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Meeting Report

A Dead Cool Meeting in Keystone 2001

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Keystone meeting: Molecular Mechanisms of Apoptosis, Keystone Resort, Colorado, USA January 16–22, 2001

Even though temperatures fell below −23°C, (known to slow caspases *in vitro*), this year's Keystone meeting on the 'Molecular Mechanisms of Apoptosis, held at the Keystone Resort, 16–22 January 2001', and organized by Doug Green and Gerard Evan was the 'best cell death meeting ever'. Or at least in keeping with the Sydney Olympics, it was so declared by some of the Australian delegates. This was due to the overall organisation, the outstanding presentations and the participation of over 500 delegates bringing almost as many posters. The clear crisp weather, a good blanket of snow, a frozen lake and a skin-full of beer helped.

While it is generally accepted that the death of a cell by apoptosis involves the activation of caspases, the complexities of the signalling pathways that lead to caspase activation, the regulation of these proteases and the consequences of their activation are still unfolding. At this meeting a broad range of topics were discussed from upstream signalling events specific to individual death stimuli and diseases to the final removal of the dead cell. A variety of new molecules were reported while single and double knockout studies advanced our knowledge of the time-honoured players in this field. In addition to classical caspase-dependent apoptosis, there was much interest in alternative pathways to cell death. We heard presentations about death that is 'not apoptosis but may not be necrosis', a death that may be 'paraptosis', and events involved in the death of a cell in the absence of caspase activation.

Signalling pathways to apoptosis: the forest or the tree

The tree

The study of individual diseases has revealed interesting private pathways to cell death that may be modulated upstream of the common apoptotic pathways. Patients with Peutz Jeghers Syndrome (PJS) have a predisposition to tumors in various tissues. J Yuan described her studies on a novel pro-apoptotic gene product of LKB1 responsible for PJS. Her studies showed different expression patterns of LKB1 in the lower intestine of control *versus* PJS patients and demonstrated a loss of apoptosis in areas where LKB1 was expressed.

The signalling pathways of two new members of the TNF-R family, XEDAR and EDAR (downless in mice), were described by V Dixit. Mutations in the EctoDysplasin-A (EDA) ligand are observed in Hypohidrotic Ectodermal Dysplasia, a human disease affecting hair, teeth and sweat gland development. The EDA ligand has two isoforms (A1 and A2) that differ by two amino acids: the short isoform EDA-A2, binds XEDAR while the long isoform, EDA-A1 binds EDAR. V Dixit presented evidence suggesting that absence of EDAR signalling leads to the premature death of folicular stem cells and indeed transgenic EDA-A1 mice had more hair (that was greasy) because of more hair follicles and a greater number of sebacious glands.

The forest

Although diseases have specific signalling pathways, these 'private pathways' in turn engage molecules that play a role in apoptosis induced by various death stimuli. For example, LKB1 above signals via p53 whereas EDAR activates NF-κB. The apoptosis promoting activity of p53 is essential for the induction of apoptosis by many chemotherapeutic drugs and as a consequence p53 loss can promote drug resistance in many clinical settings. Although malignant melanoma is extremely resistant to apoptosis-inducing agents, loss of p53 is rare. It is now clear that metastatic melanomas often lose Apaf-1, the cell death effector that acts with cytochrome *c* and caspase-9 to mediate p53-dependent apoptosis (S. Lowe). The exciting news is that restoring physiological levels of Apaf-1 through gene transfer or treatment with inhibitors of methylation can markedly enhance chemosensitivity and rescue the defects in apoptosis associated with Apaf-1 loss.

Until recently the activity of the p53 homologue p73 has been largely assumed based on its structural similarity with p53. p73 is expressed as several spliced forms and is frequently deleted in neuroblastomas, suggesting that alterations in its expression may play a role in the development of tumors of the nervous system. G Melino and colleagues now propose that the role of p73 be re-evaluated as it appears to exert its primary effect during development rather than simply mimicking the tumor-suppressive effects of p53.

M. Karin reported on a potential co-operativity between p53 and JNK-mediated activation of c-*jun*, an effect which is evident during UV induced apoptosis and enables a switch between growth arrest and apoptosis. Using c-*jun*$^{-/-}$ cells, UV radiation preferentially induces p21-

mediated growth arrest rather than apoptosis. It thus appears that c-jun may act to inhibit the p53-induced transactivation of p21 thereby switching the predominant UV-induced p53 effect from growth arrest to apoptosis.

Another gene which has recently been implicated in the sustained, but not the early and transient, activation of JNK following stress-induced apoptosis is ASK-1. Using embryonic fibroblasts isolated from ASK-$1^{-/-}$ mice, A Matsuzawa reported that ASK-1-mediated sustained/prolonged activation of JNK and p38 appears to be essential for the induction of stress-induced apoptosis as ASK-1 deficient cells were unable to sustain prolonged activation of JNK or p38 and were also resistant to apoptosis.

A potent driving force for the suppression of apoptosis in tumor cells is the coupled relationship between cell proliferation and cell death, and a good example of this phenomenon is the Myc protein. In addition to its growth-promoting property, Myc is a powerful inducer of apoptosis, particularly following genotoxic damage or depletion of survival factors. Similar antagonistic duality has been described for essentially all known growth-promoting proteins, including E2F1, whose pro-apoptotic activity counteracts the proliferative effect of loss of Rb (K MacLeod). A closer examination of the pro-apoptotic effector pathways activated downstream of Myc in $Bax^{-/-}$ and $Bid^{-/-}$ mouse embryonic fibroblasts revealed that Myc-induced apoptosis triggered release of cytochrome *c*, and activation of caspase-9, an effect which required Bax (G Evan). Another effect of deregulated Myc is angiogenesis, which raises the possibility that pharmacological inhibition of Myc could result in collapse of tumour vasculature. This is a real possibility, since inactivation of Myc in switchable Myc transgenic models of skin and pancreatic β cells leads to rapid regression of tumour vasculature, triggering concomitant tumour involution (G Evan).

NF-κB has been shown to play an important role in promoting life and suppressing apoptosis. M Karin presented evidence that the level of NF-κB may be responsible for the final outcome, life or death; high NF-κB signalling for survival and low NF-κB favouring apoptosis. Using $p50/p65^{-/-}$ and $IKK\beta^{-/-}$ cells, he presented compelling evidence that IKKβ and NFκB lie on a linear pathway. The development of proteasome inhibitors that can potently inhibit the activity of IKKβ and NF-κB, and therefore potentially kill tumor cells, is now currently underway.

Several Caspase Recruitment Domain (CARD)-containing molecules have recently been identified that do not appear to participate in caspase activation but instead are implicated in promoting cell survival via NF-κB activation. Targeted inactivation of one such gene, *bcl10* revealed that Bc110, a CARD-containing protein encoded by a gene translocated in a subset of B cell lymphomas, is absolutely required for T or B cell receptor-mediated activation of NF-κB (T Mak). In embryos from $Bc110^{-/-}$ mice, neural tube closure defects were observed as a direct result of more extensive apoptosis in these animals. However, no differences were observed in the ability of $Bc110^{-/-}$ cells to undergo apoptosis, suggesting that the primary role of Bc110 is activation of NF-κB. The viral homologue of bc110 (vCLAP) activates NF-κB via its N-terminal CARD domain, which is necessary for both self-association and direct interaction with the IKKγ (NEMO) subunit of the NF-κB activating IKK complex (E Alnemri). One could envisage that cellular Bc110 may operate in a similar manner by assembling the IKK complex around a Bc110 scaffold. Two other CARD-containing molecules were also described, Nod1/CARD4 and Nod2/NAC, which in addition to possessing CARD motifs, also contain a nucleotide-binding domain (NBD) similar to that found in Apaf-1 (G Nunez). However, unlike Apaf-1, Nod1 and Nod2 are potent activators of NF-κB. Nunez and colleagues propose that Nod proteins may be involved in the detection of intracellular pathogen components and thus act as intracellular sensors of bacterial invasion by initiating NF-κB activation and an associated inflammatory response.

Another important pro-survival signal is Akt (PKB), which when activated in neuronal cells can protect against apoptosis induced by a wide range of stimuli. In cerebellar granular neurons Akt protects via phosphorylation of two candidate substrates, BAD and the forkhead transcription factor, FKHRL1 (M Greenberg). Work is currently underway to generate a phosphorylated BAD knock-in mouse, which should reveal the *in vivo* significance of Akt-mediated BAD phosphorylation at Serine residues 112, 136 and 155. The significance of FKHRL1 phosphorylation is revealed from analysis of its subcellular localisation. In the absence of survival factors FKHRL1 resides in the nucleus where it is transcriptionally active and can induce apoptosis. Phosphorylation of FKHRL1 by Akt induces its translocation to the cytoplasm thereby inhibiting its transcriptional and pro-apoptotic activity. The transcriptional targets of FKHRL1 include the death-inducing ligand FasL, but microarray analysis of 12,000 target genes is currently underway in order to identify other potentially novel targets for this transcriptional activator of death genes.

Evolution signalling towards the mechanism of cell death

Apoptosis is relatively conserved through evolution and much of what we know about signalling pathways in apoptosis stems from studies on lower organisms. At this meeting we heard various advances in our knowledge of apoptosis in *Drosophila*. *Reaper, Grim* and *Hid* map to the same 300 Kb region and overexpression of these proteins leads to apoptosis in the *Drosophila* eye. These proteins all contain a similar N-terminal motif that contains the required information to block *Drosophila* Inhibitor of Apoptosis Proteins (DIAPs) resulting in activation of the *Drosophila* caspases (J Abrams). After treatment with ionizing radiation, *Reaper* is upregulated in dying cells. In related experiments, Abrams reported on a *Drosophila* homologue of p53 (ARK) which functions during radiation-induced apoptosis but not the cell cycle. New evidence was then presented that ARK binds to and transactivates a radiation-responsive enhancer element upstream of *Reaper*, suggesting that *Reaper* is a direct target for ARK *in vivo*. S Kornbluth reported the identification of a human homologue of *Reaper* (hrpr) which is upregulated

upon treatment of ML-1 cells with ionizing radiation. Hrpr induced caspase activation in Xenopus egg extracts, induced apoptosis in HeLa cells and also sensitized cells to ionizing radiation. Like *Reaper*, hrpr binds to a 150 kD protein from Xenopous extracts called Scythe, which is required for caspase activation. Given the requirement for *Reaper* in apoptosis in *Drosophila*, one would anticipate that human reaper may also be a critical component of apoptotic signalling in humans.

S Kornbluth also presented work that implicated a role for the cell cycle in apoptosis. The SH2 domain of CTP-regulated kinase (CRK) was found to inhibit apoptosis in the Xenopus cell-free system. Kornbluth and colleagues showed that Wee-1 can bind the CRK-SH2 domain *in vitro* and that endogenous Wee-1 and CRK interact in Xenopus egg extracts. Addition of exogenous Wee-1 accelerated caspase activation in egg extracts via a mitochondrially linked pathway, while addition of anti-Wee-1 blocked apoptosis with the apoptotic response being restored by the addition of more Wee-1. Although Wee-1 is known to phosphorylate cdc2, cdc2 is apparently not the apoptotic target of Wee-1 since Myt-1, which also phosphorylates serine15 on cdc2 had no effect on the apoptotic response.

Regulation of apoptosis by the Bcl-2 family, is it all a load of Bmf?

Many of the apoptosis signalling pathways are integrated by pro- and anti-apoptotic Bcl-2 family proteins. While it is accepted that these proteins play an integral role in regulating apoptosis, it is not clear why there are so many family members and why mice deficient in specific Bcl-2 family members have no apparent phenotype. C Thompson showed data obtained from mice lacking two of the pro-apoptotic Bcl-2 family members Bak and Bax. These mice had various phenotypic abnormalities that would be expected in the absence of apoptosis, for example, interdigital webs and enlarged spleens, whereas mice deficient in a single gene or mice that had a single copy of either gene displayed no such abnormalities. Strikingly, these mice did not contain some defects that would be predicted to be associated with a complete loss of apoptosis suggesting that Bcl-2 regulation of apoptosis is more complex than originally thought.

Four of the newer Bcl-2 family members, Bim (A Strasser and S Cory), Bcl-2 modifying factor [Bmf (A Strasser)], Bcl-$B_{L/S}$ and Bcl-$G_{L/S}$ (J Reed) now add to the level of complexity. Bcl-B is a mitochondrially localised protein which protects against Bax, but not Bak-mediated apoptosis, while Bcl-G is a BH3-only protein (containing only the third Bcl-2 homology region) that enhances apoptosis. Bim and Bmf are also pro-apoptotic BH3-only proteins. Bmf associates with the myosin-based dynein motor complex via an LC8 binding motif of dynein light chain-2. It is released upon cytokine withdrawal or when cells are deprived of support and interacts anti-apoptotic Bcl-2 family members to block their protective effect. A role for Bmf in detecting these changes in the cellular environment was supported by the observation that there is a lower level of Bmf in metastatic tumours. Bim, on the other hand, is localised to the dynein motor complex on microtubules via cytoplasmic $LC8^{-1}$ cytoplasmic dynein light chain and is released after addition of the death stimulus. It is required for growth factor-induced cell death but is not required for DNA damage or glucocorticoid-induced death. Bim interacts only with pro-apoptotic Bcl-2 family members and 'knocking out' Bim leads to a partially lethal phenotype (about 60% of Bim deficient mice die before birth due to an immunoproliferative disorder, however the 40% that survive only have lymphoid problems). Similarities between $Bim^{-/-}$ mice and vav-p-Bcl-2 transgenic mice suggest that Bim is a major physiologic antagonist of Bcl-2 and indeed, loss of Bim compensated for the loss of Bcl-2 in $Bim^{-/-}$ Bcl-$2^{-/-}$ mice. Similarities between the $Bim^{-/-}$ mice and the $Bak^{-/-}$ $Bax^{-/-}$ mice also suggested that Bim may indirectly activate Bak or Bax.

Regulation of apoptosis at the mitochondrial level

During apoptosis cytochrome *c* is released from mitochondria into the cytoplasm where it forms part of a large protein complex known as the apoptosome. This complex then recruits and activates caspases, which in turn orchestrate the death of the cell. Bcl-2 family proteins have been shown to regulate the release of cytochrome *c*, however it is still unclear how they control this event. During growth factor withdrawal-induced apoptosis, ATP levels decline and creatine phosphate accumulates in the mitochondrial inter-membrane space. Bcl-X_L prevents the drop in ATP levels and the creatine phosphate accumulation thus protecting the cell from growth factor withdrawal-induced apoptosis. Despite the ability of Bcl-X_L to inhibit death these same cells do not proliferate and eventually undergo atrophy. Bck-X_L can therefore facilitate cell survival by regulating mitochondrial metabolism and allowing efficient recirculation of ADP/ATP but cannot prevent cellular atrophy (C Thompson).

Bcl-2-regulated events which cause changes in mitochondrial transmembrane potential ($\Delta\Psi$m), either an increase (hyperpolarization) or decrease (permeability transition) have also been proposed to cause cytochrome *c* release. Time-lapse movies of single cells showed that fluctuations in $\Delta\Psi$m were not required for cytochrome *c*-GFP release during apoptosis, and that loss of $\Delta\Psi$m commenced within minutes after cytochrome *c* release (D Green). Green and colleagues also demonstrated that the mechanism of cytochrome *c* release does not involve disruption of the mitochondrial inner membrane since, in the absence of caspase activation, mitochondria regenerate any lost $\Delta\Psi$m and remain capable of ATP production via oxidative phosphorylation following cytochrome *c* release. This was supported by studies where exogenous cytochrome *c*, similar to the level found within cells, was sufficient to regenerate $\Delta\Psi$m in mitochondria treated with tBid. S Korsmeyer presented similar results showing that in mitochondria isolated from the livers of mice treated with anti-Fas, the addition of exogenous cytochrome *c* was sufficient to restore $\Delta\Psi$m and state 3/state 4 transitions similar to those observed in intact mitochondria. Maintenance of mitochondrial function following cytochrome *c*

release was further supported by D Newmeyer who presented a 3D animated-representation of electron micrographs from slices through mitochondria that had released cytochrome *c*. Interestingly, these mitochondria were not swollen and the mitochondrial inner and outer membranes remained generally intact.

There has always been great interest in identifying proteins, other than cytochrome *c*, that are released from mitochondria during apoptosis. At this meeting we heard about the results of the long awaited AIF knockout (G Kroemer). AIF is located on the X chromosome and therefore manipulation of male germ cells was used to generate a knockout of exon 3 of the AIF gene. No difference was observed in the drug-induced apoptotic response of $AIF^{+/+}$ or $AIF^{-/-}$ embryonic stem cells in the presence or absence of the caspase inhibitor zVAD-fmk. However, in the case of serum withdrawal, $AIF^{-/-}$ cells survived longer and AIF null cells treated with paraquat or menadione in the presence of zVAD-fmk also survived longer than control cells. Unfortunately, AIF null mice could not be generated in a pseudo development assay, as AIF null embryonic bodies were unable to undergo cavitation. Cavitation of the inner mass of the embryonic body was apparently caspase-independent but AIF-dependent.

Post-mitochondrial regulation of apoptosis: the apoptosome reaches the space age

Upon release from the mitochondria, cytochrome *c* signals the activation of caspases via formation of the apoptosome. Initial reports suggested that each apoptosome contained eight molecules of Apaf-1 and as many of caspase-9. This oligomerization effect led to the cart-wheel model of the apoptosome which was depicted in a humorous animated graphic by J Reed. Since the apoptosome contains so many molecules of Apaf-1, a rocket model of the apoptosome where all of the molecules lie side by side – not unlike the way in which the death inducing signalling complex (DISC) is depicted – may be a more appropriate way of thinking about this complex (S Martin). It is also emerging that, similar to the DISC, the apoptosome may contain many (perhaps more than twenty) interacting molecules, present as an active 700 kDa complex (K Cain). Two of these interacting proteins NAC and CARD 12 which may potentially regulate caspase activation by the apoptosome were reported by J Reed. NAC binds directly to Apaf-1 while CARD 12 binds to caspase-9 and disrupts its binding to Apaf-1. Further, it appears that formation of the apoptosome may be subject to ionic regulation since both K^+ and Na^+ inhibit caspase activation by abrogation of the formation of the 700 kDa apoptosome (K Cain). Addition of cytochrome *c* can overcome the inhibitory effects of high ionic strength to facilitate the formation of the active apoptosome. Many questions regarding regulation of the apoptosome remain to be explored, and since removal of Apaf-1 can contribute to the transformation potential of cells (S Lowe), the study of factors that regulate apoptosome assembly and function is currently a hot topic.

Caspase activation and regulation

Activation of caspases was originally believed to involve proteolytic processing, however, it appears that cleavage is neither necessary nor required for activation of caspase-9. Rather, activation is achieved by co-factor recruitment indicating that recruitment of caspase-9 to the apoptosome is sufficient to activate caspase-9 (G Salvesen). Furthermore, the active species of caspase-9 (which exists primarily as a monomeric zymogen) is a dimer formed in a novel way (using a priming bulge and a conformational change) in which only one of the two active sites is catalytically active. In contrast, both the zymogen and active forms of caspase-3 are dimers.

Baculovirus IAP repeat (BIR)-containing proteins have been shown to bind and inhibit caspases. Recent work presented by XD Wang showed that Smac/DIABLO, a mitochondrial protein that is released into the cytoplasm during apoptosis, binds to the BIR3 domain of XIAP thus promoting caspase activation. While XIAP disrupted the interaction of active caspase-9 with Apaf-1-GST in pull-down experiments, both Smac and Smac peptides disrupted XIAP/caspase-9 interactions (XD Wang). Strikingly, the N-terminus of Smac is similar to the N-terminus of the *Drosophila* proteins Reaper, Grim and Hid, which are known to inhibit *Drosophila* IAPs. The importance of Smac was further demonstrated by M Deshmukh, who showed that in the absence of NGF but in the presence of cycloheximide, microinjection of cytochrome *c* resulted in the death of neurons. By contrast, in the presence of NGF, microinjection of cytochrome *c* alone was not sufficient to activate caspase-3. However, co-microinjection of Smac and cytochrome *c* was sufficient to overcome the presence of NGF. This suggests that the competence to die by a cytochrome *c*-mediated pathway can be attained in neurons via Smac-mediated removal of IAPs from caspases. S Martin, also studying the interaction of Smac and IAPs, presented evidence that even although Smac preferentially binds XIAP and weakly potentiates caspase-3 activation in cellular extracts treated with cytochrome *c* and dATP, caspase-3 activation proceeds at the same rate in XIAP-depleted extracts. This suggests that XIAP may not be the primary target of Smac/DIABLO, but that Smac may instead lower the threshold of caspase-3 activation through factors other than XIAP.

Similarly, XIAPs may also function in ways that do not involve caspase inhibition. S Reffey presented data showing that XIAP binds to members of the TGFβ receptor family and may regulate the threshold of response to TGFβ; increased levels of XIAP may lead to TGFβ-induced cell cycle arrest while decreased levels of XIAP may result in TGFβ-induced apoptosis. Another BIR-containing protein that has attracted considerable attention in the field of apoptosis is Survivin. D Vaux showed that Survivin functions in concert with an inner centromere protein (INCENP), localizing to centromeres until metaphase and then remaining at the equator after the metaphase-anaphase transition. The Survivin knockout is embryonic lethal, however *in vitro* culture of the embryos revealed an identical phenotype to the INCENP knockout and a similar phenotype to the *C. elegans* BIR knockout where there is

no cytokinesis but there is DNA replication. These studies revealed two types of BIR-containing protein, those that are involved in apoptosis such as the IAPs and those that are involved in mitosis such as Survivin. Another level of complexity was added when P Gallagher and colleagues, while studying death associated protein kinase (DAPK), discovered a novel 116 kDa protein containing three BIR repeats. This protein, named Muerte, binds to DAPK, and enhances both TNF-induced apoptosis and caspase-3 activity *in vitro*. These experiments suggest that there may be both pro- and anti-apoptotic BIR-containing proteins.

Walking the path least trodden

Studies on cell death focus mainly on caspase-dependent cell death, however, alternative forms of cell death are now attracting more attention. These forms of cell death are often referred to as caspase-independent-death, however this term encompasses various pathways to death.

The digits of mice deficient in Apaf-1$^{-/-}$ form correctly but since the Apaf-1-dependent activation of caspase-9 is impaired the cells in the webs between the digits must die by an alternative mechanism; a death that could be called Death In the ABsence of Apaf (DABA). At this meeting we heard that foetal liver Apaf$^{-/-}$ and Caspase 9$^{-/-}$ cells placed in lethally irradiated recipients produced normal numbers of haemopoetic cells (A Strasser), indicating a regulated death in these cells. This is in contrast with the elevated number of cells produced when Bim$^{-/-}$ cells were used. Apaf$^{-/-}$ and Caspase 9$^{-/-}$ T cells were sensitive to death by various stimuli. Many morphological and biochemical features of apoptosis were observed when these cells died, and z-VAD.fmk blocked the death in a cell-free system, however little or no caspase-2 or -9 activity was observed. These cells may therefore use an alternative pathway to death other than the conventional caspase-dependent pathways we have discussed or they may use an as yet undescribed mammalian Ced-4 homologue to activate an 'undefined' caspase.

Although caspases are known to mediate apoptosis, when caspase activity is abrogated by the addition of z-VAD.fmk or viral inhibitors, cell death is only delayed. Cytochrome *c* is still released during this Death in the Presence of z-VAD.fmk (could be called DiPz), suggesting that impaired mitochondrial metabolism may contribute to the eventual death of the cell. In support of this, neuronal cells deprived of NGF can recover after the release of cytochrome *c* but they cannot be rescued after the loss of $\Delta\Psi$m. Further, microinjection of cytochrome *c* does not kill neuronal cells unless they have developed the competence to die (M Deshmukh). Time-lapse movies showed that, in the presence of z-VAD.fmk, mitochondria lost transmembrane potential following cytochrome *c*–GFP release, however this could be regenerated over the next 30–60 min and ATP production was unaffected (D Green). These studies indicate that although the outer membrane of the mitochondrion becomes permeabilised, the inner membrane is unaffected and the function of mitochondria is maintained at least in the early stages of DiPz. Mitochondria were also reported to be selectively eliminated during DiPz, but it is not clear how late in the DiPz process this occurs or whether it contributes to, or is required for, the death of the cell by this process (L Xue).

In the absence of caspases, it is hard to avoid the probability that other non-caspase proteases are involved in the ultimate death of the cell. FasL induces apoptosis via caspase-8 and in transformed cells this can be blocked by zVAD.fmk. In caspase-8$^{-/-}$ Jurkat T cells, FasL triggers death that for the sake of argument was not reported as necrosis (J Tschopp). This death was not characterised by chromatin condensation or cytochrome *c* release. In this case, Fas interacts directly with RIP, which in turn induces a death pathway that is inhibited by the protease inhibitor TLCK but not by the caspase inhibitor zVAD-fmk. A different cysteine protease, cathepsin B, may also be involved in receptor-mediated cell death. Cathepsin B was reported to be released from lysosomes and induce release of cytochrome *c*, upstream of caspase 9 activation, during TNF-α-induced hepatocyte apoptosis. In support of this role, cathepsin B$^{-/-}$ mice were more resistant to TNF-α-induced liver damage (ME Guicciardi). Cathepsin B-induced cell death occurred even in the presence of z-VAD.fmk, whereas an inhibitor of cathepsins, cystein A, could protect against TNF-α-induced death. It still remains to be established how, and when, cathepsin B becomes activated since it requires an acidic environment for activation. It also needs to be clarified whether cystein A can additionally inhibit caspases.

We also heard reports of a cell death in neurons dubbed 'paraptosis' (DE Bredesen). Neurons may avoid death at all costs and therefore when the apoptotic process is abrogated, the cell attempts to live but eventually dies by an as yet undefined pathway. This alternative form of cell death is characterised by cytoplasmic vacoulation and mitochondrial swelling and, despite its lack of response to caspase inhibitors or Bcl-X_L, is apparently driven by an alternative caspase-9 pathway that is Apaf-1-independent. Further characterisation of such alternative forms of cell death has important implications for determining their role in neurodegeneration.

The road to heaven, hell or phagocytosis

The ultimate fate of an apoptotic cell is clearance by phagocytosis. If cells are not cleared, they may persist as ghost cells in some sort of purgatory, and thereby interfere with tissue development or contribute to a persistent inflammatory response. Cells have a variety of signals that tell macrophages when they are ready to be phagocytosed. One of these, commonly used as a marker for apoptosis, is the exposure of phosphatidylserine (PS) on the outside of the cell. PS binds to a 48 kDa receptor (PSR) on the surface of the phagocytosing cell thus inducing a ruffling of the membrane and engulfment of the dying cell by the 'macropinocytosis' model of phagocytosis (V Fadok). The PSR is sensitive to cleavage by neutrophil elastase and the removal of the PSR in this way may contribute to inflammation in cystic fibrosis. The PSR also acts as a molecular switch, which may regulate the presence or

absence of the acquired immune response. If the road to hell is paved with good intentions, the enthusiasm Dr Fadok showed for this line of investigation may be sufficient to categorise phagocytosis as some kind of hell. Strikingly, in the absence of Apaf-1 or in the presence of caspase inhibitors, sculpting of the forepaws occurs without detectable phagocytosis or an inflammatory response. By the same analogy, if the road less traveled leads to heaven, it will be interesting to determine whether the PSR or some other miraculous event contributes to the removal of cells that die by a caspase-independent or rather a DiPz or DABA mechanism.

Conclusion

We have heard about the commitment point to cell death upstream, downstream, or parallel to the mitochondrion. We heard discussions on whether oncogenes such as Bcl-X_L can rescue a cell from death or prolong the life of the cell until it atrophies. We heard about regulation of cell death after cytochrome *c* release, that mitochondria remain more or less fully functional, that loss of Apaf-1 enhanced the transformation potential of primary cells, and that the apoptosome may be regulated by up to twenty other proteins. We also heard about the ability of cells to die in the absence of detectable caspase activation or Apaf-1 and in the presence of caspase inhibitors. These studies have expanded our view of cell death to encompass a wide range of pathways, some where we know the major players and some that we know little or nothing about. The stimulating discussions at this conference have no doubt triggered many further questions about how cells die, how they avoid death and the consequences of both.