Cell Death and Differentiation (2005) 12, 411–414

www.nature.com/cdd

Meeting Report

Cell death by the sea

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Cell Death and Differentiation (2005) **12,** 411–414. doi:10.1038/sj.cdd.4401570
Published online 18 February 2005

12th Euroconference on apoptosis and 1st training course on 'Concepts and Methods in Programmed Cell Death', Chania, Greece, 17–20 September 2004

The European Cell Death Organisation (ECDO), with the support of the EU-Marie Curie Actions, organized the 12th Euroconference on Apoptosis in Chania, Greece, from 17 to 20 September 2004. The meeting, which was held in the 'Arsenali' conference centre, a recently restored Venetian building situated in the picturesque old harbour of Chania, was attended by 310 participants from all continents. For the first time, the Euroconference was combined with a training course, aiming to familiarize young scientists and newcomers to the field with some of the basic concepts, methodologies, and tools used in the study of programmed cell death. Thus, the first training course on 'Concepts and Methods in Programmed Cell Death', to which most of the first day was dedicated, consisted of seven 1-h presentations focused on the mechanisms behind programmed cell death and the apoptotic responses (R Lockshin, G Salvesen, P Krammer, W Bursch), as well as the technological approaches that can be employed in their study (P Bernardi, E Golemis, P Silver). Details on the presentations can be found on the conference web-site, http://ecdo.maich.gr.

In his keynote lecture, Boris Zhivotovsky focused on the interplay between nuclei and mitochondria and the nature of the signals travelling between them during the apoptotic process. Caspase-2 is unique among caspases in that it has nuclear localization and different cleavage specificity (requiring the pentapeptide VDVED). Upon DNA damage, cleavage of the pentapeptide occurs earlier than activation of other caspases, while inhibition of procaspase-2 inhibits cyt *c* release, annexin externalization, and DNA fragmentation. Expression of p53 was shown to be important for caspase-2 activation. Incubation of permeabilized Jurkat cells with recombinant caspase-2 induces the release of cyt *c*, suggesting a direct action of caspase-2 on the mitochondria, which cannot be rescued by overexpression of Bcl-2. No other caspase could effect cyt *c* release in cells or cell extracts, and apoptosis inducing factor (AIF) was only released subsequent to mitochondrial permeability transition. Moreover, processed caspase-2 could still release cyt *c* in permeabilized Bax/Bak double-knockout MEFs. Proteolytic activity was not required for this effect and experiments with liposomes indicated that processed caspase-2 could be competing with cardiolipin for cyt *c* release. On a different note, in non-small lung cancer cells (NSCLC), which are resistant to DNA damage-induced apoptosis, cyt *c* was released but no active caspase-3 translocated to the nucleus. Staurosporin could still induce cell death by causing the release and migration of AIF to the nucleus. Blocking AIF migration also blocked staurosporin-induced cell death, indicating that a real dialogue exists between the organelles for both signalling and execution of cell death.

Programmed cell death across the eukaryotic kingdoms

Eric Baehrecke examined autophagy, an alternative form of cell death, which depends on a unique degradation machinery that is likely conserved from yeast to mammals. Inhibition of apoptosis in *Drosophila* salivary glands by overexpression of Baculovirus p35 could not avert eventual death with an autophagic morphology. An RNAi approach in transgenic flies showed that inhibition of Atg 3, 6, or 12 prevented the destruction of salivary glands and that the PI3K pathway regulates the process, suggesting that the nutritional pathway is directly related to the ability of the cell to die. John Abrams has been dissecting the 'gas and brake' events in apoptosis using the *Drosophila* embryo model. Removal of IAP antagonists (Reaper, Grim and Hid) blocks PCD in embryonic development. The Dark (Apaf-1 homologue) mutation in the fly prevents defects in the PCD of salivary glands and, to an extent, inhibits the massive autophagic process. Removal of IAPs leads to catastrophic cell death and the phenotype is reversible in the Dark background. Moreover, mutation in the *Drosophila* orthologue of p53 exhibits a defect in Reaper induction, indicating that apoptosis is an ancient feature of the p53 network. Drug-induced apoptosis in *Plasmodium falciparum* was shown by Stephane Picot. The parasites exhibit the hallmarks of apoptosis, including DNA fragmentation and exposure of PS on the external leaflet of *P. falciparum*

plasma membrane. Lilli Stergiou presented on the role of the *C. elegans* ATM homologue. UV-treatment of ATM mutants severely compromised the cell death response, unlike ionizing radiation (IR) that had no effect. UV radiation elicits a cell cycle arrest and an apoptotic response in germline tissue, which is mediated by the p53 homologue. ATM transduces the signal to the cell cycle machinery and partially contributes to apoptosis, possibly acting downstream of *hus-1* and *clk-2*.

Sharad Kumar presented data on the mechanism of caspase activation in flies. Dronc, the most apical caspase, is bound by DIAP. Removal of DIAP by ubiquitination and degradation allows Dronc to interact with Dark. Cytochrome *c* species do not appear to play any role in the cell death process. The levels of Dronc are critical for sensitizing different *Drosophila* cells to apoptosis. Edysone treatment of isolated salivary glands causes massive Dronc transactivation. Dronc is a direct target of p53. Deletion of p53 binding sites abolishes Dronc upregulation by DNA damage. A Dronc P-element deletion mutant is lethal at the pupal stage. In the midgut of the Dronc knockout flies, unlike in the salivary glands, downstream activation of caspases takes place independently of Dronc. Miguel Torres presented on the PCD process occurring in plants during the defense response against pathogens (Hypersensitive Response). Upon pathogen recognition, plants produce signals such as ROI, NO, and salicylic acid. Through the use of transposon mutants, plant NADPH oxidases were shown to produce most of the ROI during pathogen recognition. Using the double mutants, it was shown that in response to bacterial infection, ROI act as positive regulators of cell death, whereas upon fungal infection, ROI function as negative regulators limiting the extent of cell death. Plants also uniquely contain a Zinc-finger protein LSD1, which acts to suppress the self-amplification of the death signal. Macroautophagy was shown to be required for *C. elegans* neurodegeneration in Popi Syntichaki's presentation. Degeneration was induced by two different hyperactive proteins *mec-4* and *deg-3*. Mutation of the autophagy related gene *unc-51* suppressed neurodegeneration. This inhibition of cell death was also observed with other autophagy-related genes, such as *lgg-1* and *bec-1*. Mauro Degli Esposti pointed at the nose of zebrafish, which is full of mitochondria that can be visualized *in vivo*. Knockdown of tafazzin, a gene essential for cardiolipin metabolism and defective in Barth syndrome, due to the reduction of cell death, causes mitochondrial proliferation, proliferation of neurosensory cells, and dilated cardiomyopathy.

Survival signalling

Phil Tsichlis presented data on the role of Tpl-2 in cancer and inflammation. Tpl-2 is a gene originally cloned by MoMuLV proviral integration-induced T-cell lymphomas in mice. Overexpressed Tpl-2 is constitutively active, transforms the cells, and causes tumours in transgenic mice. Tpl-2 knockouts are healthy animals but are resistant to LPS toxic shock. Tpl-2 normally targets the 3′UTR of TNFα mRNA and mediates its nuclear export. Tpl-2 is also shown to mediate signals from TNFR1. Depending on the context, Tpl-2 can function either as an oncogene or as a tumour suppressor. The absence of Tpl-2 causes a significant delay in tumour development by Akt. On the other hand, in crosses of Tpl2$-/-$ with mice expressing a TCR transgene receiving constant stimulation by an endogenous antigen, the mice were shown to develop lymphomas by inhibition of the induction of cell death, suggesting a function in an inhibitory loop that turns off the immune response. Patrick Mehlen focused on the dependence receptors, which become activated in the absence of ligand and promote cell death. DCC receptor, a tumour suppressor gene, is one such receptor. In the absence of its ligand, DCC is cleaved by caspases, which leads to exposure of a domain termed ADD (addiction dependence domain). The human homologue of *C. elegans* Unc-5 is also shown to be a dependence receptor that interacts with DAPK to regulate its autophosphorylation and transduce the death signal. Mice overexpressing the ligand netrin show a 50% decrease in cell death in the gut epithelium, whereas netrin knockout increased cell death, pointing to an important role in tumourigenesis. In Raf signalling against cell death, Manuela Baccarini showed that in the conditional knockouts of Raf-1, upregulation of Fas on the surface is the critical event for embryonic lethality and is independent of Raf kinase activity. B-Raf conditional knockouts, on the other hand, uncovered the importance of the placental phenotype caused by lack of ERK activation and production of VEGF, as well as massive neuronal defects. Frataxin, a nuclear encoded mitochondrial gene, was shown by Natascia Ventura to delay Fas-induced apoptosis. Frataxin was also shown to be localized in the cytosol, and this cytosolic pool was also capable of suppressing cell death induced by ceramide and Fas. The neuroprotective activities of erythropoietin were dissected by Pietro Ghezzi. It was shown that the mechanism of protection by inhibition of apoptosis is independent of the hematopoietic capacity of the molecule. Administration of EPO in chronic conditions of the CNS, such as diabetic neuropathy, experimental autoimmune encephalomyelitis, and multiple sclerosis, significantly contributes to clinical improvement. To disengage the protective function from the hematocrit increases, EPO was modified by mutagenesis and chemical modification. EPO carbamylated in lysines (CEPO) retained neuroprotection but lost its ability to bind the EPO receptor and trigger erythropoiesis.

Phagocytosis

Kodi Ravichandran examined the role of the mammalian homologues of the *C. elegans* ced genes in phagocytosis. Elmo (ced-12 homologue) and Dock180 (ced-5 homologue) were shown to physically interact, activate Rac to its GTP form, induce membrane ruffling and promote phagocytosis. Dock180 acts as a GEF for Rac activation. Elmo contains a PH domain in the C-terminus, which is necessary and sufficient for binding to Dock180, and armadillo repeats in the N-terminus, which have a role in binding to RhoG, a small GTP binding protein placed upstream on the signalling pathway. The significance of CD14 as a tethering receptor in macrophages during phagocytosis was presented by Chris Gregory. CD14 knockout mice show an increased number of apoptotic cells due to a defect in clearance. Macrophages from the knockouts are less effective at interacting with apoptotic cells, indicating the importance of CD14 as a

tethering molecule in multiple tissues. The persistence of apoptotic cells did not harm the animals, as there were no signs of autoimmune disease. On a different note, in tumour associated macrophages, IL-10 was shown to enhance the capacity of macrophages to produce B cell survival proliferative factors. Wei Xu examined the phagocytic capacity of three different sets of macrophages DC, Mφ1, Mφ2. Dendritic cells exhibited a preference for necrotic cells, Mφ2 most efficiently cleared apoptotic cells and produced amounts of IL-10, whereas Mφ1 did not. Kimon Doukoumetzidis used a high throughput RNAi approach for engulfment suppression in *C. elegans*. A pilot screen identified ced-6 and the two subunits of actin related protein complex ARP 2/3 as essential mediators of actin polymerization. The induction of apoptosis as a survival mechanism of the *Leishmania* parasite was proposed by Giulia Getti. Infection of monocytic cell lines and PBMs showed that all three *Leishmania* species tested induced apoptosis and PS externalization, which led to increases in the percentage of infection, suggesting the use of phagocytosis as a spreading process.

The ECDO award lecture was presented by John Savill on how corpse clearance defines the meaning of cell death and adds value to it. Uptake of apoptotic cells by macrophages can turn off the release of TNFα and other proinflammatory molecules. This suppression could be reversed by blocking TGFβ signals. In SV vasculitis there is production of antibodies against neutrophil cytoplasmic components and perturbation of the safe clearance of dying neutrophils that may be promoting persistent inflammation. The capacity to be suppressed by TGFβ, released from a macrophage taking up an apoptotic cell, appears to be critically dependent on having actually touched an apoptotic cell. During phagocytic clearance, dying cells signal to promote growth of neighbouring cells, indicating that very important decisions are made by phagocytes and target cells, even before those phagocytes engage in the apoptotic programme.

Stress response and apoptosis

The role of calcium signalling during apoptosis was analysed by Rosario Rizzuto. Overexpression of Bcl-2 in HeLa cells was shown to lower calcium levels by 20–30% in the ER, which translated into lower mitochondrial levels. Ca^{2+} reduction in the ER mimics the effects of Bcl-2. Lowering mitochondrial Ca^{2+} levels has a protective effect against apoptosis. In the context of disease, Hepatitis B virus X protein exerts its proapoptotic function by increasing calcium levels in the ER. The state of mitochondrial fusion and fission interferes with calcium uptake from mitochondria. Fragmented organelles block travelling of the signal. PKC_β signalling through phosphorylation of p66Shc can effect such mitochondrial shape changes. DNA damage signalling that leads to p53 activation was the focus of Thanos Halazonetis' presentation. The 53BP1 protein, which shares highest homology to yeast RAD9, was shown to be recruited to sites of DNA breaks and to subsequently activate ATM. The sensing mechanism is accomplished by the recognition of exposed methylated histone 3, which is bound by 53BP1 and can be visualized as foci at the sites of DNA breaks. In some cancer cells such foci and ATM phosphorylation can also be seen. In lung and breast tumours examined, chk2 activation occurs, implying an intrinsic association of cancer with dsDNA breaks. Anton Gartner showed the identification of GLD-1, a translational repressor of many *C. elegans* developmental genes, which also regulates *cep1*/p53 levels by binding to its 3′UTR. Knockout of GLD-1 leads to very high levels of p53. Angel Martin pointed at the proapoptotic roles of NF-κB signalling. MEFs from p65$-/-$ knockouts showed resistance to apoptosis induced by DNA damaging agents and lack of cyt *c* release or caspase activation. Noxa is the only Bcl-2 family member regulated by p65. Introduction of Noxa in p65$-/-$ cells restored sensitivity to etoposide treatment.

Yoshihide Tsujimoto presented data showing that autophagic, or type II, cell death is also regulated by Bcl-2 family members. Bax/Bak DKO MEFs, even though they exhibit total resistance to apoptosis, lose their capacity to proliferate and die by autophagic cell death with characteristic formation of autophagosomes. During this process of cell death, apg5 and apg6/Beclin, a Bcl-2 interacting protein, are expressed at high levels. Bcl-2 and Bcl-x are shown to positively regulate autophagic cell death. Two programmes can essentially be activated in response to cytotoxic drugs, one leading to apoptosis and another to autophagy. In normal cells, apoptosis is faster but upon inhibition autophagy is activated. Katja Prokrovskaja presented data on the mechanism of IFNα-induced apoptosis in tumour cells. In her work, apoptosis depended on the PI3K, and not the JAK-STAT, pathway. Nico Hendrickx focused on efforts to block survival signals during photodynamic therapy (PDT) in cancer. Light-activated hypericin produces ROS and activates PLA_2. The p38 MAPK is also activated, mediating an antiapoptotic signal. Blockage of the p38 signal shifts the balance towards apoptosis. The autophagic process came back to centre stage in Mike Lenardo's talk, focusing on death triggered by the caspase inhibitor zVAD. This type of death could be blocked by PI3K inhibitors and suppression of autophagic genes Beclin1/Atg6 and Atg7 using RNAi. RIP, an S/T kinase associating with CD95, plays a role in triggering the signal. A model was proposed suggesting that low-level activity of caspase-8 is required to prevent the autophagic process. Caspase-8 can cleave RIP and block signalling to JNK. The autophagic process has to proceed all the way to lysosomal fusion in order to kill the cells.

Laura Johnston, in her presentation in the role of cell death on growth control mechanisms in *Drosophila* wings, showed that cell competition and elimination of slow growing cells by fast growing cells is important for developmental plasticity and size regulation. Myc activation is shown to actively induce competition and cause growth disadvantage to neighbouring cells. Myc expressing cells induce apoptosis in surrounding cells. Induction of Hid is important for the cell death signal. In the proposed model, a signal is sent from the myc expressing cell to cells expressing less myc, resulting in their elimination and overpopulation of myc expressing cells. Fausto Fazzini showed that in small vessel vasculitis, a disease characterized by vessel wall injury and systemic inflammation, there is lack of clearance of apoptotic cells. Low doses of TNFα trigger PMNs to release high amounts of PTX3 *in situ*. Binding of apoptotic cells to PTX3 blocks phagocytosis and leads to leucocytoclasia.

The identification of a novel vaccinia virus apoptosis inhibitor, localizing in the Golgi, was presented by Caroline Gubser. The protein shares homology with human Bax Inhibitor I, and upon overexpression confers resistance to Fas, TNFα, and genotoxic drug-induced apoptosis. In the mouse intradermal model of the disease, infection with a mutant virus is shown to exacerbate disease symptoms. Lotta Leveelathi focused on the role of cathepsins in Fas-mediated apoptosis. Release of cathepsins B and D occurs before nuclear fragmentation. When examined in parallel with cyt *c* release, cathepsins were diffused in the cytoplasm prior to cyt *c*, while their release precedes the loss of MPT by several hours. Lieselotte Vande Walle pointed at the interaction of Omi and XIAP. Omi overexpression caused caspase-independent cell death. The identification of Omi proteolytic substrates, which are also cleared during Fas-induced apoptosis, was hinted at.

Pleiotropic functions of apoptotic proteins

Tak Mak showed that conditional knockouts of survivin in T cells arrest these at the pre-T-cell receptor signal stage. Crossing the mice into a p53/p21 knockout background resulted in increased cell death by mitotic catastrophe. Survivin is overexpressed in many tumours and given that p53 mutations are found in half the cancers, this makes survivin an ideal target to induce mitotic catastrophe in tumour cells. The role of cyt *c* was also examined in mice expressing a mutated form of cyt *c* that cannot bind Apaf-1. The mice can perform oxidative phosphorylation but die from atopic protrusions due to the lack of neuronal cell death. The few mice that survive to the third week have no lymphocytes. Growth hormone administration could rescue some animals. Guido Kroemer presented data on the functions of AIF in normal metabolism and during apoptosis. AIF has a DNA-binding capacity, which is required for its proapoptotic function. Cyclophilin A binds to AIF and the complex acquires DNAase activity. Knockdown of cyclophilin A in Jurkat cells blocks AIF-induced apoptosis. AIF is evolutionarily conserved; upon stress induction, the yeast AIF homologue relocates from mitochondria to the nucleus and requires the cyclophilin A homologue for killing. AIF$-/-$ MEFs show respiratory defects that appear to be due to defects in the biogenesis of complex I. A hypomorphic retroviral insertion in the first intron of AIF, causing the form of ataxia observed in Harlequin mice, confirmed a defect in complex I abundancy at the protein level. A conditional muscle specific AIF knockout shows enormous muscle atrophy and heart dilated cardiomyopathy.

The significance of mitochondrial fission in apoptosis and cell proliferation was the focus of Jean-Claude Martinou. Bax overexpression triggers fission of the organelles. Overexpression of the HsFis1 caused extreme fission of mitochondria, release of cyt *c*, and caspase-dependent cell death. Downregulation of Drp1 by RNAi inhibited mitochondrial fission, causing large vesicular mitochondria that functioned normally. No significant protection from apoptosis is conferred by Drp1 downregulation. However, less cyt *c* is released into the cytoplasm, suggesting that for the release of the membrane bound cyt *c* mitochondrial fission is required. Inhibition of fission during cell proliferation arrests the cells in G1 phase and eventually leads them to senescence. The p27 CDK inhibitor is highly expressed in these cells. Ekaterina Vassina presented data showing that in the hypereosinophilic syndrome (HES), eosinophils exhibit delayed apoptosis and are resistant to ceramide-induced apoptosis. The protein levels of cIAP2 and survivin are upregulated in these patients and huge increases in serum IL-5 levels are seen. Mohamed Lamkanfi, aiming to identify additional functions of caspase-1, showed that the CARD domain of the molecule could mediate NF-κB and MAPK activation, probably through recruitment of RIP. INCA, a CARD only protein, is shown to efficiently bind to procaspase-1 and block LPS-induced IL1-β release.

David Vaux presented data from experiments with mouse IL-3 dependent myeloid cell lines. Bcl-2 overexpression rescues the cells from GF withdrawal and retains their proliferative capacity. IL-3 dependent cell lines on an Apaf-1 and Casp-9 knockout background do not show signs of cell death upon GF withdrawal. However, their clonogenic capacity is lost. Unlike in Bcl-2 expressing cells, cyt *c* release can be observed in Apaf-1 and Casp-9 knockouts. Bim and Bad are not required for IL-3 withdrawal killing. However, Puma knockout allows cells to maintain viability and clonogenicity, indicating that it acts prior to the commitment step of IL-3 cell death. Geert Van Loo presented the effort to conditionally inactivate the FADD gene, aiming to study the *in vivo* signalling pathways to NF-κB. Andreas Schweizer presented the development of ankyrin-based caspase-2 specific inhibitors, using a library encoding 10^{11} different ankyrin repeat proteins and successive rounds of ribosome display and enrichment for caspase-2 binding. F8, a selected molecule, was shown to bind specifically to caspase-2 and block its activity. The function of Omi/Htra as a stress sensor and effector was dissected by Emad Alnemri. The protease activity of Omi is regulated by its PDZ domain, which interacts strongly with the protease domain. Binding of the PDZ domain to a peptide from another protein will liberate the active site. MND2 (motor neuronal disease 2) is a human disease where Omi is mutated. The loss of Omi activity in MND2 mice causes high levels of cell death. MND2$-/-$ MEFs are more sensitive to toxic agents and show mitochondrial dysfunction. During apoptosis, Omi is released in the cytoplasm where it can interact with presenilin via a 15 aa region which can subsequently activate Omi protease. The capacity of mutant presenilins to induce cell death is shown to be dependent on the presence of Omi.

Acknowledgements

We would like to thank the ECDO secretary Veronique Vandevoorde for her invaluable support in organizing the Euroconference, Wim Declerq for his helpful tips from previous meetings, Victor Tzougrakis and Nikos Psillakis for technical assistance, and all the members of our laboratory for their help and support. The Training course and the Euroconference were supported by the Marie Curie Conferences and Training Courses programme. The organizers would also like to thank the municipality of Chania for permitting the use of the old custom house. Additional support was provided by Blackwell Publishing, BD Biosciences and LMBP.