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Meeting Report

'Centennial' Nobel Conference on apoptosis and human disease

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Nobel Conference, Apoptosis: Mechanisms and Implications for Human Disease
Stockholm, Sweden, October 4–7, 2001

More than a hundred years ago, a sharp-eyed German anatomist described a unique form of spontaneous cell death in regressing ovarian follicles that he termed chromatolysis for the breaking up and disappearance of nuclei.[1] Reports of cell death with similar characteristics in other tissues followed, and in the middle of the last century it was clear that programmed cell death (PCD) during embryonic development was associated with the same morphological features (for review see[2]). In 1972, Kerr and colleagues described a form of physiological cell death based on the appearance of specific morphological criteria.[3] It was given the name apoptosis and suggested to be responsible for maintenance of tissue homeostasis in the adult organism. The first biochemical evidence of apoptosis followed a few years later when the appearance of oligonucleosomal-length DNA fragments in irradiated lymphocytes was reported;[4] this effect was subsequently linked to increased endonuclease activity and for many years to come the 'DNA ladder' was the prime hallmark of apoptosis.[5] Work on the genetic regulation of cell death was initiated about the same time when it was discovered that cells of diverse origins in the nematode *Caenorhabditis elegans* undergo the same morphological changes at specific times in development.[6] Following relatively few developments in the field during the early 1980s, advances over the last 15 years have been swift and a role for apoptotic cell death in embryonic development, tissue homeostasis, and the onset of different diseases, including neurodegenerative disorders and cancer, has been demonstrated. At the recent Nobel Conference, entitled 'Apoptosis: Mechanisms and Implications for Human Disease' held in Stockholm, October 4–7, 2001, leaders in apoptosis and cell death research met to discuss the latest developments in the field. The conference covered a veritable *smörgåsbord* of topics ranging from basic mechanisms of apoptosis to the role of apoptosis in human disease. What emerged was a sense that efforts to understand basic mechanisms of apoptosis are beginning to pay off in the form of novel strategies for treating disease. In this review, we summarize the meeting's major concepts and themes as well as discuss some unresolved controversies in this rapidly evolving field.

Mechanisms of cell death

Programmed cell death in *C. elegans*

R Horvitz's (Cambridge, MA, USA) pioneering work on the genetic regulation of PCD during the development of *C. elegans* made him an obvious choice for the introductory lecture. There are 959 somatic cells in the adult nematode and an additional 131 cells undergo PCD during development. The function of three genes – *egl-1* (*egl*, *egg laying* defective), *ced-3* (*ced*, *cell death* abnormal), and *ced-4* – is required for PCD to take place. Loss-of-function aberrations in these genes result in the survival of the 131 cells that are normally predetermined to die. A fourth gene, *ced-9* (similar to human *Bcl-2*), protects cells from PCD, and loss-of-function mutations in this gene result in the death of cells that would normally survive.

The *ced-3* gene encodes a protein that was a defining member of the caspase (*c*ysteine-*asp*artate prote*ase*) family. CED-3 and other caspases are synthesized as pro-enzymes that must be proteolytically cleaved in order to be activated. Genetic experiments determined that the killing activity of *ced-4* depends on *ced-3* activity, whereas *ced-3*-mediated killing is independent of *ced-4* activity. Horvitz presented a model for PCD in *C. elegans* whereby EGL-1 binds CED-9, which, in turn, releases CED-4. This results in CED-4 oligomerization and the activation of CED-3. The CED-3 protease then is responsible for the execution stages of the process by cleavage of key death substrates. In the model, CED-4 moves from a mitochondrial to a perinuclear localization before it binds and activates CED-3, which appears to be important for PCD, though the mechanism is unclear. He also presented data on the regulation of EGL-1 indicating that in sexually dimorphic HSNs (*h*ermaphrodite-*s*pecific *n*eurons), which normally undergo cell death in males, the *egl-1* death gene is transcriptionally repressed by TRA-1 (TRA, *tra*nsformer). Horvitz and colleagues have identified and characterized *C. elegans* genes *dpl-1* (*DP-l*ike) and *efl-1* (*E2F-l*ike) encoding proteins that antagonize RTK (*r*eceptor *t*yrosine *k*inase)/Ras signaling during vulval induction by interacting with *lin-35*, a *C. elegans* homologue of the mammalian tumor suppressor pRB. Horvitz reported that these genes also facilitate PCD parallel or downstream of *ced-9*.

Seven of the *ced* genes (*ced-1*, *ced-2*, *ced-5*, *ced-6*, *ced-7*, *ced-10*, and *ced-12*) are involved in the engulfment of cell corpses.[7] They are divided into two groups: group 1 (*ced-1*, *-6*, and *-7*) and group 2 (*ced-2*, *-5*, *-10*, and *-12*). Defects in both groups, but not in one or the other, result in impaired engulfment. CED-1, homologous to human SREC (*s*cavenger *r*eceptor from *e*ndothelial *c*ell), and CED-7, homologous to ABC (*A*TP-*b*inding *c*assette) transporters, work together in corpse recognition and may regulate phosphatidylserine-induced engulfment. Interestingly, CED-7 is the only phagocytic protein that is required in the dying cell for proper engulfment. CED-6 encodes an adaptor-like protein and may transmit signals from CED-1. CED-2, homologous to CrkII, CED-5, homologous to DOCK180 (*d*ownstream *o*f *Crk*), and CED-10, homologous to Rac-like GTPase, are involved in cytoskeletal reorganization during engulfment. Horvitz also discussed the recent finding that phagocytosis may promote the execution of cell death; cells expressing a partial loss-of-function *ced-3* mutation seem to be poised between life and death and can recover completely if engulfment is prevented.[8,9] These observations challenge the previously held notion that activation of *ced-3* is the point of no return, beyond which cells cannot survive.

Taken together, studies of the development of *C. elegans* have clearly identified and characterized the basic genes and proteins involved in PCD. Furthermore, studies from Horvitz's and other laboratories demonstrate an impressive universality in this respect among different organisms. This fact indicates that the molecular mechanisms of PCD are, indeed, conserved from worm to man (Figure 1).

Apoptosis in mammalian cells

In recent years, it has become increasingly clear that mitochondria are the target of different cytotoxic stressors. Initiation of the mitochondrial pathway is accompanied by the release of numerous intermembrane space proteins, and a role for cytochrome *c*, AIF (*a*poptosis-*i*nducing *f*actor), and Smac/DIABLO (*s*econd *m*itochondria-derived *a*ctivator of *c*aspase/*d*irect *IA*P *b*inding protein with *lo*w pI) has been established (for review see[10]). As revealed by the pioneering studies by X Wang (Dallas, USA) and colleagues, cytochrome *c* binds and activates Apaf-1 (Apaf, *a*poptotic *p*rotease-*a*ctivating *f*actor), a mammalian homologue of CED-4, in the presence of dATP followed by recruitment and activation of pro-caspase-9.[11] Mechanisms of cytochrome *c* release are

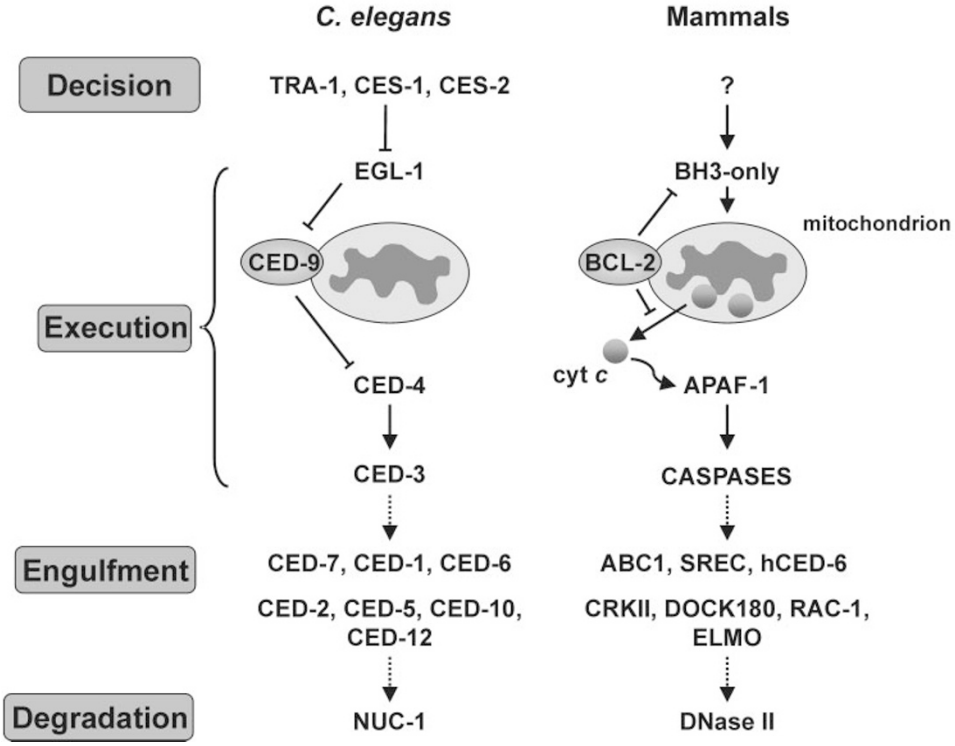

Figure 1 Conservation of apoptosis genes from worm to man. The four sequential stages of programmed cell death or apoptosis, and the genes that govern them, are remarkably conserved throughout evolution from the nematode *C. elegans* to mammals. However, key differences in apoptosis signaling also exist, including the expulsion of cytochrome *c* from the mitochondrial intermembrane space an event that seems to occur only in mammalian cells. Importantly, this highly simplified model does not include many of the other proteins and alternate pathways that are important for mammalian apoptosis, including additional pro- and anti-apoptotic Bcl-2 homologues, IAPs (*i*nhibitor of *a*poptosis *p*roteins), and the activation of the caspase cascade through ligation of cell surface receptors such as Fas. ABC, *A*TP-*b*inding *c*assette; APAF, *a*poptotic *p*rotease-*a*ctivating *f*actor; BH, Bcl-2 homology domain; CED, *c*ell *d*eath abnormal; CES, *c*ell death *s*pecification; cyt *c*, cytochrome *c*; DOCK, *d*ownstream of *crk*; EGL, *eg*g *l*aying defective; ELMO, *engulf*ment and cell *mo*tility; NUC, *nuc*lease, SREC, *s*cavenger *r*eceptor from *e*ndothelial *c*ells; TRA, *tra*nsformer

controversial, though it is likely to occur either via pores in the outer membrane formed by pro-apoptotic Bcl-2 family proteins or as a result of outer membrane rupture following mitochondrial swelling induced by permeability transition (PT) pore opening. D Green (La Jolla, USA) presented data indicating that cytochrome *c* release at the single cell level is sudden, rapid, and complete. Loss of mitochondrial membrane potential ($\Delta\psi$) follows within minutes of cytochrome *c* release and depends on caspase activity, although the underlying mechanism is unknown. He and J-C Martinou (Geneva, Switzerland) suggested that in the absence of caspase activity, mitochondria are able to use cytoplasmic cytochrome *c* to restore $\Delta\psi$. Evidence for Ca^{2+}-dependent PT pore opening and the release of cytochrome *c* came from a presentation by J Robertson (Stockholm, Sweden). He also provided data describing a mandatory two-step process for cytochrome *c* release.[12] The process first involves a breaching of this protein's electrostatic or hydrophobic interaction with cardiolipin followed by permeabilization of the outer membrane and extrusion of cytochrome *c* into the cytosol.

Pro- and anti-apoptotic Bcl-2 family proteins regulate mitochondrial outer membrane permeabilization.[13] All members of this family contain up to four conserved domains denoted Bcl-2 homology domains (BH1-4). Anti-apoptotic proteins generally contain all four domains, whereas pro-apoptotic proteins are divided into those that contain multi-domains and those that are BH3-only (for review see[14]). According to S Korsmeyer (Boston, USA), all BH3-only proteins, including Bim, Bad, Noxa, and Bid, appear to require either, but not both, of the multi-domain pro-apoptotic molecules Bax and Bak to stimulate apoptosis. He also presented data indicating that Bax and Bak double knock-out cells are refractory to perhaps all intrinsic death stimuli and thus constitute the commitment point in the mitochondrial pathway. Anti-apoptotic Bcl-2 and Bcl-X_L function, at least in part, to sequester BH3-only proteins in stable complexes, preventing the activation of Bax or Bak. As will be seen later in this review, recent evidence supports a role for Bax and mitochondria in HIV-induced lymphocyte cell killing. Y Tsujimoto (Osaka, Japan) presented data indicating that VDAC (*v*oltage-*d*ependent *a*nionic *c*hannel) can be converted to a hybrid channel for the passage of proteins, like cytochrome *c*; various pro- and anti-apoptotic Bcl-2 family members exert their effect by regulating VDAC. In fact, he suggested that VDAC is required for the anti-apoptotic activity of Bcl-2 and for both Ca^{2+}- and Bax-induced cytochrome *c* release and PT induction. It is clear that the cytochrome *c*-releasing activity of Bax or Bak often involves Bid. Martinou showed that caspase-8-mediated cleavage of Bid to tBid, which directs this protein to mitochondria, is inhibited following phosphorylation of Bid by casein kinases 1 and 2. In addition, C Dive (Manchester, UK) presented data indicating that Bid is normally involved in mitochondrial phospholipid transfer. In the presence of a pro-apoptotic signal, when lipid metabolism is disrupted, Dive suggests that Bid may begin transporting lysolipids that, in turn, accumulate in the outer membrane and promote cytochrome *c* release.

While anti-apoptotic Bcl-2 proteins regulate cytochrome *c* release, members of the IAP (*i*nhibitor of *a*poptosis *p*rotein) family control downstream catalytic processing of pro-caspases.[15] G Cohen (Leicester, UK) presented data on the 700 kDa apoptosome, which predominates in apoptotic cells. He reported that catalytically active processed or unprocessed caspase-9 is critical for the recruitment of pro-caspase-3 to the apoptosome via an interaction between cysteine (C287) of caspase-9 and aspartate (D175) in caspase-3. In addition, Cohen indicated that XIAP (*X*-linked *i*nhibitor of *a*poptosis *p*rotein) not only exerts its effect by binding caspase-9 and -3, it also binds and inhibits Apaf-1-mediated activation of pro-caspase-9. Wang reported that Smac released from mitochondria following ultraviolet radiation relieves XIAP activity by binding to the first four amino acids in its BIR3 (BIR, *b*aculovirus *I*AP *r*epeat) domain. He also presented a three-dimensional structure of the apoptosome and suggested that pro-caspase-9 molecules binding to the apoptosome complex are catalytically active, though the significance of this is presently unclear.

Fas (also known as APO-1 or CD95) is a member of the so-called death receptor family and imparts an extrinsic apoptosis signal upon binding of its cognate ligand.[16,17] Fas ligation leads to the recruitment of FADD (*F*as-*a*ssociated protein with *d*eath *d*omain) which, in turn, recruits pro-caspase-8; since FADD oligomerizes following Fas ligation, it is believed that this facilitates auto-activation of pro-caspase-8 by bringing these molecules into close proximity. However, J Tschopp (Epalinges, Switzerland) presented data indicating that Fas can efficiently kill human primary T cells also in the absence of active caspases. Under these conditions, cells exhibit necrotic morphology and mitochondrial damage without cytochrome *c* release. Fas ligand-induced caspase-independent cell death requires kinase RIP (*r*eceptor-*i*nteracting *p*rotein). He also said that RIP was required for TNF- or TRAIL (*T*NF-*r*elated *a*poptosis-*i*nducing *l*igand)-induced necrosis. Taken together, it appears that members of the TNF family, including Fas ligand, TRAIL, and TNF, can trigger cell death via distinct pathways, one involving caspase-8 and the other kinase RIP. Finally, Tschopp reported on the protective effects of injected soluble Fas ligand against both Fas-mediated fulminant hepatitis and acetaminophen-induced liver damage in mice.

The clearance of apoptotic cells by macrophages and other neighboring cells constitutes the final stage of apoptosis. Phagocytosis of apoptotic cells occurs prior to the rupture of the plasma membrane and thus serves to preclude the release of cellular constituents and subsequent inflammation and tissue scarring. Moreover, surface blebbing is believed to provide a novel context for the presentation of autoantigens and the rapid removal of apoptotic cells prevents undesirable immune responses against 'self'. Recent studies have emphasized that phagocytosis of cell corpses is not merely a matter of passive waste disposal. Hence, the ingestion of apoptotic bodies by dendritic cells and subsequent presentation of antigen derived from these cell corpses may induce tolerance.[18] In addition, a horizontal transfer of genetic information has been shown to occur upon phagocytosis of apoptotic bodies.[19]

A plethora of recognition molecules involved in the clearance of apoptotic cells has been identified in mammals.[20] These include $\alpha_v\beta_3$ and other integrin receptors, class A and class B scavenger receptors such as CD36, the oxidized LDL receptor CD68, and the glycosylphosphatidylinositol (GPI)-linked ligand CD14. The most common 'eat me' signal is phosphatidylserine (PS) externalization on the surface of the apoptotic cell. V Fadok (Denver, USA) underlined that the existence of a vast array of ligands and receptors is not necessarily a matter of redundance, but may be reflective of a sequential receptor engagement, resulting in an initial tethering of the apoptotic cell followed by its engulfment. Fadok also suggested that the recently identified PSR (*PS r*eceptor) acts as a crucial molecular switch that determines the outcome of macrophage ingestion of dying cells. In effect, PSR engagement by apoptotic, PS-exposing cells exerts an immunosuppressive and anti-inflammatory effect. Engulfment of necrotic or otherwise damaged cells, which fail to activate the PSR, does not elicit a similar protective response. S Nagata (Osaka, Japan) discussed DNA fragmentation in macrophages after engulfment of apoptotic cells, and suggested that lysosomal DNase II, a mammalian homologue of NUC-1 in *C. elegans*, degrades DNA of engulfed apoptotic cells. He also indicated that DNase II in macrophages is essential for destroying DNA expelled from erythroid precursor cells during erythropoiesis.

Cell death in disease

Apoptosis in infection, autoimmunity, and lymphoproliferation

In recent years, emerging evidence indicates a role for the dysregulation of apoptosis in the pathogenesis of human disease, and the potential for apoptosis-based therapy was a major focus of the conference. K Elkon (Seattle, USA) discussed the role of defective cell clearance in autoimmunity, focusing, in particular, on the complement cascade and CRP (*C-*reactive *p*rotein) in the uptake of apoptotic cells. CRP is an acute phase reactant that increases 1000-fold during inflammation and binds to the surface of dying cells; this enhances complement opsonization of these cells and subsequent macrophage clearance. Binding of CRP also attenuates the assembly of the terminal complement components thereby protecting apoptotic cells from inadvertent lysis and leakage of cellular constituents. CRP and the components of the complement cascade thus appear to act in concert to promote the non-phlogistic clearance of apoptotic cells. Similarly, Fadok provided evidence for C1q and MBL (*m*annose *b*inding *l*ectin) opsonization of apoptotic cells with subsequent engulfment of these cells through engagement of calreticulin and CD91 on the surface of phagocytes. These data thus concur with recent *in vivo* studies in which defective clearance of apoptotic debris was implicated in the initiation of autoimmunity. Specifically, accumulation of unengulfed apoptotic cells can be observed in the kidneys of C1q-deficient mice and these animals develop a lupus-like autoimmune disease.[21]

Studies in *lpr* and *gld* mice have elucidated the role of the Fas system in the maintenance of immune tolerance. As a result of mutations in the Fas and Fas ligand gene, respectively, these mice develop autoimmune disease, presumably due to the lack of apoptotic deletion of autoreactive lymphocytes. F Rieux-Laucat (Paris, France) discussed the role of death receptors in a human disease termed ALPS (*a*utoimmune *l*ympho*p*roliferative *s*yndrome), which closely resembles the murine phenotype. These children display profound lymphadenopathy, splenomegaly and, to a variable degree, autoimmune manifestations such as hemolytic anemia and thrombocytopenia. Cells obtained from these patients are defective for Fas-triggered apoptosis *in vitro* and heterozygous Fas mutations have been identified in most, but not all, cases.[22] A variable clinical penetrance of these mutations is seen and a second factor, environmental or genetic, has been postulated to account for the manifestation of ALPS. Interestingly, Rieux-Laucat described an ALPS patient with a Fas gene mutation, who also harbored a mutation in the related death receptor known as TRAIL-R2. Other investigators have described two families with abnormal Fas-mediated apoptosis in the absence of Fas mutations. In these ALPS patients, mutations in caspase-10 were identified, suggesting that caspase-10 is, in fact, involved in Fas signaling. Interestingly, children suffering from the related, yet fatal, immunological disorder termed FHL (*f*amilial *h*emophagocytic *l*ymphohistiocytosis) harbor mutations in the gene encoding perforin, an important effector molecule of natural killer and cytotoxic T cells.[23] Taken together, these findings demonstrate that apoptosis resistance (ALPS) as well as lack of apoptosis induction (FHL) can result in lymphoproliferation in humans; this fact underscores the importance of apoptosis for the 'programmed survival' of the organism.

There are certain sites in the body, such as the testis and the anterior chamber of the eye, where allogenic or xenogeneic tissue grafts exhibit prolonged survival. This immune privilege was originally considered a passive process relying on physical barriers and isolation ('immune ignorance'), but recent data support the view that immune privilege is an active phenomenon depending, at least in part, on apoptotic killing of infiltrating immune cells.[24] Indeed, T Griffith (Iowa City, USA) provided data in support of a role for constitutive Fas ligand expression in the eye in ocular immune privilege. There is currently some controversy surrounding this concept as Fas ligand has also been shown to elicit inflammation and rapid rejection of various murine transplants. However, as pointed out by Griffith, the outcome of Fas ligand expression may depend on the cellular context and on the presence or absence of certain co-factors such as TNF. The level of expression of Fas ligand may also be important since an excessive amount of this death factor could trigger massive apoptosis, which might then overwhelm the phagocytic capacity of the tissue resulting in secondary necrosis and inflammation. Nevertheless, the current data could have important implications for transplantation insofar as allografts or xenografts may be endowed with immune privilege, or resistance to rejection, by expression of Fas ligand in the donor tissue.

HIV infection is characterized by the gradual depletion of $CD4^+$ T cells followed by the emergence of opportunistic infections and other manifestations of immunodeficiency, collectively termed AIDS. It is therefore axiomatic that elucidation of the pathogenesis of HIV infection requires an understanding of the mechanism of $CD4^+$ T cell loss. Importantly, an increased level of apoptosis is seen both in cells directly infected with HIV and in uninfected 'bystander' cells; this propensity for apoptosis has been shown to correlate with disease progression. In addition, HIV infection is frequently associated with dementia and neuronal apoptosis triggered directly by viral proteins or indirectly through the release of macrophage, microglial, and astrocyte toxins.[25] A number of HIV proteins, including env (*env*elope glycoprotein complex), have been proposed to contribute to the induction of apoptosis in lymphocytes as well as in neuronal cells. G Kroemer (Villejuif, France) discussed the ordering of molecular events in env-induced apoptosis and provided compelling evidence for a role of mitochondria in this process. In particular, phosphorylation of p53 was shown to precede Bax upregulation, the insertion of Bax into mitochondrial membranes, and the release of cytochrome *c* from the mitochondrial intermembrane space. Moreover, Kroemer showed that p53 phosphorylation is mediated by the activation of mTOR (*m*ammalian *t*arget *o*f *r*apamycin), a regulator of cell size. These data indicate that mitochondrial or pre-mitochondrial events in env-induced apoptosis may yield novel strategies for therapeutic intervention in HIV infection.

Apoptosis in neurodegenerative disease

There is accumulating evidence implicating a role for apoptosis in neuronal death during acute disorders, such as stroke, as well as in chronic neurodegenerative disease. Both S Lipton (La Jolla, USA) and P Nicotera (Leicester, UK) reported a critical role for apoptosis in neuronal death mediated by glutamate-related pathways.[26] Each also stressed that excessive Ca^{2+} influx through the NMDA receptor can cause caspase activation and apoptotic cell death via the mitochondrial pathway; this can also involve an enhanced production of nitric oxide (NO) and reactive oxygen species. Further, Lipton described patch-clamp and neuroprotection studies with the NMDA receptor open-channel blocker drug memantine. In recent phase III clinical trials, memantine has been shown to be effective for severe Alzheimer's disease and other neurodegenerative conditions. An alternate approach for neuroprotection was discussed in which nitric oxide (NO)-generating drugs decrease channel opening by transferring NO to a redox site on the NMDA receptor. Even more effective, according to Lipton, may be the employment of a novel combination of these two mechanisms using the compound NitroMemantine. Nicotera, in turn, described some interesting novel observations demonstrating that the plasma membrane Ca^{2+} pump is cleaved and inactivated by caspase-3 during apoptosis. This leads to impaired Ca^{2+} extrusion and subsequent Ca^{2+} accumulation in the dying cell, which may promote secondary necrosis of apoptotic cells.

Whereas the core region of the ischemic brain lesion is primarily necrotic, apoptotic cell death is an important component in the adjacent tissue. Accordingly, caspase inhibitors are protective and reduce the size of the ischemic lesion in several animal models.[27] Although apoptosis has been assumed to involve mitochondria under these conditions, recent observations from the group of P Krammer (Heidelberg, Germany) indicate that blockade of the Fas-mediated pathway is also protective in an animal stroke model suggesting that both the extrinsic and intrinsic pathways contribute to apoptosis signaling in brain ischemia. T Wieloch (Lund, Sweden) presented evidence for the involvement of apoptosis in both focal and global ischemia. Moreover, he reported that treatment of animals with cyclosporin A, or N-Me-Val-4-cyclosporin A, causes significant reduction in infarct size suggesting that apoptosis under these conditions may be mediated by Ca^{2+}-induced mitochondrial PT, though there also seems to be a role for calpain activation in this model. P Brundin (Lund, Sweden) discussed the present state of neuronal grafting in the treatment of Parkinson's disease. Poor survival of the transplants constitutes a major problem, and cell death occurs during all phases of the transplantation process. Several factors contribute to this cell death, including mechanical trauma, hypoxia, and lack of growth factors. Brundin showed that caspase inhibitors are protective while inhibition of the mitochondrial pathway, Bcl-2 overexpression, and NMDA receptor inhibitors have no effect.[28]

Numerous studies have suggested that dysregulation of apoptosis plays an important role in cell death in chronic neurodegenerative disease, e.g., Alzheimer's disease, amyotrophic lateral sclerosis, and Huntington's disease (HD). Discussions at this conference focused on the mechanisms of neuronal death in HD, which is associated with the accumulation of intracellular inclusions of Huntingtin (Htt) fragments in the HD brain, as well as in the transgenic mouse brain. Cleavage of Htt by caspases is perturbed by the large number of polyglutamine (PolyQ) repeats and leads to the formation of aggregates and to cell death. PolyQ-induced cell death is mediated by caspase-8 and blocked by caspase inhibitors, as well as dominant-negative mutant FADD.[29] D Nicholson (Montreal, Canada) presented work on a new mechanism of caspase-8 activation in the HD brain. His group has identified a novel protein, Hippi, that collaborates with Hip-1 (Htt-associated protein) to recruit and activate caspase-8 by a receptor-independent mechanism.[30] Disease-associated expansion of Htt PolyQ weakens the binding of Hip-1 (normally sequestered by wild-type Htt) and allows Hip-1 to associate with Hippi, resulting in the initiation of this novel apoptotic pathway. Apart from caspase involvement in HD, Nicholson also presented data indicating that selective caspase inhibitors are protective in animal models of stroke and myocardial infarction, although problems with poor penetration of the blood-brain barrier remain to be solved. Interestingly, he also reported that caspase inhibitors are protective in a clinically relevant CLP (*c*ecal *l*igation and *p*uncture) mouse model of sepsis. This effect seems to be linked to an ability of caspase inhibition to reduce massive lymphocyte apoptosis or overburdening of macrophage

engulfment normally triggered by sepsis. Taken together, it appears that the 'next generation' of peptoid and non-peptoid caspase inhibitors may be effective in the treatment of different human pathologies.

Apoptosis in cancer

A Wyllie (Cambridge, UK) discussed a model in which most cancers arise due to a corruption of apoptosis. Whereas many types of tumor cells possess intact effector machinery for carrying out apoptosis, Wyllie indicated that these same cells lack the 'sensor' responsible for coupling injury to the effector process. He suggested that the tumor suppressor p53 is the critical 'sensor'. While approximately 50% of human cancers express mutant forms of p53, this protein may be inactive in all tumors due to a loss or up-regulation of other genes in the p53 pathway.[31] The original observation of p53-dependent apoptosis gave rise to the hypothesis that deficiencies in this protein lead to a persistence of DNA-damaged cells, the potential 'founders' of malignancy. Direct evidence of this can be seen in $p53^{-/-}$ mouse models where bone marrow cells treated with radiation sustain DNA damage but largely fail to undergo apoptosis. Similar data exist for tumors deficient in mismatch repair. Specifically, mice deficient in the MSH-2 (*M*ut*S* *h*omologue) gene are resistant to apoptosis induced by methylation damage. Moreover, surviving cells exhibit a pronounced increase in the incidence of mutations in response to p53 or MSH-2 gene deficiencies, though this effect is highly dependent on cell origin. Thus, in the absence of key proteins that detect or 'sense' DNA damage, cells that would otherwise die may survive. Considerable interest in restoring or reactivating normal function to mutant p53 has emerged recently. K Wiman (Stockholm, Sweden) presented data on the ability of a novel peptide to restore DNA binding of a wide range of p53 mutants, which, in turn, increased the sensitivity of these tumors to apoptosis. Similarly, recent studies from other investigators have described a novel family of proteins termed ASPP (*a*poptosis-*s*timulating *p*rotein of *p*53), which is able to restore or enhance p53 function.[32]

A different, albeit complementary, approach to increasing tumor cell sensitivity to cancer treatment might involve caspase activation.[33] K-M Debatin (Ulm, Germany) reported that several tumor cell lines that are resistant to receptor-mediated killing exhibit decreased levels of caspase-8, or its corresponding mRNA, without a loss of the caspase-8 gene. Interestingly, hypermethylation of caspase-8 regulatory sequences was observed in cells with impaired caspase-8 expression. Treatment with demethylating agents restores caspase-8 expression and increased sensitivity of tumors to death receptor- or drug-induced apoptosis. Debatin also presented data indicating that lowering intracellular glutathione levels, often elevated in tumor cells, with buthionine sulfoximine sensitizes cells to apoptosis. Moreover, Debatin discussed the difficult, but important, task of monitoring apoptosis in patients, as opposed to cell lines, upon treatment with chemotherapeutic agents. S Dimmeler (Frankfurt, Germany) addressed the same topic and provided data on endothelial cell apoptosis in individuals suffering from congestive heart failure, as evidenced by the detection of apoptotic membrane particles in plasma. A different approach to *in vivo* monitoring of apoptosis may involve the use of magnetic resonance imaging and a targeted contrast agent.[34]

Resistance to treatment is a strategic concern for many types of cancer. Increasing evidence indicates that resistance may be associated with defects or dysregulation of different steps within the apoptotic pathway. Aberrant p53 signaling may undermine cell killing by altering the expression of different pro- and anti-apoptotic Bcl-2 family members.[35] Alternatively, resistance to treatment may involve defects in the apoptotic pathway downstream of mitochondrial cytochrome *c* release but upstream of caspase-3 activation. In fact, a reduction in the level of Apaf-1 has been reported in human leukemic blasts and bladder cancer cells, while inactivation of Apaf-1 was described in malignant melanoma.[36,37] Moreover, it was shown that Apaf-$1^{-/-}$ and caspase-$9^{-/-}$ mice are resistant to several apoptotic stimuli, whereas E1A oncogene-transformed cells exhibit increased levels of Apaf-1 and procaspase-9 and are sensitized to DNA damage-induced apoptosis. Finally, recent studies reported by B Zhivotovsky (Stockholm, Sweden) demonstrate that NSCLC (*n*on-*s*mall *c*ell *l*ung *c*ancer cells) are resistant to radiation-induced apoptosis downstream of cytochrome *c* release and caspase activation, but upstream of nuclear events. Specifically, this inhibition is associated with an absence of caspase-3 translocation into the nucleus.[38] However, restoration of NSCLC sensitivity to anti-cancer treatment can be achieved by targeting mitochondria, resulting in the release of AIF that translocates to the nucleus and fragments DNA in a caspase-independent manner.

D Altieri (New Haven, USA) reported that many cancer cells express elevated levels of the IAP protein survivin.[39] Survivin is expressed during mitosis in a cell-cycle dependent manner. Using several novel antibodies, Altieri found that survivin is present in immunochemically distinct pools, though most of the protein is present in the cytosol.[40] Microinjection of cells with an antibody that recognizes all survivin pools had no effect on cytokinesis. Instead, the primary phenotype observed was the formation of short collapsed mitotic spindles, severely depleted of microtubules leading to prolonged metaphase arrest and apoptosis. Cytosolic survivin associates with $p34^{cdc2}$-cyclin B1, which phosphorylates it on Thr^{34}, and Altieri's group recently generated a replication-deficient adenovirus encoding a nonphosphorylatable $Thr^{34} \rightarrow Ala$ mutant of survivin (pAd-T34A) to target tumor cell viability *in vitro* and *in vivo*. Remarkably, infection with pAd-T34A caused spontaneous apoptosis accompanied by cytochrome *c* release and caspase activation in cell lines of breast, cervical, prostate, lung, and colorectal cancer, whereas it did not affect cell viability of normal proliferating human cells. In addition, pAd-T34A was as effective as taxol at inducing tumor cell apoptosis and significantly more effective than adriamycin.

Future directions

One of the most striking features of the Nobel Conference was how much has been accomplished in the last few years to

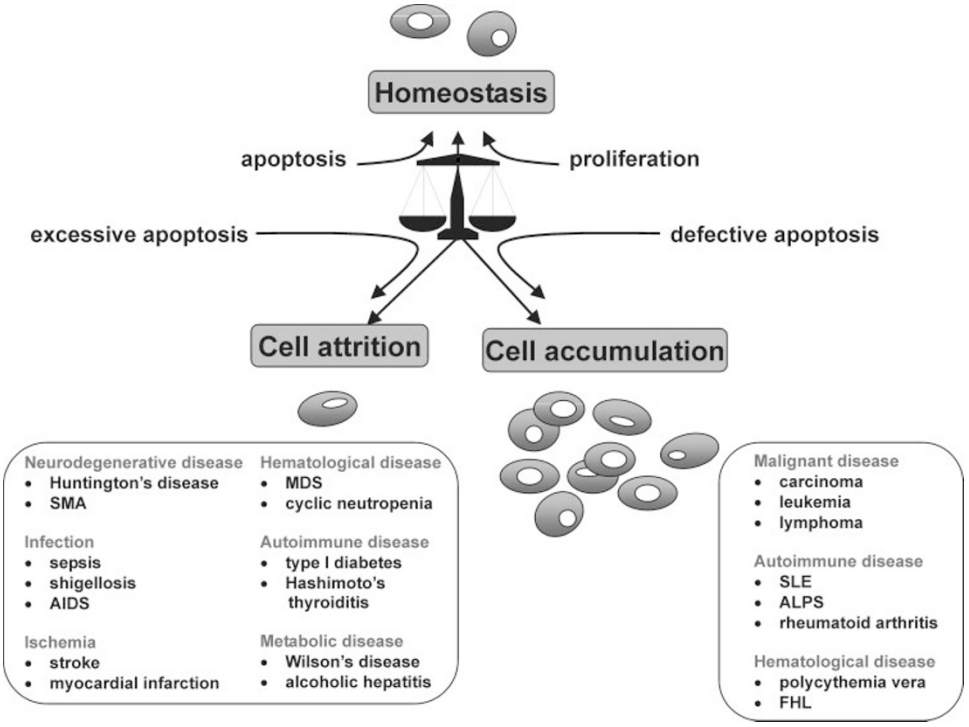

Figure 2 Apoptosis in human disease. Apoptosis is required for the homeostatic regulation of cell number in the adult organism. Some examples of degenerative and proliferative diseases in which dysregulation of apoptosis is believed to play a role are listed. Specific mutations in apoptosis genes have been identified in a number of these pathologies, e.g., NAIP (*n*euronal *a*poptosis *i*nhibitory *p*rotein) in SMA, p53 in many forms of human cancer, Apaf-1 (Apaf, *a*poptotic *p*rotease-*a*ctivating *f*actor) in malignant melanoma, Fas in ALPS, and perforin in FHL. ALPS, *a*utoimmune *l*ympho*p*roliferative *s*yndrome; FHL, *f*amilial *h*emophagocytic *l*ymphohistiocytosis; MDS, *m*yelo*d*ysplastic *s*yndromes; SLE, *s*ystemic *l*upus *e*rythematosus; SMA, *s*pinal *m*uscular *a*trophy

provide a link between aberrant apoptosis and the onset of various diseases (Figure 2). If the argument could be made previously that apoptosis was merely of academic interest, the work presented here went a long way to offset that. Despite this, it was also clear from the conference that a number of outstanding questions remain unresolved.

While it is probably safe to state that we have now advanced beyond a rudimentary understanding of the core apoptotic pathway, obvious differences in the machinery between *C. elegans* and mammals should not be ignored. Few of us would argue against the notion that mitochondrial cytochrome *c* release is indispensable for Apaf-1 activation in mammalian systems, whereas cytochrome *c* does not release and is therefore considered unimportant for PCD in the worm. If this is correct, then how precisely is CED-4 activated? While it is clear that activation involves oligomerization of this protein, is EGL-1-mediated release of CED-4 from CED-9 all that is required for this to occur or are there other yet-to-be-identified pro-apoptotic factors that must be recruited? A different, albeit related, future direction in *C. elegans* should be the identification of key CED-3 death substrates that are required for the execution stages of PCD, since this would almost certainly, if the past several years are any indication, yield additional mechanistic information about apoptosis in mammalian cells. As mentioned earlier, release mechanisms for cytochrome *c* are controversial. In fact, this and the regulation of the apoptosome complex remain two of the most contentious issues in the field. To study cytochrome *c* release mechanisms in greater detail, several investigators have recently employed GFP (*g*reen *f*luorescent *p*rotein)-labeling technology to monitor cytochrome *c* release profiles using confocal microscopy. However, questions were raised at the conference about the physiological relevance of using GFP-cytochrome *c* to study release mechanisms. In addition, an interesting study recently demonstrated that the distribution of GFP-cytochrome *c* in unstressed Bcl-2-overexpressing cells was inconsistent with endogenous cytochrome *c*, suggesting that there may be inherent problems in the use of this technology to study release patterns.[41] To further complicate matters, there is no evidence indicating whether GFP-cytochrome *c* can support respiration or if GFP-cytochrome *c* can substitute for endogenous cytochrome *c* in the activation of the apoptosome complex. These issues, as well as questions concerning the relative contribution of *de novo* pores formed by Bax or Bak *versus* hybrid channels formed by VDAC in the permeabilization of the outer membrane, should be resolved.

Despite these and other outstanding questions within the scientific literature, it seems that we are rapidly approaching a stage where our understanding of apoptotic pathways and their intrinsic regulation will be exploited to develop novel strategies for treatment. Here, too, however, a number of issues need to be addressed. For instance,

can apoptosis be selectively targeted in one organ or cell type without adverse effects to 'bystander' cells? Indiscriminate inhibition of apoptosis could lead to the survival and accumulation of genetically damaged cells that would otherwise die, whereas inappropriate promotion of apoptosis might lead to undesirable tissue degeneration. A better understanding of the molecules that govern the initiation and execution of apoptosis will be important to identify selective targets for apoptosis-modulating therapeutics. For instance, while the administration of TNF or Fas ligand is limited by their toxicity to normal tissues, selective TRAIL-induced killing of tumors was demonstrated *in vivo*, sparing normal tissues that express decoy receptors.[42] Apoptosis-based therapeutics could also be used to lower the threshold of apoptosis in cancer cells, i.e., to overcome resistance to conventional treatment, by suppression of anti-apoptotic genes such as survivin or Bcl-2, using gene therapy and antisense-based strategies (for review see[43]). Another important question is whether cells that are spared an apoptotic fate will function normally or are merely 'undead' and, as a result of inadequate expression of recognition signals, not readily available for phagocytic clearance? Furthermore, will the prevention of apoptosis merely switch the mode of cell death to necrosis as several *in vitro* studies have demonstrated? An important understanding to emerge from the conference was that disease may arise from a mismatch between apoptotic cell death and clearance of cell corpses; thus, the engulfment stage of apoptosis may yield additional targets for therapeutic intervention. Indeed, as discussed by J Savill (Edinburgh, UK), constitutive apoptosis of neutrophils serves to rectify hypercellularity at sites of inflammation, and the subsequent clearance of these apoptotic cells is essential for the resolution of inflammation. Enhancement of phagocytic clearance could therefore provide an additional means of dealing with chronic inflammation, while also, as was discussed in a previous section, serve to prevent the induction of autoimmune disease. Furthermore, if it is true that cells fluctuate between life and death and that engulfment promotes the execution of cells in humans, as in *C. elegans*, then phagocytosis becomes a feasible target for therapy.

These examples reflect the considerable potential to apply our expanding knowledge of basic apoptotic mechanisms to the treatment of human disease. However, as pointed out by P Golstein (Marseille, France) in comments made during the closing reception, we must take care not to forget basic science as the apoptosis field progresses from bench to clinic.

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