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Meeting Report

Death on the beach: a rosy forecast for the 21st century

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The Third European Workshop on Cell Death (***Death on the Beach***): Salobreña, Spain, 23–28 February 2002.

The Third European Workshop on Cell Death (*Death on the Beach*) was held in Salobreña, Spain, in February 2002. One hundred participants, mostly PhD students and young postdocs, presented novel unpublished data as 70 short talks and 30 posters. Ample time was allocated for discussions, and informal discussions among participants took place throughout the five days of the workshop. The major results and conclusions are summarized below.

TNF family members: biology and novel signaling

Members of the TNF family participate in a variety of biological processes including proliferation, differentiation and apoptosis. APRIL, a new member of the TNF ligand family involved in tumor cell proliferation, was the subject of two studies. Desiree Bonci (Rome, Italy) described the massive upregulation of APRIL during terminal megakaryocytic differentiation and its role as a growth factor for megakaryocytes. Marta López Fraga (Madrid, Spain) reported that APRIL is processed in the Golgi apparatus and, following release, can then act as a secreted factor. TRAIL is a TNF like ligand known to induce cell death primarily in tumor but not normal cells.[1] Raymond Daniels (Oxford, UK) generated antibodies against the various TRAIL receptors to investigate the distribution in normal and pathological tissues. He detected increased expression of TRAIL-R1 (DR4) and TRAIL-R2 (DR5) in malignant melanomas suggesting that these two receptors can confer an increased susceptibility to apoptosis. In support of this notion was the finding of Carmen Ruiz de Almodóvar (Granada, Spain) that breast tumor cells can be sensitized to TRAIL mediated apoptosis by exposure to the genotoxic reagent doxorubicin, that causes the upregulation of TRAIL-R1 and TRAIL-R2. Moreover, sensitivity to TRAIL-induced apoptosis can be regulated at the receptor level by a different mechanism, as indicated by Kristina Archer (Maryland, USA) who described that TRAIL- R2 can pre-associate to form heterocomplexes not only with TRAIL-R1 but also with the decoy receptor TRAIL-R4. Significantly, the TRAIL-R2-R4 pre-association was shown to inhibit apoptotic signaling induced by TRAIL.

The association of death receptors with their respective ligands leads to the formation of the death-inducing signaling complex (DISC). Adriana Eramo (Rome, Italy) described that upon ligation, CD95/Fas mainly localized in lipid rafts – caveolae-like glycosphingolipid- and cholesterol-rich membrane microdomains – where recruitment of DISC components and activation of caspase-8 seem to occur. The two splice variants of FLICE-inhibitory protein (c-FLIPl and c-FLIPs) can inhibit caspase-8 activation at the DISC although at a different level (Andreas Krueger, Heidelberg, Germany): while c-FLIPl allows the first step of pro-caspase-8 processing, leading to the generation of the p10 subunit, c-FLIPs completely prevents its cleavage. Martin Sprick (Heidelberg, Germany) observed that caspase-10 is also recruited to DISC complex and subsequently activated on T cells upon stimulation with TRAIL and CD95L, although its activation does not seem to be necessary for apoptosis. One novel death receptor association was described by Francesco Lozupone (Rome, Italy) who showed that CD95 co-localizes and immunoprecipitates with the ezrin/radixin/moesin (ERM) family member Ezrin, that links the actin cytoskeleton to the plasma membrane, only in CD95-sensitive cells. These data suggest that the CD95-cytoskeletal interaction may play a key role in the regulation of CD95-induced cell death.

TNF-like ligands cannot only induce biological responses by binding to their cognate receptors, but can themselves transmit signals via their cytoplasmic domains (reverse signaling). By employing a yeast-two-hybrid screen, Wiebke Baum (Frankfurt, Germany) identified a number of adapter molecules that associate with the cytoplasmic tail of CD95L. Among them she found the PSTPIP protein, a tyrosine-phosphorylated protein involved in the organization of the cytoskeleton.

From death stimulus to the mitochondria and beyond: regulation of cell death

After DISC formation, caspase-8 becomes activated. In some cell types the death signal is transmitted to mitochondria through the translocation of a proapoptotic Bcl-2 family member, Bid.[2] Arlette Werner (Amsterdam, the Netherlands), showed that Bfl-1/A1, an antiapoptotic Bcl-2 family member, binds the BH3 domain of cleaved Bid at the mitochondria and blocks tBid/Bax and tBid/Bak mediated mitochondrial changes. Other death stimuli can activate mitochondrial death pathways: interleukin-3 withdrawal induces activation of the Forkhead transcription factor FKHR-L1; Paul Coffer (Utrecht, the Netherlands) demonstrated an implication of Bim, a target of FKHR-L1, in apoptosis induced by this stimulus. Another signal that triggers cell death is loss of cell attachment, that unleashes Bmf from a cytoskeletal structure, thus allowing it to translocate and bind Bcl-2 homologues, as shown by Andreas Villunger (Melbourne, Australia).

Identification of the proteins released from mitochondria during apoptosis was the subject of Geert van Loo's presentation (Gent, Belgium), who employed a mass spectrometric approach to identify 16 proteins that are released from mitochondria after exposure to truncated Bid. Among them were Endonuclease G and the serine protease Omi/HtrA2 that binds to the caspase inhibitor XIAP. The mechanism of release of those proapoptotic proteins is a matter of contention at present and requires further study. Although Reactive Oxygen Species (ROS) have been suggested to mediate this phenomenon, Nigel Waterhouse (Melbourne, Australia) showed that this is not the case during genotoxic drug-induced apoptosis, as ROS scavengers did not prevent outer membrane permeabilization, while caspase inhibitors blocked the generation of ROS.

There is now increasing evidence of the lack of a single 'point of no return'. Instead, several control points have been detected after the apoptotic machinery is engaged. Inhibitors-of-apoptosis proteins (IAPs) may inhibit cell death even after cytochrome *c* has been released from mitochondria, and Smac can overcome this protection. This complicated interplay among IAPs, Smac and caspases was addressed by three participants. Marion MacFarlane (Leicester, UK) detected a rapid degradation of Smac following its release from the mitochondria, which was due to the ubiquitin-ligase activity of XIAP, as demonstrated by removing the RING finger domain of XIAP. Pascal Meier (London, UK) found that the *Drosophila* homolog of XIAP, DIAP1, can no longer inhibit apoptosis when the RING domain is mutated. John Silke, from Melbourne (Australia), generated a panel of XIAP mutants and found that individual mutants that interfere with the ability of endogenous XIAP's to bind Smac, Omi/HtrA2, caspase-3 or caspase-9, were all able to inhibit apoptotic death.

Other intracellular signals can modulate apoptotic cell death. PED is a death effector domain (DED) containing protein that inhibits death-receptor induced apoptosis when overexpressed. Gerolama Condorelli (Naples, Italy) showed that overexpression of this protein also reduces apoptosis following serum deprivation or cell stress, through increases in ERK1 and ERK2 phosphorylation and activity, coupled with reduced JNK and p38 activity. Jiang-Yan Yang (Lausanne, Switzerland) presented a novel model in which caspase-mediated cleavage of RasGAP, releases a proapoptotic and an antiapoptotic fragment. Further cleavage of the antiapoptotic fragment makes it a potentiator of apoptosis, suggesting that this protein functions as a sensor of caspase activity.

Sensitising tumor cells to apoptosis

A holy grail for cell death research is to trigger apoptosis specifically in tumor cells. Since tumor cells display a high rate of glucose uptake and glycolisis, the glycolytic pathway may be an important target for therapeutic intervention of tumor cells. Cristina Muñoz-Pinedo (Granada, Spain) demonstrated that inhibition of glucose metabolism enhanced apoptosis induced by TNF-α, CD95 agonistic antibody and TRAIL in several human tumor cell lines. Glucose deprivation enhanced the death receptor-triggered early activation of caspase-8. TRAIL may also have great potential in the treatment of human cancer, as it selectively kills tumor cells.[3] At the moment, it is not known what factors are critical for this sensitivity. Martin Leverkus (Würzburg, Germany) showed that proteasomal inhibitors sensitize human primary keratinocytes to TRAIL downstream of DISC formation and caspase-8 activation. Since XIAP levels are higher in primary compared to transformed keratinocytes and proteasome inhibition sensitized primary cells at the level of caspase-3 activation, he proposed that higher XIAP levels are responsible for the resistance. Although TRAIL is a promising anti-cancer therapy, about 60% of tumor cell lines are resistant to TRAIL. With the aim of identifying agents that sensitise tumor cells to TRAIL, Tom Ganten (Heidelberg, Germany) showed that the chemotherapeutic drug 5-fluorouracil (5-FU) rendered TRAIL-resistant hepatoma cell lines sensitive to TRAIL-induced apoptosis by down-regulating cFLIP and up-regulating TRAIL-R1 and TRAIL-R2. One protein that has been reported to induce apoptosis selectively in tumor cells is Apoptin, originally derived from the chicken anaemia virus (CAV). As shown by Jennifer Rohn (Leiden, the Netherlands), this protein binds to DNA and exerts its proapoptotic activity in the nucleus; however, *de novo* protein synthesis is not required for its killer function and so the possibility remains that Apoptin acts as a transcriptional repressor. On the other hand, tumor cells have developed mechanisms of resistance to apoptotic stimuli. Astrid A Ruefli (Melbourne, Australia) showed, using retrovirally transduced CEM cells over-expressing the ATP-dependent efflux pump and drug transporter P-glycoprotein (P-gp), that P-gp inhibits the processing and activation of caspase-8 at the DISC level, thereby inhibiting CD95-mediated apoptosis. Concetta Conticello (Catania, Italy) demonstrated that treatment of human prostate, breast and bladder tumor cells with the Th2 cytokine, IL-4, whose production is enhanced in some tumors, significantly reduced CD95L and drug-induced apoptosis by up-regulating cFLIP and Bcl-xL. Consistent with this mechanism of tumor resistance, Matilde Todaro (Palermo, Italy) found that while thyroid cancer cells expressed high levels of cFLIP and Bcl-xL and were protected from CD95-mediated apoptosis, the

neighbouring epithelial follicular cells over-expressed caspase-8 and caspase-3 and were susceptible to CD95-induced apoptosis. She also found that IL-4 and IL-10 were expressed in thyroid cancer cells but not in surrounding tissue, and that these two cytokines were responsible for the increased levels of cFLIP and Bcl-xL.

Several talks covered the biology and gene regulation of the p73, a homolog of the tumor suppressor p53. Tobias Grob (Bern, Switzerland) and Carine Maisse (Rome, Italy) found that p73 isoforms that lack the transactivation domain (DNp73) and transcriptionally active isoforms (Tap73) are transcribed from different promoters within the same gene. Since TAp73 and p53 regulate DNp73 transcription and DNp73, in turn, inhibits p53 and p73 function, they proposed that DNp73 can control a negative feedback loop that may be important for both the regulation of development and the tumor suppression.

Apoptosis in the nervous and immune systems

There is great controversy surrounding the role of nitric oxide (NO) in biological processes as it has been implicated in cell survival as well as in cytotoxicity. Huseyin Mehmet (London, UK) analysed the effects of different redox-related species of NO on the nerve growth factor (NGF)-dependent cell line PC12. Low concentrations of NO and NO^+, but not NO^-, inhibited NGF withdrawal-induced apoptosis while higher concentrations induced apoptosis. In contrast, NO^- was able to cause necrosis at high concentrations. This necrotic cell death changed to apoptosis when NO^- was oxidized to NO by addition of potassium ferricyanide. Other talks also focussed on cell death in the nervous system. Staurosporine (SSP) causes changes in actin-dependent cell morphology at apoptotic concentrations. Grisha Pirianov (London, UK), using mutants of the Ras family protein Rac1, demonstrated its involvement in the SSP induced reorganisation of actin in a neuroblastoma cell line. He also demonstrated that Jun N-terminal kinase (JNK) activity is not correlated with death or survival signaling through Rac. Jacqueline Beesley (London, UK) showed that myelin-deficient (MD) oligodendrocytes die via caspase-3, as well as calpain and caspase-12 activation, in response to the ER stress induced by the accumulation of mutant proteolipid protein (PLP).

Several presentations discussed the induction and regulation of apoptosis in the immune system. Glucocorticoid (GC) hormone produced in the intestine was shown to mediate the apoptosis of intraepithelial lymphocytes as discussed by Igor Cima (Bern, Switzerland). T cell activation positively regulated GC production although it antagonised GC-induced cell death, indicating an interplay between both events in the control of cell death of intestinal lymphocytes. Ann Zeuner (Rome, Italy) studied the protection by stem cell factor (SCF) of erythroid precursor cells from cytotoxic drug-induced apoptosis. This is important since chemotherapy-treated patients frequently develop anemia due to destruction of these erythrocytes and their precursors. She suggested that the up-regulation of Bcl-XL and Bcl-2 in response to SCF may be involved in SCF-mediated antiapoptotic effects on erythroblasts. The PKC inhibitor bisindolylmaleimide VIII (BIS) is known to block autoimmune diseases that are mediated by $CD4^+$ T cells. Christoph Wasem (Bern, Switzerland) found that BIS downregulates cFLIP in $CD8^+$ T cells and facilitates apoptosis of $CD8^+$ and $CD4^+$ T cells *in vitro*, indicating a role for PKC in the regulation of the immune response.

It has been proposed that expression of CD95L can confer immune privilege to organs such as the eye and testis.[4] Initial studies suggested that tumors may use a similar mechanism, known as tumor counterattack, to evade the immune system. In melanoma cells, CD95L has an intracellular localisation in melanosomes and it is eventually secreted extracellularly as CD95L-expressing microvesicles that can induce apoptosis in T cells (as reported by Veronica Huber, Milan, Italy). However, other studies have now shown that when tumors, transplants or transgenic organs express CD95L, they can be rapidly rejected in mice[5] as a consequence of the pro-inflammatory functions of CD95L. Katja Simon (Oxford, UK) presented a murine melanoma model in which the expression of CD95L breaks the immune system's tolerance to self-antigens. Importantly, tumor immunity was mediated by antibodies as it could be transferred by serum or the purified antibody fraction from protected mice. Finally, Frederik H Igney (Heidelberg, Germany) used a model of CD95L expressed under an inducible promoter in a transplantable tumor cell line, to demonstrate that rejection of CD95L-expressing tumors does not depend on the level and the kinetics of ligand expression.

Caspase and non-caspase proteases

Members of the caspase family of cysteine proteases play a critical role not only in apoptotic pathways but also in inflammatory signaling. Mohamed Lamkanfi (Ghent, Belgium) reviewed our current knowledge of caspases and demonstrated by phylogenetic analysis that caspase function is reflected in its gene sequence. Indeed, inflammatory caspases and apoptotic caspases contain different CARD domains. Fabio Martinon (Epalinges, Switzerland) described a new signaling complex, the inflammosome, which is involved in activation of inflammatory caspases. It is formed by NALP1 (a pyrin domain-containing protein that shares structural homology with Apaf-1), caspase-1 and caspase-5. NALP1 recruits these two inflammatory caspases and subsequently triggers their activation.

In addition to caspases, other proteases have also been demonstrated to play an important role in programmed cell death (PCD). Maria Hoyer-Hansen (Copenhagen, Denmark) described how, in MCF-7 cells, vitamin D compounds activate the Ca^{2+}-dependent cysteine protease-calpain and induce the formation of large Bax clusters. Different calpain inhibitors attenuated vitamin D-induced cell death. Cathepsin B is a lysosomal protease that displays an increased expression in primary tumors and was the subject of Nicole Fehrenbacher's presentation (Copenhagen, Denmark). Working with primary and transformed murine embryonic fibroblasts (MEFs) she demonstrated that TNF-induced cell death becomes cathepsin-B dependent upon immortalisa-

tion. Related to this was the talk of Marja Jäätelä (Copenhagen, Denmark), who described that depletion of the heat shock protein 70 (Hsp70) induces apoptosis-like death in tumor cells and exerts an anti-tumor activity in tumor-bearing mice. This type of death was associated with an increase in cytosolic cathepsin expression and activity and a decrease in the level of the serine threonine kinase AKT (PKB).

Tumors have also developed different ways to escape apoptosis induced by perforin/granzyme B. Michael Bots (Leiden, The Netherlands) found that the serine protease inhibitor, PI-9/SPI-6, which is expressed in CTLs and dendritic cells, forms a complex with and neutralizes the activity of granzyme B in tumor cells, thus inhibiting the downstream activation of Bid, caspase-8 and effector caspases. However, SPI-6 cannot protect cells against perforin-dependent CTL-induced membrane disruption. Ingrid Kolfschoten (Leiden, The Netherlands) showed that the colon carcinoma cell line CMT93 is resistant to CTL-induced lysis. She described a close homolog to SPI-6, named SPICI, which is expressed not only in tumor cells but also in immune-privilege sites, possibly conferring resistance to perforin activity.

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