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Meeting Report

The European Cell Death Group

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Fifty European scientists specifically interested in death/life decision making met in late spring, at Villa Vigoni. This workshop on 'Cell Death' was held under the auspices of the Deutsche Forschungsgemeinschaft and the journal 'Cell Death and Differentiation', supported by a research grant from the European Commission (QLG1-1999-00739), coordinated by Guido Kroemer, and another grant from MURST (Cofin 1998), coordinated by Enrico Garaci.

Death Receptors and Immunity

A hot topic of the workshop was the session on Death Receptors and Immunity, with a specific focus on tumour-immunity. For several decades immunologists have been trying to detect specific molecular alterations that would distinguish tumour cells from normal cells, with the ultimate goal to create therapies tailored to the biochemical properties of an individual patient's tumour. That apoptosis is the fashion in cancer research is also shown, for example, by the enormous number of publications in this field. This discussion opened with the recent developments on death receptors, and it was followed by the latest data on its implications in the immune system with its relationship to cancer.

Peter H Krammer (Heidelberg) showed that during an immune response the activation of T cells results in a transient increase in cell numbers linked to the emergence of apoptosis-resistant cells. Following this, the number of T lymphocytes gradually declines, through an enhanced sensitivity to apoptosis. Interleukin-2 is a co-regulator of this phenomenon via the PI(3)K–Akt survival pathway, and its consequent induction of Bcl-2 in the proliferation phase.[1] In the death phase, JAK1-STAT5 upregulate CD95L. Costimulation of T cells (TCR and CD28) prevented cell death through the expression of cFLIP and Bcl-xL and the inhibition of CD95L. A clinical example of dysregulated costimulation occurs in sepsis. Finally, he outlined the role of the CD95 system in tumour counter-attack.

In view of a clinical use of TRAIL for the treatment of cancer, Henning Walczak (Heidelberg), who co-discovered TRAIL, showed that the TRAIL trimer kills 50% of human tumour cell lines, but does not affect the majority of normal human tissues.[2] He elucidated the molecular events of TRAIL-induced apoptosis, which similarly to CD95 involves the formation of a death-induced signalling complex (DISC) and the activation of caspases 8 and 3. Interestingly, TRAIL also induced apoptosis in chemoresistant tumours with high bcl-2 and bcl-x_L levels, suggesting that mitochondria have little impact on this type of cell death.

David Wallach (Rohovot) expanded the discussion on Death Receptors to p55 TNF-R and DR3 (or WSL-1), and their signalling, discussing the role of caspase 8 in CD95, DR3 and TNF signal transduction. He explained the rationale of conditional knockouts for both caspase 8 and FADD and the use of chimeric KO/wt mice. By the 2 hybrid method he was able to identify new interacting proteins for caspases, such as UCB9 and TIP60. The latter one inhibits apoptosis (CD95) in the full length form, but can be cleaved by caspases 3 or 8. TIP60 shows histone acetyltransferase (HAT) activity, binds the proto-oncoprotein Bcl-3, a member of the IkappaB family and forms quaternary complexes with Bcl-3 and NF-κB p50. It also binds to the α-chain of the IL9 receptor. TIP60 can be both anti-apoptotic (the case of full length protein) and pro-apoptotic (the case of the cleaved protein). Full length TIP60 protects from apoptosis downstream of caspase 8, and amplifies the NF-κB-mediated inhibition of death. On the contrary, caspase 8-cleaved TIP60 is unable to block CD95-death, and by interfering with the TNF/NF-κB survival signal, facilitates CD95-death.

Moving into *in vivo* settings, Carlo Riccardi (Perugia) discussed the role of apoptosis in the thymus, and in particular two genes, GILTZ and GITR induced by glucocorticoids. Subsequently, Antonio Mastino (Messina) discussed the role of BHV4 virus in controlling cell death.

A peculiar form of cell death occurs in erythropoiesis with the formation of proerythroblasts and mature erythrocytes, where the nucleus is eliminated. Ruggero De Maria (Roma) discussed the regulation of this process and demonstrated that proerythroblasts express death receptors (CD95, TNF-R1, TRAIL-R1 and -R2). CD95L and TRAIL produced by mature erythroblasts induced the reversible arrest of erythroblast expansion. The mechanism requires CD95 and involves the cleavage of the transcription factor GATA-1, mediated by caspases 3, 7 and 8. The inhibition of the cleavage of GATA-1 caused survival of erythroid progenitors, and their differentiation independent of erythropoietin. TAL1/SCL, and its heterodimeric partner E47, are also involved.

DNA damage and the p53/p73 family

Andrew H Wyllie (Cambridge) introduced the concept of DNA damage and the role of p53 in controlling both G1 arrest, to allow repair, and apoptosis.[3] To this end, he illustrated the role of APC (Adenomatous Polyposis Coli) in colorectal cancer and the involvement of the mismatch repair genes MSH, MLH, PMS-1, PMS-2. ATM belongs to the PI(3)K-related kinases together with DNA-pKs and ATR. It is activated by the double-strand breaks elicited by DNA damaging agents, and regulates checkpoints and repair proteins, including p53, predominantly by phosphorylation. Wyllie described the potential role of large nuclear macromolecular complexes in controlling DNA damage responses, as well as their mutations in cancer development. New technologies such as spectral karyotyping, allowing for the detection of chromosomal aberrations, will soon be applied to clinical samples.

Gerry Melino (Rome) expanded the discussion of p53 to its family members p73 and p63.[4,5] p73 is clearly able to induce apoptosis upon DNA damage. It requires the expression of the mismatch gene MLH1, and a physical interaction with c-Abl. This interaction is still not clarified since in different models it requires a phosphorylation in Tyr-99 or a protein stabilisation. The expression of different p73 splicing isoforms in cancer samples where presented by Urban Novak and Jean-Francois Cajot (Bern), while Ada Sacchi and Giovanni Blandino (Rome) demonstrated that mutant p53 can interact with and affect the function of p53, expanding the complexity of this gene family.

The biochemical aspects of the nuclear events in apoptosis, and their relation to DNA damage, were introduced by Vincenzo Giancotti (Trieste) who described his work on HMG proteins.

Death Effectors: activation and regulation

Several proteins are released from mitochondria during apoptosis, including Cytochrome *c*, Smac/DIABLO, and AIF.[6] The role of mitochondria as an integration point of apoptotic signals originated from the membrane, nucleus, and cytosol was discussed by Guido Kroemer (Villejuif).[7] AIF is a caspase-independent death effector which translocates from mitochondria to the nucleus. Once in the nucleus, AIF causes 50 kb DNA fragmentation and initial chromatin condensation. A more pronounced stage of chromatin condensation, as well as oligonucleosomal DNA fragmentation, requires caspase activated DNase (CAD). Surprisingly, mitochondia release AIF before Cytochrome *c*, at least in some models, and AIF neutralisation can prevent subsequent Cytochrome *c* release and consequent caspase activation.[8]

Francesco Cecconi (Goettingen) demonstrated, through a gene trap approach, that Apaf-1 is required for apoptosis to occur in some cell types. He presented recent data on the involvement of apoptosis in the development of the eye and the inner ear.

Chemotherapy agents eliciting DNA damage induce the formation of free radicals and upregulation of CD95L on the cell surface, through JNK/SAPK pathway, as shown by Klaus Michael Debatin (Ulm).[9] Doxorubicin requires the formation of the DISC to induce apoptosis. Bcl-2 or Bcl-x_L were unable to inhibit apoptosis (type I cell death). Caspase 8 is crucial in this process, as shown in neuroblastoma cells which often lack this enzyme. These cells are resistant to both TRAIL- and doxorubicin-induced apoptosis. However, in such cells betulinic acid activates the mitochondrial death pathway. Betulinic acid seems specific for neuroectodermal tumour cells and acts independently from p53 and death receptors. Thus, this compound could overcome drug resistance in cancers in which apoptosis normally relies on p53 and/or death receptor activation.

Involution of the mammary gland, characterised by extensive apoptosis of the epithelial cells, is accompanied by Stat3 activation, as described by Christine J Watson (Cambridge). She generated a conditional knockout of Stat3 using the Cre-lox recombination system.[10] Such mice showed a delay of involution following weaning. IGFBP-5 levels did not increase as in wt controls, suggesting that it is a target of Stat3, required to induce apoptosis by sequestering IGF-1 casein micelles, thereby inhibiting its survival function. Regulation of Stat1, p53 and p21 occurred, possibly as a compensatory mechanism. Finally, Watson described the activation of NF-κB during post-lactational involution of the mouse mammary gland.[11] Active NF-κB localised exclusively to nonapoptotic epithelial cells. Activation of NF-κB in KIM-2 cells paralleled a decrease of cytosolic I-κBa. Therefore, although coincident with induction of apoptosis, NF-κB appeared to exert a selective survival role in epithelial cells.

Death Effectors: caspases

A major breakthrough after years of research in apoptosis has been the identification of common mechanisms, and in particular the identification of caspases.

Gerry Cohen (Leicester) showed that while the 700 kDa apoptosome is active, the 1400 kDa isoform is not. This seems to be the result of an inappropriate Apaf-1 oligomer due to a cleaved Apaf-1. In fact caspases are able to cleave Apaf-1, generating an aberrant apoptosome. Seamus Martin (Dublin) outlined the sequence of caspase activation downstream of the apoptosome, by using caspase-immunodepleted extracts in a cell-free system. Caspase 9 activates both caspases 3 and 7. Caspase 3 activates caspases 9, 6 and 2. Caspase 6 in turn can activate caspases 8 and 10. He finally discussed the role of DRADD and CARP-1, two proteins that interact with the CARD domain of caspase 9. Wim DeClerq (Gent) showed that normal human keratinocytes stimulated with TND exhibit cleavage and activation of caspase 3 (not 14). In sharp contrast, caspase 14 (not 3) is cleaved in terminal keratinocyte differentiation, suggesting that caspase 14 is involved in the cornification of sqamous epithelia, rather than classical apoptosis. In keeping with this hypothesis, caspase-14 is lacking in parakeratotic plaques of psoriasis lesions.

Klaus Schultze-Osthoff (Muenster) showed how keratin 18 is cleaved by caspases 3 and 6, generating a new epitope detected by a specific antibody. This antibody could

become a useful tool for the detection of apoptotic cells in clinical samples. Furthermore, he outlined that Cytochrome *c* can be released into culture fluids of apoptotic cells. Cytochrome *c* could be detected in the serum of patients with AML (acute myeloid leukaemia).

Death Effectors: other mechanisms

Tissue transglutaminase (tTG or type 2) is a transamidating enzyme catalysing the irreversible crosslinking of proteins by isodipeptide cross-linkages. The enzyme is regulated both at the transcriptional (retinoids) and post-transcriptional (GTP, Ca^{2+}, nitric oxide) levels.[12] tTG is induced and activated in cells undergoing apoptosis. Mauro Piacentini (Rome) reported that cell lines overexpressing tTG, either constitutively or under the control of inducible promoters, exhibited a mitochondrial hyperpolarisation, before and unrelated to the release of Cytochrome *c*. The cells overexpressing tTG were highly susceptible to apoptosis induced by different death agents. Sub-cellular fractionation revealed tTG to be associated with mitochondrial membranes. Furthermore, mitochondria of tTG-overexpressing cells showed striking differences at the structural and functional levels. tTG contains a BH3-like motif which could contribute to the tTG-induced Bax oligomerization on mitochondria, and the polymerisation of glutathione-S-transferase P1. These data suggest that tTG regulates apoptosis similarly to the BH3-only members of the Bcl-2 family.

The biochemical alteration in apoptosis had two further highlights, closing the circle on transglutaminase. Vincenzo De Laurenzi (Rome) presented the recent knockout of transglutaminase 2, with no obvious phenotype,[13] and Eleonora Candi (Rome) elucidated the role of transglutaminase 5 in the differentiation and death of keratinocytes.

Peter Vandenabeele (Gent) focused on the molecular differences between necrosis (induced by TNF) and apoptosis (induced by CD95 ligand), using the same L929 fibrosarcoma cell line.[14] TNF-induced necrosis was due to excessive formation of reactive oxygen species (ROS) with consequent membrane permeabilisation. At difference with CD95-induced apoptosis, necrosis induced by TNF did not show activation of caspases 3 and 7, nor cleavage of Bid. Furthermore, phosphatidylserine exposure was less evident, and necrotic cells were not phagocytosed by competent macrophages. Interestingly, Bcl-2 was able to prevent both apoptosis and necrosis. As expected, zVAD.fmk prevented apoptosis (CD95 triggers apoptosis via FADD and caspase 8) but not necrosis. zVAD sensitised cells to TNF-induced necrosis by induction of ROS. Inhibition of caspase 8 also promoted necrosis.

The evolutionary view of cell death in different kingdoms was brought in by Pierre Golstein (Marseille) with his pioneering work on Dictostelium. Here he showed a caspase-independent death in the multicellular phase that follows aggregation of the unicellular phase. Death is vacuolar, with some chromatine condensation, no DNA fragmentation, nor PS externalisation. Insertional mutagenesis to identify death genes, and selection for survival, led to the isolation of more than 10 molecules some of which are under extensive investigation. He described a new gene, Delirium-A, with 14 leucine-rich repeats, which controls PKA expression, which in turn is necessary for aggregation and further development.

Altogether, this workshop, and particularly its informal part, not discussed here, fostered new cooperations between leading European scientists working in the apoptosis field. Indeed, this has already created[3,6,8,11,15] and will further stimulate collaborations. No doubt there should be a follow-up meeting in the tantalizing scenery of death and life.

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