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Meeting Report

Keystone 1997: Return of the worm

Andrew Fraser[1] and Arul M. Chinnaiyan[2]

[1]Imperial Cancer Research Fund Laboratories, 44, Lincoln's Inn Fields, London WC2A 3PX; [2]University of Michigan Medical School, Department of Pathology, Ann Arbor, MI 48109

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Abbreviations: PCD, programmed cell death; PT, permeability transition; AICD, activation-induced cell death; EM, electron microscopy; ICE, interleukin-1β converting enzyme; GB, granzyme B; DISC, death inducing signaling complex; IgG, G immunoglobulins; IGF-1, insulin-like growth factor 1

The Keystone Symposium on Apoptosis and Programmed Cell Death was held in Tammarron, Colorado from February 18–23, 1997 and organized superbly by John Cohen and John Cidlowski. The keynote address was by Andrew Wyllie and Pierre Golstein who discussed the origin of apoptosis, its failure in normal tissues, and its relationship to the course of evolution. Here we attempt to survey the salient features of the meeting, hopefully not digressing too much into the quality of the ski slopes.

The conceptual highlight of this meeting has been the tremendous progress towards a molecular understanding of how CED-9, CED-3 and CED-4 mutually interact and the implications in terms of a basal molecular framework of cell death. Genetic analysis of programmed cell death in the nematode by Horvitz and colleagues led to the identification of the genes for the pro-apoptotic *ced-3* and *ced-4* and the anti-apoptotic *ced-9*, opening up the field of apoptosis to molecular biology. While mammalian homologues of *ced-9* (bcl-2 family) and *ced-3* (caspases) have been identified, there has been, until now, no molecular mechanism to explain the anti-apoptotic activity of *ced-9* or the death effector function of *ced-4*.

The most comprehensive model for CED-3 : CED-4 : CED-9 interactions was presented by Arul Chinnaiyan from the Dixit lab. Using coimmunoprecipitation following overexpression in mammalian cells as an assay for molecular interaction, they demonstrate the CED-9 interacts with CED-4 which in turn interacts with CED-3, all three coexisting in a 'primed' death complex. The relevance of this model to mammalian cell death is provided by experiments in which bcl-x_L, but not an inactive mutant, can coimmunoprecipitate with FLICE (a caspase) and that a mutant form of CED-4 can attenuate this interaction, suggesting the existence of a functional homologue of CED-4 in mammals. The pro-apoptotic bcl-2 family members bax and bak abolish the bcl-x_L: CED-4 interaction suggesting a potential mechanism for their function.

The CED-9 : CED-4 interaction was confirmed by a number of other groups including those of Nuñez, Evan and perhaps most compellingly by Michael Hengartner's group who combine an *in vivo* genetic approach to identify point mutants of *ced-9* that no longer prevent cell death in the nematode, with the biochemical demonstration that these CED-9 null mutants no longer interact with CED-4 *in vitro*.

The simplest model from the worm is therefore that CED-4 binds and is required for activation of CED-3, while CED-9 exerts its anti-apoptotic effect by a direct interaction with the CED-4:CED-3 complex. This model still leaves many questions. How this primed CED-3 : CED-4 : CED-9 'bomb' is detonated in the 131 somatic cells that die in nematode development is unclear, and further, if this model is applied to a mammalian cell, how cytokine signaling DNA damage response and 'death signaling' (e.g. by Fas/APO-1) impinge on an analogous mammalian complex will prove very exciting in the coming months. In addition, the growing body of data suggesting that caspase-independent programmed cell death can occur is at variance with the simplest 'CED-3 is the effector' implication of the above model.

In relation to this latter question of caspase-independent PCD, Gerard Evan reported data showing that overexpression of CED-4 in *S. pombe* causes profound chromatin condensation, that CED-4 colocalises with the condensed chromatin and that this 'nuclear death' requires a previously undescribed ATP-binding motif in CED-4. The CED-4 killing cannot be prevented by coexpression of p35, suggesting that CED-4 itself may contain a caspase-independent proapoptotic activity. When CED-4 was expressed with CED-9, colocalization was observed in *S. pombe* as well as in mammalian cells (Nuñez lab).

The interaction of CED-3, CED-4 and CED-9 presented at this meeting suggests a compelling, yet simple, molecular paradigm for cell death. It will be interesting to see how other cell death models which emphasize pore-formation by bcl-2 family members, cytochrome c release, and mitochondrial permeability transition will be integrated into this simple framework.

A poster presented by Andy Minn of the Craig Thompson lab showed that bcl-x_L, by virtue of its structural homology to diptheria toxin and the colicins, forms ion channels in synthetic lipid membranes. Similarly, Stan Korsmeyer and John Reed independently observed the formation of ion conductance pores *in vitro* by both bcl-2 and a death-promoting member of the bcl-2 family, bax.

Korsmeyer reported that in addition to mitochondrial alterations, bax overexpression induces the activation of caspases. How the *in vitro* pore-forming ability of bcl-2 family members relates to the cell death pathway will be of future interest.

Doug Green reported an elegant set of experiments involving the release of cytochrome c from mitochondria during cell death using an *in vitro Xenopus* system. He reported that while bcl-2 appears to act upstream of cytochrome c release, caspases are downstream (as monitored by the ability of zVAD to prevent chromatin condensation but not to block cytochrome c release into the cytosol). Intriguingly, he also shows that bcl-2 can only prevent release of cytochrome c from mitochondria to which it is physically bound, suggesting that in this *Xenopus in vitro* system at least, bcl-2 acts directly on mitochondria themselves and not on some global set upstream of cytochrome c release. Many questions regarding the role of cytochrome c in dell death remain to be answered including: (1) does cytochrome c release function upstream in apoptosis signaling or does it act as a downstream amplifier of the death cascade, (2) how does cytochrome c engage the suicide apparatus leading to the activation of caspases, and (3) how does cytochrome c fit into the CED-3 : CED-4 : CED-9 framework for cell death.

Of late, the role of mitochondria in the suicide pathway has drawn much attention in the cell death field, from cytochrome c release to the ability of bcl-2 to form membrane pores. Here Guido Kroemer demonstrated that apoptosis induces mitochondrial permeability transition (PT) which can be blocked by bcl-2. Once PT occurs, a 50 kDa single chain protein called AIF is released from the mitochondria. *In vitro*, AIF can induce caspase activation and is blocked by zVAD, but is not blocked by bcl-2. According to Kroemer, caspases can also function upstream of PT, as recombinant ICE can induce the phenomenon *in vitro*. It remains unclear where cytochrome c release functions relative to PT and AIF release. Furthermore, formation of ion conduction pores by bcl-2 *in vitro* must be reconciled with the ability of bcl-2 to prevent PT *in vivo*.

Suzanne Cory demonstrated that bcl-2, along with inhibiting cell death, can also influence cell cycle entry and exit. Expression of the bcl-2 transgene *in vivo* reduced proliferation and slowed the turnover of thymocytes. Ectopic expression of bcl-2 significantly delayed growth factor induced S-phase entry of quiescent NIH3T3 fibroblasts. Bax can antagonize the regulation of cell cycle by bcl-2. Critically, she also showed that the cell-cycle and anti-apoptotic functions of bcl-2 could be dissected by a single point mutation. In addition, Cory emphasized the importance of the N-terminal BH4 domain of bcl-2, characterized a novel death suppressing member of the bcl-2 family named bcl-w, and introduced a death-promoting, BH3-containing protein called bim.

On the caspase side, Don Nicholson presented data from studies using a random peptide library and purified caspases to build up a comprehensive and highly detailed picture of caspase specificity. Caspases fell into three principal 'substrate preference' groups, all of which being critically determined by the P4 position. Caspases 1, 4 and 5 had a WEHD specificity, although this was relatively loose; 3, 7 and 2 cleaved DExD preferentially with an absolute requirement for D at the P4; 6, 8 and 9 had a less strict P4 requirement, cleaving I/L/VExD were termed 'activators'. In addition, the serine protease granzyme B, which is known to be capable of activating caspase 3 *in vitro* was found to have a strong IExD preference, which ties in with its ability to cleave caspase 3 at an IExD-conforming sequence. Finally, data were presented to suggest that the critical activating cleavage for a caspase is the processing between the large and small subunits and not the N-terminal prodomain release.

Yuri Lazebnik described an *in vitro* system for studying the biochemistry of cell death in normal and transformed cells. Caspases activated during apoptosis were purified and identified with a biotinylated caspase-specific inhibitor. In Jurkat cells caspase-3 and caspase-6 were identified using this method. A drug resistant 293 cell line was shown to be resistant to etoposide. Interestingly, when extracts were made from untreated cells they spontaneously underwent apoptosis and activated caspases suggesting that the endogenous machinery was still intact in these resistant cells. When cells of diverse genetics linkages were tested, it was determined that the adenovirus E1A oncogene was responsible for triggering this apoptotic activity. E1B, on the other hand, kept E1A 'in check' and was not present in the cell extracts (as it partitioned into the insoluble fraction), suggesting a mechanism for the phenomena observed. Taken together, these studies emphasize the link between oncogene expression and the cell death machinery and may be exploited to 'coax' transformed cells to die.

At the substrate end of caspases, Eileen White constructed a lamin A mutant in which the caspase cleavage site is mutated to analyze the importance of lamin cleavage in the execution of cell death. The lamina as expected remained intact at all stages of apoptosis in cells overexpressing mutant lamin and the kinetics of nuclear collapse were slightly delayed. However, EM analysis showed that the morphology of the apoptotic cells was identical to that of wild-type cells, thereby showing that lamin cleavage is not required for apoptosis.

Arnold Greenberg and Chris Bleackley independently discussed their fascination with the so-called 'molecular-scissors', also known as cytotoxic T cell derived granzyme B (GB). Greenberg showed that mutant versions of ICE and ICH-1, but not ICH-3, blocked GB-induced death. Bleackley presented elegant work describing how a replication deficient adenovirus can substitute for the pore-forming protein, perforin allowing GB to induce DNA fragmentation and membrane damage. Once GB exits the endosomal compartment it is translocated to the nucleus where it triggers DNA fragmentation.

Aside from the 'basal machinery', the other key area of interest and progress at Tamarron was the receptor-mediated death signaling of the type exemplified by the Fas-FasL system. Arul Chinnaiyan reported the characterization of a new 'death-receptor':'death ligand' pair, DR4 and TRAIL/APO-2L respectively. Signaling via DR4 does

not activate NF-κB and is not blocked by overexpression of FADD-DN (unlike TNF-R1 and Fas signaling for example), suggesting that DR4 may utilize a novel death adaptor molecule to engage the apoptotic machinery. Lynch presented a beautiful piece of work demonstrating the involvement of both Fas and, surprisingly, TNF-R2 in activation-induced cell death (AICD) in HIV. While a substantial component of AICD can be attributed to signaling via Fas, he also demonstrated a TNF-dependent component which was shown by the use of TNF-R1 and TNF-R2 knockout mice to act via TNF-R2, a receptor with no previous described role in cell death.

Peter Krammer discussed the signaling machinery of Fas/APO-1 called the DISC (Death Inducing Signaling Complex). He demonstrated that day 1 primary T cells were resistant to Fas-induced apoptosis while by day 6, activated T cells regained susceptibility. The DISC of day 1 resistant T cells did not contain the caspase FLICE, unlike the day 6 T cells in which FLICE could be recruited. Interestingly, bcl-x_L expression correlated with resistance to apoptosis and ability to recruit FLICE – it was up-regulated in day 1 T cells and down-regulated in day 6 cells.

In collaboration with Krammer, Elisheva Yonish-Rouach showed that p53 induces expression of Fas suggesting that death receptor activation may be a component of p53-induced apoptosis. p53 binding sites in the Fas promoter were also characterized. Similarly, Evan presented intriguing data suggesting that c-myc-induced apoptosis may also utilize the Fas pathway. Both Fas-Fc (the extracellular domain of Fas fused to the Fc region of IgG) and FADD-DN significantly attenuated myc-induced apoptosis. Furthermore, IGF-I was shown to inhibit Fas killing.

While work on p53 played a relatively small role in the meeting, Eileen White presented interesting work involving a temperature-sensitive p53 mutant in which an N-terminal proline-rich region was deleted. While this mutant could induce cell-cycle arrest but no detectable apoptosis, transcriptional induction of BAX occurs as normal, demonstrating that this is not sufficient for p53-induced apoptosis.

In conclusion, the 1997 Keystone Symposium on Apoptosis and Programmed Cell Death will be remembered for how the *C. elegans* death proteins CED-3, CED-4 and CED-9 were shown to biochemically interact as a multicomponent death complex. These mutual associations by core components of the suicide apparatus provide a molecular framework for which various death signals will likely interface. How these signals impinge upon the core machinery will occupy cell death aficionados for the near future.

Acknowledgements

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