Mechanisms of cell death: molecular insights and therapeutic perspectives

LF Díaz[1], M Chiong[1], AFG Quest*,[1], S Lavandero*,[1] and A Stutzin*,[1]

[1] Centro FONDAP de Estudios Moleculares de la Célula, Facultades de Medicina y Ciencias Químicas y Farmacéuticas, Universidad de Chile, Independencia 1027, Santiago 838-0453, Chile

* Corresponding authors: A Stutzin, AFG Quest, S Lavandero, Centro FONDAP de Estudios Moleculares de la Célula, Facultad de Medicina Universidad de Chile, Independencia 1027, Santiago 838-0453, Chile. Tel: +56-2-6786494; Fax: +56-2-7376240; E-mail: astutzin@bitmed.med.uchile.cl, aquest@med.uchile.cl, slavander@uchile.cl

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The International Symposium 'Mechanisms of Cell Death: Molecular Insights and Therapeutic Perspectives', Conference Town, Reñaca, Chile, 13–16 April 2005, was held at the beachside Conference Town resort of Reñaca, close to Valparaíso and some 100 km west from the capital city of Santiago. About 25 invited speakers discussed in a comprehensive manner mechanisms of cell death, their association with pathological situations and future therapeutic developments. Given the mix of participants, topics were chosen to meet the interests of both basic and clinical scientists. Here, we summarize some of the salient features that emerged from the meeting.

Mechanisms of Cell Death, Kinases and ATP

One of the defining features of cell death via apoptotic mechanisms is the formation of multiprotein complexes that provide the molecular scaffolding for the activation of initiator caspases, such as caspase-8 (extrinsic pathway) or caspase-9 (intrinsic pathway). The protein complexes involved are the death-induced signaling complex (DISC) and the apoptosome, respectively. Once activated by autoproteolytic cleavage facilitated by procaspase subunit proximity, initiator caspases then process executioner caspases, such as caspase-3, that induce the organized disassembly sequence characteristic of apoptosis. Conspicuously absent in this scenario is a role for the otherwise omnipresent kinases in other signaling pathways. However, the fact that staurosporine, a broad-spectrum kinase inhibitor, is frequently employed to induce apoptosis in a variety of cell settings would appear to underscore the need for a better understanding of the kinase connection. At the meeting, mechanisms of activation as well as the potential role of the protein kinase C (PKC) superfamily and the death-associated protein (DAP) kinases were discussed.

The development of a function-based gene trapping strategy based on the use of antisense cDNA libraries leads to the identification of novel death-promoting (DAP) genes. Galit Shohat and Adi Kimchi (Weizmann Institute of Science, Rehovot, Israel) discussed the family of DAP kinases (DAPk) and the regulation of the various DAP family members. One of the DAP genes is a Ca^{2+}/calmodulin (CaM)-activated Ser/Thr kinase named DAPk, the founding member of a class of death-associated serine/threonine kinases, including DRP-1 and ZIP kinase (ZIPk). These members share 80% homology in their catalytic domains, but differ in their extracatalytic regions and cellular localization. DRP-1 is the closest DAPk relative, in that it shows a high degree of homology in both the catalytic and CaM-binding domains. The two proteins differ predominantly at the COOH-terminus where DAPk has a domain responsible for binding to the cytoskeleton, while DRP-1 has a dimerization motif. Additionally, DRP-1 shares with DAPk a unique autoinhibitory mechanism responsible for preventing the DAP effects of both kinases. Interestingly, autophosphorylation at Ser308 in the CaM-binding domain keeps the kinase silent in proliferating cells and is specifically removed by an as yet to be identified phosphatase when cells are exposed to various apoptotic stimuli. Recently, evidence for the existence of a functional and physical interaction between DAPk and ZIPk was obtained. The trans-phosphorylation of ZIPk by DAPk at six sites in the C-terminal domain of ZIPk controls the shuttling of the latter between nuclear and cytoplasmic compartments and amplifies the DAP signal.[1]

The PKC superfamily of Ser/Thr protein kinases are a broad class of lipid and protein effector-regulated transducers implicated in controlling multiple processes in cells. The family is related to the single PKC1 gene product of *Saccharomyces cerevisiae* and in mammals comprises the classical (cPKC; Ca^{2+}, diacylglycerol (DAG) dependent), novel (nPKC; DAG dependent), atypical (aPKC; DAG-independent) and the PKC-related kinases (PRK/PKN; Rho, Rac activated). Knockout studies have indicated that there are

some specific nonredundant functions of individual PKC isoforms. In particular, PKCε (epsilon) has been implicated in the protection from damage following cardiac ischemic/reperfusion injury, whereas PRK/PKN-associated JNK activation contributes to tissue damage following cardiac stress. In this context, Peter Parker (Cancer Institute at London, UK), discussed two areas of work related to the relationship between members of the PKC Ser/Thr protein kinase superfamily and the control of cell death. In the first presentation, a signaling cascade engaging multiple upstream kinases was described; this was shown to converge on PKCε conferring a protective effect during stress-induced cell death. In the second, PKN1 was shown to act in an activity-independent manner as a scaffold to the mitogen-activated protein kinase pathway.[2] Elimination of this process correlated with stress-induced cell survival. The participation of PKN1 is particularly notable bearing in mind that PKN3, a related kinase, is a validated target in invasive prostate cancer.

Model systems that have been widely used to study and dissect the mechanisms of apoptosis include the fruit fly, *Drosophila melanogaster*. Mechanisms controlling apoptosis in *Drosophila* were presented by Hyung Don Ryoo and Hermann Steller (Howard Hughes Medical Institute, The Rockefeller University, New York, USA). In *Drosophila*, three genes clustered in a single genomic locus are required for virtually all cell death events during embryogenesis. These three genes, *hid*, *grim* and *reaper*, encode antagonists for the *Drosophila* inhibitor of apoptosis 1 (DIAP1), an essential antiapoptotic factor. In living cells, DIAP1 inhibits apoptosis in part by binding to, and ubiquitinating the apical caspase Dronc, and failure to do so leads to Dronc accumulation and apoptosis. In cells destined to die, expression of DIAP1 antagonists stimulate the DIAP1 autoubiquitinating activity, leading to the degradation of this caspase inhibitor. Cells lacking DIAP1 ubiquitin-ligase activity can be rescued by introducing a heterologous caspase inhibitor p35, which revealed a caspase-independent role of DIAP1 in tissue growth. Cells lacking DIAP1-induced Jun amino-terminal kinase (JNK) signaling and secretion of the developmentally important factors Wingless and Dpp. Expression of such secreted factors correlated with the growth and proliferation of neighboring cells. Based on this, Ryoo and Steller propose that apoptotic cells signal to the neighboring cells to induce compensatory cell proliferation. These events may be important in maintaining tissue homeostasis following cell death.

Novel Forms of Cell Death

Autophagy, the process by which cells recycle cytoplasm and dispose of defective organelles, also referred to as type II programmed cell death (PCD), plays a central role in the maintenance of cellular homeostasis, and participates in the turnover of intracellular organelles as well as in the regulation of proteins with a long half-life. The main criteria to define a cell death process as autophagy is the appearance of double membrane-containing vacuoles in the cytosol, and fusion of autophagosomes with the lysosomes. In addition, several genes relevant to autophagy, such as the *atg* genes, are upregulated and used as indicators of this process.[3] Despite a recent burst of enthusiasm sparked by reports implicating the Atg family member beclin-1, a key player in early events leading to the formation of autophagosomes, as a tumor suppressor, as well as others suggesting that a number of anticancer drugs elicit their cytotoxic effects by triggering autophagy, it is important to bear in mind that under conditions of nutrient deprivation, autophagy is thought to operate, at least initially, as a survival pathway.

Guido Kroemer (Institut Gustave Roussy, Villejuif, France) delivered the keynote lecture on the pathophysiology of mitochondrial cell death regulation. The fact that mitochondrial membrane permeabilization (MMP) controls cell death has both diagnostic and therapeutic implications. Thus, ongoing or imminent cell death may be diagnosed in tissue sections by revealing signs of MMP such as the translocation of mitochondrial intermembrane proteins to extramitochondrial sites. Similarly, the detection of a reduced mitochondrial transmembrane potential can be used to predict irreversible cellular damage, for instance, in circulating blood cells. Moreover, therapeutic induction of MMP may restore apoptosis in cancer cells in which these pathways have been disabled. Conversely, drugs designed to suppress excessive MMP may avoid pathological cell death. This appears particularly important in a series of acute pathologies including septic shock, myocardium infarction and stroke. In addition, Guido Kroemer discussed two topics linked to MMP. On the one hand, he outlined recent attempts to delineate a p53-dependent molecular pathway through which HIV-1 infection causes apoptosis. On the other hand, he showed how impediment of MMP that precludes cell death can contribute to genomic instability in early oncogenesis.

In a somewhat controversial lecture, Guido Kroemer also predicted the 'end of autophagic cell death', provided that cell death can be considered to occur *through* autophagy. He provided clear evidence in mammalian cell lines that, under conditions of starvation, autophagy is induced as a cytoprotective mechanism and that inhibition of autophagy then sensitizes cells to death induced by nutrient depletion. Cell death occurs via classical apoptosis, with MMP, caspase activation and nuclear chromatin condensation, when autophagy is inhibited at an early step. Alternatively, when autophagy is inhibited at a late step, such as at the level of fusion between autophagic vacuoles and lysosomes, nutrient depletion causes spectacular autophagic vacuolization. This phenotype, which resembles textbook examples of autophagic cell death, switches after a latency phase to an apoptotic morphology. Inhibition of MMP or caspases can delay cell death in this latter model, by inhibiting the switch from autophagic to apoptotic morphology. Cells bearing the autophagic phenotype may recover as long as the inner mitochondrial transmembrane potential remains intact. Guido Kroemer speculated that this may be a relatively general feature of the so-called autophagic cell death, which would occur *with* but not *through* autophagy.[4]

María Isabel Colombo (Universidad Nacional de Cuyo, Mendoza, Argentina) discussed cellular signaling events involved in the development of autophagy and a role in microorganism survival in host cells. Two protein conjugation systems are key components involved in the initial steps of the autophagic pathway: the Atg12-Atg5 and Atg8/LC3 systems.

LC3 is conjugated to the lipid phosphatidylethanolamine and changes its distribution to both the outer and inner autophagosomal membranes following processing. Evidence was presented showing that members of the Rab family of GTPases, such as Rab24 and Rab7, are involved in autophagy. Rab24 colocalizes with LC3 upon induction of autophagy by starvation or rapamycin treatment and seems to be associated with formation of autophagosomes. Rab7 localizes to the limiting membrane of autophagic vacuoles and is required for autophagic vacuole maturation, since a functional Rab7 is required for fusion with the lysosomal compartment to degrade incorporated material. A role for autophagy in infectious diseases was also discussed. In particular, autophagy inhibits survival of *Mycobacterium tuberculosis* in infected macrophages. Thus, autophagy represents an underappreciated mechanism of innate immunity employed by host cells to eliminate intracellular pathogens.[5] Alternatively, results were discussed showing that induction of autophagy favors the generation and maturation of the *Coxiella burnetti*-parasitophorous vacuole. Furthermore, overexpression of LC3 and Rab24 were found to promote the generation of the *Coxiella* replicative niche and the transit through this pathway is beneficial for the survival of this bacterium. Therefore, autophagy can act as a defense mechanism against invasion by certain bacteria and viruses, and also can be subverted by pathogens to establish a replicative niche.

Galit Shohat also discussed evidence implicating DAPk as an important element in the cellular decision between apoptosis and autophagic cell death. In primary embryonic fibroblasts, DAPk activates p53 in an ARF-dependent manner leading to classical caspase-dependent apoptosis. In such primary fibroblasts, DAPk expression was strongly antioncogenic. Hence, the status of DAPk in tumors was evaluated. DAPk expression was found to be lost at high frequency in many human carcinomas and B-cell lymphomas, mainly due to DNA methylation. In epithelial cells, the activated form of DAPk triggers two major cytoplasmic events: membrane blebbing characteristic of several types of cell death and extensive autophagy, which is typical of type II PCD. Both membrane blebbing and autophagy are independent of caspase activity and represent two additional functional consequences of DAPk activation that are p53 independent. One of the substrates of DAPk is myosin light chain, the phosphorylation of which may induce cortical contractions leading to membrane blebbing. Other substrates linked to the autophagic pathway are currently being identified by high-throughput proteomics screening. The available data implicate DAPk as a molecular link between the evolutionarily ancient process of autophagic type II PCD and the more recent process of type I PCD or apoptosis.[6]

During development, multiple forms of cell death are distinguished including apoptosis (type I), autophagy (type II) and nonlysosomal cell disintegration (type III). Thus, in addition to type I and type II PCD, other forms of cell death are likely also to prevail at later stages of life. Indeed, evidence in favor of the existence of another regulated form of cell death, reminiscent of necrosis, is emerging. Junying Yuan (Harvard Medical School, Boston, MA, USA) used chemical genetics to dissect a nonapoptotic mechanism and discussed evidence suggesting that an intrinsic necrotic cell death pathway may exist. The mechanism of apoptosis has been extensively characterized over the past decade, yet little is known about the mechanisms of alternative regulated cell death. Although stimulation of Fas/TNFR receptor family by their corresponding ligands triggers the canonical 'extrinsic' pathway of apoptosis, stimulation of Fas/TNF receptors in the absence of intracellular apoptotic signaling (for instance, in the presence of caspase inhibitors) has been shown to lead to necrotic cell death in multiple cell types. Junying Yuan proposed that such cell death is mediated by a cellular pathway referred to as necroptosis.[7] Yuan's laboratory has identified a small molecule which they named necrostatin-1 (Nec-1), which inhibits necroptosis in multiple cell types that were induced by FasL and TNFα in the absence of caspase activity. Junying Yuan also presented data showing that necroptosis is characterized by necrotic cell death morphology and activation of autophagy. Furthermore, necroptosis appears to contribute to delayed mouse ischemic brain injury *in vivo* through a mechanism distinct from that of apoptosis. Necroptosis is proposed to offer a novel therapeutic target for stroke with an extended window for neuroprotection.

Chemical genetics refers to any methodology used to screen for compounds that perturb a biological molecule, function or process of interest. The term is meant to emphasize certain parallels with true genetics: both techniques permit unbiased, functional screens for changes in an observable biological process without *a priori* knowledge of the regulatory machinery controlling that process; both can be subjected to high-throughput analysis, allowing the testing of many mutated genomes or small molecules, respectively; and both can provide new biological information on the process of interest via target identification (i.e. a mutated gene in a genetic screen or a small molecule target – usually a protein – in a chemical screen). Chemical genetics has been used successfully to probe numerous basic biological processes, including cytoskeletal dynamics, histone deacetylation, cell cycle regulation and apoptosis.[8,9] In this meeting, Junying Yuan reported on the use of chemical genetic approaches to probe a novel pathway of cell death termed necroptosis and endoplasmic reticulum (ER) stress-induced cell death.[10] These studies demonstrate that chemical genetics provides a powerful tool to study the mechanisms underlying these processes in mammalian cells. In one example, Junying Yuan described their work analyzing the mechanism of ER stress-induced apoptosis that has been implicated in pathological conditions such as diabetes, Alzheimer's disease (AD) and viral infection, but effective therapeutic strategies targeting the relevant ER stress pathways are lacking. Yuan's laboratory identified a small molecule which they named salubrinal that protects cells from ER stress by inhibiting the cellular eIF2α phosphatase complex without affecting global protein phosphorylation status.[10] Salubrinal also inhibits a homologous eIF2α phosphatase complex encoded by the herpes simplex virus and blocks viral replication. These results indicate that inhibition of virus-mediated eIF2α dephosphorylation may be a potent novel anti-viral therapeutic strategy and suggest that the specific pharmacological inhibition of eIF2α phosphatase complexes may have therapeutic benefit for diseases involving ER stress.

Andrew Quest (FONDAP Center for Molecular Studies of the Cell at the University of Chile, Santiago, Chile) also discussed an alternative form of cell death downstream of the Fas receptor. By a variety of criteria, this alternative form was shown to be distinct from apoptosis and RIP kinase-mediated cell death.[11] The characteristic features observed in A20 B-lymphoma cells included absence of DNA fragmentation, apoptotic changes in nuclear morphology, requirement of lipid scrambling and increased cell volume. Particularly, the latter property led to a definition of this form of cell death as necrosis. In common with apoptosis are a requirement for caspase-8 activation and the fact that phosphatidylserine (annexinV-positive cells) also is present on these cells prior to membrane permeabilization (propidium iodide-impermeable cells). In contrast to necroptosis observed downstream of the TNF receptor in the presence of caspase inhibitors, this form of necrosis is actually caspase-8 dependent. However, low doses of the inhibitor zVAD preferentially block Fas-induced apoptosis rather than necrosis. This experiment suggests that Fas-induced necrosis may be preferentially associated with 'inefficient' caspase-8 activation. A connection between Fas-induced necrosis and production of the lipid second messenger ceramide was discussed. Ceramide production is delayed somewhat with respect to caspase activation, consistent with the notion that it occurs as a downstream event. However, ceramide production follows closely the appearance of phosphatidylserine at the cell surface. This observation provided a crucial link between lipid scrambling-associated cell necrosis and production of ceramide. Indeed, treatment of cells with cell-permeable ceramides (C2, C6) led predominantly to cell death by necrosis without caspase-3 activation, DNA fragmentation, cell shrinkage and chromatin condensation. Taken together, these data favor a model whereby following Fas triggering, ceramide production downstream of caspase-8 may be temporally delayed compared with the kinetics of caspase-3 activation and apoptosis induction, so necrosis is only triggered in those cells not already committed to apoptosis.[12,13] More recently, experiments have focused on analyzing the events associated with necrosis triggered by cell-permeable ceramides. Interestingly, ceramide-induced necrosis, but not apoptosis, was found to be blocked by some (but not all) compounds that sequester reactive oxygen species (ROS). Indeed, using a fluorescence-based assay, ROS formation was shown to be rapid and transient following C6-ceramide exposure. These experiments link ceramide-induced necrosis in A20 cells to the ROS formation and, in doing so, distinguish necrosis observed here from the previously mentioned phenomenon of necroptosis.

Organelles and Ca^{2+} in Cell Death

Ca^{2+} is a ubiquitous eukaryotic second messenger that participates in many signal transduction pathways. On the other hand, Ca^{2+} signals are also associated with the development of pathological situations in humans via the modulation of membrane receptors and/or channels. Indeed, the alteration of Ca^{2+} homeostasis is now widely appreciated as a crucial step in the genesis of various forms of cell death, including apoptosis and necrosis. Within eukaryotic cells, mitochondria and the ER are the key organelles involved in calcium handling. Interestingly, intracellular signals are able to induce profound alterations in organelle structure and function that are particularly striking in the case of mitochondria.

Rosario Rizzuto (University of Ferrara, Italy) discussed how, in HeLa cells, ceramide treatment triggered Ca^{2+} release from the ER. Alternatively, Ca^{2+} loading of the mitochondria together with ceramide administration causes fragmentation and swelling of this organelle, followed by release of cytochrome *c* and other caspase activators. The amount of Ca^{2+} released from the ER and the ability of mitochondria to take up released calcium appear to represent critical determinants in the decision-making process leading to cell death. Antiapoptotic proteins of the Bcl family, such as Bcl-2, protect cells from C_2-ceramide-induced cell death by decreasing the pool of releasable Ca^{2+} from the ER. Alternatively, the ER-resident fraction of the proapoptotic Bcl-2 family member Bax increases the steady-state Ca^{2+} storage levels. As a consequence, Ca^{2+}-mediated signaling events linked to cell death are amplified.[14]

Also, Rosario Rizzuto described how mitochondrial structure is regulated and provided interesting insights to the relationship between mitochondrial morphology and its participation in Ca^{2+} signaling. Drp-1 and hFis1 are two key proteins involved in mitochondrial fusion and fission events, respectively, that additionally participate in mitochondrial and cellular Ca^{2+} homeostasis and in different apoptotic pathways. A striking general feature of apoptotic cells is the concomitant breakdown of the mitochondrial network. Hence, a connection is becoming apparent between Bax-mediated mitochondria-linked apoptosis and Drp-1-mediated mitochondrial fragmentation and inhibition of mitochondrial fusion.[15]

The importance of mitochondrial architecture in events related to cellular metabolism and Ca^{2+} homeostasis were further highlighted by showing how the genetic program of the nucleus determines mitochondrial biogenesis. There, the peroxisome proliferator gamma-activated receptor-coactivator 1 alpha (PGC-1) plays a crucial role by interacting with different nuclear receptors, thereby activating nuclear and mitochondrial transcription factors. In turn, modification of the gene expression profile augments the mitochondrial capability to produce ATP and heat during cellular activation, differentiation and adaptive thermogenesis. Further investigation is needed to clarify the significance of this new mode of mitochondrial regulation in the life and death of the cell.

A few examples demonstrating how the activity of cellular kinases can drastically change the capacity of mitochondria to accumulate Ca^{2+} upon physiological stimulation were reviewed. In particular, the role of different PKC isoforms was discussed in this context. Overexpression of PKCε does alter Ca^{2+} homeostasis in either the ER, the cytosol or the mitochondria. With PKCα (alpha) overexpression, a global alteration of the Ca^{2+} signal was observed. PKCβ (beta) and PKCζ (zeta) overexpression affected only mitochondrial Ca^{2+} homeostasis, but in opposite ways: PKCβ (beta) overexpression reduced Ca^{2+} mitochondrial transients, while PKCζ (zeta) increased them. These results imply that signaling pathways operating in a cell can specifically modulate mitochondrial responsiveness to Ca^{2+} while leaving cytoplasmic signaling unaffected.

Claudio Hetz (Harvard Medical School, Boston, MA, USA) presented evidence supporting a model whereby ER-located BAX and BAK play important roles in maintaining the ER Ca^{2+} stores necessary for Ca^{2+}-dependent cell death. Indeed, $BAX^{-/-}BAK^{-/-}$ double-knockout (DKO) cells have reduced resting ER Ca^{2+} levels due to increased Ca^{2+} leakage as the consequence of an increase in the Ca^{2+}-permeable, hyperphosphorylated state of the inositol trisphosphate receptor type 1 (IP_3R-1). Conversely, under nonapoptotic conditions, expression of antiapoptotic Bcl-2 decreases the ER Ca^{2+} concentration, a phenomenon that is negatively regulated by phosphorylation at the ER membrane.

Calcium overload is a central feature of necrotic cell death. A series of recent papers has indicated that neuronal Ca^{2+} overload results from the activation of Ca^{2+}-permeable channels. At this meeting, Felipe Barros (CECS, Valdivia, Chile) showed, in two epithelial cell lines, that the onset of the necrotic Ca^{2+} overload cannot be explained by channel activation, but rather reflects the collapse of the plasma membrane calcium ATPase (PMCA).[16] Most strikingly, PMCA inhibition was not due to enzymatic inactivation but rather resulted from a dramatic drop in ATP via the Na^+/K^+ ATPase, which competes with the PMCA for ATP utilization. This phenomenon, which was termed 'ATP Steal', provides a novel explanation for the permissive role of Na^+ in epithelial cell necrosis. The sequence discussed here, namely stress → Na^+ overload → Na^+/K^+ ATPase activity → local ATP depletion → PMCA inhibition → Ca^{2+} overload → necrosis, differs sharply from the classic textbook view that goes as follows: stress → ATP depletion → Na^+/K^+ ATPase inhibition → Na^+ overload → VOCC activation/reverse Na^+/Ca^{2+} exchange → Ca^{2+} overload → necrosis.

Cancer, Immune System and Cardiac Apoptosis

Abnormalities in cell death control can contribute to a variety of diseases, including cancer, autoimmunity, cardiovascular and neurodegenerative disorders. The importance of apoptosis in the development of some specific pathologies was also addressed at this Symposium.

Deregulation in apoptosis signaling may contribute to the development of resistance to cancer therapy. Therefore, apoptotic pathways represent interesting targets to sensitize cells to either conventional or novel therapeutic antitumor strategies. The identification of clear-cut prognostic markers for a specific cancer often depends on the strategy used to kill tumor cells, which occurs mostly by apoptosis. Thus far, mutations in apoptosis- regulating genes are rare events, with perhaps the exception of p53. On the other hand, only Bax shows prognostic significance in cancer therapy. Klaus-Michael Debatin (University Children's Hospital, Ulm, Germany) discussed studies showing how targeting apoptosis signaling can become a relevant approach in the treatment of cancer, specifically in acute leukemias. In patients, $CD34^+$ and $CD34^-$ leukemia cell populations were identified. During chemotherapy, $CD34^+$ leukemia cells were more rapidly depleted than $CD34^-$ cells. Furthermore, a significant increase in leukemia cell apoptosis *ex vivo* was detected in $CD34^+$ cells, while this was not the case for the $CD34^-$ subpopulation, suggesting that $CD34^+$ leukemia cells are the main targets of apoptosis-promoting drugs in leukemia cells.[17] Klaus-Michael Debatin also reported that transfer of the Smac gene sensitized various tumor cells, including resistant neuroblastoma or melanoma cells, *in vitro* and *in vivo* to induction of apoptosis. Smac peptides enhanced the antitumor activity of TRAIL in an intracranial malignant glioma xenograft model *in vivo*. Complete eradication of established tumors and survival of mice was, however, only achieved upon combined treatment with Smac peptides and Apo2L/TRAIL without detectable toxicity to normal brain tissue.[18] Thus, Smac agonists are promising candidates for cancer therapy that potentiate available cytotoxic therapies. Therefore, molecular insights into the regulation of apoptosis will improve our understanding of responses to conventional treatment strategies and help in devising more rational approaches to cancer treatment as well as identifying novel targets for future therapeutic intervention.

The rational design of vaccines initially involves identification of the immune effector mechanisms responsible for protection against disease (cellular immune responses for cancer) and the subsequent selection of an antigen capable of eliciting the desired adaptive response. Laurence Zitvogel (Institut Gustave Roussy,Villejuif, France) discussed aspects that determine the immunogenicity of dying tumor cells. The preponderant type of cell death induced by chemotherapy is apoptosis, and apoptosis is frequently but not unanimously viewed as a nonimmunogenic or tolerogenic form of cell death. This lack of immunogenicity is explained by the 'silent' macrophage-mediated (as opposed to dendritic cell-mediated) clearance, active suppression of inflammatory responses or the lack of a 'second signal', also called 'danger signal'. Thus, as an unwarranted side effect, anticancer chemotherapy could specifically reduce the antitumor immune response. As 'apoptosis' is itself nonuniform with respect to the signaling events that trigger cell death, it would appear important to compare specifically the elements defining these pathways with the immunologically nonuniform outcomes of cell death. Laurence Zitvogel highlighted the minimum requirements in order to generate anticancer vaccines by immunizing with dying tumor cells as follows: the cells must be engulfed by dendritic cells, they must stimulate the maturation of dendritic cells and they must elicit long-lasting, T-cell-dependent antitumor immunity. Using these criteria, Laurence Zitvogel demonstrated that tumor cell death can be immunogenic, depending on the specific inducer of apoptosis.[19] It may be anticipated that studies of this kind will help identify novel, immunogenic chemotherapeutic approaches.

Fabio Rossi (University of British Columbia, Vancouver, Canada) presented data revealing the first described components of the molecular machinery that regulate the entry of T-cell progenitor cells into the thymus. The thymus is the adult organ where most apoptosis takes place during clonal selection of T cells. Novel T cells that undergo selection are generated from bone marrow-derived precursors that need to reach the thymus to survive and function. Fabio Rossi's laboratory has conducted a large number of *in vivo*

experiments to demonstrate that P-selectin is not only required for efficient progenitor homing to the thymus but also that P-selectin expression is regulated by the availability of progenitor niches within this organ.[20]

Cardiovascular diseases, in particular cardiomyopathies, are the leading cause of death in all developed countries. The loss of cardiomyocytes due to cell death is an important causative factor in the development of such diseases. However, the mechanism by which cardiomyocytes die and then disappear from the cardiac tissue is not clear. Osmotic stress is an important mechanism that leads to cell damage under physiological conditions. In the heart, osmotic stress occurs during myocardial ischemia/reperfusion. Sergio Lavandero (FONDAP Center for Molecular Studies of the Cell, University of Chile, Santiago, Chile) described how cardiac myocytes respond to osmotic stress by activating two opposing cellular signaling cascades that either lead to cell death or promote cell survival. The balance between these two pathways determines cell fate. Different osmolytes (sorbitol and mannitol) that induced hyperosmotic stress, measured as cell shrinkage, to the same extent, differentially activated stress pathways and apoptosis-related signaling events. Hence, apoptosis induced by hyperosmotic stress in cardiac myocytes depends on the nature of the osmolyte rather than the degree of osmotic stress *per se*.[21] Sorbitol-induced hyperosmotic stress stimulated a fast and transient increase in intracellular Ca^{2+} levels due to both extracellular Ca^{2+} influx and Ca^{2+} release from intracellular stores by a phospholipase C/IP_3 pathway. Sorbitol-dependent Ca^{2+} increase induced mitochondrial depolarization followed by the activation of caspase-9 and -3, DNA fragmentation and a decrease in cell viability. Hyperosmotic stress by sorbitol also induced oxidative stress in cultured cardiac myocytes. Hydroxyl radicals were identified by electron spin resonance spectrometry as the main ROS that was generated. In these cells, ROS-mediated NF-κB activation was also detected. NF-κB inhibition decreased cell survival and promoted cell death induced by hyperosmotic stress. Activation of other transcription factors was also described. Sorbitol activated CREB and NFAT3 but not MEF2. Overexpression of dominant-negative forms of CREB, calcineurin or CAIN (an endogenous calcineurin inhibitor) did not modify cell death.

Ion Channels and Cell Death

Appropriate regulation of cell volume and intracellular osmolarity is important for cell survival. Increases in cell volume generally are the result of passive water influx that is driven mainly by NaCl influx and KCl efflux. Cell volume decreases are achieved in the opposite manner. Hence, ion channels play a significant role in events relevant to normal cell-volume regulation and perturbations in the function of these proteins lead to inappropriate changes in cell volume that are linked to apoptosis and necrosis.

John Cidlowski (National Institute of Environmental Health Sciences, NIH, North Carolina, USA) analyzed the role of regulatory volume decrease in the activation of apoptosis. He discussed how potassium efflux was necessary for apoptosis to occur and showed that inhibition of K^+ efflux blocked cell death. Loss of K^+ from cells was linked to activation of K^+ channels and inhibition of the Na^+/K^+ ATPase. Also, cell shrinkage, one of the hallmarks of apoptotic cell death, was shown not to be necessary for apoptosis to occur, suggesting that molecular crowding was not a prerequisite to apoptotic cell death. Finally, he provided evidence indicating that plasma membrane depolarization is one of the earliest defining features of this form of cell death.

From a slightly different perspective, Yasunobu Okada (National Institute for Physiological Sciences, Okazaki, Japan) discussed the roles of the volume-sensitive, outwardly rectifying (VSOR) anion channel, cytosolic ATP and protein kinases in apoptotic cell death in epithelial cells, highlighting three main conclusions. First, activation of the VSOR anion channel is involved in induction of the apoptotic cell volume decrease, an early event in apoptosis. Second, cytosolic ATP within cells undergoing apoptosis is maintained at a level higher than in control cells even during the execution stage of apoptosis, and an increase in cytosolic ATP plays a critical role in apoptotic cell death. Third, protein kinases, including ASK and Akt, couple dysfunction in cell volume regulation to the apoptotic cell death process.

Andrés Stutzin (FONDAP Center for Molecular Studies of the Cell, University of Chile, Santiago, Chile) also discussed the role of ion channels in cell death. Persistent elevation of intracellular ROS observed in response to severe stress conditions or after exposure to toxic agents can cause necrosis. Intracellular calcium and sodium overload are recognized as critical steps in the events leading to necrosis. A fenamate-sensitive Ca^{2+}-activated, ATP-inhibited, nonselective cation channel (NSCC) has previously been implicated in free radical-induced rat liver cell necrosis.[22] Based on those observations, Stutzin's group went on to address the effect of reactive oxygen species (ROS) and oxidizing agents on the gating behavior of this channel. He showed that hydroxyl radicals increased the open probability (P_o) by $\sim$40% without affecting the unitary conductance. Also, exposure of the channel to the oxidizing agent 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) had a similar effect to $^{\bullet}$OH. The increase in P_o induced by $^{\bullet}$OH or DTNB was not reverted by preventing $^{\bullet}$OH formation or by DTNB washout, respectively. However, the reducing agent dithiothreitol completely reversed the effects on P_o of both $^{\bullet}$OH and DTNB. A similar increase in P_o was observed by applying the more physiologically relevant oxidizing agent GSSG. GSSG-oxidized channels showed enhanced sensitivity to Ca^{2+} and the effect of GSSG was fully reversed by GSH. These results implicate cysteine residues of the fenamate-sensitive NSCC protein as intracellular target site(s) for the action of oxidizing agents that induce epithelial cell necrosis.[23]

From a clinical perspective, Maurizio Taglialatela (University of Naples Federico II, Naples, Italy) analyzed the role of ion channels in neurodegenerative processes. He reported on recent studies showing the involvement of K^+ channels in neuronal cell death in *in vitro* models of AD. The results obtained suggest that cell death observed upon exposure of clonal and primary neurons to neurotoxic beta-amyloid peptides derived from the amyloid-associated protein involves Ca^{2+}-dependent increases in ROS, NF-κ*B* activation and, as a consequence enhanced expression of voltage-gated K^+

channels. Since decreased cytoplasmic K^+ concentrations represent an important prerequisite for cell death via apoptosis upon exposure to several neurotoxic insults, these observations are of considerable relevance to understanding AD, as well as for other neurodegenerative disorders.

Inflammation, Infection and Cell Death

Glucocorticoids mediate an impressive number of physiological responses in vertebrates. Both natural and synthetic glucocorticoids are widely used to control a variety of inflammatory diseases including asthma, inflammation, rheumatoid arthritis and many others. The actions of glucocorticoids are mediated via the glucocorticoid receptor (GR), commonly regarded as a hormone-dependent transcription factor, for which cortisol is the natural, endogenous ligand. The GR is the product of a single receptor gene expressed in all cells, yet glucocorticoids induce a plethora of diverse and tissue specific responses. The mechanisms responsible for generating this diversity of physiological actions are currently under intense scrutiny and recent studies have shown that multiple receptor isoforms can be generated from this gene via alternative splicing and alternative translation initiation mechanisms. The GR has three different promoters, each one regulated by different transcription factors, which give rise to three different mRNAs in humans. Complexity is increased by the alternative use of one out of eight initiator methionines present in the first $\sim$350 amino acids, resulting in isoforms A, B, C and D. John Cidlowski (National Institute of Environmental Health Sciences, NIH, North Carolina, USA) also discussed exciting new developments concerning the mechanisms of glucocorticoid action. In particular, he provided evidence that eight functionally distinct GRs are produced from a single gene, and furthermore that different cell types contain different forms of GRs.[24]

Gerald Pier (Harvard Medical School, Boston, MA, USA) addressed the topic of inflammation from a rather different perspective, by presenting data concerning the inflammatory response observed upon infection of cells from cystic fibrosis (CF) patients. CF is the most common autosomal recessive disease in Caucasians and is due to mutations on a single gene encoding a chloride channel termed CF transmembrane conductance regulator (CFTR). Inflammation is a critical early host response to infection; however, once the pathogen has been eliminated the tissue must return to its initial basal state. Apoptotic death of those cells on mucosal surfaces that encounter pathogens is key to reverting the initial inflammation process. To study the processes underlying initiation and termination of inflammation in response to infection, Pier and co-workers used cells and tissues from patients and transgenic mice with CF. Loss of functional CFTR protein leads to increased susceptibility to infection by the Gram-negative bacterium *Pseudomonas aeruginosa*. The presence of the intact CFTR was shown to be required both to initiate an appropriate, coordinated innate immune response to *P. aeruginosa* infection as well as to terminate the inflammatory response by inducing apoptotic death of lung epithelial cells and PMNs. CFTR initiates inflammation by activating nuclear translocation of NF-κB following *P. aeruginosa* binding to CFTR and entry of this complex into cells via lipid rafts. CFTR activity is also needed to change this initial proinflammatory cellular response to a proapoptosis response by increasing Fas-FasL expression in cells infected by *P. aeruginosa*. Thus, CFTR mutations and *P. aeruginosa* infection represent important tools to dissect the molecular components of effective inflammation and resolution on a mucosal surface and to understand how this is defective in CF.

Concluding Remarks

As highlighted in this brief meeting review, and perhaps not surprisingly, cell death is an extremely complex process. A plethora of different signaling pathways and genes were discussed, which lead to morphologically very distinct endpoints with often significant variations in the pathophysiological consequences for an organism. The discussion of this rapidly expanding field documented how existing views of cell death are being revisited. Perhaps exemplary in this respect was the unresolved discussion as to whether autophagy should be considered a cell survival strategy or a form of cell death. In recent years, insights to these processes have increased dramatically in complexity and diversity. Despite such mind-boggling conceptual heterogeneity, some key aspects and players common to different forms of cell death are emerging. Changes in the activity of ion channels and associated ion fluxes were attributed an important, but often neglected, role in events associated with apoptosis and necrosis at this meeting. Mitochondrial function or dysfunction is generally recognized as a key element in determining cellular well being. Many signaling pathways linked to cell death that either impinge on or are modulated by the mitochondria were discussed. An, until recently, unappreciated number of changes in mitochondrial morphology are now recognized as being intimately associated with decisions concerning cell life or death. Intricacies of the ER–mitochondria connection that are central to 'management' of cellular calcium levels were also highlighted. Furthermore, ROS were implicated as important modulators or effectors of cell death pathways. Insights to these different facets of cell death are opening up new exciting possibilities for therapeutic intervention in cancer, infectious and inflammatory diseases as well as neurodegenerative disorders, which were also addressed at the meeting.

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