Meeting Report

Challenging the dogmas . . .

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Abbreviations: Ψ m, mitochondrial membrane potential; cPLA2, cytosolic phospholipase 2; HSP, heat shock protein; ICAD, inhibitor of caspase-activated DNase; NS, nuclear scaffold protease; ROS, reactive oxygen species

The Sixth Euroconference on apoptosis was held in September 1998 in Saltsjö-Boo, a beautiful suburb of Stockholm. Despite unusually nice weather, the lectures were well-attended, thanks to the interesting program and outstanding team of speakers. It has been a tradition of the Euroconferences to focus each meeting on one aspect of apoptosis. This time participants' attention was directed towards the role of different proteolytic systems in the apoptotic process, including caspases, calpains and proteasomes. There was discussion of targets for proteases, the role of mitochondrial and non-mitochondrial signals in protease activation and association of various proteolytic activities with different diseases. In this report we have summarized some highlights of the meeting.

In an elegant and stimulating introductory lecture, Dr. Andrew Wyllie (University of Edinburgh, Edinburgh), who could perhaps be considered the instigator of the past three decades' research on apoptosis by numerous laboratories, discussed the role of mutation in subverted apoptotic cells following therapy. It is well known that upon treatment with ionizing radiation, UV-light or methylating agents, the resulting DNA damage can be repaired, or can signal cell cycle arrest or apoptosis. The factors which determine the route taken are poorly understood. Dr. Wyllie described two phases of apoptosis following injury to DNA. The first is initiated relatively early after DNA damage, is dependent on p53 and (very likely) transactivation of Bax. This pathway is very efficient in the removal of mutant cells. The second one is p53-independent, appears relatively late after damage and is less efficient. Deficiency in the second pathway might lead to high mutation frequency in radiation-treated cells. These data have considerable relevance to the outcome of tumor therapy with DNA-damaging agents.

Caspases

A detailed review of caspase function and modulation was given by Dr. Donald W. Nicholson (Merck Frosst Center for Therapeutic Research, Montreal). He described the crucial role for this family of proteases in apoptosis and suggested

that multiple pathways of caspase activation, their different subcellular localizations and regulatory control by macromolecular inhibitors determine the circumstances under which apoptosis proceeds.

Dr. Nicholson described some of the work by his group on caspase-3. It has recently been shown that pro-caspase-3, in addition to being present in the cytoplasm, localizes to the mitochondria. In this compartment pro-caspase-3 is catalytically competent and can be activated by autoproteolysis, although the precise mechanism of its intramitochondrial activation is unclear. Dr. Nicholson described the isolation of a complex containing pro-caspase-3, pro-caspase-6 and heat shock protein 60 (HSP60) from whole cell extracts. *In vitro* activation of pro-caspase-3 was markedly enhanced in the presence of recombinant HSP60, which suggests a novel function for this chaperone molecule. Dr. Afshin Samali (Karolinska Institutet, Stockholm) reported on the presence of an *intramitochondrial* complex containing pro-caspase-3, HSP60 and HSP10. At an early stage in apoptosis this complex dissociates and HSP60 and HSP10 are released from mitochondria concomitantly with cytochrome *c*. It was proposed that, given the known chaperone function of HSPs, changes in the conformation of proteins induced by HSPs might influence the activation process.

Considering the central role different caspases play in the apoptotic process, relatively little is known about their intracellular localization in normal and apoptotic cells. This is of interest with regard to the different subcellular localizations of the various proteins that are cleaved by caspases. These aspects were discussed in two lectures. Dr. Sharad Kumar (Hanson Center for Cancer Research, Adelaide) presented interesting data showing that both the precursor and processed forms of caspase-2 localize to the cytoplasmic and the nuclear compartments. Moreover, the nuclear localization of caspase-2 is strictly dependent on the presence of the prodomain. An amino-terminal fusion of the prodomain of caspase-2 to caspase-3 mediates nuclear transport of caspase-3, which is normally only found in the cytoplasm and mitochondria. These results suggest that, besides its roles in dimerization and recruitment through adaptors, the caspase-2 prodomain has a novel function in nuclear transport. Dr. Boris Zhivotovsky (Karolinska Institutet, Stockholm) showed that pro-caspase-2, in addition to being present in the cytoplasm and nuclei, also localizes to the

mitochondria. However, upon induction of apoptosis, active caspase-2 is found mainly in the nucleus. Regarding other caspases, Dr. Zhivotovsky also reported that mitochondrial pro-caspase-3 can potentially be activated within the mitochondria. Although, in addition to pro-caspase-3 there are at least two other pro-caspases (pro-caspase-2 and -9) found in mitochondria of normal cells, their role in the activation of mitochondrial pro-caspase-3 is unclear. It is also unclear whether active caspase-3 acts directly on mitochondrial proteins or is exported to target extra-mitochondrial proteins. One potential substrate for mitochondrial caspase-3 is Bcl-2, which is located on the outer mitochondrial membrane and is cleaved during apoptosis. The main conclusion from these presentations is that the localization of pro-caspases to different subcellular compartments may play an important, though not yet completely clear, role in their activation, substrate cleavage pattern and thus, in development of the apoptotic process.

It is very likely that all components required for caspase activation are present in normal cells but are either sequestered or blocked by specific inhibitors. One such inhibitor, Usurpin, was recently identified by Dr. Nicholson's group and independently by seven (!) other groups. Usurpin heterodimerizes with pro-caspase-8 and was found to prevent pro-caspase-8 recruitment by the FADD/MORT1 adaptor protein. Cell death induced by CD95 ligation was attenuated in cells transfected with Usurpin. Following ischemia/reperfusion injury in cardiac tissue, Dr. Nicholson reported that a Usurpin deficit in TUNEL-positive myocytes was observed in the infarct area, which had prominent active caspase-3 expression. In contrast, abundant Usurpin expression (and a caspase-3 deficit) occurred in the surrounding unaffected cardiac tissue, suggesting reciprocal regulation of these pro- and anti-apoptotic molecules.

The fact that inappropriate apoptosis is prominent in several human diseases suggests that therapeutic approaches which modulate caspases may have promising clinical potential. Dr. John H. Silke (The Walter and Eliza Hall Institute, Melbourne) described the design of a series of CrmA pseudo-substrate variants. These substrates effectively protect *S. pombe* from death induced by overexpression of caspase-3. Dr. Silke suggested that some of these inhibitors could be used in the endeavour to protect transgenic mice from apoptosis following ischemic shock.

In several presentations targets for caspases were discussed. One substrate is ICAD (Inhibitor of Caspase-Activated DNAse) described by Dr. Shigekazu Nagata (Osaka University Medical School, Osaka). ICAD is present in cells in a complex with CAD. Both proteins were purified from mouse lymphoid cells and their respective cDNAs were isolated. Mouse ICAD is homologous to human DFF45 and contains two recognition sites for caspase-3. In apoptotic cells, ICAD is cleaved by active caspase-3 and CAD is released from the complex to permit oligonucleosomal fragmentation of chromosomal DNA. Overexpression of wild-type ICAD or a non-cleavable ICAD mutant was found to prevent DNA degradation during apoptosis. However, the cells were still killed,

suggesting that DNA degradation itself is not a prerequisite for apoptosis.

Another caspase substrate discussed was cytosolic phospholipase A2 (Dr. Marja Jäättelä, Danish Cancer Center, Copenhagen). This protein is cleaved by caspase-3 and mutation in the cleavage site protects it from cleavage *in vivo* and *in vitro*. Expression of cPLA2, particularly the caspase-3 cleavage fragment, kills cells and was shown to sensitize cells to TNF-induced death. Dr. Jäättelä also reported that overexpression of HSP70 inhibits downstream events in apoptotic cascade. The precise site(s) and mechanism(s) of HSP70 protection are unclear although cleavage of cPLA2 and other substrates in TNF-treated cells was not affected.

A new look at the Bcl-2 family: a link to caspase activation

As previously mentioned, caspases can regulate the function of the anti-apoptotic protein Bcl-2. Using the Sindbis virus genome as a vector to express Bcl-2 family members, Dr. J. Marie Hardwick (Johns Hopkins School of Hygiene and Public Health, Baltimore) has been investigating the molecular mechanisms by which Bcl-2 family proteins and caspases modulate each other's activities in the cell death pathway and how these mechanisms alter viral pathogenesis. Bcl-2 and/or Bcl-x_L are cleaved by caspases during cell death induced by a variety of agents. The cleavage sites are located in the loop domain but the position of these cleavage sites within the loop is not conserved between Bcl-2 and Bcl-x_L, consistent with the lack of sequence similarity between their loop domains. Overexpression of N-terminal deletion mutants of both proteins leads to induction of apoptosis, suggesting that cleavage of these proteins unleashes a latent pro-death activity. Pro-apoptotic activity induced by N-terminal truncation of Bcl-2 and Bcl-x_L can be inhibited by p35, suggesting that the pro-death activity of cleaved proteins lies upstream of amplification of the caspase cascade. Furthermore, the function of Bax may not merely be to promote cell death. In fact, overexpression of Bax in the brain (by neurotrophic Sindbis virus with a Bax insert) protects neurons from virus-induced death *in vivo*. Hippocampal neurons cultured from these animals exhibited increased survival, in contrast to cultured dorsal root ganglion neurons which underwent increased death. This suggests that there are additional factors which determine whether Bax is a killer or a survival protein.

The model whereby Bcl-2 and Bax function by heterodimerization and mutual neutralization was shown to be a simplification of the truth in an excellent lecture given by Dr. Christoph Borner (University of Fribourg, Fribourg). These proteins also have additional anti-/pro-apoptotic functions, respectively, independent of each other. His group has also been interested in whether the subcellular localization of these two proteins affected their function. They generated Bcl-2/Bax chimera containing interchanged BH domains. The results described support the idea that the death protective actions of Bcl-2 are not restricted to the mitochondria, but may also be partly due to its additional localization at the endoplasmic reticulum and

nuclear membrane. Bcl-2 targeted to the ER by a specific targeting sequence was still as death-protective as a Bcl-2 targeted to mitochondria. In fact, the best protection was achieved by wild-type Bcl-2 that associates with both mitochondrial and ER/nuclear membranes when over-expressed. Preliminary data indicate that Bcl-2 may slow down the transport of proteins into these organelles. While all the pro-apoptotic chimera exhibited a punctuated mitochondrial staining reminiscent of damaged, clustered mitochondria, the neutral and anti-apoptotic chimera exhibited a reticular ER/nuclear membrane association. Another model of Bcl-2 action involves its interaction with other molecules which are then attracted to the sites where Bcl-2 normally resides. Of the long list of these molecules including, Raf, calcineurin, BAG-1, R- and H-Ras, the strongest association was with Bax, suggesting that the subcellular localization of Bcl-2 and of Bax affects the localization of the other and thus its function.

Other proteases

In addition to caspases, other proteolytic activities have been implicated in apoptosis. Dr. George Kass (University of Surrey, Guildford) presented a comprehensive overview of current knowledge concerning calpains in apoptosis. David H. Burgess (Karolinska Institutet, Stockholm) reported the interesting observation that μ -calpain can induce cleavage of pro-caspase-3 to a 29 kD peptide. This cleavage results in the inhibition of cytochrome *c*-mediated activation of type-II caspases via caspase-9 *in vitro*. These results suggest that under certain conditions μ -calpain could act as a regulator of pro-caspase activation. Alteration of such regulation may result in apoptosis.

The role of lysosomes in cell death was discussed by Dr. Ulf T. Brunk (Linköping University, Linköping). Lysosomes are the principle site of intracellular digestion, containing many degradative enzymes. Dr. Brunk reported that lysosomal rupture leads to decompartmentalization of lysosomal enzymes, which might initiate/promote cell death. Although the role of lysosomes in necrosis is well documented their exact function in the apoptotic process remains to be elucidated.

Apoptosis can also be regulated by activation of serine protease(s) and the proteasome (Dr. David McConkey, M.D. Anderson Cancer Center, Houston). The nuclear scaffold protease (NS) is a serine protease located in the nuclear scaffold, which can cleave lamins. Microsequencing analysis has revealed that NS and the proteasome might be structurally and functionally related. Moreover, inhibitors of the proteasome also prevent thymocyte apoptosis, consistent with a role for one or both of these proteases in the response. Peptide-based inhibitors of these protease activities blocked the release of cytochrome *c* from mitochondria as well as several downstream events. However, disparate effects on glucocorticoid-induced exposure of phosphatidylserine (PS) were observed. Proteasome inhibitors largely prevented PS externalization, whereas the NS inhibitor promoted PS exposure in the absence of an apoptotic stimulus, an effect that was associated with disruption of mitochondrial membrane

potential (Ψ m). In fact, Dr. Hannes Drexler (Max Plank Institute of Physiology, Bad Nauheim) investigated the induction of apoptosis by inhibitors of proteasomal function in several experimental systems. He suggested that some proteasome inhibitors might be useful as a novel class of therapeutic anti-proliferative agents. Taken together, these data suggest that non-caspase proteases also play an important role in the regulation of apoptosis. However, precisely where these proteases act within the apoptotic pathway and how they interact with other components of the pathway is unclear.

Caspase-independent cell death

An interesting discussion concerning the biochemical definition of apoptosis was initiated by Dr. Sten Orrenius (Karolinska Institutet, Stockholm). A number of researchers alluded to 'caspase-independent apoptosis', describing cells whose demise was morphologically indistinguishable from 'classic apoptosis' but did not appear to contain detectable caspase activity. However, experimental data has provided evidence that activation of the caspase cascade is a common biochemical pathway that explains both the morphological features and the irreversible commitment points in apoptotic cell death. Dr. Pierluigi Nicotera (University of Konstanz, Konstanz) emphasized that the absence of activation of only one class of caspases (i.e., DEVDases) should not lead to the general conclusion of an apoptotic mechanism without caspase activity. Activity of other classes of caspases should be investigated together with detailed analysis of cellular morphology. In fact, Dr. Nagata described the caspase-independent death of a Jurkat clone resistant to CD95 treatment (due to a lack of caspase-8). These cells can be killed by oligomerization of FADD, where no activation of caspase-8 or other downstream caspases was observed. The morphology of these cells was more related to necrosis rather than apoptosis (in that the cells and mitochondria swell). Dr. Borner also referred to caspase-independent cell death that still occurred when execution caspases were blocked by various specific caspase inhibitors. Dr. Bengt Fadeel (Karolinska Institutet, Stockholm) demonstrated involvement of caspases in constitutive and CD95-induced apoptosis in neutrophils, but not in activation-induced death of these cells. Moreover, Dr. Stein-Ove Døskeland (University of Bergen, Bergen) presented evidence for the presence of several distinct forms of death in a single cell type. It will be interesting to see whether we are on the verge of a further refinement of the modes of cell death.

Cytochrome *c* release

The production of reactive oxygen species (ROS) is a common phenomenon in many cells undergoing apoptosis; however, whether this is a cause or effect has, until now, never been clarified. Dr. Dean P. Jones (Emory University, Atlanta) reported on the ability of cells, which lack a functional respiratory chain (ρ^0 cells), to release mitochondrial cytochrome *c* in response to apoptotic stimuli. This occurs without the redox change that ordinarily accompanies the induction of apoptosis in cells with normally respiring

mitochondria. Thus, superoxide and other ROS produced during apoptosis originate from the disrupted respiratory chain from which cytochrome *c* has been lost. Dr. Jones further suggested that redox signaling may function in apoptosis by amplification of mitochondrial permeability transition, marking proteins/organelles/apoptotic bodies for phagocytosis and/or inactivation of caspases to terminate the apoptotic program.

In a later lecture Dr. Caroline Dive (University of Manchester, Manchester) certainly challenged dogma with her data demonstrating that two of the potential commitment events in apoptosis, namely, the reduction in Ψ_m and release of cytochrome *c* are reversible events. To show this an IL-3-dependent cell line was transfected with temperature-sensitive *v-abl*. Clonogenicity could be fully restored by activation of *v-abl* tyrosine kinase activity up to 18 h after the reduction in Ψ_m . These tantalizing data, if reproduced in other systems, should compel us to reconsider currently accepted commitment points in the induction of apoptosis. This brings us back to caspases – does their activation represent a more infallible indication of commitment to apoptosis?

Dr. Tom G. Cotter (University of Cork, Cork) discussed the connection between oxidative stress, mitochondria and apoptosis in photoreceptor cell death. Dr. Christoph Richter (Swiss Federal Institute of Technology, Zurich) presented evidence for a link between mitochondria and ceramide-induced apoptosis. He showed that ceramides (which are elevated early in TNF-mediated killing) have varying affinities for cytochrome *c* and can induce its release from mitochondria in a Bcl-2-inhibitable manner. It was noted that ceramides are more effective in causing cytochrome *c* release when the protein is oxidized, suggesting that oxidative stress is not merely a consequence of the loss of cytochrome *c* from mitochondria. Perhaps this is a specific amplification loop employed during instances of apoptosis that involve production of ceramide.

Clinical aspects of apoptosis

Dr. Peter H. Krammer (German Cancer Research Center, Heidelberg) gave an excellent update on current research concerning CD95-induced apoptosis. He described different CD95-signaling pathways in Type I and Type II cells in which DISC formation does or does not occur, respectively. This was illustrated by activated T cells, which at day 1 are resistant to CD95 (and do not form a DISC complex despite the presence of all the components) while at day 6 DISC formation occurs and the cells are sensitive to CD95. Elevated Bcl-x_L levels were observed at day 1 although this does not fully explain the resistance. In addition, Dr. Krammer demonstrated a role for mitochondria in amplification of CD95-induced cell death, due to the ability of caspase-8 to cleave the Bcl-2 family member Bid and hence facilitate cytochrome *c* release.

At a previous Euroconference (Bingen, Germany, 1997) exciting new data was presented showing that the CD95 system could be upregulated by anticancer drugs, in particular CD95 ligand expression. At this year's meeting Dr. Claudia Friesen from K.-M. Debatin's group (German Cancer Research Center, Heidelberg) showed further

evidence in support of ROS regulation of CD95 ligand expression describing inhibition of this phenomenon by increasing the intracellular concentration of glutathione. Furthermore, the CD95 ligand gene contains binding sites for AP1 and NF κ B (two redox-modulated transcription factors). Interestingly, upregulation of CD95 ligand expression was reported to be acutely cell- and drug-specific (Dr. M. Ruiz Ruiz, Institute of Parasitology and Biomedicine, Granada; Dr. Ivan Uray, University Medical School, Debrecen). Moreover, CD95-blocking antibodies are not universally protective against drugs that upregulate the CD95 system. In addition to CD95 and CD95 ligand, the adapter molecule FADD was also reported to be upregulated by an anticancer drug, namely cisplatin (Dr. O. Micheau, Faculty of Medicine, Dijon). Upregulation of CD95 ligand is also observed in many tumor cells which enables them to kill activated T cells ensuring they are protected from killing by immune cells (Dr. Krammer). A new member of the CD95 ligand family, APRIL, was described by Dr. M. Hahne (BIL Research Center, Lausanne) to induce proliferation in a variety of cell lines, thus emphasizing the important role that members of this family may play in tumor biology.

Continuing the theme of anticancer drugs, the mechanism of action of mistletoe lectins (which are commonly used in adjuvant chemotherapy) was described by Dr. Heike Bantel (University of Tübingen, Tübingen). It involves triggering of mitochondrial cytochrome *c* release and potentiation of anticancer drug-induced apoptosis. Dr. Raymond E. Meyn (University of Texas, Houston) found that in etoposide-treated small cell lung cancer cells Bcl-2 expression correlates with apoptosis inhibition but not clonogenic survival. Whereas the Bcl-2 protein appears to be fully functional in these cells, their resistance to treatment with chemotherapeutic drugs may be mediated by other mechanisms. Relationships between the development of resistance to another anti-tumor drug, cisplatin, susceptibility to drug-induced apoptosis and function of the p53 transduction pathway in cisplatin-resistant variants were discussed by Dr. Evelyn Segal-Bendirdjian (Institute Gustave-Roussy, Villejuif). The data showed that resistance to cisplatin and cross-resistance to other DNA-damaging agents correlates with a defect in apoptosis. In addition, it was suggested that in response to treatment with drugs having different targets, the apoptotic cell death may operate through distinct signaling pathways involving different protease activities.

While anti-cancer treatment focuses on therapies to induce cell death, the opposite is the case with HIV infection. Dr. Marie-Lise Gougeon (Pasteur Institute, Paris) presented a thorough update concerning T cell death during HIV infection and its modulation by current anti-retroviral therapies. In AIDS patients mostly non-infected T lymphocytes undergo apoptosis leading to impoverishment of the pool of effector and memory cells. This is due to upregulation of the CD95 pathway by HIV viral proteins, gp120 and tat. Activated T cells (in HIV) are highly sensitive to apoptosis and can be rescued by IL-2. In fact IL-2 levels are found to be decreased in HIV infected patients. New anti-retroviral combination therapies, which

target both the viral protease and reverse transcriptase, are effective in suppressing viral load to undetectable levels, in elevating IL-2 production and increasing CD4⁺ T cell levels. However this does not represent a recovery of naive T cells and the long term survival of the patients is not known.

Excitotoxicity (which is probably the main cause of neuronal death during stroke) was the subject of Dr. Pierluigi Nicotera's lecture, with particular focus on the participation of the mitochondria. Mild stress to mitochondria, for example as a result of exposure to low levels of nitric oxide (which can target complexes I, III and IV of the respiratory chain) or MPP⁺ (which inhibits complex I) results in deregulated ATP homeostasis. One consequence of this in cerebellar granule cells (CGCs) is persistent glutamate release leading to excitotoxicity. Neuronal cells which do not express NMDA receptors (CGCs in culture for 2 days) are relatively insensitive to the toxicity of nitric oxide compared with CGCs that have been cultured for at least 7 days and express NMDA receptors. The importance of intracellular energy levels in the decision of a cell to die by apoptosis or necrosis was also underscored by Dr. Nicotera who showed that ATP levels affect the cell death pathway triggered by staurosporine or CD95 treatment of Jurkat lymphocytes. Up to 1.5 h after induction of cell death the process can be switched between apoptosis and necrosis by adjusting intracellular ATP levels as appropriate.

Another mechanism of blockage of apoptosis was described by Dr. Gerry Melino (University of Rome 'Tor Vergata', Rome). He discussed the possibility of S-nitrosylation of different components of the apoptotic machinery, such as AP-1, several members of the transglutaminase family of enzymes and also caspases. Interestingly, at least seven members of caspase family of proteases are susceptible to reversible inhibition by nitric oxide. Thus, in addition to other proposed mechanisms of apoptosis inhibition, such as the alterations in ATP level, Dr. Melino suggested that nitric oxide can regulate cell death process by direct interaction with the apoptotic machinery and could also induce switch between apoptosis and necrosis. These mechanisms of inhibition of cell death might play an important role in several pathologies.

Conclusion

Life (and death, or at least the mechanisms thereof) seems to have been much simpler in the early years when our level of knowledge was that Bcl-2 inhibited and Bax promoted apoptosis. One of the most notable themes running through much of the research presented at this meeting was that the complexity of apoptosis and the variations between different cell types and inducers ensure that many more years of research are required to fully elucidate the many pathways and regulators.