Cell Death and Differentiation (1997) 4, 343–346

Meeting Report

Death in Capri

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The Fourth Euroconference on Apoptosis,
Capri, Italy, 25–29, September 1996

Abbreviations: PCD, programmed cell death; SM, sphingomyelin; PC-PLC, phosphatidylcholine-specific phospholipase C; TNF, tumour necrosis factor; PBL, peripheral blood lymphocyte; DD, death-domain; APL, acute promyelocytic leukaemia; RA, retinoic acid; tTg, tissue transglutaminase; ICE, interleukin converting enzyme

In late September 1996, around 130 scientists and graduate students from around the world gathered at the beautiful island of Capri to attend the Fourth Euroconference on Apoptosis. Keeping with the tradition of the Third Euroconference on Apoptosis, this meeting was also held in a monastery (Certosa di S. Giacomo), an appropriate venue to discuss matters of life and death. The meeting was sponsored by the Training and Mobility of Researchers Program of the European Commission and the European School of Haematology, and was organised by Drs. Gerry Melino and Mauro Piacentini. As in previous Euroconferences, the program consisted of several plenary and short talks, and two poster sessions covering a broad spectrum of apoptosis research.

The meeting began in the afternoon of Wednesday September 25 and the first session included two plenary talks by Dr. Robert Horvitz (Boston) and Dr. Peter Steinert (Bethesda). In the opening talk Horvitz summarised the work on the characterisation of various genes involved in the regulation of programmed cell death (PCD) in *Caenorhabditis elegans*. Three genes involved in one of the two pathways required for the engulfment of cell corpses, *ced-2*, *ced-5* and *ced-10*, also participate in another developmental process, the migration of distal tip cells. *ced-7*, another gene required for the efficient engulfment of corpses, encodes a protein similar to mammalian ABC transporters. Two genes, ces-1 and ces-2, which regulate death of specific pharyngeal cells, encode putative transcription factors. CES-1 protein is similar to the snail family of Zn finger transcription factors and inhibits PCD, while CES-2 is a putative member of the bZIP PAR subfamily of transcription factors and is a repressor of CES-1. This pathway operates by affecting the expression of *ced-9*, a *bcl-2* homologue that blocks PCD by inhibiting *ced-4* and *ced-3* function. Horvitz also detailed the work on four *C. elegans* genes, *ced-3*, *ced-4*, *ced-8* and *ced-11*, involved in the execution phase of PCD. ced-3 and ced-4 are both required for PCD in the worm, whereas *ced-8* and *ced-11* appear to be involved in specific aspects of the killing process downstream of *ced-3*, and encode novel proteins. *Ced-8* appears to play a role as a death effector: corpses are made more slowly in *ced-8* mutant worms, and *ced-8* can synergise with weak *ced-3* mutants. *Ced-11* mutants show abnormal corpse morphology, suggesting a role in the structural changes of dying cells. Findings from these studies indicate that the molecular mechanisms responsible for PCD are broadly conserved among organisms as diverse as nematodes and humans.

Dr. Peter Steinert focused on cornification in epidermis and discussed the relationship between cornification and apoptosis. Although apoptotic cells can be found in cornifying epidermis, it is not known whether PCD is required or involved in keratinocyte differentiation. Nevertheless, cornification involves several specialised proteins, including three different transglutaminases and a number of tissue-specific substrates, including loricin, trichohyalin, elafin, filaggrin, and several keratins, all of which contribute to the strength and elasticity of the cornified envelope. Impermeability to water is achieved through a specialised process of synthesis and attachment of lipids, including ceramides, to cell envelope proteins. Steinert also reviewed various molecular aspects of the disorders associated with cornification. These disorders occur due to mutations in various keratin genes, or in other molecules involved in the formation of cell envelope, such as transglutaminase 1. Although cornification shares many features with classical apoptosis, there are differences in the mechanism, post-mortem stages, the way cells are removed, and the use of distinct transglutaminases.

The second day of the meeting was dominated by Fas/APO-1/CD95 mediated cell death. In the first talk, Dr. Peter Krammer (Heidelberg) summarised what is currently known about the molecular mechanism of APO-1 mediated death signalling. He described the identification of several proteins (CAP1-CAP4) that immunoprecipitate with APO-1. CAP1 and CAP2 were identified as phosphorylated MORT1/FADD. CAP3 and CAP4 associated with aggregated APO-1 and were detected immediately (<1 s) after crosslinking of APO-1. As it turned out, CAP3 and CAP4 represented the prodomain and precursor forms of a caspase (Ced-3/ICE family protease), FLICE. These findings, together with those of Wallach, provide the first evidence of a direct link between the activation of a cell surface death receptor and the execution phase of apoptosis. Once FLICE is activated, it can further activate other members of the caspase family such as CPP32.

Interestingly, Bcl-X_L can block the activation of CPP32 without affecting the activation of FLICE, suggesting that Bcl-2 family members function somewhere between FLICE and CPP32.

Dr. Roberto Testi (Rome) reviewed the role of the sphingomyelinase pathway in Fas/APO-1/CD95-mediated apoptosis. Crosslinking of CD95 results in the hydrolysis of sphingomyelin (SM), a major membrane phospholipid, by the action of sphingomyelinases, resulting in the release of ceramide. Ceramide, the hydrophobic portion of sphingomyelin, triggers apoptosis. Using an elegant experimental model involving CD95-sensitive and -resistant HuT78 cells, Dr. Testi presented evidence that sequential activation of phosphatidylcholine-specific phospholipase C (PC-PLC) and acid sphingomyelinase rather than the neutral sphingomyelinase, could be responsible for the propagation of the death signals from the CD95 death domain. Data were also presented to establish a role for acid sphingomyelinase in the death of normal lymphocytes in the immune system. The relationship between the sphingomyelinase and caspase pathways remains obscure however. Indeed, mutations of acidic sphingomyelinase in mice and humans (Niemann Pick disease) do not lead to the immunological abnormalities that would be expected if there were a defect in CD95 signalling.

Dr. Klaus-Michael Debatin (Heidelberg) discussed the role of the APO-1/CD95 system in human disease, particularly lymphoproliferative disorders, T cell leukaemia and T cell depletion in AIDS. Germline mutations of CD95 in humans have been recently discovered and clinical presentation of the patients was similar to the lpr phenotype in mice. These mutations lead to a non-malignant lymphoproliferative pathology. Somatic mutations of CD95 or its ligand in leukaemias or lymphomas have not been reported, but increased levels of soluble CD95 have been detected in patients with various lymphoid malignancies.

CD95 seems to play a role in several PCD pathways, possibly as kind of amplifier to potentiate the cell death triggered by the main inducer. Viral gene products such as gp120 and Tat can accelerate the autocrine death of T cells by upregulating the CD95 ligand (K-M. Debatin), and there is strong evidence that p53 can induce functional CD95 and some indication that it can also induce CD95L (presented by C. Choisy-Rosy, Villejuif, France), even though CD95 is not required for p53-mediated PCD induced by DNA damage. Chemotherapeutic agents (e.g. doxorubicin, methotrexate, cytosine arabinoside) appear to induce PCD in T cells by upregulating and activating CD95, with subsequent activation of caspases (K-M. Debatin). In these studies resistance to doxorubicin and anti-CD95 treatments were associated.

Three short talks continued the CD95 theme. Dr. Deena Gibbons (London) discussed the role of the TNF receptor family in regulating apoptosis in murine pre-B cells. She described how pre-B cells expressing TNF receptors, Fas or CD40 show enhanced apoptosis when exposed to their respective ligands. The increase in apoptosis was accompanied by a decrease in the expression of Bcl-2. Dr. Giovina Ruberti (Rome) talked about several naturally occurring soluble isoforms of Fas generated by alternative splicing of the primary Fas transcript. All these proteins were shown to block apoptosis induced by Fas ligand or the agonistic antibody, probably by forming inactive trimers with the primary membrane bound Fas. Dr. Hans-Uwe Simon (Zurich) discussed defective Fas mediated apoptosis in patients with eosinophilia. In a patient with idiopathic hypereosinophilic syndrome and in an HIV-1 infected individual with associated eosinophilia, high numbers of CD4- CD8- T cells in peripheral blood (PBL) were observed. T cells from these patients did not express functional Fas receptor, resulting in a defect in T-cell apoptosis. Dr. Simon also presented data indicating that Fas is involved in the regulation of eosinophil apoptosis, with possible relevance to allergic and asthmatic diseases.

Dr. Pierre Golstein (Marseille-Luminy) changed the subject from CD95 to the general question of the evolution of PCD from simpler unicellular organisms. Dr. Golstein discussed how *Dictyostelium discoideum* might provide a link between unicellular and multicellular organisms. They display both unicellular and multicellular forms in their life-cycle and proliferate as free-living amoeboid cells when conditions are favourable. When starved, the cells aggregate and develop into a fungus-like structure, consisting of a stalk and a fruiting body, which contains large numbers of viable spores. The stalk cells are dead, having died as a normal part of their differentiation program, and they display some features in common with apoptotic animal cells, but the mechanism of the cell death is unknown. Dr. Golstein's group is using a mutagenesis approach to search for *Dictyostelium* genes required for the death of cells under starvation conditions; mutants with such genes inactivated are expected to survive.

During the third day of the meeting, some talks focused on the Bcl-2 family of proteins. Dr. Robin Brown (London) is investigating cell death in another unicellular organism, the fission yeast *S. pombe*. Bak expression kills *S. pombe*, with the same phenotype as has been previously observed for Bax-killing in *S. pombe*, and BclX_L can protect against Bak-induced death. Although the phenotypes of these Bcl-2 family members in yeast parallels their functions in animal cells, however, it is not clear whether this phenotype is due to a conserved death programme shared with animal cells. Brown and colleagues are using this yeast system to search for cDNAs that can suppress Bak-lethality in *S. pombe* in an effort to discover novel proteins that functionally interact with the Bcl-2 family.

Dr. Brown also presented the results of two yeast two-hybrid screens. One, using the E1B-19k protein as bait, yielded two Bcl-2 family members, Bak and Bik. Bak is a killer protein that binds to BclX_L and is located mainly in the ER. Bik (=NBK) has the same interactions as Bak, but overexpression does not induce PCD although it accelerates PCD induced by Fas activation. Normal expression of Bik was found to be tissue-restricted. Another screen used the death-domain (DD) of TNF-R1 as bait and yielded another DD-containing protein, named weasel. Weasel (55 kD) looks similar to TNF-R1 and contains a DD homologous to TNF-R1 and TRADD, suggesting that it is

a receptor. It is expressed in hemopoietic tissues and overexpression induces NFκB activity and recruits TRADD, but its ligand is not known.

Dr. Robert Friis discussed PCD signalling during tissue remodelling in the mammary gland, prostate and ovarian corpus luteum in response to hormonal changes. His group is looking for genes that are induced during all three models by using a differential screening method in an attempt to pick up genes specific for apoptosis and excluding those involved in tissue remodelling. Of five genes identified, four encode extracellularly exposed products and are being studied further. One possibility is that some of these gene products are involved in the phagocytosis of dead or dying cells.

Dr. Giuseppe Pelicci (Milan) considered the regulation of survival by a fusion protein of PML and RARα, the product of a frequent translocation in acute promyelocytic leukaemia (APL). The most common reciprocal translocation (t15;17) generates a PML/RARα fusion protein which promotes the survival of hemopoietic precursor cells and blocks their terminal differentiation. The expression of this protein in nonhemopoietic cells, however, results in cell death. Less frequent translocations in APL involve the RARα gene on chromosome 17 and PLZF and NPM genes on chromosome 11 and 5, respectively. Wild type PML, PLZF and NPM exhibit growth suppressor activity in all cell types. Similar to PML/RARα, NPM/RARα and PLZF/RARα fusions induce apoptosis in cells of nonhemopoietic lineages. These studies provide an example of how cell survival can be affected in a lineage specific manner by chromosomal translocations in hemopoietic malignancies. Dr. Pelicci also showed data which suggest that PML can associate with Rb and E1A and colocalises in nuclear bodies. Expression of PML/RARα disrupts the localisation of PML to nuclear bodies.

Dr. Hugh Brady (London) described the phenotype of transgenic mice designed to express Bax in their T cells. T cells from these mice show accelerated apoptosis, consistent with the killer activity of this Bcl-2 family protein. Interestingly, Bax expression facilitated the re-entry of T cells into the cell cycle (tested using a paradigm in which the cells are first arrested by conA stimulation and withdrawal of IL-2 and then stimulated to re-enter the cell cycle by adding back IL-2). Not only did the Bax-transgenic T cells enter S-phase more rapidly (compared to cells from wild-type mice) in this paradigm, the cyclin-dependent kinase inhibitor p27 decreased faster after IL-2 was added. Transgenic mice that expressed Bcl-2 in their T cells had the opposite phenotype: S-phase entry was slowed and p27 did not drop as rapidly after the addition of IL-2. Other studies have reported similar effects of Bcl-2 on the cell cycle, but the mechanism for this is not known, and it is not clear whether this phenotype has any relationship to the ability of these proteins to modulate PCD.

Dr. Laszlo Fesus (Debrecen) discussed the role of tissue transglutaminase (tTg) in apoptosis. tTg is a calcium-dependent protein cross-linker that is induced and activated during PCD in a large number of *in vivo* systems, including *C. elegans*. Its function in PCD is not known, although it may be involved in maintaining the stable structure of apoptotic bodies. tTg can be induced by several apoptosis inducers that work via transcriptional activation, including dexamethasone, T-cell receptor activation, irradiation and retinoic acid (RA), but not by Fas, which does not require transcription. In order to investigate the function of tTg, Fesus and colleagues have labelled substrates of tTg in whole cells by using a DNP-labelled hapten. When labelled protein was extracted and analysed, a major component turned out to be actin, consistent with the cross-linking of abundant cytoskeletal proteins during those PCDs in which tTg is activated.

Dr. Pier Luigi Nicotera (Konstanz) examined the relationship between apoptosis and necrosis in neurons, arguing for a continuum between the two forms of cell death, with the switch between the two dependent on ATP levels. According to this hypothesis, apoptosis occurs by default, but if ATP levels fall too far necrosis takes over. A physiological example of both apoptosis and necrosis occurs during brain ischemia, in which an apoptotic penumbra surrounds the necrotic region of initial ischemic damage. Many of these deaths are mediated by excess glutamate release activating neuronal glutamate receptors of the NMDA type, which results in increased intracellular calcium levels and is followed by loss of mitochondrial function and a fall in ATP levels. In cultured neurons, these glutamate-induced changes are followed by the (necrotic) lysis of a subpopulation. Those that survive recover their ATP levels and mitochondrial function and subsequently undergo apoptosis. It is not entirely clear whether ATP levels are required for the execution of apoptosis or to maintain the homeostatic integrity of the cells and so avoid necrotic lysis.

Caspases are at the heart of the death programme in animal cells, and are required for PCD to occur in a number of systems. Several talks on the last day of the meeting focused on the role of these proteases in PCD and on the details of their mechanisms of action. Dr. Michael Jacobson (London) suggested that all nucleated animal cells have a caspase-based death programme and discussed the utility of caspase inhibitors as tools to probe the role of PCD in developmental or disease processes in whole tissues or animals, using examples from interdigital cell death, lens epithelial cell differentiation and neural tube closure. As there are multiple caspases (at least 11 in humans), an important task is to work out which ones become activated for each cell type and PCD inducer. Dr. Marion MacFarlane (Leicester) discussed evidence that multiple caspases are cleaved and activated during PCD. In THP.1 cells treated with etoposide, Cpp32, Mch3, Ich-1 and Mch2 were all found to be cleaved, and the caspase inhibitor zVAD-fmk inhibited these cleavages and PCD. It is not known, however, which specific caspases are required.

Another important task is to find all of the substrates of the various caspases and to determine the functional significance of these cleavages in the death programme. Dr. Sharad Kumar (Adelaide) discussed protein cleavage in cells undergoing apoptosis. One novel substrate was hnRNP-C, an RNA-binding protein. It is cleaved *in vivo* during apoptosis and *in vitro* by CPP32, Mch2 and Mch3 but not by ICE, Nedd2, Tx or granzymeB. zVAD-fmk blocks this cleavage in cells and DEVD-CHO but not YVAD-cmk is

effective *in vitro*, indicating that it is a CPP32 subfamily substrate. Another substrate is DNA-dependent protein kinase, which is cleaved by CPP32 but not by Mch2, Tx, ICE, and ICErel-III. These data add to the accumulating evidence indicating that there are a limited number of substrates with various functions in the nucleus, cytoplasm, cytoskeleton, and intracellular membranes. Presumably cleavage of these substrates together accounts for many of the changes in the cell as it undergoes PCD, many of which we do not understand or even know about. It remains a mystery, however, exactly how such substrate cleavages account for the most obvious morphological changes in apoptosis.

Cyril Broccardo from Dr. G. Chimini's group (Marseille-Luminy) discussed work on ABC1, a mammalian ATP-binding cassette transporter required for the engulfment of apoptotic corpses. This protein is homologous to the protein encoded by the *C. elegans* gene *ced-7*, which is also involved in the engulfment process. It is expressed in macrophages and functions as an anion transporter when expressed in oocytes. Blockade of its function, by injecting anti-ABC1 antibodies into macrophages, selectively prevents the phagocytosis of apoptotic bodies but not of other particles. Dr. Chimini's group is in the process of knocking out the gene in ES cells (which can be induced to differentiate into macrophages in culture) and in mice.

Dr. Marie-Lise Gougeon (Paris) explained why peripheral blood lymphocytes from HIV-infected individuals are more susceptible to apoptosis (they die more readily for example when simply cultured *in vitro*, or when treated with an apoptosis inducer such as Fas). The primary reason seems to be the chronic activation of the immune system throughout HIV infection, which results in chronically-stimulated T cells. For example, $CD8^+$ T cells from HIV-infected donors express markers corresponding to anergic end-stage CTLs; cells at this stage would normally be deleted by PCD. The high Fas expression in these cells helps explain their increased sensitivity to Fas activation. The immune activation idea is supported by a comparison between HIV-infected humans and chimpanzees: although chimpanzees can become chronically infected with HIV they do not develop AIDS. The difference is that HIV does not provoke the chronic immune response in chimpanzees that it does in humans.

How can our understanding of PCD be used to develop therapies, for example to prevent unwanted PCD? Dr. David Tomei (Richmond, California) discussed one strategy, the development of soluble extracellular molecules that act on cell-surface receptors involved in modulating apoptosis. In an *in vitro* screen for such molecules it was found that an extract from soy flour was particularly effective at inhibiting PCD. The active ingredient was a mixture of lysophospholipids. These needed to be bound to protein, suggesting a carrier function. An optimised formulation of lysophospholipids (Elirex™ and Lexirin™) blocked PCD in several culture and whole-organ systems. Applications include chemotherapy (to reduce the death of intestinal crypt cells) and as an organ preservative for use in transplantation. Clinical trials to test its effectiveness as a heart preservative look promising.

The substance of this conference reflected the current state of the field: in some areas recent discoveries are being consolidated and research is becoming more focused, as in the CD95 and caspase fields; in others there are more discoveries but major unsolved mysteries remain (consider the Bcl-2 family). A consensus seems to have emerged about the general nature of the death programme and the central importance of caspases. It is also apparent that the conceptual and practical advances of the last few years are beginning to be applied to the understanding and treatment of various diseases. We now keenly look forward to the next Euroconference on Apoptosis to be held in Germany in late 1997, to see what another year of research yields.