syndecan-2 was associated with PKC δ in osteoblastic cells. We also found that syndecan-2 overexpression induced cytosolic accumulation of PKC δ protein and inhibited PKC δ activation and kinase activity. We demonstrated that specific inhibition of PKC δ activity with Rottlerin or a dominant negative strongly activated effector caspases and increased the number of apoptotic cells as evaluated after chromatin staining with Hoechst. Moreover, when PKC δ activity was rescued by cotransfecting syndecan-2 with a construct coding the catalytic domain of PKC δ , cell death induction was reduced. These results clearly indicate that PKC δ is a prosurvival kinase in osteoblastic cells and that syndecan-2 induces apoptosis by inhibiting PKC δ activity in these cells. In contrast, transfection with wild type PKC δ also induced apoptosis and suppression of PKC δ protein accumulation in syndecan-2 overexpressing cells by siRNA targeting the kinase, resulted in reduced syndecan-2-induced apoptosis. These results indicate a pro-apoptotic role for PKC δ .

Altogether, our data point out a dual involvement of PKC δ in the control of apoptosis in osteoblastic cells both as a prosurvival kinase and as a proapoptotic molecule. Signaling mechanisms that allow PKC δ to fulfil these conflicting functions are now under investigation.

Roscovotine treatment facilitates early events in TRAIL receptor signaling and sensitizes breast tumor cells to TRAIL-induced apoptosis

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TRAIL is one of the several members of the TNF gene superfamily that induces apoptosis through engagement of death receptors. TRAIL selectively induces apoptosis in many transformed cells but is relatively non-toxic to normal cells. However, breast tumor cells are particularly resistant to TRAIL. Interestingly, it has been described that combinatory therapy of TRAIL and different treatments as a solution to the resistance to TRAIL in tumor cells.

Here we report that treatment with TRAIL in combination with subtoxic doses of roscovotine, a specific inhibitor of Cdk1, Cdk2 and Cdk7 induced rapid apoptosis in the majority of TRAIL-resistant breast cancer cells examined. Roscovotine sensitized breast tumor cell lines to TRAIL-induced apoptosis by facilitating DISC formation and activation of caspase-8, without affecting TRAIL-receptor expression at the cell surface. On the other hand, it has been described that cFLIP, a protein that is recruited to the TRAIL DISC, has an important role regulating TRAIL sensitivity in different cell lines. Over-expression of cFLIP confers resistance to TRAIL-induced apoptosis and its reduction by siRNA has been described to sensitize tumor cells to TRAIL. Here we also demonstrate that roscovotine treatment reduces the expression of cFLIP large and small variants at both the mRNA and protein levels. In summary, our results indicate that roscovotine sensitizes breast tumor cells to TRAIL-induced apoptosis by facilitating early events in TRAIL receptor signaling.

Incorporation of a N-methyleneamido group to N¹-phenyl-N²-(2-pyridinyl)-diazenecarboxamide induces necrosis instead of apoptosis-like cell death

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We have synthesised various diazenecarboxamides (shortly diazenes) that were cytotoxic to several tumor cell lines. To increase their solubility and biological activity, the structure has been properly modified. In the present study we examined the biological activity of N¹-phenyl-N²-(2-pyridinylmethyl)diazenedicarboxamide (RL-337) and compare it to already examined cytotoxic compound N¹-phenyl-N²-(2-pyridinyl)diazenecarboxamide (JK-279) that possesses similar structure. Using a modified colorimetric MTT assay, the cytotoxicity of RL-337 was determined on human cervical carcinoma HeLa cells, glioblastoma A1235 cells, and prostate adenocarcinoma PC-3 cells. The possible synergistic effect of diazene RL-337 with cisplatin, doxorubicin and vincristine, and its influence on intracellular GSH content (determined by Tietze's method) was examined on HeLa cells. Diazene RL-337 was cytotoxic against all three human tumor cell lines, being more cytotoxic to HeLa cells than diazene JK-279. Higher efficacy of RL-337 with regard to JK-279 can be connected with a higher basicity of the 2-picoline moiety, present in the former diazene, comparing with the pyridine fragment that is a part of the latter one. The diazene RL-337 acted synergistically with cisplatin, doxorubicine and vincristine, while diazene JK-279 exhibited synergistic effect only with cisplatin. Glutathione was not a target molecule of diazene RL-337, but was for JK-279, as shown earlier. After just one hour of treatment with diazene RL-337, the cells started to loose membrane integrity, suggesting necrosis as possible mechanism of cytotoxicity, while previously for diazene JK-279 apoptosis-like cell death was detected. Thus, although diazenes JK-279 and RL-337 are very similar in their structure, they exhibited largely different biological activity.

Investigating a role for Inhibitors of Apoptosis (IAP) proteins in developmental apoptosis

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Throughout development, apoptosis shapes tissues and appendages as they form, deletes unwanted structures, and maintains homeostasis. Failure to control apoptosis tightly can produce a range of disease states, with insufficient and excess apoptosis common to hyperplastic and neurodegenerative disorders respectively. Inhibitors of apoptosis (IAPs) are a family of proteins that can regulate caspases. Exogenous expression of IAPs in many cellular models can prevent cell death induced by both extrinsic and intrinsic death stimuli, but there is little evidence of their anti-apoptotic role *in vivo*. The mouse mammary gland is a useful model for studying developmental apoptosis because it exhibits repeated cycles of proliferation and apoptosis. The potent apoptosis-inhibiting potential of IAPs suggests that they may have a role in controlling apoptosis in the mammary gland. Thus, this tissue provides a powerful system for investigating whether IAPs have an important role in developmental apoptosis regulation. Our initial studies have shown that the levels of several members of the IAP family are altered during mammary gland development and that this co-incides with commitment to apoptosis by its epithelial cells in lactation and post-weaning involution. IAP expression also alters in response to physiologically relevant anti- and pro-apoptotic stimuli in cultured mammary epithelial cells.

Expression of caspase-3 in the rat spinal cord after injury; neuroprotective effect of caspase-3 inhibitor

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Objective and Aim: Apoptosis has been showed for several serious pathologies including neurodegenerative diseases, ischemia, and traumatic injuries in the nervous system. Caspase-3 as an important intracellular protease contributes to apoptosis. The aim of this study is to investigate the effects of Z.devd.fmk as a caspase-3 inhibitor in the spinal cord trauma models of rat.

Material and Method: 26 female Wistar albino rat was used for three groups. (1) Control group, (2) the group that have spinal cord trauma by dropping a bar weighing 4g from a height of 10 cm after 8, 9, 10 thoracic laminectomy, (3) the treatment group that used Z.devd.fmk after spinal cord trauma. All the spinal cord tissue samples of all three groups were taken at 4th and 24th hours, fixed in the neutral formalin and embedded in paraffine. TUNEL method and immunohistochemistry for caspase-3 were performed to evaluate apoptosis. The labelled apoptotic cells were counted on the TUNEL applied sections. The immunostaining intensity of caspase-3 was correlated with TUNEL.

Results: In the trauma group widespread hemorrhage, necrosis and degeneration in the motor neurons were seen in the grey matter of spinal cord. Apoptosis observed mostly motor neurons and also glial cells in surrounding tissue of the lesion core. Labelled apoptotic cells were observed significantly decreased in Z.devd.fmk group compared with traumatic group both on 4th h and 24th h (respectively, $p < 0.01$, $p < 0.05$). Caspase-3 expression was increased in both trauma group where as the down-regulated expression caspase-3 was seen in the treatment groups.

Conclusion: Our findings indicate that this trauma model triggers the apoptotic cell death in the spinal cord. The suppression of the expression of caspase-3 in the spinal cord was probably related to the protective effect of Z.devd.fmk on this experimental spinal cord trauma model.

Anti-apoptotic effect of HMGB1 on stem cells

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High mobility group box 1 is a multifunctional cytokine playing an important role in inflammation, cell migration, differentiation, and tumorigenesis. Stem cells are pluripotent cells that retain the ability to proliferate, migrate and differentiate in response to growth factors contributing to regeneration of diverse tissues. Our recent studies demonstrated that extracellular HMGB1 acts as a damage signal promoting *in vitro* and *in vivo* migration of stem cells and inducing their proliferation *in vitro*, even in the absence of serum. This proliferation is likely to involve distinct mitogenic and survival-promoting effects of HMGB1. After documenting the mitogenic properties of HMGB1 (Palumbo), we decided to address its anti-apoptotic effects using vessel-associated stem cells (mesoangioblasts) that are able to differentiate into mesodermal cells and to home to damaged muscle tissue. We treated mesoangioblasts with different pro-apoptotic stimuli, including serum deprivation, UV irradiation and exposure to TNF (plus cycloheximide). Cell viability and DNA fragmentation were evaluated after 16 hours. Cell survival increased in the presence of HMGB1 compared to untreated cells. Analysis of DNA fragmentation showed that HMGB1 has an anti-apoptotic effect protecting mesoangioblasts from cell death. The protective effect of HMGB1 in this cell type correlates with increased expression of Bcl-2 family proteins.

Roberta Palumbo¹, Maurilio Sampaolesi, Francesco De Marchis, Rossana Tonlorenzi Sara Colombetti, Anna Mondino, Giulio Cossu and Marco E. Bianchi.
Extracellular HMGB1, a signal of tissue damage, induces mesoangioblast migration and proliferation. J Cell Biol. 2004 Feb 2;164(3):441-9

Sclareol (labd-14-ene-8, 13-diol) induces cell cycle arrest and apoptosis in human colon cancer cells via a p53 independent pathway

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Sclareol is a labdane-type diterpene isolated mainly from the plant *Salvia sclarea* as well as members of the *Cistus* genus. Like other diterpenes, sclareol has demonstrated a significant cytotoxic activity against human leukemic cell lines. Here we report the effect of sclareol against human colon cancer cells (HCT116^{wt} or ^{p53^{-/-}}) in a p53-independent manner. Sclareol was found to induce apoptosis via a p53 independent pathway, in a dose and time dependent manner at cells cycling normally. Sclareol is shown to activate both caspases 8 and 9, thus suggesting the implication of both apoptotic pathways. Additionally, flow cytometry analysis of the cell cycle indicated that sclareol is able to arrest cells at G₀/G₁ phase. This is accompanied by an increase of p21, which is also p53 independent. Intracellular levels of neither Mdm2 nor Bax were found to be altered.

Calmodulin binding enhances the fragmentation of the plasma membrane calcium ATPase isoform 4b by caspase-3

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The calmodulin stimulated plasma membrane Ca²⁺ATPases (PMCA) have a regulatory domain at their carboxyl-terminus that interacts with the catalytic core thus inhibiting their calcium transport activity. Ca²⁺-calmodulin binds to the calmodulin-binding region of this domain, abolishing the intramolecular interactions and activating the pump. One of the most widely expressed isoforms, PMCA4b, contains a caspase-3 consensus sequence (¹⁰⁷⁷DEID¹⁰⁸⁰) five residues upstream of the calmodulin-binding region. We have demonstrated that caspase-3 cleaves the pump right after Asp¹⁰⁸⁰ and removes the whole autoinhibitory domain resulting in a single 120 kDa proteolytic fragment which is fully activated. Further, we studied the effect of calmodulin binding on the fragmentation of hPMCA4b by recombinant caspase-3. Our experiments show that the cleavage by caspase-3 depends highly on the conformation of the pump. The pump was fully converted to the 120 kDa fragment in the presence of Ca²⁺-calmodulin while in the absence of calmodulin the digestion was slow and incomplete even after prolonged incubation with the protease. In accordance with these findings we found that calmodulin antagonists (trifluoperazine and W-7) greatly reduced PMCA fragmentation during staurosporine or anoikis induced apoptosis of COS-7 cells expressing hPMCA4b. Our results suggest that at low intracellular Ca²⁺ the carboxyl terminus of PMCA4b should be protected from caspase-3 like proteases. When cytosolic Ca²⁺ increases, calmodulin will bind to the pump, making cleavage by caspase-3 possible.

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Angiotensin II delays FLS decision to undergo apoptosis

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Angiotensin II is an octapeptide produced by ACE-mediated cleavage of angiotensin I and, being initially identified for its action on blood pressure, showed later its ability to stimulate the release of proinflammatory cytokines and increase oxidative stress, hence playing a pivotal role in inflammation. It has been reported that angiotensin converting enzyme (ACE) is overexpressed in synovia from rheumatoid arthritis patients. Rheumatoid arthritis is an autoimmune disease characterized by an uncontrolled expansion of fibroblast-like synoviocytes (FLS) due to a loss of response to apoptotic stimuli and of cell cycle control, leading to pannus formation and cartilage invasion. An important role in this mechanism is played by the transcription factor NF- κ B, overactivated in course of rheumatoid arthritis.

In this study we show that FLS express angiotensin II receptors AT1 and AT2 and that their expression is downmodulated by switching off NF- κ B through a specific siRNA against the p65 subunit of the transcription factor. Moreover, whereas under optimal culture conditions (10% FBS), a 24 hours exposure of the cells to 100nM angiotensin II increases bromodeoxyuridine incorporation (12%) in serum starvation conditions (1% FBS) angiotensin II halves cell proliferation rate. This cell cycle slowing is reflected in a delay in apoptosis induction. In fact, angiotensin II treatment results in a 26% reduction in oligonucleosomes in cell lysates resulting from DNA digestion, a feature typical of apoptotic cell death. Angiotensin II exerts a similar protective effect in presence of 150mM nitric oxide decreasing apoptosis induction of 10% as shown by Hoechst staining.

Our experiments highlight the protective effects of angiotensin II on FLS apoptosis induced by different stimuli. This observation, together with the fact that AT1 and AT2 expression is controlled by NF- κ B, support the hypothesis of a role played by angiotensin II in rheumatoid arthritis.

NU6140, a novel cyclin-dependent kinase inhibitor, potentiates paclitaxel-induced apoptosis through the down-regulation of the anti-apoptotic protein survivin

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Dysregulation of cell cycle control is one of the major characteristics of tumour cells, and molecules, such as cyclin-dependent kinases (CDKs), which play a crucial role in cell cycle progression, are promising targets for anticancer therapy.

We investigated the cellular effects of the novel CDK inhibitor NU6140 with respect to inhibition of cell proliferation and cell cycle progression and the ability to increase spontaneous and paclitaxel-induced apoptosis in the HeLa cervical carcinoma cell line in comparison to purvalanol A. Exposure to both CDK inhibitors resulted in a concentration-dependent cell cycle arrest at the G₂/M phase and in an increase in the apoptotic rate, with a concomitant down-regulation of the anti-apoptotic protein survivin, a member of the inhibitor apoptosis protein (IAP) family. Notably, the addition of NU6140 to paclitaxel-treated cells resulted in markedly increased cytotoxic effect and apoptotic response compared to the paclitaxel-purvalanol A combination. Similarly, the extent of caspase-9 and caspase-3 activation in paclitaxel-NU6140 treated cells was about 4-fold higher than after the paclitaxel-purvalanol A combination. Moreover, an almost complete abrogation of the expression of the active Thr³⁴-phosphorylated form of survivin was observed in cells exposed to the paclitaxel-NU6140 combination. A synergistic effect of the paclitaxel-NU6140 combination, as a consequence of survivin inhibition and increased activation of caspase-9 and caspase-3, was also observed in the OAW42 ovarian cancer cell line but not in the derived subline, OAW42/Surv, which ectopically expresses survivin.

Overall, results from the study indicate that NU6140 significantly potentiates the apoptotic response to paclitaxel in cancer cells and support the hypothesis that inhibition of survivin expression/phosphorylation is a major mechanism.

The proteasome inhibitor Bortezomib induces apoptosis in mantle cell lymphoma through generation of ROS species and noxa activation independent of p53 status

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Mantle Cell Lymphoma (MCL) is a mature B-cell lymphoma with an aggressive course and generally poor prognosis. Conventional chemotherapy has little efficacy. Bortezomib (PS-341, VELCADE) is a novel, reversible and high specific proteasome inhibitor that appears as a new hope for MCL treatment. We have analyzed the *in vitro* sensitivity to bortezomib in four MCL cell lines and in primary tumoral cells from ten MCL patients. Bortezomib induced phosphatidylserine exposure, mitochondrial depolarization, ROS generation, Bax and Bak conformational changes and caspase activation. In addition, ROS scavengers, but not pan-caspase inhibitors, blocked all apoptosis hallmarks. Protein and mRNA expression analysis, revealed marked upregulation of the BH3-only protein Noxa, between 4-6 h after bortezomib addition, independent of p53 status. However, this upregulation was faster and higher in cells with functional p53. Noxa RNA interference markedly decreased sensitivity to bortezomib, pointing this protein as a key mediator between proteasome inhibition and mitochondrial depolarization in MCL cells. Noxa interacts with the anti-apoptotic protein Mcl-1 suggesting that upregulation of Noxa might counteract the Mcl-1 accumulation after bortezomib treatment. These findings should be useful to extend the therapeutic strategies in MCL patients and to improve their prognosis.

Raf-1 modulates Fas signaling through Ezrin regulation

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The Raf-1 serine/threonine kinase relays signals inducing cell proliferation, differentiation, and survival. By using conventional gene ablation techniques, we have shown that Raf-1 is essential in counteracting apoptosis during embryonic development and that it does so independently of its ability to activate MEK. Embryos lacking Raf-1 die between E10.5 and E12.5 with increased fetal liver apoptosis and placental defects. In addition, ablation of Raf-1 sensitizes embryonic fibroblasts to Fas-induced, but not TNF-alpha-induced apoptosis. Recently, we have found that this increased sensitivity is due to the accumulation of Fas on the surface of Raf-1-deficient fibroblasts. Here, we investigated the molecular mechanism at the basis of this process. We find that Ezrin, a cytolinker connecting Fas and the actin cytoskeleton, is hyperphosphorylated and mislocalized in Raf-1-deficient cells. Using a dominant negative version of Ezrin we could confirm that Ezrin deregulation leads to increased Fas sensitivity. Moreover, the stable expression of kinase-dead Raf-1 restores Ezrin phosphorylation levels and Fas localization. Thus, the essential, kinase-independent role of Raf-1 in the modulation of the Fas signaling is mediated through Ezrin regulation.

Analysis of cooperation between two TNF receptors in the U937 cell variants responding to soluble and transmembrane TNF

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TNF exerts its wide range of activities via two receptors TNF-R55 and TNF-R75. The latter is efficiently activated by transmembrane TNF (tmTNF), but not by soluble TNF (sTNF), while TNF-R55 can respond fully to both TNF ligands. Very little is known about cooperation of TNF receptors on the level of initiation of activation by tmTNF. It is also of interest how cell death and life decisions are made on the level of receptors as it may be crucial for treatment of cancer and autoimmune diseases. We observed a distinct pattern of cytotoxic response to tmTNF in a model of U937 cell variants characterized by different TNF sensitivity. U937M cells were sensitive to sTNF and tmTNF, while U937ATCC cells were resistant to both forms of TNF. Surprisingly, CHX did not enhance cytotoxic effect of tmTNF in U937M cells suggesting the impaired cytotoxic mechanism probably dependent on TNF-R75 function or its cooperation with TNF-R55. Blocking of TNF-R55 by antibodies in U937M cells was not efficient enough to inhibit cytotoxic effect of sTNF and tmTNF. However it was possible to fully block the cytotoxic effect exerted by the p55-specific TNF mutein in U937M cells. This effect could suggest that TNF-R75 may transduce cytotoxic effect itself; however it was not confirmed by direct activation by p75 mutein or by different agonistic antibodies. The effect of sTNF and tmTNF was blocked effectively by anti-TNF-R55 antibodies in U937ATCC cells. Mobilization of NF-κB from cytoplasm to nucleus was estimated to measure pro-survival response in U937 cells. Activation of NF-κB was blocked by anti-TNF-R55 antibodies but not by anti-TNF-R75 antibodies upon stimulation with sTNF in U937M and U937ATCC cells. On the other hand tmTNF activation was blocked effectively by anti-TNF-R55 antibodies but not anti-TNF-R75 antibodies in U937ATCC cells. In U937M cells blocking by anti-TNF-R55 antibodies was weak. Similar mild effect was exerted by anti-TNF-R75 antibodies. It was also possible activate NF-κB by TNF-R75 specific TNF mutein in the presence of stabilizing antibodies 80M2 in U937M cells. Taken together, we suggest that cooperation between TNF receptors in exerting cytotoxic effect by tmTNF in U937 cells is required. While TNF-R75 seems to have no effect on NF-κB mobilization in U937ATCC it is involved in induction of this activity by tmTNF in U937M cells.

***Mcl-1* gene encoding antiapoptotic member of Bcl-2 family is repressed by wild type p53**

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Mcl-1 plays an important role in the regulation of mitochondrial apoptotic pathway. Formation of *Mcl-1*-Bak heterodimers inhibits Bak's proapoptotic activity. A rapid decrease of *Mcl-1* protein amount at the early stage of apoptosis initiation has been observed. In addition, wild type p53 disrupts *Mcl-1*-Bak heterodimers. As a result of both mechanisms Bak protein is released, forms homodimers, and initiates mitochondrial apoptotic pathway. p53 initiates cell cycle arrest and apoptosis *via* different mechanisms. In addition to the protein-protein interactions, it acts as transcription regulator. p53 binds to its recognition site located in target genes promoter regions and activates their expression. p53 can repress gene expression *via* disruption of transcription activator's function (Sp1, C/EBP etc.), interaction with basal transcriptional machinery, or chromatin structure change.

We show here that 1826 bp human *Mcl-1* promoter is repressed about 20-fold by wild type p53. Transcription activation assay with different amounts of p53 expression vector showed that this was a dose-dependent effect. To find the region responsible for p53-dependent repression, deletion mutants were created. Transcription activation assays showed that p53 inhibitory effect is mediated by 293 bp promoter fragment proximal to putative transcription start site. EMSA showed that p53 disrupts Sp1 transcription activator binding to its three recognition sites located within this promoter fragment. However, overexpression of Sp1 reduced p53-dependent repression only by approximately 2,2-fold. The majority of p53 inhibitory effect was mediated by 57 bp promoter fragment containing putative GATA binding site. Therefore we conclude that that *Mcl-1* promoter is repressed by p53 partly by interference with transcription activator Sp1 binding, partly by interaction with basal transcription machinery.

Taken together, our results show another mechanism of p53-dependent apoptosis, namely repression of *Mcl-1* gene transcription. Our results complement current knowledge regarding interactions between p53 and Bcl-2 family proteins, that are either activated (like proapoptotic Bak and Bax), or repressed (like antiapoptotic Bcl-2 and *Mcl-1*) by this protein. Such interactions finally lead to cytochrome C release from mitochondria, and initiation of apoptosis.

Irradiation-induced translocation of p53 to mitochondria in the absence of apoptosis

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The tumor suppressor protein p53 promotes apoptosis in response to death stimuli by transactivation of target genes and by transcription-independent mechanisms. Recently, it was shown that during apoptosis p53 can specifically translocate to mitochondria, where it physically interacts with and inactivates prosurvival Bcl-2 proteins. In the present study, we therefore investigated the role of mitochondrial translocation of p53 for the radiation response of tumor cells. In both MCF-7 and HCT116 carcinoma cells ionizing irradiation triggered a robust translocation of a fraction of p53 to mitochondria. Nevertheless, the cells remained resistant to apoptosis and became senescent, although irradiation triggered a functional p53 response and induced expression of the target genes p21 and Bax. Interestingly, even the targeted expression of p53 to mitochondria was insufficient to launch apoptosis, whereas overexpression of wildtype p53 induced Bax activation and apoptotic alterations. Together, these results suggest that, in contrast to previous reports, mitochondrial translocation of p53 *per se* does not lead to cell death, but constitute a mechanism that could contribute to the radioresistant phenotype of MCF-7 and HCT116 tumor cells.

Apoptosis and endometrial carcinoma

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Introduction: Apoptosis is a regulating mechanism of self-suicide of damaged, aged or unuseful cells. The pro-apoptotic and anti-apoptotic's gene expression differs in association with the ovulatory cycle and their role in endometrial tumorigenesis may be important. We studied the expression of pro-apoptotic genes p53, bax, bim, bad (Group 1) and anti-apoptotic genes bcl-2, bag-1 (Group 2).

Material: 21 endometrial adenocarcinoma (frozen tissues)

Methods: RNA isolation, RT-PCR, Multiplex-PCR, DNA sequencing and statistical analysis: test for two proportions and Fischer's exact test (χ^2)

Results: p53 42,9%, bax 42,9%, bim 4,8%, bad 23,8%, bcl-2 33,3% and bag-1 52,4%

Conclusions:

- 1) statistically significant difference between the expression of bim–bcl-2 (P: 0,011), bim–bag-1 (P: 0,001) and bad–bag-1 (P: 0,046), thus maximum expression of Group 2
- 2) Group's 1 genes follow the trend to be expressed at least one of them
- 3) statistically significant correlation between p53–bad (P: 0,045) and bax–bad (P: 0,045). Incompatibility between these genes. One's expression minimizes the possibility of other's expression.

Apoptosis modulation on porcine macrophages infected with African swine fever virus (ASFV) of different virulence

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ASFV is the agent of a threatening and highly contagious pig disease without effective vaccine. This large dsDNA virus replicates on pig monocytes and macrophages and possesses genes potentially involved in apoptosis control, namely *bcl-2* and *iap* homologues, shown to prevent apoptosis when expressed in mammalian cell lines. Although such genes may confer valuable tools for infection and immune evasion, their effect is still unknown on pig macrophages. Moreover, death mechanisms induced with different virulence isolates and its implications in pathogenesis are unknown.

Our aim was to characterize the apoptotic process on blood derived pig macrophages infected with highly virulent ASFV/L60 versus low virulent ASFV/NH/P68, through the assessment of (i) apoptosis levels at early (ongoing viral replication) versus late (accomplished replication) infection phases (ii) viral induced protection to apoptosis triggered by exogenous inducers staurosporin (ST) or cycloheximide (CHX) and (iii) expression of viral *bcl-2* and *iap* along infection by RT-PCR. At 8h (early phase) and 18h (late phase) post infection (p.i.), DNA internucleosomic fragmentation, caspase-3 activity and Hoechst stained nuclear morphology were determined.

No protection from apoptosis was observed on L60 or NH/P68 infected macrophages treated with ST or CHX. Nevertheless, clear induction of apoptosis was obtained only at 18h with both viruses, supporting an apoptosis protection at early infection when replication is ongoing. L60 infection revealed lower levels of caspase-3 activity 18h p.i. when compared to NH/P68 and expressed higher levels of both viral *bcl-2* and *iap* at very early times post infection (1,30h, 4h). Moreover, viral *iap* described by other authors as a late expression gene with Vero cell line adapted ASFV, revealed to be clearly expressed in pig macrophages very early after infection (1,30h). Ongoing studies may bring new insights for the comprehension of ASFV apoptosis modulation and thus for the clarification of viral evasion mechanisms.

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Differentiation and cell death of leukaemic HL-60 cells co-induced by cytokines and inhibitors of arachidonic acid metabolism

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Cytokinetics of hemopoietic cells was influenced both by cytokines (TNF- α and TGF- β 1) and inhibitors of arachidonic acid (AA) metabolism. Alteration of cytokine signalling pathways induced by AA inhibitors were studied with the aim to either decrease proliferation or increase cell death of leukaemic cells HL-60. The cell death is often a consequence of terminal differentiation, which was induced and studied in this project. The TNF- α signalling pathway involved the activation of AA metabolism, which was modified by action of inhibitors of 5-lipoxygenase (5-LPO) MK886, cyclooxygenase - proadifen and indomethacin, and NDGA, that inhibited mainly 5-LPO (Rizzo 2002). Point of intersection of TNF- α and TGF- β 1 pathways represented a transcription modulator NF- κ B (Aggarwal 2004), that can transactivate anti-apoptotic Bcl-2 protein (Tamatani et al. 1999).

Promyelocytic HL-60 cells treated simultaneously with TNF- α and MK886 or proadifen expressed in five days the differentiation-associated markers CD11b and CD14, whereas the influence of indomethacin and proadifen was negligible. This differentiation was accompanied by increased number of apoptotic cells. They were the most numerous in samples with inhibited AA metabolism co-treated by both cytokines. Cells affected by both cytokines simultaneously accumulate in G₀/G₁ phase of the cell cycle, that was the point where cells left the cycle and commit the death. Samples treated by TGF- β 1 (all combinations) showed increased expression of 5-LPO after three days even when incubated with its inhibitors. All the combinations of treatments involving TNF- α contained just minimal amount of non-degraded I κ B during whole five days of incubation, which reflected the increased amount of free activated NF- κ B. Even though NF- κ B could be the direct activator of Bcl-2 expression, in samples containing activated NF- κ B we observed decreased expression of Bcl-2 after three days of incubation. This suggests that the pro-survival effect of NF- κ B was overcome by cell death accompanying the terminal differentiation.

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Spatio-temporal analysis of apoptosis cascade events by confocal fluorescence imaging of live cells

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During apoptosis Bax translocates to mitochondria and multimerizes while cytochrome c is released into the cytosol. Oligomeric Bax has been found to be associated with the formation of the mitochondrial apoptosis-induced channel (MAC), which is the putative cytochrome c release channel induced early in the intrinsic apoptosis pathway. Using fluorescent probes in time-lapse confocal microscopy experiments we simultaneously visualized the key molecular events taking place in live cells during apoptosis, like Bax relocalization, cytochrome c release, caspase 3 activation and loss of mitochondrial transmembrane potential ($\Delta\Psi_m$). Single-cell analysis of HeLa and Hct116 Bax^{-/-} cells challenged with staurosporine showed that early clustering of Bax on the mitochondrial outer membrane (MOM) occurred only after cytochrome c release, then followed by caspase 3 activation and eventually, loss of $\Delta\Psi_m$. However, a second phase implying a complete sequestration of Bax from the cytosol building-up the clusters at the MOM was found to strictly depend on caspase 3 activation. Furthermore using a specific subcellular probe for caspase 3 we monitored the progressive appearance of caspase 3 activity in the cytosol, nucleus and mitochondrial intermembrane space. Using our approach we could thus reconstitute a spatio-temporal sequence of the major key apoptotic events at the single live cell level.

hTERT inhibits p53-induced apoptosis at the mitochondrial level

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The p53 tumor suppressor is a key regulator of cell growth and survival that controls fundamental cellular events through transcriptional regulation of multiple target genes. We previously found that the human telomerase reverse transcriptase (hTERT) gene is downregulated by p53 at the mRNA-level. hTERT is a ribonucleic protein that synthesizes telomeres. Telomerase, activated in many tumors allowing escape from senescence and crisis has been shown to have an anti-apoptotic role in addition to its ability to maintain telomere length. We hypothesized that p53-mediated repression of hTERT is critical for p53-induced apoptosis. To test this we have investigated the effects of constitutive expression of hTERT and catalytically inactive DN-hTERT on p53-dependent apoptosis in two cell systems, BL41 Burkitt lymphoma cells carrying an exogenous temperature-sensitive p53 (BL41-ts p53) and HCT116 colon carcinoma cells with or without endogenous wild type p53. We found that constitutive expression of hTERT in both cell lines inhibits p53-induced apoptosis. Both FACS analysis and TUNEL staining showed decreased apoptotic response in cells expressing hTERT after activation of p53. The catalytically inactive DN-hTERT was as efficient as wt hTERT in antagonizing p53-mediated apoptosis. Furthermore, we found that constitutive hTERT expression prevents p53-mediated mitochondrial changes and cytochrome c release. Similar result was obtained upon expression of telomerase-inactive hTERT mutant. Our data demonstrates that hTERT inhibits p53-dependent apoptosis at the mitochondrial level independently of its telomerase activity.

Protective effect of antioxidant enzymes on UVB-induced apoptosis in normal human keratinocytes

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UV-induced apoptosis in keratinocytes is a highly complex process in which two different caspase-dependent molecular pathways are involved. These include an extrinsic pathway via triggering of death receptors and an intrinsic one via DNA damage and reactive oxygen species (ROS) formation.

In this study we investigated the overexpression of antioxidant enzymes (Catalase and Cu/ZnSOD), on apoptosis induced by UVB exposure of normal human keratinocytes. Cells were irradiated at room temperature (RT) or at 4°C. Cell death (apoptosis and necrosis) was measured by following the activation of caspases with FITC-VAD-FMK (which binds to activated caspases) and plasma membrane permeability using propidium iodide (PI). DNA fragmentation was studied by PI labelling and cytofluorimetry. Caspase-3, -8 and -9 activities were quantified by western blotting. Intracellular formation of ROS was detected by the fluorescent probe CM-H₂DCF-DA.

Irradiation at low temperature compared to irradiation at RT reduced UV-induced apoptosis by 40% in normal keratinocytes: this was explained by the inhibition of the aggregation of death receptors leading to a decrease in caspase-8 activation. Catalase overexpression decreased apoptosis by 40% in the cells irradiated at RT with a reduction of caspase-9 activation; at 4°C, there was a 80% reduction in UVB-induced apoptosis accompanied by decrease in caspase-8 and -9 activation. In addition, in the irradiated cells overexpressing catalase, the accumulation of p53 and serine-15 phosphorylated p53 was reduced in comparison with non transduced irradiated cells. Cu/ZnSOD overexpression had no effect on UVB-induced apoptosis. ROS amounts increased transiently at irradiation time even in cells overexpressing catalase. Furthermore, the addition of H₂O₂ on the cells only at irradiation time reduced UV-induced apoptosis.

We conclude that ROS augmentation just at irradiation time has an anti-apoptotic effect. The protective role of catalase overexpression against UVB irradiation takes place through the reduction of ROS following irradiation by preventing ROS-induced damages especially on DNA.

Immunotoxins containing *Pseudomonas* exotoxin A induces apoptosis via the caspase-dependent pathway in cancer cells

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Aim: In order to obtain better therapeutic immunotoxins (ITs) for cancer therapy, we have to understand the molecular mechanisms responsible for IT-mediated induction of apoptosis and cell death.

Introduction: Two of our ITs consist of antibodies covalently linked to the bacterial toxin *Pseudomonas* exotoxin (PE). The antibodies we utilize, 425.3 and 9.2.27, recognize antigens that are highly expressed on respectively breast cancer and malignant melanoma cell lines. ITs containing PE induce cell death by arresting protein synthesis and also induce apoptosis, although very little is known about the underlying mechanisms.

Results and discussion: Our immunotoxins induced a rapid inhibition of protein synthesis, a decrease in mitochondrial membrane potential and an induction of apoptosis in a cathepsin-caspase-dependent pathway. The caspase-9 specific inhibitor (z-LEDH.fmk) obliterated the IT-induced caspase-3, -8 and -9 protease activity, suggesting that IT-induced apoptosis is dependent of caspase-9 activation. The broad-spectrum caspase inhibitor, z-VAD.fmk (1-5 μ M) had a pronounced cell rescuing effect even after IT treatment for 72 hours, suggesting that caspases play an important role in IT-induced apoptosis in MA11 breast cancer cells. Combining z-VAD.fmk and z-FA.fmk (an inhibitor of cathepsin B/L) completely prevented IT-induced cell death, indicating that cathepsin activation are important for optimal induction of IT-induced cell death.

To further analyze upstream regulators of IT-induced apoptosis we measured mitochondrial membrane potential ($\Delta\Psi_m$) and performed Western blot analyses on Bcl-2 family members. The $\Delta\Psi_m$ decreased before caspases were activated, clearly showing that the $\Delta\Psi_m$ is an upstream regulator of IT-induced apoptosis. Western blot analyses demonstrated the anti-apoptotic protein Mcl-1, a Bcl-2 family member, dramatically decreased in 425.3PE-treated MA11 breast cancer cells whereas the levels of Bcl-2, and Bcl-X_L were unchanged. Decreased expression of various anti-apoptotic proteins like Mcl-1, survivin, Bcl-x_L and phosphorylation of Bcl-2 were observed in 9.2.27PE treated malignant melanoma cells. Furthermore, the proteasome inhibitor Lactacystin protected the cells against IT-induced down-regulation of Mcl-1, caspase-3 activation and PARP inactivation in MA11 cells. The rapid turnover of Mcl-1 protein caused by proteasome degradation contributed at least to two of the hallmarks of apoptosis; the caspase-3 activation and PARP inactivation, supporting the importance of Mcl-1 down-regulation from IT-treatment. This suggests that IT-treatment changes the balance between pro-apoptotic proteins in excess of anti-apoptotic proteins. In addition, the decreased level of Mcl-1 may lead to the observed changes in mitochondrial membrane permeabilization, and subsequently a decrease in $\Delta\Psi_m$.

The role of apoptosis in reperfusion injury

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Despite decades of intensive research, the mechanism of reperfusion injury following cardiac ischemia is still subject of debate. This lack of understanding of the damage inflicted upon the heart following restoration of its blood supply seriously hampers efforts of both prevention and treatment.

Apoptosis due to the reperfusion process has been recently implicated as a direct cause of myocardial injury commonly observed to occur after surgical or medical restoration of the impaired myocardial blood supply. This theory has been investigated in the canine model using homologous working cervical donor heart preparation. Inducing asystolic ischemia of various length (both protected and unprotected) parameters of function were monitored and the degree of tissue damage including the occurrence of apoptosis and necrosis were determined using conventional and electron microscopy, HPLC and western block analysis and immunohisto-chemistry for lumin B and activated caspase-3.

The conclusion obtained of the above measurements was that apoptosis, which ultimately results in reperfusion damage is initiated during ischemia and completed during reperfusion, therefore, measures of prevention should start during the period of ischemia.

Inhibition of NFκB pathway allows mantle cell lymphoma (MCL) cells to undergo TRAIL-induced apoptosis by transcriptional regulation of TRAIL receptors, c-FLIP and XIAP down-modulation

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Mantle cell lymphoma (MCL) is a lymphoid malignancy derived from mature B-cells. MCL cells are characterized by the chromosomal translocation t(11;14)(q13;q32) which results in cyclin D1 overexpression, and also present a constitutive activation of the NFκB pathway which leads to the overexpression of several anti-apoptotic regulators. As a consequence, these cells have an aggressive course and poorly respond to common chemotherapeutic agents acting via the intrinsic mitochondrial pathway. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a potent activator of the extrinsic cell death pathway and has been shown to exert *in vivo* an anti-tumoral activity via its receptors TRAIL-R1 and -R2. On the other hand, it shows minimal toxicity on normal cells mainly expressing the inhibitory decoy receptors TRAIL-R3 and -R4. Our purpose was 1) to assess the sensitivity of MCL cells to recombinant human TRAIL on primary and established MCL cell lines and 2) to potentiate its effects in the less sensitive cells by pharmacological co-treatment cells. On the 6 MCL cell lines tested, three (Jeko, HBL-2, UPN-1) presented a high sensitivity to TRAIL, two (Rec-1 and Granta-519) were less sensitive and one cell line (JVM-2) remained resistant, without apparent correlation either with TRAIL-R1 and -R2 receptors or Bcl-2 family protein levels. TRAIL also exerts a cytotoxic activity in primary cells from 3 out of 6 (50%) MCL patients. TRAIL-induced apoptosis was characterised by a time- and dose-dependent loss of mitochondrial membrane potential, Bax and Bak activation, caspase activation and phosphatidylserine ecto-exposure. Primary and established MCL cell lines with reduced response to TRAIL were characterised by high TRAIL-R3 /TRAIL-R1 mRNA ratios and elevated protein levels of the DISC inhibitor c-FLIP. Both features presumably impeded caspase-8 cleavage upon TRAIL treatment. Co-treatment of primary and established MCL cell lines with sub-toxic doses of the proteasome inhibitor Velcade or inhibitors of the NFκB activation pathway decreased TRAIL-R3 /TRAIL-R1 mRNA ratios and also c-FLIP and XIAP protein levels. These effects were associated with a higher cell sensitivity to TRAIL and with an increased formation of TRAIL-dependent DISC and caspase activation. These results indicate that MCL cells sensitivity to TRAIL is regulated by NFκB-induced anti-apoptotic factors that act at both DISC activation and caspase regulation and could be increased by co-treating cells with NFκB pathway inhibitors.

Hypericin-mediated photocytotoxic effect on HT-29 adenocarcinoma cells is reduced by light fractionation with longer dark pause between two unequal light doses

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The present study demonstrates the in vitro effect of hypericin-mediated PDT with fractionated light delivery. Cells were photosensitized with unequal light fractions separated by dark intervals (1 h or 6 h). We compared the changes in viability, cell number, survival, apoptosis and cell cycle on HT-29 cells irradiated with a single light dose (12 J/cm²) to the fractionated light delivery (1+11 J/cm²) 24 h and 48 h after photodynamic treatment. We found that a fractionated light regime with a longer dark period resulted in a decrease of hypericin cytotoxicity. Both cell number and survival were higher after light sensitization with a 6 h dark interval. DNA fragmentation occurred after a single light dose application, but in contrast no apoptotic DNA formation was detected with a 6 h dark pause. After fractionation the percentage of cells in the G1 phase of the cell cycle was increased, while the proportion of cells in the G2 phase decreased as compared to a single light dose application i.e. both percentage of cells in the G1 and G2 phase of the cell cycle were near control levels. We presume that the longer dark interval after the irradiation of cells by first light dose makes them to resistant to the effect of the second illumination. These findings confirm that the light application scheme together with other photodynamic protocol components is crucial for the photocytotoxicity of hypericin.

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Amelioration of cardiovascular functions by the suppression of myocardial apoptosis in left ventricular remodeling

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Cardiac remodeling occurs after myocardial infarction (MI) and plays a major role in the progression to heart failure. It has been suggested that apoptotic death of cardiac myocytes is frequently observed in remodeled hearts and is critically involved in the process of post-infarction cardiac remodeling. If myocardiocyte apoptosis plays an important role in the process of cardiac remodeling, it would be meaningful to clarify the functional and/or pathological changes induced by modulating the apoptotic signal transduction pathways. In the present study, we have tried to clarify the pathophysiological link between myocardial apoptosis and cardiovascular function by inhibiting an apoptotic signal transduction factor, caspase-3 or calpain, in left ventricular remodeling after MI.

Either a caspase-3 inhibitor (CasI) or calpain inhibitor (Call) was administered immediately after MI in a rat model of MI. Blood pressure, heart rate (HR), and blood flow velocity (BFV) were measured and pressure-rate product (PRP) was calculated for estimating the changes in cardiovascular function. BFV showed no remarkable changes in any of the groups. Systolic blood pressure (SBP) and PRP were significantly reduced in the MI group compared to the sham group, whereas HR was significantly greater in the MI group than sham group. However, although the decrease in SBP and the increase in HR for both MI + CasI and MI + Call were observed at 1 day after MI, these variables returned to baseline levels. These results suggested that both caspase-3 and calpain inhibitors had the effect of improving cardiovascular function in left ventricular remodeling after MI. The administration of apoptotic inhibitors immediately after MI is expected to ameliorate left ventricular remodeling and cardiovascular function in remodeled hearts.

Suppression of apoptosis by Coxsackie B virus infection

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It is well known that virus infection may induce host cell death by apoptosis, but some (mainly DNA) viruses are capable of preventing this process. RNA viruses were thought not to display anti-apoptotic activity but instead evade host cell suicide by rapid replication. Recently, anti-apoptotic activity was demonstrated in poliovirus. We have therefore investigated the ability of the closely related Coxsackievirus B4 (CVB4), an enterovirus in the family Picornaviridae, to antagonise apoptosis in human cells.

CVB4 infection of HeLa cells resulted in negligible induction of apoptosis over a period of 24h. However, CVB4 infected cells showed a significant delay in the induction of apoptosis by staurosporine (STS), TRAIL or actinomycin D as evidenced by an inhibition of phosphatidyl serine exposure and chromatin condensation. Investigations into the anti-apoptotic mechanism revealed that the apoptotic machinery was fully activated in STS-treated infected cells as evidenced by the levels of pro-caspase-3 processing to the p20/p17 fragments. However, under these same conditions cellular DEVDase activity was significantly inhibited in the virus + STS-treated cells compared with STS-treated cells alone. The addition of CVB4-infected cell lysates to recombinant caspase-3 significantly suppressed its activity. Immunoprecipitation studies using [³⁵S]Met-labelled CVB4-infected cells with an anti-caspase-3 antibody revealed a 48 kDa protein that was identified as viral protein 2BC. Likewise, immunoprecipitation experiments conducted on recombinant viral proteins showed that 2BC but not its cleaved form 2B could be co-immunoprecipitated with an anti-caspase-3 antibody. DEVDase assays confirmed that this interaction by 2BC inhibits the activity of recombinant caspase-3 with no suppression occurring with 2B. Interestingly, sequence analysis of 2BC shows that the 2C portion has serpin motifs and has close homology to the poxvirus IAP CrmA. These results suggest that 2BC is acting as a viral IAP and this is the first report of such an ability for an RNA virus.

Differential mechanisms for apoptosis induced by dental monomers

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Methacrylate monomers have been identified in aqueous extracts of freshly cured dental comonomers. Others and we have shown that these compounds are able to induce apoptosis and necrosis in a concentration dependent manner. To further elucidate mechanisms involved in the apoptotic response induced by these compounds, we have tested four commonly used monomers: HEMA, TEGDMA, GDMA and MMA in a salivary gland cell line.

The results show that the concentrations of monomers needed for an apoptotic response in a 24 h exposure experiment varied significantly between the different monomers with GDMA being most potent (1 mM) and MMA as least potent (35 mM). Furthermore, formation of ROS appeared to be a necessary initial incident in the development of the apoptotic response. Interestingly, when increasing exposure time from 24 to 48 hrs, the concentration of monomers necessary to induce apoptosis appeared to be significantly reduced (75 μ M GDMA and 10 mM MMA). We were not able to detect increased levels of ROS at these concentrations.

Moreover, the results indicate that the mechanisms involved in mediation of apoptosis varied depending on exposure conditions (time and concentration) of the tested monomers. Preliminary results also indicate that this conferred activation of different pro-caspases.

Programmed cell clearance: phosphatidylserine-dependent macrophage engulfment of activated neutrophils is defective in chronic granulomatous disease patients

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Chronic granulomatous disease (CGD) is characterized by severe bacterial and fungal infections due to a genetic defect in different components of the NADPH oxidase in phagocytes and neutrophils. Our previous studies have shown that neutrophils from CGD patients are defective for phorbol myristate acetate (PMA)-induced plasma membrane exposure of phosphatidylserine (PS), an important recognition signal for macrophages (Fadeel et al., Blood, 1998). Similarly, treatment of normal neutrophils with a pharmacological inhibitor of the NADPH oxidase blocked PMA-induced PS exposure. Here, we have tested a hypothesis that the absence of a functional NADPH oxidase in CGD patients could result in defective macrophage engulfment of apoptotic cells. Four CGD patients harboring defects in the p47, p67, or gp91 subunits of the NADPH oxidase were included in the study, and ethical approval was obtained from the local ethical committee at Karolinska Institutet. Our studies demonstrate that monocyte-derived macrophages engulf normal apoptotic neutrophils. In contrast, the degree of phagocytosis of normal PS-positive target cells was minimal or absent when CGD macrophages were investigated. Moreover, as expected, CGD neutrophils were not efficiently engulfed by normal macrophages, most likely due to the absence of PS externalization. Taken together, these studies suggest that programmed cell clearance is dependent on a functional NADPH oxidase not only in the target cell (PMA-stimulated neutrophils), but also in macrophages. Defective cell clearance could perhaps contribute to the formation of tissue-destructive granulomas and chronic inflammation evidenced in CGD patients.

Ceramide, a novel player in photoreceptor apoptosis

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Reactive oxygen species (ROS) play a critical role in photoreceptor apoptosis. However, the exact molecular mechanisms triggered by oxidative stress in photoreceptor cell death remain undefined. The present study demonstrates that the sphingolipid ceramide is the key mediator of oxidative-stress induced apoptosis in 661W retinal photoreceptor cells. Treatment of the retina-derived 661W cells with the nitric oxide donor sodium nitroprusside (SNP) activates the sphingomyelin (SM) pathway. As a result, SM is hydrolysed by acid SMase, which leads to an increase in the concentration of ceramide. The current work shows that ceramide is responsible for the activation of the mitochondrial apoptotic pathway in the retina-derived 661W cells. Thus, ceramide induces the collapse in the mitochondrial transmembrane potential, ROS overproduction, cytochrome c release from the mitochondria and subsequent activation of the caspase cascade. Furthermore, ceramide is responsible for the increased calcium levels in the mitochondria and cytosol that precedes activation of the calpain-mediated apoptotic pathway in SNP-treated 661W photoreceptor cells. Interestingly, recent work from our laboratory has demonstrated a critical role for radical species in an *in vivo* model of retinal degeneration, in which calpains are also active. We are currently investigating the role of ceramide this *in vivo* model.

The effect of dominant-negative Bcl-2 on BI-1 mediated ER calcium reduction and protection from apoptosis

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Bax inhibitor-1 (BI-1) is an evolutionarily conserved protein capable to suppress apoptotic cell death induced by Bax and other agents. The antiapoptotic mechanism seems to include changes in intracellular calcium handling. BI-1 reduces the agonist-induced cytosolic and mitochondrial calcium rise by a reduction of the amount of calcium stored within the endoplasmic reticulum. BI-1 consists of six transmembrane domains and was shown previously to interact with the protooncogene Bcl-2. In this study, we investigated the effect of supposedly dominant-negative Bcl-2 constructs (BH1, BH4 and G145A) on calcium homeostasis and cell death. We also present conflicting results between stable and transient transfection. In our hands, CHO cells stably expressing BI-1 are more sensitive to cell death induced by tunicamycin and HA14-1, whereas transient transfection is protective. These changes are associated with opposing changes in calcium handling.

DNA fragmentation, but not caspase-3 activation or PARP-1 cleavage, combined with macrophage immunostaining as a tool to study phagocytosis of apoptotic cells in situ

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Efficient phagocytosis of cells undergoing apoptosis by macrophages is important to prevent immunological responses and development of chronic inflammatory disorders, such as systemic lupus erythematosus, cystic fibrosis or atherosclerosis. To study phagocytosis of apoptotic cells (AC) by macrophages, we validated different apoptosis markers (DNA fragmentation, caspase-3 activation and cleavage of its substrate poly(ADP-ribose)polymerase-1) in combination with macrophage immunostaining. Human tonsils were used as a model because they show a high apoptosis frequency under physiological conditions as well as efficient phagocytosis of AC by macrophages. On the other hand, advanced human atherosclerotic plaques were examined to evaluate phagocytosis of AC in a pathological condition. Our results demonstrate that the presence of non-phagocytized TUNEL-positive AC represents a suitable marker for poor phagocytosis by macrophages in situ. Other markers for apoptosis, such as cleavage of caspase-3 or PARP-1, should not be used to assess phagocytosis efficiency, since activation of the caspase cascade and cleavage of their substrates can occur in AC when they have not yet been phagocytized by macrophages.

Mitochondrial carrier homolog 2 is a target of tBID in cells signaled to die by TNF α

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BID, a pro-apoptotic BCL-2 family member plays an essential role in the TNF α /Fas death receptor pathway *in vivo*. Activation of the TNF-R1 receptor results in the cleavage of BID into truncated BID (tBID), which translocates to the mitochondria and induces the activation of BAX or BAK. In TNF α -activated FL5.12 cells, tBID becomes part of a 45kD cross-linkable mitochondrial complex. Here we describe the biochemical purification of this complex, and the identification of mitochondrial carrier homolog 2 (Mtch2) as part of this complex. Mtch2 is a conserved protein that is similar to members of the mitochondrial carrier protein (MCP) family. Our studies using mouse liver mitochondria indicate that Mtch2 is an integral membrane protein exposed on the surface of mitochondria. Using blue-native gel electrophoresis we revealed that in viable FL5.12 cells, Mtch2 resides in a protein complex of approximately 185kD in size, and that addition of TNF α to these cells leads to the recruitment of tBID and BAX to this complex. Importantly, this recruitment was partially inhibited in FL5.12 cells stably expressing BCL-X_L. These results implicate Mtch2 as a mitochondrial target of tBID, and raise the possibility that the Mtch2-resident complex participates in the mitochondrial apoptotic program.

TGF-beta1 induced apoptotic mechanism in human B-lymphoma cells

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Transforming growth factor beta1 (TGFb1) has antiproliferative and/or proapoptotic effect on normal B-cells. The B-lymphoid tumor cells often are less sensitive or insensitive to TGFb1. In HT58 B-cell NHL lymphoma cell line, exogenous TGFb1 did not inhibit the proliferation, however was able to induce apoptosis. In these cells the activation of Smad and alternative TGFb signaling pathways were studied.

Lymphoma cells were treated with recombinant TGFb1. Apoptotic effect and depolarization of the mitochondria were detected by flow cytometry. Expression, localisation and activity of different molecules (Smads, MAPKs, members of Bcl-2 family, survival factors) were studied by PCR, Western-blot and confocal microscopy. The role of PP2A phosphatase, MEK1 kinase and caspase activity was estimated by specific inhibitors.

Smad2 phosphorylation, nuclear localization and the increased expression of TGFb1 induced early gene (TIEG) proved the rapid activation of Smad signal. The role of PP2A phosphatase activation followed by decreased expression of active phospho-JNK and phospho-Erk1/2 was supported by the antiapoptotic effect of PP2A inhibitors and the proapoptotic effect of specific MEK1 inhibitor. The activation of apoptosis was death receptor independent, however, both main initiator caspases (caspase8 and 9) took part in the apoptosis induction amplifying the ROS-dependent mitochondrial pathway. This induced apoptosis was accompanied by inactivation of survival signals in lymphoma cells.

It seems that exogenous TGFb1 had double but interrelated actions on HT58 cells:

- a) suppression survival signals by protein phosphatases, which
- b) allowed the Smads to induce apoptosis. Therefore, it is possible that the lost sensitivity of malignant lymphoid cells to proapoptotic regulators (such as TGFb1) could be reactivated especially by lowering the survival threshold.

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Detection of apoptotic cells in root apical meristem of saffron (*Crocus sativus*) after treatment with fusaric acid and determination of apoptotic bodies' fate

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Programmed cell death (PCD)- a selective and genetically controlled cell death- after exhibiting specific morphological features is described as apoptosis. It has been shown that some apoptotic hallmarks are observed in dying plant cells. Fusaric acid (FA), a *fusarium* mycotoxin, is known to alter mitochondrion and plasma membrane permeability which has a crucial role in apoptosis of animal cells. For this reason, we treated Saffron (*Crocus sativus*) roots with different FA doses and studied the induced cell death. Test of cell viability was carried out using Vybrant Apoptosis Assay Kit#2 on root-derived protoplasts. DNA was extracted from roots by CTAB method, and DNA fragmentation was studied on gel electrophoresis. FA-treated roots were fixed and subjected to either transmission electron microscopic study or TUNEL assay. Flow cytometric results showed that in 25-100 μmol , the prominent cell population is apoptotic cells. Study of DNA fragmentation revealed raise of DNA fragmentation by increase of FA doses. The ladder pattern observed in 25-100 μmol doses is of apoptotic features. There are five morphogenic regions in root apex, namely meristem, cap and caliptra, epidermis, cortex, and pericycle. Root cap, epidermis and 1-2 sub-epidermal layers as well as pericycle contain TUNEL-positive cells in normal condition. These cells undergo developmental programmed cell death. In concentrations upper than 25 μmol FA, TUNEL-negative cells of root meristem and cortex asynchronously become TUNEL-positive. In these nuclei, spheres of condensed chromatin are observed which then are packed in nucleus envelope and form what we named internal apoptotic bodies. Electron microscopy showed that these bodies travel to vacuoles where they are digested.

Key words: Apoptosis, Internal apoptotic body, PCD, Plant, Root.

Neuroprotection by estrogen and estrogen-like compounds by regulating the apoptotic factors

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Estrogen responses have not only been associated with endocrine function, but also with cognitive function. Several studies have indicated that estrogen replacement therapy has favorable effects on memory and cognition, and may have potential in the prevention and treatment of degenerative brain diseases. In our experimental studies on rat brain we have demonstrated presence of estrogen receptor (ER) subtypes ER α and ER β in hippocampus, an area of brain associated with memory and cognition. A significant down-regulation of the two proteins was observed as a consequence of depleted hormone levels in aged female rat hippocampus. Whereas ovariectomy in young adult rats resulted in up-regulation of the above two receptor proteins, but it also led to up-regulation of pro-apoptotic protein Bax and down-regulation of anti-apoptotic protein Bcl-2. Selective estrogen receptor modulator (SERM) tamoxifen therapy resulted in reversal of these ovariectomy induced changes in hippocampus similar to the estradiol treatment. The results of our investigations unravel the neuroprotective role of estrogen in hippocampus and explain the reduced incidence of Alzheimer's disease and better performance in cognition by post-menopausal women on hormone replacement therapy.

ARAP1, a regulator of downstream signaling from TRAIL-R1/DR4?

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TRAIL, the ligand of the TNF α family induces upon binding to its receptors (TRAIL-R1/DR4 or TRAIL-R2/DR5) apoptosis of tumor cells and thus seems to be a candidate anti-tumor agent of the new generation (1). Activated TRAIL receptors trigger formation of DISC (Death Inducing Signaling Complex), activation of pro-caspases 8/10 and eventually apoptosis (2). However, exact mechanism of the signal transduction from TRAIL receptors and regulation of this signaling is not yet fully understood.

In order to uncover new regulators of the proximal signaling from TRAIL receptors, we screened several yeast 2-hybrid cDNA libraries for proteins interacting with the intracellular part of TRAIL-R1. Among specifically interacting proteins we also identified the C-terminal part of recently described Rho and Arf GAP protein ARAP1 (3). Their interaction was confirmed by co-immunoprecipitation of overexpressed proteins. Apparently, TRAIL-induced caspase-dependent processing of endogenous ARAP1 in NCTC cells is required for its interaction with TRAIL-R1. Both proteins also co-localize in post-DISC, LC3 positive, endosome-like bodies. Downregulation of ARAP1 via siRNA accelerates TRAIL-induced apoptosis of NCTC cells. Overexpressed ARAP1 per se does affect TRAIL-triggered death of 293FT cells but upon inhibition of autophagy, it significantly enhances TRAIL-induced, caspase-dependent apoptosis. Thus, ARAP1, might affect TRAIL-induced post-DISC signaling through regulating autophagocytosis.

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Exposure of human leukemic MOLT4 cells to imidazoacridinone derivative C-1311 (Symadex) induces G2/M arrest and apoptosis

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The imidazoacridinone derivative C-1311 (Symadex), with promising antitumor effects in vitro and in vivo, has been synthesized in Gdansk University of Technology and is currently undergoing Phase I clinical studies. Previous studies indicated that C-1311 intercalates to DNA, inhibits catalytic activity of topoisomerase II, covalently binds DNA after metabolic activation. At pharmacologically relevant doses, C-1311 inhibits cell cycle progression in the G2/M phase. G2/M arrest is followed by varying degrees of apoptosis as well as mitotic catastrophe, depending on cell line. Human leukemic MOLT4 cell line is one of the most susceptible cell lines to C-1311 treatment. In attempt to understand the impressive potency of C-1311 to MOLT4 cells, a detailed time analysis of the events leading to cell death after C-1311 exposure was evaluated. Cell death studies were carried out over continuous exposure times varying from 3 to 72 h at EC₉₀ concentration of the drug. Treatment of MOLT4 cells with C-1311 led to time-dependent accumulation of cells in the G2/M phase of the cell cycle, beginning from 12 h and becoming maximal by 24 h. After 12 and 24 h cells exhibited maximal cyclin B1 content, corresponding to their accumulation in the G2/M phase. Starting about 30 h after exposure, G2/M block released partially and cells further resumed DNA synthesis, giving rise to polyploid cells with DNA content above 4C. Between 39 and 72 h C-1311 exposure, there was an increasing frequency of TUNEL positive cells indicative of apoptosis. Several others features of apoptosis also were observed, including chromatin condensation, internucleosomal DNA fragmentation, caspase-3 activation, disruption of mitochondrial membrane potential ($\Delta\Psi_m$) and annexin V binding. The general caspase inhibitor (Z-VAD.fmk) partially blocked C-1311-induced apoptosis. However, it didn't affect drop of $\Delta\Psi_m$ which may suggest that C-1311 induced apoptosis proceeds mostly through a mitochondrial (intrinsic) pathway.

Leptin affects the p53 expression in the gut mucosa of neonatal piglets

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First enteral feedings enforce the rebuilding of small intestinal epithelium in the neonate from a fetal-type into adult-type of markedly modified structure and function. The rebuilding is thus associated with enhanced proliferation of the crypt stem cells, simultaneous with high apoptosis rate, and only during the first 24 hours the apoptosis is transiently reduced resulting in an increased weight and size of the gut mucosa. In proliferating cells, the cell-cycle checkpoints are known to be tightly correlated with the regulation of apoptosis, in which p53 protein plays an important role. The present study aimed to investigate the mitotic and apoptotic indexes and the expression of p53 in the epithelial cells of the small intestine in 7 d old piglets using confocal microscopy. Three groups of piglets were studied: sow reared for 7 days (C7SR), fed with artificial milk formula (C7), and fed with milk formula plus intragastric leptin (10 µg/kg b. wt.) administration (L10). In formula feed piglets the mitotic index was reduced and the apoptosis rate increased in the mid jejunum as compared to the sow reared pigs. The p53 expression was present in the crypts located on the mucosal folds. Nearly no p53 immunofluorescence was observed between the folds and on the intestinal villi. Nevertheless, the p53 expression in the mid-jejunum was 6-times higher in C7SR than in C7 piglets. Interestingly, leptin supplementation resulted in 3-times higher expression of p53 in L10 as compared to C7 piglets. In the distal jejunum the mitotic index was slightly reduced in C7 as compared to C7SR. Accordingly, the p53 expression in C7 was reduced only 2-times as compared to C7SR piglets. Leptin supplementation was ineffective. Concluding, the p53 expression correspond with mitotic and apoptotic indexes, and exogenous leptin seems to restore in part the p53 expression debilitated by milk formula feeding.

Mustard gas induce caspase-dependent anoikis in human bronchial epithelial cells

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Sulfur mustard (SM) is a warfare agent which causes severe damage in exposed areas of the body. Understanding the mechanism involved in cell death associated with detachment (anoikis) is of critical value in developing treatments for upper airway respiratory tract lesions following SM exposure. To this end, the toxicity of SM (0.1 to 0.5 mM) and its structural analogue, mechlorethamine (HN2, 0.1 to 0.5 mM), was investigated in a human bronchial epithelial cell line (16HBE). After 6h of treatment, some cells detached from the culture flask and presented apoptotic features, whereas attached cells remained alive without any cell death characteristic. This death process identified as anoikis reached 40% after 24h of incubation and depended upon the drug concentration. Indeed, at higher concentrations, anoikis switched into necrosis, this switch occurring at lower concentrations for HN2 (0.25 mM) than for SM (0.5 mM). In detached cells, both mustards induced a similar apoptotic process, involving mitochondrial depolarization and caspase activation such as caspase-2, -9 and -13. In addition, caspase-8 and -3 were specifically activated in response to SM but not to HN2. Interestingly, the early activation of caspases, as soon as 30 min after treatment, and the decrease of the number of detached cell in the presence of z-VAD suggested that activation of caspase(s) is involved upstream of cell detachment. Among specific caspase inhibitors tested (caspase-1, -2, -3, -4, -6, -8, -9, -10, 13), caspase-2 inhibitor was the most active in reducing the detachment, underlying once more the key role of this caspase in our study. Altogether, our results demonstrate that mustards triggered the anoikis of human bronchial cells, a phenomenon which requires caspase activation and which is mediated by the mitochondrial pathway.

Overexpression of thymosin β -4 renders SW480 colon carcinoma cells more resistance to apoptosis triggered by FasL and two topoisomerase II inhibitors via down-regulating Fas and up-regulating Survivin expression, respectivelyYeu Su*Institute of Biopharmaceutical Science, School of Life Science, National Yang-Ming University, Shi-Pai, Taipei 11221, Taiwan, R.O.C.**E-mail: yeusu@ym.edu.tw*

Downregulated Fas expression and a consequential reduction in susceptibility to the cytotoxicity of an agonistic anti-Fas IgM (CH-11) in SW480 colon cancer cells overexpressing the thymosin β -4 ($T\beta_4$) gene has recently been reported by us. The present work was conducted to examine the effects of $T\beta_4$ on the apoptosis of SW480 cells induced by other cytotoxic agents. As expected, $T\beta_4$ overexpressers were also more resistant to the toxic effect of FasL-bearing Jurkat T cells. On the other hand, both the Fas levels and the sensitivity to CH-11 in the $T\beta_4$ overexpressers were restored by pretreating them with an MMP inhibitor. Interestingly, while the susceptibilities of the $T\beta_4$ overexpressers to 5-FU and irinotecan remained unchanged, these cells were more resistant to doxorubicin and etoposide which triggered apoptosis via a mitochondrial pathway. Concordantly, activation of both caspase-9 and caspase-3 by the two aforementioned topoisomerase II inhibitors was dramatically abrogated in the $T\beta_4$ overexpressers which could be attributed at least in part to an increased expression of Survivin, a critical anti-apoptotic factor. Finally, poor survival was found in colon cancer patients whose tumors were stained positively by the anti-Survivin antibody. Thus, advantages such as immune evasion and resistance to anticancer drug-induced apoptosis acquired by colon cancer cells through $T\beta_4$ overexpression might facilitate their survival during metastasis and chemotherapy.

Involvement of localized apoptosis for papillary structure formation

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Human epididymal protein 1 (HE-1) controls intracellular cholesterol trafficking. We found that HE-1 is present in all papillary tissues including papilloma and papillary carcinoma. Transfection of non-papillae forming cancer cell line (anaplastic thyroid carcinoma cells—ARO cells) with *HE-1* and injection of *HE-1*-enriched cells into nude mice caused *de novo* papillae formation in the xenograft. Inhibition of *HE-1* by *siRNA* in the vector abolished papillae formation. Thus, *HE-1* plays an important role to form papillae. Papillary proliferation is unique because it retains papillary structure rather than packed uniform cell proliferation. The mechanism of papillae formation has never been studied. Since all papillary structures have open spaces we speculated that localized apoptosis is involved to create spaces for papillae to grow. To examine this hypothesis, slices of the xenograft from the *HE-1*-enriched ARO cells were analyzed by the TUNEL assay. Apoptotic cells were found along the out-layer of epithelial cells of papillae and not in the core of papillae or non-papillary portions of the xenograft. Human tissues from papillary adenocarcinoma of the lung and breast expressing HE-1 protein were also examined by the TUNEL assay. Indeed, the two tissues showed localized apoptosis along the out-layer of papillae, where HE-1 positive cells were present. Next, we examined whether enrichment *HE-1* triggers apoptosis. Wild ARO cells, *HE-1*-enriched ARO cells and *HE-1*-depleted ARO cells by *siRNA* were cultured. Caspase-3 activity in cells was measured by the colorimetric method. Cells enriched with *HE-1* showed 147 ± 24 % (mean \pm SD of 5 samples) increase in caspase-3 activity, whereas *HE-1* depleted ARO cells showed 76 ± 10 % of the control activity ($P < 0.01$). Conclusions: Formation of papillary structure involves local apoptosis. This apoptosis appears to be mediated by HE-1.

Regulation of MAP Kinase-dependent apoptotic pathway. Implication of reactive oxygen and nitrogen species

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Mitogen-activated protein (MAP) kinase signaling cascades are multi-functional signaling networks that influence cell growth, differentiation, apoptosis and cellular responses to stress. Apoptosis signal-regulating kinase 1 (ASK1) is a MAP kinase kinase kinase that triggers apoptogenic kinase cascade leading to the phosphorylation/activation of c-Jun N-terminal kinases (JNK) and p38-MAP kinase, which are responsible to induce apoptosis. This pathway plays a pivotal role in transduction of signals from different apoptotic stimuli. In normal cells ASK1 is directly inhibited by thioredoxin (Trx), a 12-kDa protein ubiquitously expressed in all living cells, which has a variety of biological functions related to cell proliferation and apoptosis. Reactive oxygen species (ROS) were reported to oxidize Trx leading to dissociation/activation of ASK1 in different cell types. We have verified this finding in the *in vivo* studies with liver and brain of Wistar rats.

Recently we have discovered a novel mechanism for induction of apoptosis by nitric oxide. We have found that Trx reactive SH-groups are sensitive to nitrosation. Stimulation of HEK-293 cells with NO donors caused Trx S-nitrosation, which showed straight correlation with ASK1 activation. Treatment of cells with N-acetyl-cysteine after pretreatment with GSNO caused an increase in glutathione and nullified ASK1 activation. We have also investigated S-nitrosation of the Trx in NO/superoxide system. We have found that Trx thiol groups are the targets for S-nitrosation by N₂O₃-like species generated in the system containing xanthine/xanthine oxidase (superoxide producing system) and DEA/NO though they have shown low sensitivity to the •NO radical derived from DEA/NO. N₂O₃-dependent S-nitrosation of Trx resulted in dissociation and activation of ASK1.

In addition, our recent findings concerning S-nitrosation and oxLDL-dependent accumulation of hypoxia inducible factor 1 α (HIF-1 α) combined with current knowledge gives an opportunity to suggest possible participation of HIF-1 α in apoptotic cell death induced by NO/ROS via MAP kinase dependent pathway.

A ubiquitin conjugating enzyme variant, *UEV1A*, activates NF- κ B and prevents stress-induced apoptosis in mammalian cells

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Post-translational modification via Gly76-Lys63 poly-ubiquitination allows target proteins to participate in diverse cellular functions. Poly-ubiquitin chains linked to proteins via Lys63 of ubiquitin requires a unique ubiquitin-conjugating enzyme complex composed of Ubc13 and a Ubc enzyme variant (Uev). Mammalian cells contain at least two such Uev proteins, namely Mms2 and Uev1. While Mms2 is known to play a role in DNA post-replication repair, the precise function of Uev1 is currently unclear. *UEV1* was isolated by its ability to transactivate the *c-fos* promoter and by two independent mRNA differential display screens. *UEV1* transcript level has been shown to be down-regulated in HT-29-M colon cells undergoing chemical-induced differentiation. We have previously shown that *UEV1* is up-regulated in all tumor cell lines examined and when SV40-transformed human embryonic kidney cells undergo immortalization. *UEV1* is mapped to chromosome 20q13.2, a region where DNA amplification is frequently reported in cancer pathogenesis. The above evidence links *UEV1* to tumorigenesis and implicates *UEV1* as a putative proto-oncogene. The Ubc13-Uev1 heterodimer was also isolated during the study of the TRAF6 signaling pathway and is indeed required for the TRAF6-mediated activation of I κ B kinase complex by poly-ubiquitination of NEMO/IKK γ , a regulatory subunit of I κ B kinase in the NF- κ B signaling pathway. Here we show that constitutive high-level expression of *UEV1A* alone in cultured human cells was sufficient to cause an increase in NF- κ B activity and that this effect was reversible upon suppression of *UEV1*. Overexpression of *UEV1A* conferred prolonged cell survival under serum-deprived conditions, and protected cells against stress-induced apoptosis. Taken together, these observations present convincing evidence that Uev1A is a critical regulatory component in the NF- κ B signaling pathway in response to environmental stresses and identifies *UEV1A* as a potential proto-oncogene. Further studies will be undertaken to elucidate the precise biochemical function of Uev1 in the above processes.

TRAIL sensitization by arsenic trioxide is caspase-8 dependent, involves death receptor 5 and inhibition of akt

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Tumour necrosis factor-related apoptosis inducing ligand (TRAIL) selectively induces apoptosis in a variety of cancer cells by interacting with death receptor 4 and 5 (DR4 and DR5), but it spares normal cells. In contrast, binding of TRAIL to decoy receptors 1 and 2 (DcR1 and DcR2) and the soluble receptor OPG prevents the induction of apoptosis. Use of TRAIL receptor selective variants could permit more tumor specific systemic therapies by eliminating the competition with the decoy receptors. Use of TRAIL and receptor-specific mutants is however restricted due to TRAIL resistance of numerous cancers. We found, that sublethal concentrations of arsenic trioxide (ATO) enhanced TRAIL-mediated apoptosis in myeloid leukemic cell lines but not in other tumour cell lines. The combination of ATO and TRAIL enhanced cleavage of caspase-8 and the synergistic effect was blocked by the caspase inhibitor IETD.fmk as well as in cells deficient for caspase-8, suggesting a requirement of the DISC complex. Furthermore, ATO increased cell surface expression of DR5, inhibited phosphorylation of Akt/PKB and led to downregulation of the short isoform of FLIP (FLIP_S). Inhibition of the phosphatidyl inositol 3-kinase (PI3K) was equally efficient in sensitizing leukaemic cells to TRAIL with similar effects on DR5 and FLIP_S, suggesting that ATO-mediated sensitization may require DR5 and acts through inhibition of the PI3K/Akt signalling pathway. The requirement of DR5 rather than DR4 was further explored using DR5 specific mutants of recombinant TRAIL designed by computational strategy. Selectivity towards DR5 was achieved with one or two amino acid substitutions. The designed DR5-selective TRAIL variants showed a significant increase in biological activity when compared to wild-type TRAIL in DR5 responsive cancer cells. We also found that ATO treatment sensitized cells to the DR5 mutants to the same degree as it did for wild-type TRAIL. These findings offer a promising and novel strategy involving a combination of DR5 specific TRAIL mutants and ATO, or more specific Akt inhibitors in the treatment of various haematopoietic malignancies.

Characterization of cell death type in transgenic and mutant *Drosophila* stocks with neurodegenerative phenotypes

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Drosophila is an appropriate model in which we can study fundamental cellular pathways in the context of developing and functioning nervous system. Recently *Drosophila* has been applied to examine different disorders, including Alzheimer's, Parkinson's and Huntington's diseases. In the fly's nervous system we can easily observe the effect of different mutant and missfolded proteins, and find novel genes participating in characteristic neurodegenerative phenotypes. The common features of these models are the pronounced cell death in the brain and shortened life span, which highly resemble the human disorders mentioned above. Studies on model organism require exact information about the cell dying mechanisms to make these animal model systems comparable with human neurodegenerative diseases.

In our work we demonstrate cellular death in known mutant and transgenic *Drosophila* stocks' central nervous system (including human amyloid precursor protein, alfa-synuclein transgenes or vap, swiss-cheese and blue-cheese mutations). We further try to characterize the cell death type by using known markers of autophagy, UAS-GFP-Atg8 transgenes that label autophagosomes, and UAS-Hrp-LAMP1 that label endosomes and lysosomes with GAL4 gene activator expressed in the central nervous system. We also used some lysosome detecting techniques: LysoTracker Red staining, acridine orange staining or acidic phosphatase detection. We used TUNEL-reaction for apoptosis detection. Our work help to make clear the cell death type in various *Drosophila* models of human neurodegenerative diseases, which is essential to use these models as a powerful tool to study human central nervous system disorders.

Regulation of apoptosis by IAPs - Insights from insects

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Certain members of the Inhibitor of Apoptosis (IAPs) protein family regulate cell death by interacting and blocking caspases. *Drosophila* carries four IAPs, two of which (DIAP1 and DIAP2) contain baculovirus inhibitory repeat (BIR) domains and a C-terminal RING finger domain. In *Drosophila*, DIAP1 represents the last line of defence against caspase-mediated damage. This is evident because loss of DIAP1 instigates spontaneous and caspase-mediated cell death. While the BIR1 region of DIAP1 is critically important for DIAP1's ability to bind to the effector-caspases drICE and DCP-1, it is the BIR2 domain, which binds to the initiator caspase Dronc. For its full anti-apoptotic potential, DIAP1 requires N-terminal cleavage and a functional RING finger domain.

To explore the possibility that DIAP1's mode of action is evolutionarily conserved we compared the anti-apoptotic mechanism of DIAP1 with the ones of IAPs from other insects. In particular, we compared DIAP1 with IAPs from *Anopheles gambiae*, *Aedes aegypti*, *Bombix mori*, *Spodoptera frugiperda* and the viral (*Orgyia pseudotsugata multicapsid nucleopolyhedrovirus*) Op-IAP. Our data support the notion that DIAP1's mode of action is evolutionarily conserved throughout the insect kingdom.

Are the lung cells resistant to oxidative stress?

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The lung is the main target of oxidative stress. Evolutionally lung cells have developed defense mechanism against oxidative stress injury - alveolar surfactant components enriched in unsaturated fatty acids and specific proteins. Recently it has been shown that some of surfactant proteins (e.g. SP A) and its receptor are involved in protection from apoptosis in pneumocytes type II. Nevertheless many lung diseases require application of oxygen therapy at doses above physiological (hyperoxia) which as a consequence causes injury on pulmonary epithelial cells. Hyperoxia generates reactive oxygen species and thus can activate stress-induced apoptosis in lung cells. Controversial data exist for lung cells sensibility to oxidative stress and H₂O₂.

The aim of our study was to assess the level of oxidative stress injury in two human lung-derived cell lines (bronchial 16HBE14o⁻ and alveolar A549) and to compare their capability of restoration. The cells were treated with 100 µm H₂O₂ for period of 20 min. The effect on genome integrity was evaluated by comet assay and nuclear morphology was followed by fluorescent microscopy up to five days.

Our results demonstrated that DNA was highly damaged in both cell lines. Alveolar cell were more sensitive (average value 76.99 % ± 1.56) in comparison with bronchial cells (average value 69.46 % ± 2.68). Our studies indicated increased nuclear fragmentation in both cell types up to the 5th day after treatment. Less than 25 % of the alveolar cells succeed to repair DNA on the third day. Simultaneously in about 30 % of the cells, DNA damage was increased on the third day of post-treatment period, which suggest induction of cell death.

Molecular effectors of cell death in light induced retinal degeneration

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Retinal degenerations are an important cause of blindness in industrialized countries. They are characterized by the death of photoreceptors, the light sensitive cell in the retina. Despite various origins (genetic, oxidative), cells follow, in retinal degenerations, the same physiopathological fate: photoreceptors die by apoptosis. Previous studies show that in most cases this apoptosis is not mediated by caspases.

Our study investigates this apoptosis caspase independent pathway in a light-induced retinal degeneration model. We first focus our study on: the LEI (Leukocyte Elastase Inhibitor)/L-DNase II, a protein of the serpin family which, has been reported to be involved in numerous caspase-independent models of apoptosis. In its native form, LEI, a cytoplasmic protein, has an anti-protease activity. Under some apoptotic conditions, like intracellular acidification or growth factor withdraw, LEI becomes L-DNase II by post-translational modification, acquires a nuclear localisation and gets an endonuclease activity. Recent studies on glutamate cytotoxicity show the involvement of protein kinase zeta in controlling cell death. As glutamate is the neurotransmitter used by photoreceptors we all investigate the activation and fate of this enzyme.

In this work two to three months old Fischer rats were exposed to a constant white light for one to nine days. On one hand, immunohistochemistry showed a nuclear translocation of L-DNase II confirmed by an increase of L-DNase II expression observed by immunoblotting in the purified nuclei of these retinas. This also was confirmed by L-DNase II activity measurements. On the other hand, PkC zeta is activated at early time points and inactivated thereafter, suggesting a two steps behaviour of the enzyme: the first one in order to counterbalance the insult, the second one inactivation the enzyme as cell death is triggered.

Role of tissue transglutaminase in the process of phagocytosis of apoptotic cells

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The rapid and efficient phagocytosis of apoptotic cells plays a crucial role in preventing secondary necrosis, inflammation as well as in tissue remodeling and regulating immune responses. However, the molecular details of engulfment are just beginning to be elucidated.

In this clearance process the professional phagocytes, so called macrophages, play a central role. Engulfment of dying cells initiates cytoskeletal reorganization in macrophages and these events are evolutionally highly conserved and well regulated. The members of Rho-family GTPases (Rho, Rac, cdc42) are involved in the regulation of actin cytoskeleton rearrangement such as phagocytosis, migration, differentiation, adhesion and mitosis. Active Rac plays role especially in migration and phagocytosis.

Tissue transglutaminase is a GTP binding protein, and it also has transamidation function as well. Previous studies in our department have shown that the engulfment process is defective in TG2 knock out mice.

Here we show that phagocytosis of TG2^{-/-} macrophages is also defective under *in vitro* condition and this is related to altered cytoskeletal reorganization. In the present study we have investigated the mechanism of the observed deficiency in the phagocytosis of apoptotic cell.

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Chromatin modification and retention of HMGB1 by apoptotic cells

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HMGB1, an abundant and very mobile nuclear protein, serves as a signal to distinguish between necrosis and apoptosis. Necrotic cells release a massive amount of HMGB1 in the extracellular medium in order to signal to neighbouring cells that tissue damage has occurred, whereas apoptotic cells immobilize HMGB1 onto chromatin, avoiding its release in the external environment.

To date, we know that HMGB1 does not appear to be modified in apoptotic cells, while chromatin undergoes chemical or structural transitions, including histone hypoacetylation, that allow irreversible HMGB1 binding.

In mammalian cells, the only core histone modification unique to apoptosis known so far is histone H2B phosphorylation at serine 14 (S14).

I have now demonstrated that TSA treatment of apoptotic cells is able to inhibit both HMGB1 binding to apoptotic chromatin and histone H2B (S14) phosphorylation. Moreover, the lack of H2B phosphorylation at Ser14 in apoptotic cells treated with an inhibitor of caspase 3 allows HMGB1 to move into the nucleus with kinetics similar to those measured in living cells.

We also demonstrated that HMGB1 co-immunoprecipitates with nucleosomes enriched in H2BS14p, but *in vitro* HMGB1 has similar binding affinity for the phosphorylated or unphosphorylated H2B tail.

Evidence for induction of apoptosis in differentiating murine erythroleukemia (MEL) cells: Implications in leukemia therapy

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The concept that apoptosis (programmed cell death) may be a major process in regulation of erythropoiesis has been proposed. However, direct evidence confirming induction of apoptosis as an integral part of the erythropoiesis is still missing. We used the MEL cells (a model for erythrodifferentiation) and investigated whether differentiated cells (which undergo DNA fragmentation) are also committed to apoptosis upon erythroid differentiation (Differentiation Depended Apoptosis, DDA). Cells were exposed to both dimethylsulfoxide (DMSO; 1.7% v/v) and hexamethylene-bis-acetamide (HMBA; 5 mM) for up to 96 hr and at various timed intervals were assessed for: a) Induction of erythrodifferentiation b) Fragmentation of both nucleus and DNA; c) Accumulation of subpopulation of damaged apoptotic cells expressing annexin V and undergoing cell death (loss of viability) by cytofluorography; d) Analysis of the level of cell cycle arrest-related proteins (Cdk2, cyclin D3) and apoptotic (caspase 3, Bad) /antiapoptotic (Bcl-X_L, Bcl-2) markers. Finally, HMBA-treated cells were examined microscopically for nuclear morphology using acridine orange. Etoposide, a potent cytotoxic agent and inducer of apoptosis, was used as positive control. Our data thus far indicate that: a) MEL cells induced to differentiate by HMBA produced hemoglobin (75-80% Bz⁺ cells by 96 hr), underwent (reduction in size, nuclear condensation and growth arrest) and exhibited nuclear and DNA fragmentation (DNA ladder), characteristic of apoptosis; b) Cytofluorographic analysis revealed a substantial subpopulation of apoptotic cells in differentiated cell cultures as well as a minor discrete population of non-differentiated non-apoptotic cells. Cell death determined by trypan blue dye exclusion (TB⁺ cells) ranked up to 33% after 96 hr treatment with HMBA; Cytofluorographic analysis by using Annexin V antibody revealed that etoposide caused apoptosis extensively in MEL cells without promoting differentiation. c) Cells exposed to HMBA for 96 hr showed moderate increase in caspase 3 but not substantial changes in the steady-state protein level of other markers examined (cyclin D3, Cdk2, Bad). A decrease however, in the level of antiapoptotic proteins (Bcl-2 and Bcl-x), was observed. These data indicate that apoptosis in MEL cells can be induced by agents that promote erythrodifferentiation in leukemia. Apparently this apoptosis can function as a balancing act to regulate the kinetics of erythropoiesis. Alternatively, etoposide promoted apoptosis but not differentiation. We are in the process to delineate the molecular mechanisms of Differentiation Depended Apoptosis (DDA) since it may eventually lead to novel therapy of leukemias.

The Rai (Shc C) adaptor protein regulates the neuronal stress response and protects against cerebral ischemia

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Rai is a recently identified member of the family of Shc-like proteins, which are cytoplasmic signal transducers characterized by the unique PTB-CH1-SH2 modular organization. Rai expression is restricted to neuronal cells and regulates in vivo the number of postmitotic sympathetic neurons. We reported that Rai overexpression in neuronal cell lines promotes survival by reducing apoptosis both under conditions of limited availability of the Ret ligand glial cell line-derived neurotrophic factor (GDNF) and in the absence of Ret activation. Overexpressed Rai resulted in the potentiation of the Ret-dependent activation of phosphatidylinositol 3-kinase (PI3K) and Akt. In neurons treated with low concentrations of GDNF, the prosurvival effect of Rai depends on Rai phosphorylation and Ret activation. In the absence of Ret activation, the prosurvival effect of Rai is, instead, phosphorylation independent. Rai potentiates PI3K/Akt signaling pathways and regulates Ret-dependent and -independent survival signals.

Rai has a role also in the regulation of the neuronal adaptive response to environmental stresses. We demonstrated that primary cultures of cortical neurons derived from Rai^{-/-} mice are more sensitive to apoptosis induced by hypoxia or oxidative stress. Using an in vivo mouse model, we found that ischemia/reperfusion injury induces in Rai^{-/-} mice severe neurological deficits, increased apoptosis and size of the infarct area and significantly higher mortality than in Rai wt animals. Moreover, Rai functions as a stress-response gene that increases PI3K activation and Akt phosphorylation after hypoxic or oxidation insults. Rai protein activation is mediated by serine/threonine dephosphorylation, which permits either interaction with other effector proteins or lead to the release of Rai protein from an inhibiting survival protein.

Preliminary results from point mutation analysis indicate that two residues in the CH1 region are probably the major phosphorylation sites of Rai protein involved in stress-response. These data suggest that Rai has a functional neuroprotective role in brain injury and the characterization of its signalling pathway may result in novel therapeutic strategies in the treatment of stroke.

Role of the senescence biomarker Clusterin/Apolipoprotein J in the modulation of cellular growth and survival

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Clusterin/Apolipoprotein J (CLU) is a ubiquitously expressed heterodimeric glycoprotein which is differentially regulated in many severe physiological disturbance states including cell death, ageing, cancer progression and various neurological diseases. Despite extensive efforts CLU function remains an enigma, the main cause being the intriguingly distinct and usually opposed functions in various cell types and tissues. We investigated the effects of small interference RNA (siRNA) mediated CLU gene expression silencing on cellular growth and survival in three human osteosarcoma (OS) cell lines, namely KH OS, Sa OS and U2 OS. These cell lines originate from the same tissue but pose distinct genetic backgrounds and express distinct endogenous CLU levels. Our data demonstrate that siRNA-mediated knock down of the secreted isoform of CLU (sCLU) induces a significant reduction of cellular growth, higher rates of spontaneous endogenous apoptosis and reduced plating efficiency. These effects are enhanced in those cell lines expressing high endogenous sCLU levels. Moreover, sCLU knock down sensitizes dramatically the OS cells to both genotoxic and oxidative stress. By assaying the expression levels of several proteins involved in regulating apoptosis we found that sCLU knock down results in the down-regulation of the anti-apoptotic molecule bcl-2. In U2 OS cells, which bear a functional p53 molecule, sCLU knock down apart from bcl-2 down-regulation is also accompanied by p53 accumulation and up-regulation of its downstream pro-apoptotic effector bax and the cyclin-dependent kinase inhibitor, p21. We suggest that sensitization of OS cells following sCLU knock down largely depends on the activation of the cellular pro-apoptotic machinery. Overall, our results reveal that in the distinct cellular contexts assayed, sCLU is a central molecule in cell homeostasis that exerts a potent cytoprotective function.

Effects of olomoucine II on ODC and Bax mRNA levels in rat prostate cancer cell lines

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Polyamines (PAs) are biological cations necessary for normal cell growth. Recent evidences indicate that PAs are associated with apoptotic cell death. High incidence of prostatic cancer and the limitations of its treatment caused the necessity of new therapeutic models. A new research area is to use cyclin-dependent kinase (CDK) inhibitor treatments. CDK inhibitors are derivatives of purines and they may provide a new tool in the chemotherapy of prostate cancers with various phenotypes. The aim of this study is to determine the effects of a new CDK inhibitor olomoucine II (OLO II) on cell growth rate, PA contents and apoptosis in different rat prostate cancer cell lines.

Weakly (AT-2) and highly metastatic (MAT-Lylu) cell lines (1×10^6 cells/ml) were cultured in RPMI1640 medium, supplemented with 5% fetal calf serum, 1% penicillin-streptomycin and 15 μ M OLOII for 24 and 48 hours. PA contents were determined by HPLC; mRNA levels of PA biosynthetic enzyme ornithine decarboxylase (ODC) and pro-apoptotic protein Bax were established by RT-PCR.

It was estimated that 48 hours OLOII treatment inhibited the cell growth rate by 97% in both cell lines and also decreased total protein levels by 46% in AT-2, 57% in MAT-Lylu cells. It was also observed that MAT-Lylu was the most affected cell line with OLOII. 48 hour OLOII application reduced Put, Spd and Spm levels by 90%, 87% and 93%, respectively in MAT-Lylu cell lines. Furthermore, it was determined that OLOII has an inhibitory effect on ODC and Bax mRNA expression levels in both cell lines.

In conclusion, OLOII exhibited an inhibitory effect on PA levels and ODC mRNA transcription level; however it is also reduced Bax mRNA levels, which is not expected. In this case it is necessary to investigate the apoptotic parameters after the application of OLOII in prostate cancer cell lines.

Effects of AT1 blocker on apoptosis in experimental diabetic nephropathy

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Tubular atrophy is a major feature of most renal diseases and is closely associated with loss of renal function. Angiotensin II (Ang II) mediates progressive nephron loss in diabetes and stimulates apoptotic cell death. The aim of this study is to investigate effects of irbesartan as an Ang II type 1 (AT1) blocker on apoptosis in the Streptozotocin (STZ)-induced diabetic rat.

24 male Wistar albino rat was used for three groups. The first group (n=8) was the non-diabetic control group. Second group (n=8) was the untreated STZ-induced diabetic group, (60 mg/kg, single dose, i.p). The third group (n=8) was irbesartan (15 mg/kg/day, intragastric, for 4 week) treated STZ-diabetic rats. During the period of the experiment, blood glucose and microalbuminuria levels of the rats were measured. At the end of the study renal tissue samples were fixed in neutral formalin and embedded in paraffin. Tissue sections were examined for apoptosis is using TUNEL assay and immunohistochemical staining for pro-apoptotic protein Bax.

The microalbuminuria levels of the irbesartan treated STZ-diabetic group were found reduced when compared with the untreated STZ-diabetic group. Widespread apoptosis was seen in the tubules of untreated STZ-diabetic group. A significant decrease in the expression of the proapoptotic Bax protein and in the apoptotic cell number in the treated with irbesartan of STZ-diabetic rats.

The results suggested that irbesartan treatment has renoprotective effects in diabetic nephropathy. AT1 receptor blockade inhibites Ang II mediated apoptosis in the kidney of the STZ-diabetic rats.

Role of ERK pathway in regulation of colon cancer cell sensitivity to TRAIL-induced apoptosis

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TRAIL is a member of the Tumor Necrosis Factor superfamily with a promising clinical potential in anticancer treatment. Most of the research progress has been made on elucidating the endogenous biochemical pathway leading to TRAIL-induced apoptosis in cancer cells. However, the mechanisms of resistance of some cancer cells to TRAIL effects are still not fully understood. Clarifying these issues will help to realize its therapeutic application.

The aim of the study was to demonstrate the role of ERK pathway in regulation of the HT-29 human colon adenocarcinoma cell sensitivity to TRAIL effects. TRAIL induced a strong activation of ERK1/2 compared to untreated cells. This activation was completely blocked by pretreatment of the cells with MEK inhibitor U0126. Furthermore, inhibition of ERK1/2 signalling sensitized HT-29 cells to TRAIL-induced apoptosis as confirmed by sensitive methods of flow cytometry (phosphatidyl serine translocation - Annexin-V assay, specific cleavage of cytokeratine 18 - M30 cytoDEATH, mitochondrial apoptotic protein - Apo2.7), fluorescence microscopy (nuclear morphology, DAPI staining) and Western blotting (PARP cleavage).

As ERK pathway was shown to inhibit TRAIL-induced apoptosis of colon cancer cells in our experiments, mechanisms involved in protective effect of this pro-survival pathway against TRAIL-induced apoptosis were examined. The attention was paid to the clarifying the site of the apoptotic pathway affected by U0126.

We suppose that the balance between pro-survival and apoptotic pathways may play an important role in regulation of the colon cancer cell sensitivity to the induction of apoptosis by TRAIL. As constitutive activation of MAPK/ERK pathway has been implicated in the pathogenesis of a variety of malignancies, interruption of this signalling may represent a promising therapeutic strategy for combination with TRAIL treatment also in the colorectal cancer.

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Insertion of Bax into mitochondria through the preprotein translocase of the mitochondrial outer membrane (TOM complex)

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The association of Bax with mitochondria is an essential step in the implementation of apoptosis. We have shown that Bax associated with mitochondria through its first alpha helix which also intervene in Bid induced change of conformation (Cartron et al., JBC 276: 11633, 2003; Mol Cell 16: 807, 2004). Bax interaction with TOM is inhibited by peptides derived from its first helix which thus unraveled a new pathway to inhibit specifically Bax dependent apoptosis. By means of a bacterial two-hybrid assay and cross-linking strategies, we identify TOM22, a component of the translocase of the outer mitochondrial membrane (TOM), as a mitochondrial receptor of Bax. Antibodies raised against TOM22 or antisense treatment to knock-down its expression inhibit the association of Bax with mitochondria and prevent Bax-dependent apoptosis in human glioma cell lines. We also show that the transient interaction of monomeric Bax with TOM 22 is followed by that with TOM 40, the central element of TOM, and Bax membrane integration. Quite remarkably, once inserted in mitochondria Bax can be used as a receptor for another Bax molecules and the forced dimerization of Bax trigger its insertion in mitochondria independently of TOM.

The characterization of 86 novel protein processing sites in Fas-induced apoptotic Jurkat cells using non-gel proteome degradomics

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A global view on substrates of proteases, induced upon triggering apoptosis in human Jurkat T-lymphocytes by Fas antibody treatment, was obtained. We used an in-house developed, differential, peptide-centric proteomics approach which specifically marks cleavage sites within processed proteins. More than 1,600 different proteins were identified in the Jurkat proteome and we directly located 93 cleavage sites in 73 different proteins. In addition, indirect evidence of apoptosis-specific cleavages led to a grand total of 92 processed proteins in apoptotic Jurkat cells. Although most cleavages occurred at caspase-consensus sites, about one third was located at sites flanked by at least one basic residue, suggesting a noteworthy activation of non-caspases. Interestingly, the spliceosome complex appeared as a preferred target since at least 14 of its members were processed. Our differential proteomics strategy further revealed specific release of nucleosomal components from apoptotic nuclei. The protease substrate screening-method developed here is generally adaptable to degradome analysis in various biological systems.

The hop-flavonoid xanthohumol induces endoplasmic reticulum stress in human breast cancer cells

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In recent years, there is growing interest in chemopreventive phytochemicals like flavonoids. We demonstrated earlier that the hop-flavonoid xanthohumol (X) has an anti-invasive effect on the human breast cancer cell lines MCF-7 and T47-D. This effect could at least partially be attributed to induced cell death.

Here we demonstrate that X induces cell death of various human breast cancer cell lines (MCF-7/AZ, MCF-7/6, T47-D and BT-20) but not of a panel of human primary normal breast epithelial cells. Treatment with X initiated extensive formation of autophagic vacuoles, as visualized by monodansyl cadaverin which stains autophagic vacuoles as distinct dot-like structures and by transmission electron microscopy. In addition, X stimulated the expression of the autophagy gene product beclin 1. Finally, apoptotic events like stimulated cleavage of PARP, and the appearance of nuclear condensation of the X-treated cells were observed. In parallel, 24-hours treatment with X induced an overexpression of an 80-kDa protein in the cancer cells. We have identified it as the endoplasmic reticulum (ER) chaperone BiP (GRP78) after 2-DE separation of untreated *versus* X-treated T47-D cell lysates, *in-gel* trypsin digestion of this protein was performed and peptidic fragments were analyzed by MALDI-TOF and LC-MS-MS. Interestingly, we further showed that BiP was strongly upregulated in the X-treated breast cancer cells but unaltered in the normal breast cells. This upregulation coincided with an increased phosphorylation of the extracellular signal-regulated kinase (Erk). Furthermore, inhibition of calpain activity blocked both the stimulated BiP expression and Erk phosphorylation, which points to the involvement of this Ca²⁺-dependent protease in the ER stress induced by X.

In conclusion, we have demonstrated by a proteomic approach that overexpression of BiP is correlated with the pro-apoptotic effect of X. Moreover, our results indicate that X induced an ER stress and calpain activity specifically in human breast cancer cells but not in normal epithelial mammary cells. This stress is associated with both apoptotic and autophagic events which eventually result in cell death. Our data underline the potential of X in breast cancer treatment.

Imaging of mitochondrial apoptogenic proteins released during apoptosis in living and fixed cells

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Our project is focused on study of mitochondrial apoptogenic proteins released during apoptosis and associated with caspase-independent nuclear chromatin condensation and degradation. We introduce our automated 2D/3D/4D/FRET high-resolution image data acquisition and analysis system developed to visualize and quantify fluorescence signals in fixed and living cells. For our studies, we utilize the fluorescent proteins and various low-toxicity cell permeable fluorescent probes that make it possible to conduct the non-invasive quantitative visualization in living cells. In our experiments, cells are stably or transiently transfected or cotransfected by lipofection with DNA plasmids coding fusion proteins of mitochondrial apoptogenic proteins (cytochrom c, AIF, amid, and endonuclease g) with one of fluorescence proteins (EGFP, EYFP, or t-HcRed).

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Enhanced endocytosis in excitotoxic cell death

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Endocytosis has been shown to be enhanced in some cases of neuronal death such as in NMDA-treated hippocampal slices or in kainate-treated retinal amacrine cells. This new phenomenon may be important for understanding the causation of cell death in excitotoxicity.

The enhanced endocytosis was shown in rats that received an icv injection of 4.4 kD FITC-dextran before a transient focal cerebral ischemia. They were sacrificed 24 h later, when there was strong neuronal endocytosis restricted to the ischemic zone only. We have therefore begun to analyze the underlying mechanisms in rat cortical neuronal cultures.

Enhanced endocytosis of FITC-dextran (4.4kD) was observed in NMDA-treated neurons, as measured by fluorescence spectrometry. This endocytosis increased with the dose of NMDA and the duration of NMDA treatment. In order to study specifically the endocytotic mechanism, we determined a combination of concentration and timing that enhances endocytosis but with minimal cell death, as judged by LDH release. Moreover, we confirmed that the internalization of dextran was due to endocytosis by the fact that the dextran-labelling did not occur at 4°C. Use of commercially available inhibitors of different types of endocytosis revealed two components of dextran internalization that had completely different pharmacological profiles. One occurred constitutively (even in the absence of NMDA) and was blocked by inhibitors of classical fluid-phase dynamin-independent endocytosis (e.g. by wortmannin). The other component was induced by NMDA, insensitive to fluid-phase inhibitors, but sensitive to inhibitors of dynamin-mediated endocytosis such as 0.4M sucrose and also to the JNK pathway inhibitor D-JNKI1, a powerful neuroprotectant. The latter inhibitors had little effect on the constitutive component of endocytosis.

The induced endocytosis may provide a means for delivering neuroprotective agents specifically into the cells that need them.

Tumor therapy with ionizing radiation and TRAIL: efficacy and key molecular determinants

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The aim of the project is to evaluate whether synergy can be accomplished by combining TRAIL and ionizing radiation (IR) in cancer therapy and to investigate the key molecular determinants involved. In particular, we want to know whether combined therapy can be effective in cells with a dysfunctional p53 pathway and/or a blockade in the mitochondrial apoptosis route. This will be explored, both in vitro and in vivo, in a lymphoid malignancy, in which apoptosis is an important mode of death after treatment with DNA damaging regimens and in solid tumor types, in which the importance of apoptosis on therapy outcome remains to be proven. For this purpose, we will make use of genetic mouse models of spontaneous tumorigenesis.

We have found that in Jurkat T leukemic cells, IR induces apoptosis via a death receptor-independent pathway that, surprisingly, relies on aspartate cleaved BH3-only protein Bid. Upon blockade of the mitochondrial pathway by Bcl-2 or a non-cleavable Bid mutant, apoptosis-induction by IR is inhibited. TRAIL receptor-induced apoptosis is also inhibited to a large extent. However, TRAIL (as well as CD95 ligand) strongly synergize with IR for apoptosis-induction in cells with a blockade in the mitochondrial pathway. RNA interference-mediated knock down of p53 does not affect the apoptotic response. Therefore, in Jurkat cells, the combination of TRAIL and IR allows for the p53-independent, synergistic activation of an apoptosis pathway that can bypass resistance at the mitochondrial level. Combined treatment does not break resistance, because Cyt c release remains inhibited. Membrane expression of TRAILR-2 is only marginally increased by IR and that of CD95 is not. We surmise therefore, that receptor upregulation at the cell surface is not the basis of synergy, but rather an alteration in the mode of death receptor signaling. This possibility is currently under investigation.

Modulation of apoptosis in a murine model of chronic hepatitis C

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The hepatitis C virus causes chronic infection in about 80% of infected individuals who become at high risk for development of inflammatory liver diseases that often progress to hepatocellular carcinoma (HCC).

We have studied a transgenic mouse with liver targeted expression of the entire HCV polyprotein. Despite the absence of the immune response against the HCV proteins, the animals develop liver pathologies, including hepatocellular carcinoma. Thus, the expression of the viral proteins has a direct impact on tumour development.

A major issue of hepatitis C is the very high frequency of establishment of a chronic infection, despite a robust humoral and cell-mediated immune response. Interestingly, the immunocompetent HCV+ mice reproduce this aspect of human pathology: an adenoviral infection results in an indistinguishable immune response in the HCV+ and the control animals. Nevertheless, there is a strong increase in persistence of virally infected cells in the liver of the transgenic animals. This effect is due to an intrinsic defect of an apoptotic signal transduction pathway in cells expressing the HCV viral proteins. More specifically, expression of Bid, the rate-limiting factor of the Fas signal transduction pathway in hepatocytes, is compromised in the HCV transgenic livers.

Analysis of Bid expression in human patients suffering from HCV-linked HCC, as well as in an experimental system permissive for the HCV replication, confirmed the data obtained in the HCV transgenic mouse model.

Thus, the expression of HCV proteins down-regulates an essential component of an apoptotic signal transduction pathway and creates conditions permissive both for the establishment of the chronicity of infection and for hepatocarcinogenesis.

Inhibition of glycolysis by oxidative stress leads to necrotic-like cancer cell death through Poly(ADP-ribose) polymerase activation and subsequent NAD⁺ depletion

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Among all the different features of cancer cells, two of them are of particular interest: their nearly universal glycolytic phenotype and their sensitivity towards an oxidative stress, both resulting presumably from the combination between high anabolic needs and environmental growth conditions lacking O₂. Therefore, we took advantage of these features to develop an experimental approach selectively exposing cancer cells to an oxidant insult induced by the association of menadione and ascorbate. This association develop a synergistic antitumor activity *in vivo* as well as *in vitro*, based on the potentiation of the menadione redox cycling by ascorbate. This leads to the intracellular generation of reactive oxygen species among them H₂O₂ appears to be the main responsible of the cytolytic effect. Since lactate and ATP are severely and rapidly depressed following ascorbate/menadione exposure, we suggest that the major intracellular event is related to the impairment of glycolysis [1]. Indeed, NAD⁺ is rapidly consumed, related with a strong Poly(ADP-ribose) polymerase activation, thus explaining the glycolysis inhibition. The profile of cell death do not correspond to apoptosis (no caspase-3 nor PARP processing, DNA strand breaks with spread pattern) but is rather close to necrosis [2]. Strong morphological events occur during the cytolytic process including the formation of cytoplasmic pieces, organelles-free. On these morphological basis, such a cell death has been called *autoschizis* [3], but the mechanism by which PARP activation leads to NAD⁺ depletion resulting in necrotic cell death is also known as *programmed necrosis* [4]. Due to the high energetic dependence of cancer cells towards glycolysis, the impairment of such an essential pathway may explain the effectiveness of this association on many tumor cells. Moreover the poor antioxidant status presented by cancer cells, coupled with intracellular ascorbate accumulation, may confer the basis for differential sensitivity between normal and cancer cells towards ascorbate/menadione.

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Expression activated caspase-8 following an excitotoxic injury to the immature rat brain

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Excitotoxic cortical damage to the postnatal brain results in neurodegeneration, which includes caspase-3 expression and subsequent cell death. The main caspase-dependent apoptotic pathways include: the intrinsic mitochondrial pathway and the extrinsic pathway under the control of death receptors. Oxidative stress is a result of increased ROS which leads to activation of the intrinsic pathway with activation of caspase-9, whereas the extrinsic pathway is activated by an increase in cytokines and activation of caspase-8; both pathways end in caspase-3 activation and eventual cell death. The objective of this study was to investigate the principal caspase-dependent pathways implicated in caspase-3 activation seen after postnatal excitotoxic damage.

N-methyl-D-aspartate (NMDA) was injected intracortically in 9 day old postnatal rats and sacrificed at 4, 10, 24 and 72 hours post-lesion. The brains were processed immunohistochemically and expression of active caspases 8 and 9 was evaluated. No increase in the activation of caspase-9 was observed at any time. However, activation of caspase-8 was seen at 4 hours post-lesion, with peak expression between 10 and 24 hours post-lesion that decreased by 72 hours post-injury. Most of the active caspase-8 positive cells were identified as neurons by double labeling with neuronal nuclear antigen (NeuN).

These results suggest that caspase-8 could play an important role in apoptosis that occurs after excitotoxic injury during postnatal development and may be responsible for the caspase-3 activation seen in this model. However, the pathways involved in activating programmed cell death after excitotoxicity have yet to be fully understood. It is likely that there are also caspase-independent pathways that can lead to apoptotic death and act independently from the activation of caspase-3.

NO and NOS isoforms in the development of apoptosis in renal ischemia/reperfusion

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Nitric oxide (NO) as well as the expression of endothelial (eNOS) and inducible (iNOS) isoforms of nitric oxide synthase (NOS) are recognized as important mediators of physiological and pathological processes of renal I/R injury, but little is known on its role in apoptosis. The ability of the eNOS/NO system to regulate the iNOS/NO system and thus promote apoptosis was assessed during experimental renal I/R. Renal caspase-3 activity and the number of TUNEL-positive cells increased with I/R, but decreased when NOS/NO systems were blocked with L-NIO (eNOS), 1400W (iNOS) and N-nitro-L-arginine methyl ester (L-NAME, non-selective NOS inhibitor). I/R increased renal eNOS and iNOS expression as well as NO production. The NO increase was eNOS- and iNOS-dependent. Blockage of NOS/NO systems with L-NIO or L-NAME also resulted in a lower renal expression of iNOS and iNOS mRNA; in contrast, eNOS expression was not affected by iNOS-specific blockage. In conclusion two pathways define the role of NOS/NO systems in the development of apoptosis during experimental renal I/R: a direct route, through eNOS overexpression and NO production, and an indirect route, through expression/activation of the iNOS/NO system, induced by eNOS.

Combretastatin-A4 and -ST2125 induce mitotic catastrophe dependent on spindle checkpoint and casapase-3 activation in non-small cell lung cancer cells

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Combretastatins are tubulin binding agents, structurally related to colchicines and with a high potential as vascular targeting agents for their capability to induce vascular-mediated tumour necrosis. In a screening of a large chemical library of new combretastatin derivatives, a new molecule ST2125 has been selected. Molecular mechanisms leading to cell death have been investigated in lung cancer cells (H460) after treatment with ST2125 or combretastatin A-4 (CA-4). We found that both compounds induced depolymerisation and rearrangement of spindle microtubules and an increasingly aberrant organization of metaphase chromosomes in a dose- and time-dependent manner. H460 cells were arrested at a pro-metaphase stage with condensed chromosomes and a triggered spindle assembly checkpoint, as evaluated by kinetochore localization of BUB-1 and MAD1 antibodies. Prolonged checkpoint activation led to mitochondrial membrane permeabilization (MMP) alterations, cytochrome c release, activation of caspase-9 and -3, PARP cleavage and DNA fragmentation. Contrastingly, caspase-2, -8 and the lysosomal pathway of cell death were not activated by the drug treatment.

The presence of multinucleated cells strongly supported the notion that combretastatins, particularly the ST2125 triggers a mitotic catastrophe pathway.

In conclusion, we believe that the discovery of new agents able to trigger mitotic catastrophe cell death as a result of mitotic block and prolonged spindle checkpoint activation is particularly worthwhile, taking into account that tumor cells have a high proliferative rate and that mitotic failure occurs irrespective of p53 status.

p57 is a potential substrate for caspases-3 and its expression enhances staurosporine-induced apoptosis in HeLa cells

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p57^{Kip2}, together with p21^{Cip1} and p27^{Kip1}, belongs to the Cip/Kip family of cyclin dependent kinase inhibitors, which plays a critical role in regulating cell proliferation. Importantly, only p57^{Kip2} has been shown to play essential role during embryogenesis a function that other cell cycle inhibitors cannot compensate for. p57^{Kip2} null-mutant mice display severe developmental defects. Interestingly, many of the defects described are not directly linked to increased cell proliferation, implying that p57^{Kip2} may have a broader scope of cellular action than only being a cell cycle inhibitor. By looking at its location, biochemical activities and imprinted expression, p57^{Kip2} is a candidate tumor suppressor gene. Our objective is to understand the possible functions and structure requirement of p57^{Kip2} in the process of cell death/survival. Using a HeLa cell line, in which the expression of p57^{Kip2} is under the control of a tetracycline promoter (HeLa Tet-On^{p57}), we were able to demonstrate that p57^{Kip2} expression strengthens staurosporine (STS)-induced apoptosis. We also noted that p57^{Kip2} expression enhanced caspase-3 activity and the subsequent processing of PARP upon STS treatment. p57^{Kip2} expression also improved STS-induced mitochondrial dysfunctions, such as, loss of mitochondrial transmembrane potential $\Delta\Psi_m$, ROS production and release of AIF and cytochrome *c* into the cytosol. Time-laps confocal microscopy experiments, using transient expression of GFP tagged p57^{Kip2} and TMRE staining, also revealed that p57^{Kip2}-expressing cells loose their mitochondrial transmembrane potential upon STS treatment faster than the non-p57^{Kip2}-expressing cells. Bcl-2 is a pro-survival protein that is associated with the mitochondrial outer membrane and it has been shown to prevent release of cytochrome *c* from mitochondria and therefore apoptosis. We noticed that Bcl-2 over-expression is able to block the pro-apoptotic effects of p57^{Kip2}. Together, these results suggest that p57^{Kip2} might accelerate the apoptotic process and act upstream of the mitochondria. In addition, proteolytic degradation of p57^{Kip2} is detected with the generation of a C-terminal cleavage product of 42kDa. This cleavage seems dependent on caspase-3 activity since DEVD-fmk, a specific caspases-3 inhibitor, prevents it. We have identified a conserved potential caspase cleavage site in this region, suggesting that p57^{Kip2} is a potential substrate for caspases.

The extent of liver steatosis in chronic hepatitis C virus infection is mirrored by caspase activity in serum

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Hepatic steatosis is a frequent histological alteration in chronic hepatitis C virus (HCV) infection that sensitizes the liver to cell injury, inflammation and fibrosis by still unclear mechanisms. Although apoptosis has been implicated in various liver diseases, its importance in HCV-associated steatosis is largely unknown. In this study, we investigated the role of caspases, the key regulators of apoptosis, and employed two novel caspase assays, an immunological and a luminometric enzyme test, to detect hepatic caspase activation in sera from HCV patients with different grades of steatosis. Our data show that increased caspase activation cannot be found in only liver biopsies, but also in sera from steatotic HCV patients. Patients with steatosis exhibited significantly higher serum levels of caspase activity compared to normal healthy individuals. Moreover, the extent of steatosis closely correlated with serum caspase activity, whereas in particular in cases of low or moderate steatosis no correlation was found with aminotransferase levels. In conclusion, our results demonstrate that apoptotic caspase activation is considerably elevated in HCV-associated steatosis. More importantly, our data imply that measurement of caspase activation might be a sensitive serum biomarker to detect liver steatosis in patients with chronic HCV infection and other liver diseases.

Intravenous Ig preparations induce neutrophil death by both anti-Fas and anti-Siglec-9 autoantibodies

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Human intravenous Ig (IVIg) preparations are used for the treatment of humoral primary immunodeficiencies and a broad range of autoimmune diseases. We have previously shown that both agonistic and antagonistic anti-CD95 antibodies are present in IVIg, and that the effect on CD95 is dose-dependent. However, cell death induced by higher concentrations could not be explained by CD95-dependent mechanisms alone. Here we report that significant neutrophil death is induced by anti-Siglec-9 autoantibodies present in IVIg preparations. Siglec-9 has recently been shown to induce both apoptotic and non-apoptotic forms of cell death in neutrophils depending on the cytokine environment. Our current findings suggest an immunoregulatory role of naturally occurring anti-Siglec-9 autoantibodies by transmitting death signals into inflammatory cells. Knowledge about the exact mechanisms by which death receptors are triggered by autoantibodies has potential implications for IVIg treatment.

Macrophages detection and functional specification during the human intrauterine development

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Human prenatal development (IUD) is based on cells proliferation, differentiation and their programmed death. The role of macrophages as an innate immunity cells in the human development regulation and degradation of apoptotic cells is proved. Macrophages as a professional phagocytes participate in the cleaning up of apoptotic cells or bodies during IUD. The inflammatory reaction is absent. The role of macrophages in protection of foetus against infection or oncogenic cell transformation is unquestionable.

We used histologically normal tissues and organs of embryos and foetuses ranging from the 6th to the 30th week of IUD. The tissues were processed by routine paraffin technique.

We focused on embryonic and foetal liver, urinary system (mesonephros and metanephros), thymus, spleen and bone marrow and for comparison adult liver, kidney, thymus and bone marrow.

The first step was the detection of macrophages by a standard indirect three-step immunohistochemical method. For the detection of apoptotic cells (apoptotic bodies), TUNEL (TdT-mediated dUTP nick end labelling method) was applied.

In the second step we used a double-staining technique to detect macrophages and apoptotic cells (bodies) in the same section.

Ab-1, CD64 and CD68 positive macrophage were detected from the 6th week of IUD in all organs we studied (in the 6th week of IUD – liver, mesonephros and metanephros). Positive cells in liver were found in small isles and in mesonephros and metanephros, they were found only sporadically but in older embryos and foetuses the quantity of positive cells raise. In older foetal liver (from 12th week of IUD) we detected individual cells or small groups of positive cells, but not the isles as in 6th weeks old embryo. Macrophages and Kupffer cells were distinguished morphologically.

The thymus was studied in foetus from the 12th week of IUD and in all sections we found macrophages as a single cell or small groups.

The apoptotic cells (bodies) were detected in all sections. We detected macrophages that engulf apoptotic bodies unambiguously in the 10th week of IUD. Collocation of apoptotic bodies and macrophage markers was demonstrable in all three cases (TU/Ab-1, TU/CD64 and TU/CD68).

We detected macrophages of monocytic origin in the early phases (the 6th week of IUD) of human development and their functional ability – participation in the clearance of apoptotic bodies was proved in the 10th week of IUD.

This work was supported by grants MSMT 6198959205.

Structural architecture of a death receptor and an initiator procaspase associated with FADD in a death-inducing signaling complex

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The architecture of a DISC has been examined by determining the organization of CD95 and procaspase-8 engaged with intact FADD. The architecture has been built up through the identification of the binding surfaces of FADD DED and DD for both receptor and the procaspase prodomain biochemically and visualizing these interactions directly with the tools of structural biology. Biochemical analysis of the action of the FADD DED in the context of the full-length protein using FADD^{-/-} cells establishes that the FADD DED and FADD DD must be covalently linked to one another for successful recruitment to an activated receptor and assembly of the DISC. The meaning of the anti-apoptotic phenotype of DN-FADD has been analyzed as part of this study, establishing that DN-FADD can be inhibitory, but is not a dominant inhibitor nor does DN-FADD act by engaging the receptor itself to block FADD binding sites. Resolution of the mechanism of DN-FADD function clarifies the roles of each FADD domain in receptor recruitment. The three-dimensional structure of FADD solved as part of this study demonstrates that the domains of FADD possess a functional orientation in the intact protein, with the receptor and procaspase-binding surfaces on opposite sides of the 'cigar-shaped' molecule. The molecular basis for the procaspase prodomain interaction with FADD will also be discussed. Specific roles and binding surfaces will be described for each of the two DEDs of procaspases 8 and/or 10 and compared to the roles of the DEDs employed by the FLIP prodomain.

To illuminate the organization of a fully reconstituted DISC, we have also created constitutively active death receptor mimics whose expression is tightly regulated in stably transfected cells. These receptor mimics activate initiator caspase-mediated cell death with all the hallmarks of ligand-induced receptor activation. These receptor mimics have been used to explore the stoichiometry of the CD95 DISC, demonstrating that a dimeric receptor is both necessary and sufficient for cell death receptor activity. We summarize these and other results with the presentation of an entirely new model for the organization of a receptor DISC.

Bridge over troubled waters: milk fat globule epidermal growth factor-8 facilitates macrophage clearance of apoptotic cells

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During apoptosis, phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane of the dying cell and serves as a crucial recognition signal for macrophages (Kagan et al., J. Immunol., 2002). Recent studies have identified MFG-E8 (milk fat globule epidermal growth factor-8) as an important factor that serves to enhance the interaction between the apoptotic cell and the macrophage by binding to PS on the target cell and the integrin receptor on the phagocyte. We studied the effect of MFG-E8 on phagocytosis in an in vitro co-cultivation system using human monocyte-derived macrophages. We observed that non-blebbing, staurosporine-triggered Jurkat cells displayed high levels of PS exposure but appeared to be less efficiently engulfed by macrophages than blebbing (Fas-triggered or etoposide-treated) target cells with a similar level of PS externalization. Pre-treatment of Fas-triggered cells with cytochalasin B prevented blebbing, but did not affect PS externalization; phagocytosis of these cells was also diminished. When recombinant MFG-E8 was added to the co-cultures with staurosporine-treated or cytochalasin B + anti-Fas-treated cells the number of phagocytosis-positive macrophages increased and the number of engulfed target cells per macrophage was elevated. We also found that primary human neutrophils isolated from peripheral blood of healthy donors and forced into massive, synchronous apoptosis were able to bind MFG-E8, whereas freshly isolated neutrophils failed to do so. We are currently testing whether exogenous MFG-E8 is able to potentiate macrophage clearance of apoptotic neutrophils. Together, our results provide further evidence that MFG-E8 may act as a bridging molecule during programmed cell clearance. These results may be of relevance in human diseases characterized by defective cell clearance, or a mismatch between cell clearance and cell death.

Apoptosis-induced membrane expression of proteinase 3, a neutrophil-derived serine proteinase: a novel effector mechanism of apoptotic neutrophils

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Neutrophils are essential actors in the defense against pathogens. Because neutrophils contain highly toxic microbicidal mediators, their apoptosis, considered as a "clean disposal mechanism" is crucial for the resolution of inflammation. Proteinase 3 (PR3) and human neutrophil elastase (HNE) are two serine proteinases homologues which are stored in the azurophilic granules of neutrophils. They have microbicidal properties and can mediate connective tissue degradation when they are released in the extracellular medium upon neutrophil degranulation. Because it has been previously described that serine proteinases (such as granzyme B) can be involved in apoptosis, we studied whether PR3 or HNE could be involved in neutrophil apoptosis. In order to study each proteinase separately, we have used a model of stably transfected rat basophilic cell lines (RBL) with either PR3 or HNE. After stimulation with a ionophore (A23187), both HNE and PR3 were secreted after cell stimulation, in RBL/HNE and in RBL/PR3, respectively. However, only PR3 was expressed at the plasma membrane after degranulation. We next studied the influence of apoptosis on the membrane expression of HNE and PR3. We here showed that apoptosis triggered by etoposide, resulted in the membrane expression of PR3 in RBL/PR3, whereas no membrane expression of HNE was observed in RBL/HNE. Likewise, in neutrophils, PR3 membrane expression could also be induced by physiologic apoptosis. Apoptosis-induced PR3 membrane expression might i) represent a potential deleterious mechanisms of apoptotic neutrophils and ii) interfere with the mechanisms of clearance of apoptotic neutrophils.

In conclusion, our data strongly suggested a functional difference between HNE and PR3 in neutrophil apoptosis. This work was supported by Vaincre la Mucoviscidose (VLM) et l'Association pour la Polyarthrite Rhumatoïde (ARP).

Aberrant expression of genes related to apoptosis in ovarian serous adenocarcinoma

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Ovarian carcinoma is a leading cause of cancer death in women in Hong Kong as well as worldwide. Recurrent ovarian cancer continues to be a therapeutic dilemma. The poor prognosis has prompted major efforts to identify novel diagnostic and prognostic factors, improve early diagnosis, surgical staging, and develop adjuvant therapies that could improve patient outcome. Approximately 50% of ovarian epithelial cancer are in serous type. Recently, we examined the expression profiles of 28 primary ovarian serous adenocarcinomas and 19 normal ovarian specimens using oligonucleotide microarray U133A containing over 22,000 transcripts. DNA-Chip analyzer was used for data quality checking and high-level analysis. Supervised analysis of gene expression data identified 224 and 168 genes that exhibited >4-fold up-regulation and down-regulation, respectively, in ovarian cancer compared to normal ovary. Functional analysis of the genes of interest showed 5 genes among them are related to apoptosis. These include (1) baculoviral IAP repeat-containing 5 (BIRC5) (17q25) with 134.38-fold-up; (2) NACHT, leucine rich repeat and PYD containing 2 (NALP2) (19q13.42) with 17.69-fold up; (3) secreted phosphoprotein 1 (SPP1) (4q21-q25) with 17.1-fold-up; (4) pleckstrin homology-like domain, family A, member 2 (PHLDA2) (11p15.5) with 7.29-fold-up; and (5) NACHT, leucine rich repeat and PYD containing 1 (NALP1) (17p13) with 7.09-fold-down, in cancer compared to control, respectively. The differential expression of SPP1 and BIRC5 was further validated in another independent set of cancer and control specimens. Our results suggest the genes related to apoptosis do involve tumorigenesis/progression of ovarian serous carcinoma. Such genes might be the potential candidate of molecular diagnostic/prognostic marker(s) or novel therapeutic targets of ovarian cancer.

Apoptosis of cervical cancer cells induced by siRNA specific to SPP1 gene

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Using oligonucleotide microarray an analysis of gene expression profiles obtained from cervical and ovarian cancers in Hong Kong women was performed to find those genes most aberrantly expressed and thus with the most promise for translation into clinically useful molecular markers and/or therapeutic targets for these common female genital malignancies. SPP1 was one of the genes that exhibited >2-fold found in both cervical and ovarian cancers compared to their normal counterparts. Differential expression of SPP1 was further validated by quantitative RT-PCR on independent set of cancer and control specimens. Immunohistochemical staining of cancer specimens also confirmed differential expression of SPP1 potential marker/target on cervical cancer cells vs non-tumor cells. In vitro study, we explored silencing of SPP1 expression in both HeLa (cervical cancer) and OCC1 (ovarian cancer) cells by siRNA specific to SPP1. Quantitative RT-PCR showed the SPP1 expression in these two cell lines were suppressed by siRNA specific to SPP1 in both dose- and time-response manner. Fluorescent staining and flow-cytometer assessments showed increased apoptosis of both cancer cells induced by SPP1 specific siRNA. Our study revealed that SPP1 might be one of candidate molecular targets for cervical and ovarian cancer therapy.

Cell signaling in HepG2 cells after stimulation with chemotherapeutic drugs

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Programmed cell death (apoptosis), an important mechanism during development and T cell selection, regulates the general cell turnover in many organs. A defect in the “balance” of such death- and survival signals can lead to diseases like autoimmune disease and cancer, where malignant cells survive in spite of their genetic and morphological transformation. Cancer is also a state where apoptosis is inhibited. When employing chemotherapeutic drugs, one hopes to re-induce apoptosis and consequently, eliminate cancer cells. In this study, we have tested the effects of some chemotherapeutic drugs at the molecular level. We have established real-time PCR in order to analyze the expression of most of the anti- and pro-apoptotic transcripts. The anti-cancer drugs 5-Fluorouracil (5-FU), cisplatin (CDDP) and etoposide have been used (added at concentrations of 100 μ M), with staurosporin employed as an apoptosis-positive control (added at 1 μ M conc.).

Cells were harvested after incubations with drugs for various time-points. Gene expression of bax, bak, bid, bim, and BNIP3 as well as bcl-2, bcl-xL, mcl-1 and survivin was examined by RT-PCR. Gene expression was verified by western blot in cytosolic- and mitochondrial fractions, using actin and Cox IV as markers for the respective fractions, respectively.

Preliminary results indicate that different drugs activate different signaling pathways; e.g. both 5-FU and etoposide induce expression of the anti-apoptotic factor bax and BNIP3 in a time- dependent manner, while cisplatin gives nearly unchanged or decreasing expression, respectively, for these two factors.

Apoptosis induction in human lung adenocarcinoma cells by oil-soluble allyl sulfides: triggers, pathways and modulators

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Background: DAS (diallyl sulfide), DADS (diallyl disulfide) and DATS (diallyl trisulfide), are major oil-soluble allyl sulfides (OAS) that represent major garlic constituents. The anticarcinogenic and antimutagenic effects of these substances have been extensively studied during the last decades. Previous reports suggest that induction of apoptosis by OASs might contribute to their chemopreventive effects. However, the underlying mechanisms are largely unknown.

Aim: As a comprehensive study on apoptosis induced by allyl sulfides, the aim of present work is to reveal triggers, pathways and modulators in oil-soluble allyl sulfides mediated apoptosis.

Methods: Flow cytometry was employed to analysis cell cycle, percentage of cells in sub-G1, Fas expression, reactive oxygen species (ROS) production and change of mitochondria membrane potential (MMP). Expression of other proteins was measured by Western Blotting.

Results: DADS and DATS but not DAS induce significant apoptosis in human lung adenocarcinoma A549 cells. Differential modulation of reactive oxygen intermediates (ROI) and mitochondria membrane potential (MMP) may account for the apoptotic effects of DADS and DATS. The underlying molecular mechanisms of apoptosis induction by both compounds include activation of C-Jun N-terminal Kinase (JNK), up-regulation of p53 and down-regulation of bcl-2 expression. Up-regulation of extracellular signal-regulated protein kinase (ERK) was not dispensable for apoptosis induction; DAS, DADS or DATS did not modify expression of MAPK p38, bax and bcl-xL. Further investigation revealed that the specific JNK inhibitor SP600125, the antioxidant NAC and p53 inhibitor pifithrin- α , but not ERK inhibitors blocked DADS and DATS-induced apoptosis. We also demonstrated that activation of JNK and p53 is redox-modulated. Additionally, our data provided the first evidence that Fas-mediated cell death pathway is partly involved in DADS but not DATS-mediated cell death.

Conclusions: Taken together, our work elucidated the triggers, important modulators and signal transduction pathways in DADS and DATS-mediated apoptosis. These findings indicate that DADS and DATS are highly effective chemopreventive agents and even might be promising tumor therapeutic agents.

TNF- α inhibits asbestos induced cytotoxicity via a NF- κ B dependent mechanism

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Asbestos is a known pathogenic agent for human malignant mesothelioma (MM). However, asbestos does not induce transformation of primary human mesothelial cells (HM) in vitro. Rather, asbestos is very cytotoxic to HM in vitro, an effect that has been related to HM susceptibility to the genotoxic effect of asbestos. TNF- α is one of the major cytokines released by macrophages and some other cell types following exposure to asbestos. We observed that the expression of TNF- α receptor I was induced in human mesothelial cells exposed to asbestos in cell culture. Treatment of HM with TNF- α changed the morphology of cultured HM from epithelioid into fibroblastic. When we treated HM with TNF- α before exposing them to asbestos, TNF- α significantly reduced asbestos cytotoxicity. TNF- α did not induce apoptosis in HM but strongly activated NF- κ B. We further determined that TNF- α prevented programmed cell death of HM through activation of NF- κ B signaling. In fact, inhibition of NF- κ B with Bay11-7082, a chemical compound that blocks the phosphorylation of I κ B, suppressed the protective effect of TNF- α . To further verify the results, we performed RNA interference assay using siRNA-RelA constructed with lentivirus to knock down NF- κ B-RelA expression. The results confirmed that NF- κ B is required for the TNF- α mediated cytoprotection effect. Our data show a critical role for TNF- α signaling in mediating the HM cellular response to asbestos. Indeed, TNF- α through NF- κ B dependent mechanisms increases the percentage of HM that survives asbestos exposure, thus increasing the pool of asbestos-damaged HM that are susceptible to malignant transformation.

Involvement of ERK1/2 and (PI3-K)/Akt signal pathways in acquired resistance against neurotoxin of 6-hydroxydopamine in SH-SY5Y cells following cell-cell interaction with astrocytes

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Glial cells are known to interact with neurons and play important roles in the development, differentiation, maintenance and repair of the nervous system. We previously reported that human neuroblastoma cells (SH-SY5Y) became dramatically resistant to neurotoxin 6-hydroxydopamine (6-OHDA) when co-cultured with mouse astrocytes. We showed that this specific interaction requires direct cell-cell contact. Following interaction with astrocytes the 6-OHDA-induced increase of p53 was blocked in the SH-SY5Y cells. We further examined the activation of extracellular signal-regulated kinases (ERKs) and Akt signal pathways in 6-OHDA treated SH-SY5Y cells alone or in co-culture with astrocytes. Western blot analyses demonstrated that 6-OHDA significantly increased the phosphorylated ERK1/2 level after 12 hours of treatment in SH-SY5Y cells and this level sustained to 24 hours. While the phosphorylated Akt increased more rapidly, it peaked after 3 hours of treatment and returned to base level after 12 hours. SH-SY5Y cells after co-cultured with astrocytes for 24 hours exhibited an increase in ERK1/2 phosphorylation as early as 3 hours after 6-OHDA treatment; the response of phosphorylated Akt appeared to be more intense after 3 hours treatment compared with that in mono-cultures. Meanwhile we also determined that the expression of anti-apoptotic protein bcl-2 in SH-SY5Y cells significantly increased after 3 hours of 6-OHDA treatment under co-culture condition. Selective inhibitor of PI3-K/Akt signal pathway blocked the acquired resistance to 6-OHDA in SH-SY5Y cells following interaction with astrocytes. Inhibition of ERK1/2 signal pathway did not affect the cell survival. Our data suggest that PI3-K/Akt signal pathway, but not ERK1/2, is involved in the acquired resistance in SH-SY5Y cells following cell-cell interaction with astrocytes against the neurotoxic 6-OHDA insults.

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Key words: astrocytes, cell death and survival, signal transduction, neuronal-glia interaction, neuroprotection, ERK, AKT

Exogenous Folic Acid supplementation affects Superoxide dismutase gene expression and protein distribution of Bcl-2 and Bax in rat yolk sacs

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Background: It is suggested that the yolk sac protects the embryo from exogenous insults and that lack of proper yolk sac function results in severe outcomes for the embryo. Addition of Folic Acid (FA) to the maternal diet prevents embryonic maldevelopment in diabetic rat pregnancy. However, the effects on antioxidative capacity and rate of apoptosis in the yolk sac by FA treatment are not known.

Aims: To examine if treatment of normal (N) and manifestly diabetic (MD) rats with FA during pregnancy affects the mRNA levels of three isoforms of Superoxide dismutase (SOD) and the protein distribution of Bcl-2 and Bax in the embryonic yolk sac.

Methods: We used embryonic yolk sacs at gestational day 10 and 11 from N and MD rats treated with or non-treated with FA. The yolk sac mRNA-levels were estimated by real-time PCR. Immunohistochemistry was used for Bcl-2 and Bax protein distribution to estimate apoptotic rate.

Results: A common trend was lower SOD mRNA levels in the MD10 and 11 yolk sacs compared with nondiabetic groups. In addition SOD mRNA levels tended to be slightly lower in FA treated groups compared to non-FA treated groups. Bcl-2/Bax ratio was decreased in MD yolk sacs. FA treatment of the MD group increased the Bcl-2/ Bax ratio, indicating lower apoptosis rate.

Conclusion: FA supplementation appears to downregulate SOD gene expression concomitant with increasing Bcl-2/ Bax ratio in FA treated embryonic yolk sacs.

Autophagy is preferred pathway of camptothecin-induced programmed cell death of v-*myb*-transformed monoblasts

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A damage of programmed cell death pathways is a frequent event during malignant transformation, and it can significantly modify the response of cancer cell to a therapy. In this study, we analysed programmed cell death in chicken v-*myb*-transformed BM2 and human U937 promonocytes induced by arsenic trioxide, cycloheximide and camptothecin. The presence of functional v-Myb protein pre-determined the BM2 cells to autophagic pathway of programmed cell death in response to the DNA-damaging agents. The absence of transactivating v-Myb protein allowed activation of cell death pathway marked by mitochondrial membrane depolarization and resulting in necrosis. The fact that the antiapoptotic function of the Myb protein can be overcome by the induction of alternative cell death pathway suggests that understanding of molecular mechanisms of autophagy could improve therapy of cancer cells suffering from defective apoptosis.

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Mechanisms for TGF- β 1 - Smad7 induced apoptosis in normal and malignant epithelial cells

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Transforming growth factor- β (TGF- β) is an important regulator of cell fate during embryonal development, and maintenance of tissue homeostasis. Activation of the TGF- β -Smad7 pathway has been shown to be implicated in apoptosis in normal and malignant prostate epithelial cells, by activation of the p38 MAP kinase pathway (Landström *et al.*, 2000, Edlund *et al.*, 2003). We are investigating the targets for Smad7 and p38 in TGF- β treated normal and malignant human epithelial cell lines. The aim of our project is to study the possible contribution of p53 in TGF- β induced apoptotic responses.

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HIV infection is associated with increased skeletal apoptosis assessed by TUNEL

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Purpose of the study: Increased apoptosis in CD4⁺ T lymphocytes plays an important role in the pathogenesis of HIV infection, probably through several viral proteins which have been proposed to be directly or indirectly associated with the dissipation of mitochondrial membrane potential, thereby inducing apoptotic cell death. The aim of the study was to assess whether this increase is also present in the skeletal muscle of HIV-infected patients.

Methods: We included 18 healthy individuals without HIV infection as controls and 5 asymptomatic antiretroviral naive HIV-infected patients. Immunohistochemical reactions using monoclonal antibodies for TUNEL (deoxyribonucleotidyl-transferase-mediated-dUTP-biotin nick-end labeling) were performed on skeletal muscle samples from deltoid muscle of the non-dominant arm of each patient and control. The specimens were coded with random numbers, and read by three independent, blind observers. The percentage of apoptotic cells was determined by means of an apoptotic index (Ai) that was calculated by dividing the total number of positive staining myocyte nuclei in the TUNEL assay by the total number of the myocyte nuclei, and multiplying this value by 100.

Summary of results: The results were consigned in a semiquantitative scale (- if less than 1‰ of positive nuclei or cells were present / + if less than 1% / ++ if less than 5% / +++ if more than 5%). TUNEL assay showed data of significant increased apoptosis in skeletal muscle of 80% of HIV patients when compared to uninfected individuals (p<0.01). None of the control individuals were positive for TUNEL.

Conclusions: Skeletal muscle of HIV-infected patients exhibits increased apoptosis compared with healthy uninfected individuals. Further studies are necessary to elucidate the mechanisms by which HIV infection leads to increased apoptosis.

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(by session)



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P-42	The inhibition of nitric oxide synthesis protects brain against inflammation related apoptosis	Czapski, Grzegorz
P-48	Phosphorylated glyoxalase I as an effector molecule of nitrosative stress-induced cell death	de Hemptinne, Virginie
P-56	Trolox is a potent modulator of arsenic-mediated cytotoxicity in vitro and in vivo	Diaz, Zuanel
P-63	Tomato phytochemical but not lycopene alone is able to induce apoptosis in HL60 human leukemia cells	Ettore, Anna
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P-83	Influence of autocrine factor deficit on energy metabolism and survival of CTLL-2 cells under oxidative stress	Grechikhina, Mariya
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- P-209** Overexpression of thymosin beta-4 renders SW480 colon carcinoma cells more resistance to apoptosis triggered by FasL and two topoisomerase II inhibitors via down-regulating Fas and up-regulating Survivin expression, respectively Su, Yeu
- P-223** Effects of olomoucine II on ODC and Bax mRNA levels in rat prostate cancer cell lines Tufekci, Mehmet
- P-224** Effects of AT1 blocker on apoptosis in experimental diabetic nephropathy Tuncdemir, Matem
- P-225** Role of ERK pathway in regulation of colon cancer cell sensitivity to TRAIL-induced apoptosis Vaculova, Alena
- P-226** Insertion of Bax into mitochondria occurs through the preprotein translocase of the mitochondrial outer membrane (TOM) complex Vallette, Francois
- P-227** The characterization of 86 novel protein processing sites in Fas-induced apoptotic Jurkat cells using non-gel proteome degradomics Van Damme, Petra
- P-237** p57 is a potential substrate for caspases-3 and its expression enhances staurosporine-induced apoptosis in HeLa cells Vlachos, Pinelopi



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P-25 Human CD8 ⁺ are more sensitive than CD4 ⁺ T cell blasts to regulation by APO2L/TRAIL and by IL-2 deprivation	Bosque, Alberto
P-27 Direct cleavage of ROCK II by granzyme B induces target cell membrane blebbing in a caspase-independent manner	Bréard, Jacqueline
P-37 Role of lysosomal proteases in neutrophil apoptosis	Conus, Sébastien
P-40 Human and Murine forms of Granzyme B exhibit divergent substrate preferences: implications for the mechanism of CTL/NK killing	Cullen, Sean
P-45 A20 differentially affects apoptosis in endothelial cells and smooth muscle cells	Daniel, Soizic
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P-98 Induction of apoptosis and autophagy in ALCAM/CD166 deficient breast cancer MCF-7 cells	Jezierska, Agnieszka
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