



Cell Death Mechanisms and Their Implications in Toxicology

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Necrotic cell death was long regarded as the ultimate consequence of chemical toxicity and was thought to result from simple cell failure because of toxic interference with vital cell functions. Introduction of the novel concept of programmed cell death (PCD), or apoptosis, has changed this view dramatically. This development has been further stimulated by the characterization of several other genetically PCD modalities, such as autophagy and pyroptosis. Like apoptosis, these modes of cell death are governed by complex signaling networks, containing “switches” responsible for cross talk between them. Recruitment or repression of these cell death signaling networks by foreign chemicals can lead to acute as well as chronic toxicity. In many instances, such effects of toxicants are mediated by disruption/modulation of cellular Ca²⁺ homeostasis or increased generation of reactive oxygen species in the mitochondria or other intracellular compartments. Caspases, calpains, lysosomal proteases, and endonucleases are the main executioners of cell death, and they often co-operate during the execution stage of apoptosis. Finally, dead or dying cells are recognized and engulfed by phagocytes to prevent inflammation and associated tissue damage. Defective macrophage engulfment and degradation of cell corpses may also result from toxicity and can contribute to both the inflammatory response and dysregulation of tissue homeostasis. Hence, the cell death and phagocytosis regulatory networks offer a multitude of targets for toxic chemicals.

Key Words: apoptosis; autophagy; cell death mechanisms; toxicity; calcium; phagocytosis.

As toxicology and related basic sciences have developed significantly over the past century, the dose-response relationship for toxic agents has become increasingly complex. This relationship, which has long been regarded as part of the foundation of toxicology, can be straightforward for a measurable acute response. For example, toxic liver failure often manifests above a certain threshold and becomes progressively more severe with increasing exposure, until the liver finally succumbs to massive necrosis. Often, however, the exposure to

a chemical does not result in overt tissue injury, but may affect cell functions in a more subtle way, thereby increasing the susceptibility to other forms of damage. This can make assessment of a single dose-response related to an individual end point quite redundant. The problem becomes even more intricate when assessing the potential risk of chronic exposure to an agent that has no obvious acute effect but may still have long-term consequences. Therefore, in many cases, chronic toxicity has been inferred by extrapolating the acute effects seen at very high doses to possible delayed effects at low doses. This is particularly true for potential tumorigenic effects of chemicals. However, extrapolations of this type can be grossly misleading and, although pathways may be shared, the molecular mechanisms that result in the acute effects are often distinct from those responsible for chronic toxicity.

Cellular effects of foreign chemicals might involve recruitment or de-repression of cell death mechanisms that ultimately may turn out to be either protective (as in carcinogenesis) or deleterious (as in neurodegeneration) to the organism. Whether a cell survives or dies in the presence of a chemical insult is often determined by proliferative status, repair enzyme capacity, and the ability to induce proteins that either promote or inhibit the cell death process. The discovery that apoptosis and certain other cell death modalities can also be used to eliminate genetically damaged cells, thereby protecting the organism from cancer, has suggested the possibility that chronic exposure to potentially dangerous chemicals may act not only by triggering oncogenic reactions but also by inhibiting tumor surveillance. Conversely, the finding that components of the apoptotic program, such as caspases, can also be involved in nonapoptotic signaling pathways has pointed to the complexity of the cellular effects exerted by toxic chemicals (Li *et al.*, 2010).

With the advent of more sophisticated analytical technologies and molecular studies, it is now possible to identify chemico-biological interactions at very low concentrations. Understanding the underlying mechanisms, some of which are discussed in the following sections, allows a better prediction of the

consequences of exposure to low levels of toxicants and a safer assessment of the risks associated with environmental pollution.

APOPTOSIS: HISTORICAL PERSPECTIVES

Pathologists have long defined cell death by morphological criteria. From the classical iconography of cell death by Virchow to the current confocal and electron microscopical imaging, the appearance of the dead cell has been central to this definition. The first references to a distinct mode of cell death came from developmental studies. The concept of programmed cell death (PCD) refers to the type of cell death that occurs at specific time points as part of the normal development of the organism. Glücksmann clearly demonstrated the existence of this phenomenon already in 1951 (Glücksmann, 1951). This observation was followed by a surge of interest in PCD in the 1960s (Lockshin and Williams, 1965). In the 1970s, histochemical studies on lysosomal changes in hepatic ischemia provided experimental evidence for the existence of two distinct cell death modalities also in mammalian tissue (Kerr, 1971; Kerr *et al.*, 1972). The finding of rounded bodies in ischemic hepatic tissue suggested that this type of cell death was morphologically different from necrosis. Initially, this observation caused some confusion and the phenomenon was interpreted as a special form of necrosis, termed “shrinkage necrosis” (Kerr, 1971). The following year, Kerr, Wyllie, and Currie proposed the term “apoptosis” to denote the distinct morphological features associated with this form of cell death (Kerr *et al.*, 1972). Today, apoptosis and necrosis are viewed as separate modes of cell death with distinct morphological characteristics.

Notably, two modes of cell death with different morphologies had been recognized earlier in radiobiology and termed “reproductive cell death” and “interphase cell death” (Okada 1970). Reproductive cell death was characterized by its association with the mitotic cycle, whereas interphase cell death was independent of cell division and occurred before the cell entered the cell cycle. Interphase cell death was observed after exposure of resting and dividing and differentiated cells to a wide range of irradiation doses. Lymphoid cells were found to be the most sensitive to ionizing radiation and were characterized by death in interphase. Interestingly, both in apoptotic cell death, as defined by Kerr and colleagues, and in interphase cell death, the typical morphological appearance was attributed to DNA damage and nuclear collapse combined with cell shrinkage.

The major conceptual advance in the 1972 article by Kerr and colleagues was the postulate that apoptotic cell death was an active process involved in the physiological elimination of redundant cells as well as in pathological processes, such as cancer (Kerr *et al.*, 1972). Furthermore, cell death by apoptosis would compensate for cell proliferation and hence be responsible for the maintenance of tissue homeostasis. This link between cell death as a programmed event in tissue differentiation and homeostasis and cell death in disease was fundamen-

tally different from the prevailing idea that cell death would be caused by simple cell failure. However, the major breakthrough was the discovery that the PCD observed during the development of simple organisms, such as the nematode *Caenorhabditis elegans*, involved similar mechanisms and genetics as cell death by apoptosis in mammals, and that these programs had been conserved during evolution (Horvitz, 2003).

The early characterization of the fundamental processes involved in apoptotic cell death has since led to a variety of more or less stringent definitions of apoptosis. The discovery of caspases (cysteine-dependent aspartate-specific proteases) as the main executors of apoptosis introduced the definition of caspase-dependent and caspase-independent cell death. Although this distinction remains paradigmatic, it is now clear that executionary caspases, including caspase-3, are not activated solely during apoptosis but that they can also participate in other cellular processes. Energy requirement has been regarded as another characteristic feature of apoptotic execution (Ankarcrona *et al.*, 1995; Leist *et al.*, 1997), although other cell death modalities, such as autophagy, also require adenosine triphosphate (ATP). Further, the involvement of Bcl-2 family proteins has been yet another defining criterion of apoptotic cell death, until it became clear that Bcl-2 can also protect from both necrotic and autophagic cell death. Finally, the coexistence of different cell death modalities in pathological settings has indicated that cell death morphology may not be an appropriate defining parameter. A more general definition of cell death based on the predominant executioner pathway (e.g., the proteases that execute cell death) may be preferable to more complex definitions. This follows from the evolution of a field that started from a traditional histological description but has since evolved into an intricate cross talk of cellular signaling pathways.

APOPTOSIS: MORPHOLOGICAL CHARACTERISTICS, BIOCHEMISTRY, AND GENETICS

Apoptosis has been described as a form of cellular suicide because death appears to result from induction of active processes within the cell itself. The dying cell then undergoes rapid changes, which are reflected in both its structure and biochemistry. Morphologically, apoptosis is characterized by margination and condensation of nuclear chromatin (pyknosis), cytoplasmic shrinkage, nuclear fragmentation, and blebbing of the plasma membrane. The cell subsequently breaks up into membrane-enclosed fragments, termed apoptotic bodies, which are rapidly recognized and engulfed by neighboring cells or macrophages. Considerable biochemical changes occur within the apoptotic cell to facilitate neat packaging and removal of apoptotic bodies by phagocytosis. Modifications of the cytoskeleton and the cytoplasmic membranes are required for cell shrinkage to occur. During apoptosis, this results in the loss of cell-cell contact, untethering of the plasma membrane and rapid blebbing or zeiosis.

The efficient recognition of apoptotic cells by phagocytes requires rearrangement of the infrastructure and molecular composition of the plasma membrane in the dying cell. For example, alteration of the distribution of carbohydrates on the cell surface promotes preferential binding of macrophages to apoptotic cells. The loss of phospholipid asymmetry, resulting in the externalization of phosphatidylserine (PS), further facilitates the recognition of dying cells by macrophages. In fact, PS exposure on the apoptotic cell surface appears to be the most important “eat me” signal, although its binding to macrophages is usually not direct but mediated by a number of bridging molecules (Hanayama *et al.*, 2004). The development of fluorescently labeled Annexin-V, which binds specifically to PS residues on the cell surface, enables detection of apoptotic cells with externalized PS both *in vitro* and *in vivo*. However, it should be noted that under *in vitro* culture conditions, where phagocytic cells are normally absent, apoptotic cells and their fragments lyse in a process similar to necrosis. This is termed “secondary necrosis” or “post-apoptotic necrosis.”

Several years ago, it was reported that ATP is required for apoptotic cell death, and it was suggested that the level of intracellular ATP determines whether a cell will die by apoptosis or necrosis (Leist *et al.*, 1997; Tsujimoto, 1997). There is now abundant evidence for ATP requirement in multiple apoptosis models, although the critical ATP-dependent steps in the process have not been fully characterized. They are likely to be associated with both the signaling and the execution phases of apoptosis. For example, dATP/ATP is required for apoptosome formation leading to the activation of the caspase proteolytic cascade, and an active nuclear transport mechanism, which requires ATP hydrolysis, is involved in apoptotic nuclear changes. The latter would be in accordance with the previously published findings that ATP is required for the nuclear apoptosis induced by cytoplasmic extracts.

Our understanding of the genetic regulation of apoptosis is based primarily on studies of PCD in *C. elegans*, notably by H. R. Horvitz and his coworkers (Horvitz, 2003). This process involves a cell death protease (Ced-3), which is associated with activating (Ced-4) and inhibitory (Ced-9) proteins. Homologous proteins have been found in organisms throughout the animal kingdom, including insects, amphibians, and humans. Ced-3 is “the founding member” of the caspase family of cysteine proteases, and overexpression of these proteins results in cell death with apoptotic morphology in a variety of organisms.

TOXICITY-RELATED CELL DEATH

Although it is now evident that chemical toxicity might be associated with multiple modes of cell death, toxic cell death was initially thought to be of the necrotic type. Killing of hepatocytes by exposure to carbon tetrachloride or bromobenzene may serve as classical examples thereof. Bromobenzene-

induced cell death was studied intensely in the 1970s and found to be preceded by cytochrome P-450-mediated metabolic activation and glutathione depletion; it was usually monitored by cellular leakage of lactate dehydrogenase, or uptake of trypan blue, traditional assays of the increased plasma membrane permeability associated with necrotic cell death.

Following the recognition of apoptosis as a distinct mode of cell death, in 1987, Andrew Wyllie put forward two hypotheses related to its role in toxic cell death, namely (1) that apoptosis might be induced by injurious agents of lesser amplitude than those causing necrosis in the same cells and (2) that this might occur more readily in cell populations primed for apoptosis (Wyllie, 1987). Studies in our and other laboratories have since shown that a host of chemical agents can exert their toxicity via induction of apoptosis. Glucocorticoid-induced apoptosis in thymocytes and lymphocytes were early examples of such findings. It was subsequently reported that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin could also trigger apoptosis in thymocytes, although at concentrations much higher than those found to cause thymic atrophy *in vivo* (McConkey *et al.*, 1988). More recent studies have demonstrated that a variety of environmental contaminants, including heavy metals (Cu, Cd, MeHg, Pb), organotin compounds, and dithiocarbamates can also cause apoptotic cell death in various *in vitro* models. Interestingly, studies in lead-exposed mice have shown that retinal degeneration is associated with rod-selective apoptosis (He *et al.*, 2003). Disruption of intracellular Ca²⁺ homeostasis, or induction of mild oxidative stress, might mediate the apoptosis-inducing effects of some of these chemicals.

Activation of cell death might also contribute to the toxicity of certain representatives of the rapidly growing class of engineered nanomaterials. Hence, there are reports of nanoparticles that can trigger either autophagic, apoptotic, or necrotic cell death in primary cultures or cell lines. The doses required to induce cell death are usually high, and the relevance of these findings for *in vivo* toxicology is therefore uncertain. However, apoptotic macrophages have been observed in the airways of mice after inhalation of single-walled carbon nanotubes (Shvedova *et al.*, 2010). In most cases, enhanced generation of reactive oxygen species (ROS), leading to oxidative stress and inflammation, is thought to be responsible for nanoparticle toxicity (Nel *et al.*, 2006).

The interaction of nanomaterials with the immune system may have additional negative systemic effects. Macrophages exposed for days to superparamagnetic iron oxide nanoparticles can undergo apoptosis following the long-lasting activation of the c-Jun N-terminal kinase (JNK) pathway. JNK activation and cytotoxicity could be abolished by addition of antioxidants (Lunov *et al.*, 2010). When the macrophage scavenging ability is compromised, further cytotoxic events may follow. Insufficient scavenging of apoptotic cells can be at the base of degenerative disorders such as mucopolidosis type IV (MLIV), a lysosomal storage disease (Venkatachalam

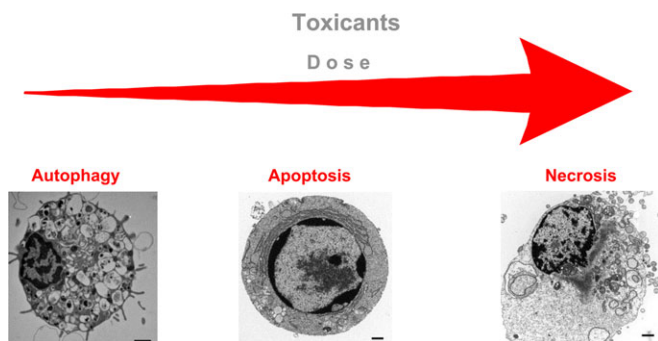


FIG. 1. Dose-response relationship of toxicant-induced modes of cell death. The mode of cell death triggered by some toxicants is dose dependent. Most often, exposure to low doses results in autophagy or apoptosis, whereas higher levels of the same toxicant might cause necrosis.

et al., 2008). Decreased clearance of apoptotic cells in MLIV models is because of disruption of the TRPML1 channel and results in both neurodegeneration and motor defects. Thus, it is reasonable to assume that low-level effects of neurotoxicants on phagocytes may cause disturbances in clearance of apoptotic cells and thereby subtle neurotoxic effects.

Also gain of function of the resident microglia, or circulating macrophages, may favor apoptosis and chronic neurodegenerative processes. One example may be the administration of the herbicide paraquat to rodents. According to Di Monte and colleagues (Purisai *et al.*, 2007), the neurotoxicity of paraquat, as well as other pro-oxidants that cross the blood brain barrier, may be because of a two-tiered effect, a priming of microglia and secondary injury to the neurons because of the combination of a proinflammatory response and a direct neuronal oxidative stress. The major source of the oxidative stress may, in fact, be the resident microglia.

Complex death paradigms may also be explained by activation of different death pathways depending on the dose of a given toxic agent (Fig. 1). An early example thereof was our finding that increasing concentrations of the redox-cycling quinone, 2,3-dimethoxy-1,4-naphthoquinone, stimulated proliferation, induced apoptosis, or caused necrosis in a pancreatic cell line (Dypbukt *et al.*, 1994). More recently, studies with arsenic trioxide revealed that low doses of this agent triggered Bax/Bak-dependent apoptosis, whereas higher doses caused mitochondrial permeability transition (MPT) and apoptotic/necrotic cell death. The latter observation illustrates the frequent finding *in vivo* that distinct cell death modalities may coexist within the same lesion and that interference with certain signaling pathways or critical cell functions might result in cell killing by a different mechanism. Postapoptotic necrosis due to intracellular Ca^{2+} overload because of caspase cleavage and inactivation of vital Ca^{2+} -extruding components in the plasma membrane is one example thereof.

SIGNALING PATHWAYS LEADING TO APOPTOSIS IN MAMMALIAN CELLS

Several major signaling pathways may lead to apoptosis in mammalian cells (for review, see Orrenius *et al.*, 2003) (Fig. 2). In the extrinsic, receptor-mediated pathway the ligation of surface receptors (e.g., CD95, TNFR1) is followed by the formation of the death-inducible signaling complex (DISC), resulting in the activation of procaspase-8. In type I cells, caspase-8 activates procaspase-3, which cleaves target proteins, leading to apoptosis. In type II cells, caspase-8 cleaves Bid, which, in turn, induces the translocation, oligomerization, and insertion of Bax and/or Bak into the mitochondrial outer membrane. This is followed by the release of several proteins from the mitochondrial intermembrane space, including cytochrome *c*, which forms a cytosolic apoptosome complex with Apaf-1 (apoptosis activating factor-1) and procaspase-9 in the presence of dATP. This results in the activation of procaspase-9, which triggers the caspase cascade by activation of procaspase-3.

In the intrinsic pathway, death signals act directly or indirectly on the mitochondria to cause the release of proapoptotic proteins from their intermembrane space. This cell death pathway is controlled by Bcl-2 family proteins (regulation of cytochrome *c* release), inhibitor of apoptosis proteins (IAPs) (inhibition of caspases), second mitochondrial activator of caspases (Smac), and Omi (negative regulation of IAPs). The intrinsic pathway may also operate via caspase-independent mechanisms, which involve the release from mitochondria and translocation to the nucleus of at least two proteins, apoptosis inducing factor (AIF) and endonuclease G (EndoG). Nuclear effects of AIF include chromatin condensation and formation of high-molecular-weight DNA fragments. The role of EndoG in cell death is still unclear. When DNA damage is the trigger of the apoptotic response, the initially activated caspase is procaspase-2. Its activation also leads to the release of cytochrome *c* and apoptosome formation, although the precise mechanisms for this are unclear.

Recently, a caspase-independent, granzyme A (GrA)-mediated pathway was described (Fan *et al.*, 2003). After being delivered into the cytoplasm of the target cell via perforin-mediated pores, GrA activates a caspase-independent pathway that is characterized by the formation of single-stranded DNA nicks and the appearance of apoptotic morphology. The endonuclease involved in the formation of DNA strand breaks in this system was identified as GrA-activated DNase (GAAD). GAAD activity is regulated by its specific inhibitor IGAAD, also known as the SET complex, which is located in the endoplasmic reticulum (ER). This complex contains an inhibitor of protein phosphatase 2A (pp32), the nucleosome assembly protein SET, HMG2, and apurinic endonuclease-1 (Ape1). In this pathway, GrA cleaves SET, HMG2, and Ape1, but not pp32, to release and activate GAAD. Active GAAD translocates into the nucleus to induce DNA strand breaks.

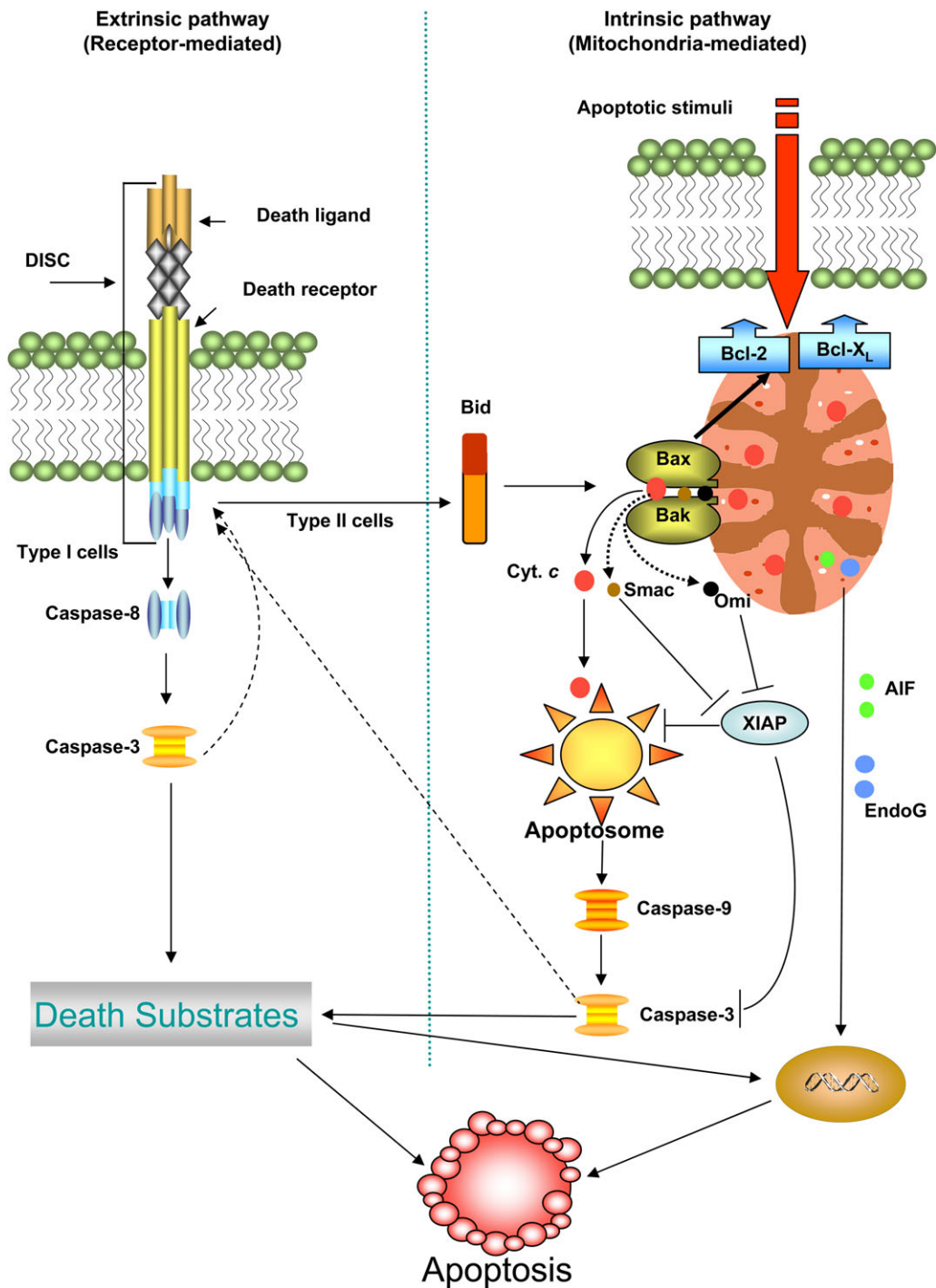


FIG. 2. Schematic illustration of apoptotic cell death pathways. The extrinsic pathway is triggered by ligands binding to receptors on the cell surface (TNFR1, CD95, TRAIL), resulting in their oligomerization, formation of DISC, and caspase-8 activation. In Type I cells, caspase-8 activates caspase-3, which results in the cleavage of a host of target proteins and apoptosis. In Type II cells, caspase-8 cleaves Bid, resulting in the engagement of the mitochondrial pathway. The latter involves the release of proapoptotic proteins (cytochrome *c*, Smac, Omi, AIF, and EndoG) from the mitochondrial intermembrane space into the cytosol via Bax/Bak-mediated pores in the outer membrane. Here, cytochrome *c*, Smac, and Omi participate in the activation of the caspase cascade, whereas AIF and EndoG are further translocated to the nucleus, where they participate in chromatin condensation and large-scale DNA fragmentation. X-Inhibitor of Apoptosis Protein is a cytosolic IAP, whose activity is blocked by Smac and Omi.

The accumulation of unfolded or misfolded proteins can lead to the activation of several ER proteins, which results in either inhibition of translation or induction of gene expression. Ca^{2+} storage and signaling, as well as folding, modifying, and sorting of newly synthesized proteins are among the main functions of the ER in mammalian cells. Disturbances in any of these functions can lead to so-called ER stress. Tunicamycin and a variety of other drugs can induce apoptosis by this mechanism. Prolonged ER stress stimulates the activation of procaspase-12 (Nakagawa *et al.*, 2000). This enzyme is localized in the ER membrane and cleaved and activated by calpain during ER stress or in response to the mobilization of intracellular Ca^{2+} stores. Procaspase-12 may also be activated by its recruitment into a complex with Ire1 α and adaptor protein TRAF-2 (tumor necrosis factor [TNF] receptor-associated factor-2), which is formed as part of the unfolded protein response pathway (Yoneda *et al.*, 2001). Once activated, caspase-12 acts on effector caspases to induce apoptosis. These findings indicate that ER stress, as caused by Ca^{2+} depletion or alterations in the Ca^{2+} transport systems, can be linked directly to caspase activation.

THE ROLE OF CALCIUM IN CELL DEATH

It has long been known that Ca^{2+} signals govern a host of vital cell functions and hence are necessary for cell survival. However, it is also well established that cellular Ca^{2+} overload, or perturbations of intracellular Ca^{2+} compartmentalization, may cause cytotoxicity and result in either apoptotic, necrotic, or autophagic cell death. Thus, apoptotic cell death can be brought about by a loss of Ca^{2+} homeostatic control, but can also be triggered, or modulated, by more subtle changes in Ca^{2+} distribution within intracellular compartments.

Historically, the role of the Ca^{2+} ion as a death trigger dates back to Fleckenstein's observation that excess Ca^{2+} entry into cardiomyocytes might be the mechanism that underlies cardiac pathology after ischemia (Fleckenstein *et al.*, 1974). Subsequent studies emphasized the general importance of this observation, as both receptor overstimulation (Leonard and Salpeter, 1979) and cytotoxic agents (Trump and Berezsky, 1995) were found to cause lethal Ca^{2+} influx into cells. More recently, it became clear that also non-disruptive changes in Ca^{2+} signaling could have adverse effects, including the induction of apoptosis. For example, interference with Ca^{2+} sequestration by the ER can be sufficient to trigger apoptosis as part of a stress response. In addition, Ca^{2+} -dependent processes can be responsible for the processing of AIF, resulting in its mobilization from the mitochondria and release into the cytosol (Norberg *et al.*, 2008), or recruited to warrant the final elimination of dead or dying cells by promoting either their phagocytosis or their lysis.

The Endoplasmic Reticulum, Ca^{2+} , and Apoptosis

An early link between Ca^{2+} and apoptosis was the finding that Ca^{2+} induced a typically apoptotic, ladder-like DNA fragmentation pattern in isolated thymocyte nuclei through the activation of a Ca^{2+} - and Mg^{2+} -dependent endonuclease (Wyllie, 1980). Subsequent dissection of the mechanism of glucocorticoid-induced apoptosis in thymocytes revealed that extracellular Ca^{2+} was necessary for cell death to occur. More recent work has shown that the type 3 inositol-1,4,5-trisphosphate ($\text{Ins}[1,4,5]\text{P}_3$) receptor was upregulated in lymphocytes undergoing IgM- or dexamethasone-induced apoptosis and that the augmented receptor population was localized to the plasma membrane (Khan *et al.*, 1996). This finding provided an explanation for the need of extracellular Ca^{2+} in lymphocyte apoptosis. Moreover, another study has documented that T lymphocytes deficient in type 1 $\text{Ins}(1,4,5)\text{P}_3$ receptors were resistant to apoptosis induced by multiple triggers and that this resistance could be reversed by pharmacologically raising cytoplasmic Ca^{2+} levels (Jayaraman and Marks, 1997). Taken together, all these observations suggest that Ca^{2+} is an important trigger of apoptosis in lymphocytes.

Mitochondria and Ca^{2+} Signaling

The mitochondria have long been known to actively participate in intracellular Ca^{2+} compartmentalization (Carafoli, 2002). They take up Ca^{2+} electrophoretically from the cytosol through a uniporter transporter and can release it again via several different routes. The affinity for Ca^{2+} of the uniporter is low, and the size of the mitochondrial Ca^{2+} pool is small under physiological conditions. However, much larger amounts of Ca^{2+} can accumulate in the mitochondria under pathological conditions, when intracellular Ca^{2+} concentrations rise (Thor *et al.*, 1984).

Hence, for many years, mitochondrial Ca^{2+} uptake was regarded mainly as a safety device in situations of temporary intracellular Ca^{2+} overload. However, now this view has changed thanks to the development of novel indicators, which can sense Ca^{2+} fluctuations in specific intracellular compartments (Rizzuto *et al.*, 1992). As a result of this technology, it has become apparent that mitochondrial Ca^{2+} fluxes are integrated parts of cellular Ca^{2+} signaling. The low affinity of the mitochondrial Ca^{2+} import system is overcome by the proximity of the mitochondria and the ER and by the creation of Ca^{2+} "hot spots" at the mouth of release channels, where the local Ca^{2+} concentration may reach very high levels (Hajnoczky *et al.*, 1995). The resulting uptake of Ca^{2+} by the mitochondria stimulates the Ca^{2+} -sensitive matrix dehydrogenases, which are key sites of nicotinamide-adenine dinucleotide, reduced form (NADH) production for the respiratory chain and thereby for stimulation of mitochondrial ATP and ROS production. The importance of this mechanism for normal cellular energy metabolism has recently been demonstrated convincingly (Cardenas *et al.*, 2010).

Ca²⁺-Mediated Mitochondrial Permeabilization

As discussed above, recruitment of the mitochondrial pathway of apoptosis signaling results in the permeabilization of the outer mitochondrial membrane (OMM) and the release of a host of mitochondrial proteins, some of which trigger distinct apoptotic events in other subcellular compartments. OMM permeabilization may be achieved by several different mechanisms, including pore formation by proapoptotic Bcl-2 family proteins (notably Bax and Bak) and membrane rupture as a result of mitochondrial swelling (for review, see Orrenius *et al.*, 2007). Except for the activation of Bad by calcineurin-catalyzed dephosphorylation, there is no established role for Ca²⁺ in the activation or translocation of the Bcl-2 family proteins during apoptosis. Instead, Ca²⁺ can trigger another mechanism of OMM permeabilization, which involves the opening of a permeability transition pore (PTP) made of a large proteinaceous complex whose exact composition is still unclear. The pore has been localized to the contact sites between the inner and OMMs and behaves as a voltage-operated channel. Pore opening is activated by high level of Ca²⁺ in the mitochondrial matrix and is further stimulated by oxidative stress, pyridine nucleotide and thiol oxidation, alkalinization, and low transmembrane potential. Initially, rapid and stochastic opening and closing of the pore is observed. Persistent pore opening allows Ca²⁺ and low-molecular-weight matrix components ($M_r < 1500$) to escape rapidly from mitochondria, whereas influx of water and solutes from the cytosol result in mitochondrial swelling and membrane rupture.

Apoptosis by Mitochondrial Permeabilization

A number of treatments have been found to trigger apoptosis via Ca²⁺-mediated mitochondrial permeability transition (MPT) in a variety of cell types. Such agents include Ca²⁺ ionophores and thapsigargin, neurotoxins, chemotherapeutics, and prooxidants (e.g., arachidonic acid and peroxynitrite) (Orrenius *et al.*, 2003). Cell death can sometimes be prevented by inhibitors of mitochondrial Ca²⁺ uptake or PTP formation, such as ruthenium red and cyclosporin A. PTP formation can result in the release from the mitochondria of cytochrome *c*, AIF, and other apoptogenic proteins. However, this is not always the case, and it has been speculated that only a fraction of the mitochondrial population may undergo permeability transition and release cytochrome *c*. Or resealing of PTP may occur, which allows the mitochondria to recover in spite of the partial loss of cytochrome *c* and other proteins from their intermembrane space (Petronilli *et al.*, 2001). Of particular interest is the observation that apoptotic stimuli, notably ceramide, can induce a switch in mitochondrial Ca²⁺ signaling at the beginning of the apoptotic process by facilitating Ca²⁺-induced opening of the PTP (Szalai *et al.*, 1999). This is in accordance with the recent observation that resistance of leukemic cells to 2-chlorodeoxyadenosine (CDA) was associ-

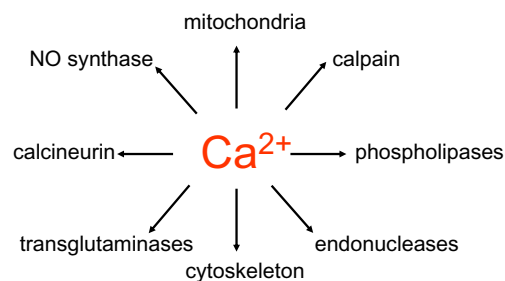


FIG. 3. Some intracellular targets in Ca²⁺-mediated cytotoxicity. Many toxicants cause modulation/disruption of Ca²⁺ signaling, which might result in the activation of multiple cytotoxic mechanisms, including MPT and disruption of cytoskeletal organization. Ca²⁺ activation of NO synthases, calcineurin, transglutaminase, calpain, phospholipases, or endonucleases has been shown to be involved in various forms of apoptotic cell death.

ated with an increased ability of their mitochondria to sequester Ca²⁺ without concomitant PTP opening. The CDA-resistant cells were selectively cross-resistant to thapsigargin but not to staurosporine- or Fas-induced apoptosis.

Ca²⁺-Activated Effector Mechanisms

There are multiple potential targets for Ca²⁺ signaling in apoptosis, including Ca²⁺-dependent protein kinases and phosphatases, nitric oxide synthases, endonucleases, transglutaminases, phospholipases, and proteases (Fig. 3). Each one of these targets has been reported to be involved in apoptosis signaling in some experimental model system. Of particular interest in this context, however, is the role of the Ca²⁺-dependent proteases, the calpains.

Although the vast majority of studies have focused on caspases, there is considerable evidence that additional proteases, including lysosomal cathepsins and members of the calpain family of Ca²⁺-activated cysteine proteases, participate in apoptosis. Results of inhibitor studies indicate that the contribution of calpain to apoptosis seems to be more prominent in certain cell types, for example, thymocytes, monocytes, cardiomyocytes, and neuronal cells (Vanags *et al.*, 1996). In addition, calpain has been reported to cleave the endogenous calcineurin inhibitor cain/cabin 1, which results in the activation of calcineurin and the promotion of Ca²⁺-triggered cell death (Kim *et al.*, 2002). Moreover, accumulating data indicate that cross talk between calpains and caspases exists in the regulation of the apoptotic process and that a host of cellular proteins can be cleaved by both calpains and caspases during apoptosis.

Recent studies have revealed a novel function of Ca²⁺/calpain in apoptosis signaling, notably in the processing of mitochondrial AIF (Norberg *et al.*, 2008). This flavoprotein is anchored to the mitochondrial inner membrane by part of its peptide chain and needs to be cleaved before a 57-kDa apoptotic fragment can be released into the cytosol for further transport to the nuclear matrix, where it promotes large-scale

DNA fragmentation. Responsible for this AIF cleavage is a calpain localized to the intermembrane space of the mitochondria. It is activated by a sustained elevation of the cytosolic Ca^{2+} level, and AIF proteolysis is further stimulated by the oxidative modification of AIF by mitochondrially produced ROS, which increases calpain affinity (Norberg *et al.*, 2010a). In certain cancer cells and cortical neurons treated with protein kinase C inhibitors, for example, staurosporine, the activating Ca^{2+} signal originates from the import of extracellular Ca^{2+} through a hyperpolarization-activated cyclic nucleotide-gated (HCN) ion channel in the plasma membrane.

Caspases and Intracellular Ion Homeostasis

As discussed above, Ca^{2+} signals can trigger apoptosis and lead to the activation of caspases, which may in turn cleave key components of the signaling system to cause perturbation of Ca^{2+} homeostasis. For example, it has been shown that caspase-3 can cleave the type 1 $\text{Ins}(1,4,5)\text{P}_3$ receptor in apoptotic cells and that such cleavage resulted in a decrease in channel activity as the receptor was degraded (Hirota *et al.*, 1999). In addition, caspase-3 can also cleave the plasma membrane Ca^{2+} -ATPase (Schwab *et al.*, 2002). This enzyme is of critical importance for the long-term control of cellular Ca^{2+} homeostasis, and cleavage-associated loss of function during apoptosis results in Ca^{2+} overload and secondary cell lysis or necrosis. Moreover, the $\text{Na}^+/\text{Ca}^{2+}$ -exchanger is also cleaved by caspase-3 during apoptosis, which further decreases the ability of the apoptotic cell to extrude excess Ca^{2+} .

The Na^+ , K^+ -ATPase is another ion pump in the plasma membrane that can act as a caspase substrate during apoptosis. Previous studies have shown that volume shrinkage in apoptotic cells is accompanied by a net efflux of ions because of the inactivation of Na^+ , K^+ -ATPase (Mann *et al.*, 2001) and that apoptosis is accelerated via a Bcl-2-sensitive activation of outward K^+ currents. The β -subunit of the Na^+ , K^+ -ATPase, which regulates the K^+ affinity of the enzyme at the extracellular site, seems to be the caspase target. Apart from a role in cell shrinkage during apoptosis, inactivation of the Na^+ , K^+ -ATPase may promote the apoptotic process by lowering the intracellular K^+ concentration to levels that are compatible with optimal caspase activity (Vu *et al.*, 2001).

Ca^{2+} and the Phagocytosis of Apoptotic Cells

Apoptotic cell death is terminated by the phagocytosis of the dying cell by macrophages or neighboring cells. Exposure of PS on the cell surface is the most important “eat me” signal used by apoptotic cells. Phospholipids are distributed asymmetrically across the plasma membrane, with the bulk of phosphatidylethanolamine, and all the PS, present in the inner leaflet of the plasma membrane in healthy cells. In apoptotic cells, this situation is changed, such that part of the PS pool is

translocated to the cell surface, whereas the distribution of the other major phospholipids is not affected. PS exposure stimulates phagocytic activity via several bridging molecules and receptors (Fadeel *et al.*, 2010; Savill *et al.*, 1992). Of toxicological interest is the recent observation that functionalization of carbon nanotubes with PS targeted the nanotubes to professional phagocytes, including monocyte-derived macrophages, dendritic cells, and microglia (Shvedova *et al.*, 2010). Hence, PS can obviously serve as an “eat me” signal not only for apoptotic/dying cells but also for engineered nanoparticles.

In the plasma membrane, the distribution of PS between the two leaflets is regulated by a Ca^{2+} -dependent scramblase that catalyzes the nonspecific randomization of phospholipids across the bilayer, and an ATP-dependent aminophospholipid translocase that mediates movement of aminophospholipids from the outer to the inner leaflet. Although their relative contribution to the regulation of phospholipid asymmetry in the plasma membrane is not known in detail, it seems that the aminophospholipid translocase is critically dependent on the intracellular ATP level for activity but is inhibited by Ca^{2+} . A comparison of PS exposure in several different cell lines that undergo Fas-mediated apoptosis has shown that it was better correlated to differences in translocase than in scramblase activity and that the upregulation of scramblase activity had little effect on the extent of PS exposure (Fadeel *et al.*, 1999). This is in contrast to findings with platelets, which also have an apoptosis-like death program (Vanags *et al.*, 1997), where elevation of the cytosolic Ca^{2+} concentration with thapsigargin triggered large amounts of PS exposure within minutes. In thapsigargin-treated Jurkat cells or U937 promonocytes, however, much fewer cells exposed PS, and it took up to 6 h for this to occur (Hampton *et al.*, 1996). In addition, in TNF- or Fas-treated Jurkat cells, chelation of intracellular Ca^{2+} had no effect on PS exposure, whereas removal of extracellular Ca^{2+} caused a ~50% inhibition. Hence, at present, one can only conclude that PS exposure in apoptosis is Ca^{2+} dependent, but not Ca^{2+} regulated, whereas the same process in platelets is Ca^{2+} regulated. However, the exact mechanisms by which Ca^{2+} regulates PS exposure in platelets remain to be elucidated.

OXIDATIVE STRESS AND CELL DEATH

Oxidative stress is generally defined as an imbalance that favors the production of ROS over antioxidant defenses. Among other effects, this results in the oxidation of protein thiols and a shift in cellular redox signaling (Kemp *et al.*, 2008). The majority of ROS are products of mitochondrial respiration. About 1–2% of the molecular oxygen consumed during normal respiration is converted into superoxide anion radicals (O_2^-), which can be regarded as the precursors of most ROS. The mitochondrial electron transport chain contains several redox centers that may leak electrons to

molecular oxygen, serving as the primary source of superoxide production in most tissues. According to one estimation, the steady-state concentration of O_2^- in the mitochondrial matrix is about 5- to 10-fold higher than that in the cytosol or nuclear matrix (Cadenas and Davies, 2000). The dismutation of superoxide anions by superoxide dismutase (SOD) results in H_2O_2 production. Subsequent interaction of H_2O_2 and O_2^- in a Haber-Weiss reaction, or Fe^{2+} - (or Cu^{2+})-driven cleavage of H_2O_2 in a Fenton reaction, can generate the highly reactive and toxic hydroxyl radical (Toninello *et al.*, 2004). Monoamine oxidase (MAO), a flavoprotein localized on the OMM, is another important source of H_2O_2 (Toninello *et al.*, 2004). Because H_2O_2 easily passes through mitochondrial membranes, MAO can contribute to an increase in its steady-state concentrations in both the mitochondrial matrix and cytosol.

Targets of ROS

ROS generated by mitochondria, or elsewhere in the cell, can cause damage to cellular macromolecules, including nucleic acids, phospholipids (oxidation of polyunsaturated fatty acids), and proteins (oxidation of SH-groups, formation of protein carbonyls, etc.). Mitochondrial DNA represents a critical cellular target for oxidative damage. It is especially susceptible to attack by ROS because of the close proximity to the respiratory chain, the major locus for superoxide production, and the lack of protective histones. For example, the level of oxidatively modified bases in mtDNA is 10- to 20-fold higher than that in nuclear DNA. Oxidative damage induced by ROS is probably a major source of mitochondrial genomic instability leading to respiratory dysfunction.

An important mechanism of O_2^- toxicity is the direct oxidation and inactivation of iron-sulfur (Fe-S) proteins, such as aconitases, and the associated release of iron (Fridovich, 1997). The inactivation of mitochondrial aconitase results in the simultaneous release of Fe^{2+} and H_2O_2 ; ingredients of the Haber-Weiss and Fenton reactions, which can result in generation of the potent hydroxyl radical that can oxidize mitochondrial proteins, DNA, and lipids, thereby amplifying O_2^- -initiated oxidative damage. Mitochondrial aconitase plays a key role in the Krebs cycle, catalyzing the conversion of citrate to isocitrate. Hence, inhibition of aconitase might result in Krebs cycle dysfunction and thereby have an impact on energy production and cell viability.

As mentioned above, a potentially deleterious effect of oxidative stress is the facilitation of Ca^{2+} -dependent MPT, which plays a key role in some modes of cell death. The basic unit of the PTP is the VDAC-ANT-CyP-D (voltage-dependent anion channel—adenine nucleotide translocase—cyclophilin D) complex. Recent studies identified the ANT as one important target for ROS induced by anticancer drugs, such as doxorubicin and arsenic trioxide. In particular, it was demonstrated that doxorubicin-induced cardiac toxicity correlates with oxidation of SH-groups in ANT and a decrease in

ANT protein concentration, leading to inhibition of mitochondrial respiration and increased probability of MPT induction.

Recent observations have questioned the importance of MPT for the release of cytochrome *c* from the mitochondria under apoptotic conditions. Thus, overexpression of cyclophilin-D had opposite effects on apoptosis and necrosis, whereas NO-induced necrosis was promoted, NO- and staurosporine-induced apoptosis was inhibited. These findings suggest that MPT leads to cell necrosis but argue against its involvement in apoptosis (Li *et al.*, 2004). Similarly, CyP-D-deficient cells died normally in response to various apoptotic stimuli but were resistant to necrotic cell death induced by ROS and Ca^{2+} overload. In addition, CyP-D-deficient mice showed resistance to ischemia/reperfusion-induced cardiac injury. These results suggest that the cyclophilin-D-dependent MPT mediates some forms of necrotic but not apoptotic cell death (Baines *et al.*, 2005; Nakagawa *et al.*, 2005).

Oxidation of Cardiolipin Facilitates Cytochrome C Release

Cytochrome *c* is normally bound to the inner mitochondrial membrane (IMM) by an association with the anionic phospholipid, cardiolipin. Cardiolipin is present only in mitochondria and is found primarily in the IMM. The molecular interaction between cardiolipin and cytochrome *c* involves electrostatic as well as hydrophobic interactions and hydrogen bonding. In fact, one of the acyl chains of cardiolipin is inserted into a hydrophobic channel in cytochrome *c*, whereas the other extends into the phospholipid bilayer (Tuominen *et al.*, 2002).

Unlike other phospholipids, the fatty acyl groups on cardiolipin are essentially restricted to C18 chains. The dominant C18 chain in mammals is linoleoyl (18:2), with oleoyl (18:1), and linolenoyl (18:3) also being present. The unsaturated nature of the acyl chains confers functional specialization to cardiolipin, which appears to be required for optimal function of many of the proteins involved in mitochondrial energy metabolism.

Because the bulk of cytochrome *c* is bound to the IMM, it appears that the electrostatic and hydrophobic interactions between cardiolipin and the hemoprotein must be “breached” in order for cytochrome *c* to leave the mitochondria. It was early found that cardiolipin oxidation decreases its binding affinity for cytochrome *c* and, more recently, that oxidative modification of cardiolipin facilitates cytochrome *c* mobilization from the IMM (Ott *et al.*, 2002). Based on these results, we hypothesized that cytochrome *c* release during apoptosis should occur by a two-step process, involving first the detachment of the hemoprotein from the IMM, followed by permeabilization of the OMM and the release of cytochrome *c* into the soluble cytoplasm.

Selective peroxidation of cardiolipin has since been demonstrated by Kagan and colleagues to precede cytochrome

c release during apoptosis (Kagan *et al.*, 2005). Investigating the mechanism of cardiolipin oxidation, the authors found that cytochrome *c*, in complex with cardiolipin, catalyzes H₂O₂-dependent cardiolipin peroxidation which, in turn, facilitates the detachment of cytochrome *c* from the outer surface of the IMM and its subsequent release into the cytoplasm through pores in the OMM. Quantitative characterization of the peroxidase activity of cytochrome *c* revealed that at low ionic strength and high cardiolipin-cytochrome *c* ratio, the peroxidase activity of the cardiolipin/cytochrome *c* complex was increased more than 50-fold. This catalytic activity correlates with partial unfolding of cytochrome *c*, and an increase in the peroxidase activity preceded the loss of protein tertiary structure. It seems that electrostatic cardiolipin-cytochrome *c* interactions are central to the initiation of the peroxidase activity, whereas hydrophobic interactions are involved when the tertiary structure of cytochrome *c* is lost (Belikova *et al.*, 2006). The finding that cytochrome *c* might change its structure during apoptosis was unexpected because several groups have shown that even small changes in the structure of cytochrome *c* resulted in abrogation of its proapoptotic function (Hampton *et al.*, 1998). In the presence of cardiolipin *in vitro*, cytochrome *c* peroxidase activity was activated at lower hydrogen peroxide concentrations than seen with cytochrome *c* in the absence of phospholipid (Belikova *et al.*, 2006). Altogether this suggests that redistribution of cardiolipin in the mitochondrial membranes combined with increased production of hydrogen peroxide can switch on the peroxidase activity of cytochrome *c* and cardiolipin oxidation in mitochondria and that this might be a prerequisite step in the execution of apoptosis. The peroxidase function of the cardiolipin-cytochrome *c* complex is compatible with the proposed “two-step” hypothesis of cytochrome *c* release and also provides an explanation for the protective effects reported for multiple mitochondrial antioxidant enzymes (Orrenius *et al.*, 2007). It also emphasizes the importance of mitochondrial H₂O₂ production as an integrated component of the apoptotic program.

CELLULAR STRESS AND APOPTOSIS

The effector phase of apoptosis typically involves signaling through protein kinase cascades, resulting in changes in substrate conformation and/or interactions as well as altered gene expression. Mitogen-activated protein kinase (MAPK) cascades are among the best-characterized signaling cascades (Hagemann and Blank, 2001). Both stress- and growth-regulating signals are transduced from the cell surface to the nucleus via these cascades. Of the two major MAPK pathways, the extracellular signal kinase pathway is activated in response to mitogens and transduces survival, proliferation, and differentiation signals. Signaling in response to chemical and environmental stresses, and by proinflammatory cytokines,

activates the stress-activated protein kinase (SAPK) pathway, which includes p38 and JNKs (Davis, 2000).

A role for MEKK, a protein in the SAPK pathways, was shown in response to survival factor withdrawal and in apoptotic signaling induced by UV irradiation, DNA-damaging agents, and TNF. All these agents induce caspase-mediated cleavage of full-length 196-kDa MEKK1 into a C-terminal 91-kDa fragment (Hagemann and Blank, 2001). It seems that the full-length protein transduces survival signals, whereas a cleavable fragment participates in, and amplifies, apoptosis signaling. The proapoptotic function of the 91-kDa fragment was indicated by its ability to induce apoptosis in several cell types and to sensitize cells to genotoxic damage. Cleavage of MEKK is not required for JNK activation (Widmann *et al.*, 1998). However, in ES cells isolated from Mekk1-deficient mice, JNK activation in response to microtubule-destabilizing drugs, serum deprivation, and cold shock was impaired. In response to DNA-damaging drugs, JNK is activated, and studies with antisense JNK, dominant-negative JNK mutants, and c-Jun-deficient cells revealed decreased sensitivity to apoptosis. In contrast, a protective function of JNK was suggested by increased sensitivity to cisplatin as a result of expression of a non-phosphorylated c-Jun mutant (Hagemann and Blank, 2001). UV treatment of wild-type (wt) MEFs induced activation of Bax but failed to do so in Jun null MEFs, indicating that JNK might be involved in the regulation of Bax. Bcl-2 family proteins may serve as potential molecular links between JNK and the mitochondrial events. Several studies have shown that JNK can phosphorylate Bcl-2 and Bcl-X_L *in vitro* and in intact cells. Mutation of the phosphorylation sites in both proteins increases the anti-apoptotic capacity of either protein (Kharbanda *et al.*, 2000).

Heat-shock proteins (HSPs) are a set of evolutionarily conserved proteins, which are synthesized, or activated in response to cellular stress. The main function of these proteins is to afford protection to the cell (Jaattela, 1999). When the stress is removed, the cells regain normal function, and the HSP level drops back to normal. There is evidence suggesting that the HSP induction results in acquisition of tolerance to higher doses of stress, which might otherwise be lethal to the cell. Hence, it was demonstrated that mild heat-shock induced rapid synthesis of several HSPs and that heat-shocked cells were less susceptible to environmental stresses, probably by resisting apoptosis. In fact, thermotolerant, HSP-overexpressing cells are known to be more resistant to cell death induced by a variety of stimuli, supporting a more general role for HSPs in apoptosis resistance.

INFLAMMATION AND CELL DEATH

It is well known that efficient phagocytosis of dead or dying cells prevents the development of inflammation during apoptosis (Kerr *et al.*, 1972). Recently, it was shown that

cellular stress or infections promote the maturation of proinflammatory cytokines, such as interleukin-1 β , to engage innate immune defence. This activation occurs on molecular platforms, known as inflammasomes (Martinon and Tschopp, 2004). Several caspases can act as proinflammatory enzymes. Among them are caspase-1, -11, and -12 in the mouse and caspase-1, -4, and -5 in humans. Caspase-12 is mutated to encode a nonfunctional protein in most human populations. Among the proinflammatory caspases, caspase-1 is the best characterized. Its catalytic activity is tightly regulated by signal-dependent autoactivation within inflammasomes that mediate caspase-1-dependent processing of cytokines, for example, IL-1 β .

Inflammasome-mediated caspase-1 activation can result in an inflammatory form of cell death known as pyroptosis, which occurs most frequently upon infection with intracellular pathogens and is likely to be part of the antimicrobial response (Fink and Cookson, 2007). Pyroptosis is caspase-1-dependent by definition and occurs independently of other proapoptotic caspases. Although pyroptosis is considered to be a form of PCD, it is distinct from the immunologically silent mode of cell death characteristic of apoptosis. Pyroptosis is accompanied by plasma membrane rupture, water influx, cellular swelling, osmotic lysis, and the release of proinflammatory cellular content. The regulation of pyroptosis is not well defined; however, the extent of pyroptosis appears to increase with increasing inflammasome stimulation, suggesting that pyroptosis might occur when cytoprotection by autophagy is lost, or when mechanisms suppressing cytosolic caspase-1 action become overwhelmed.

EXECUTION OF APOPTOSIS

The execution stage of apoptosis involves the proper function of several enzyme systems activated through elaborate signaling pathways. The proteolytic activity of caspases provides a biochemical basis for the apoptotic phenotype (Vaculova and Zhivotovsky, 2008). As mentioned above, caspases constitute a family of proteases, which are synthesized as proenzymes with very low intrinsic activity and therefore require activation, either by proteolytic maturation or by interaction with an allosteric activator. Proteolytic automaturation might be the result of activation of a so-called “high proximity mechanism.” Based on the size of the pro-domain, caspases can be divided into long and short pro-domain containing enzymes. Long pro-domain caspases, that is, caspase-2, -8, -9, and -10, belong to the group of initiator caspases, whereas short pro-domain caspases, that is, caspase-3, -6, and -7, belong to the group of effector enzymes.

The effects of caspases in apoptosis are accomplished by the cleavage of numerous proteins located in the cytoskeleton, cytoplasm, and nucleus. Structural components, such as nuclear lamins and cytoskeletal proteins, are cleaved by

caspases, and this cleavage precedes nuclear condensation and membrane blebbing. Exposure of PS on the cell surface, the prime “eat me” signal of apoptotic cells, in some experimental models is blocked by caspase inhibitors. Furthermore, caspases cleave negative regulators of apoptosis and either inactivate them or produce fragments that promote cell death.

As discussed above, the calpains are also critically involved in cell death execution, and cross talk between caspases and calpain is important in the regulation of this process (Orrenius *et al.*, 2003). Proteins that can be cleaved by both calpain and caspases include procaspase-3 and -7, Bcl-2 family proteins, structural proteins, signal transduction proteins, DNA repair and cell-cycle regulatory proteins, glutamate receptors, and several unclassified proteins, such as Ins(1,4,5)P₃R and calcineurin.

Although both calpain and caspases are cysteine proteases, their functions and cleavage specificity are different. Caspases contain a conserved QACXG pentapeptide with the active-site cysteine, and they have a unique preference for cleavage of the peptide bond carboxy-terminal to aspartate residues. This cleavage specificity is rare among proteases, and only the serine protease, granzyme B, has a similar cleavage specificity. The calpains include several tissue-specific isoforms (*n*-calpains) and two ubiquitous isozymes (μ -calpain and *m*-calpain) (Kelly *et al.*, 2009). For activation, μ - and *m*-calpains require micromolar and millimolar Ca²⁺ concentrations, respectively. Compared with caspases, calpains do not exhibit a strict cleavage specificity. Calpain activity is regulated by the endogenous inhibitor, calpastatin. Interestingly, in several experimental models of apoptosis, it has been shown that calpastatin is cleaved by caspase-3 and that this cleavage is essential for regulation of calpain activity during cell death.

Calpains can also cleave caspases, and most often, this cleavage inactivates caspase function. For example, calpain cleaves caspase-7 at sites distinct from those cleaved by the upstream caspases, generating proteolytically inactive fragments. Procaspase-8 and -9 can also be cleaved by calpains, and truncated caspase-9 is unable to activate procaspase-3. Finally, it has been reported that procaspase-3 cleavage by calpain results in the generation of a 29-kDa fragment, although it is unclear whether such cleavage causes the activation or inactivation of caspase-3 function. A recent study reports that, in the early execution phase, calpain down-regulates caspase-3 activity and slows progression of apoptotic nuclear morphology, whereas subsequent calpain activity, facilitated by caspase-mediated degradation of calpastatin, contributes to plasma membrane disruption and secondary necrosis (for review, see Orrenius *et al.* [2003]).

The lytic nature of lysosomal enzymes and their release in injured cells suggested early that they might be responsible for cell necrosis in damaged tissues. Already in the 1960s, De Duve developed the concept of the lysosome as a “suicide bag” (De Duve, 1963). This hypothesis stated that vesicle-enclosed

acid hydrolases can be released into the surrounding cytoplasm under appropriate conditions and bring about its ultimate destruction. This might occur during cellular injury, but lysosomes are relatively stable organelles, which disintegrate and release their contents after cell death rather than before. Recently, lysosomal activity was also implicated in apoptosis (Kroemer and Jaattela, 2005). Importantly, it has been shown that several cathepsins can cleave the proapoptotic Bcl-2 protein, Bid, leading to Bax activation. However, current evidence supports a role for lysosomal enzymes in the amplification, rather than initiation, of the apoptotic process.

During apoptosis, three patterns of DNA degradation are known to occur, namely single-strand nicks and chromatin cleavage with the formation of large (50–200 kbp) fragments as well as nucleosome-sized (180–200 bp) fragments, producing the ladder pattern on agarose gels that has long been regarded as a hallmark of apoptosis (Wyllie, 1980). The apoptotic endonucleases cleave DNA at the exposed linker regions, and it was suggested that cleavage activity is topologically constrained rather than sequence constrained. Although the detailed mechanisms of DNA fragmentation in apoptotic cells are still unclear, in 1988, it was reported that the endonuclease that is responsible for internucleosomal DNA cleavage is a caspase-activated endonuclease (CAD)/DNA fragmentation factor (DFF40) (Liu *et al.*, 1998; Sakahira *et al.*, 1998). The function of this enzyme is controlled by the inhibitor of CAD (ICAD)/DFF45, which is cleaved by caspase-3 to release CAD/DFF40. The enzymes responsible for the formation of the large chromatin fragments are still unknown.

ROLE OF AIF IN TOXICITY

AIF was the first protein reported to mediate caspase-independent, apoptotic cell death. As discussed above, AIF is N-terminally anchored to the inner mitochondrial membrane, and during apoptosis, AIF has to be liberated from its membrane anchor prior to being released into the cytosol and translocated to the nucleus, where it participates in large-scale DNA fragmentation and chromatin condensation. The available evidence suggests that AIF plays a critical role in cell death only in certain cell types, such as neurons and some tumor cells (Joza *et al.*, 2001; Norberg *et al.*, 2010b). Furthermore, the type of stimulus also determines whether AIF will be important in the cell death process to follow. Preferential AIF triggers should either perturb intracellular Ca^{2+} homeostasis or lead to early lysosomal permeabilization, in order to make the AIF-mediated pathway an essential mechanism of subsequent apoptosis. However, both these events are frequent components of cell death signaling, particularly in ischemia-reperfusion injury and after treatment with cytotoxic drugs.

Several studies suggest a critical role for AIF in neuronal cell death. Hence, microinjection of neutralizing AIF antibodies, or

siRNA downregulation of AIF, has been found to suppress glutamate-, hypoxia-, and N-methyl-D-aspartic acid (NMDA)-induced neuronal death in primary cultures. Further, AIF knockdown in PC12 cells reduced the neurotoxic effects evoked by MPP^+ (1-methyl-4-phenylpyridinium) (Liou *et al.*, 2005), and AIF-mediated DNA fragmentation was observed in cardiomyocytes undergoing ischemia-reperfusion injury. There are also several *in vivo* observations demonstrating the importance of the AIF-mediated pathway in neuronal cell death. For instance, as compared with wild-type mice, Hq mice were protected against NMDA- and kainic acid-induced neuronal damage in the hippocampus (Klein *et al.*, 2002). Cell death was also found to be suppressed in Hq mice subjected to hypoxia/ischemia. Embryonic stem cells deficient in AIF were less susceptible to serum withdrawal-induced apoptosis, as compared with wt ES cells. Treatment of mice with an MnSOD mimetic (MnTBAP) prevented AIF translocation and DNA fragmentation upon ischemia and resulted in neuroprotective effects. Neuroprotection was also observed in different mouse models, when AIF processing was prevented by oral administration of HIV protease inhibitors. Finally, it was reported that inhibition of the nuclear translocation of AIF was required to achieve neuroprotection in a rat model of retinal degeneration (Murakami *et al.*, 2008).

Although, additional studies are needed to clarify the precise role of AIF in neuronal cell death, there is ample evidence for its involvement in several CNS pathologies. Hence, improved knowledge about the molecular mechanisms regulating AIF release from the mitochondria and its translocation to the nucleus should be beneficial for future attempts to design more specific drugs for treatment of these diseases.

LINKS BETWEEN APOPTOSIS AND OTHER CELL DEATH MODALITIES

It is now apparent that multiple cell death programs can be activated during toxicity. Some of them are shared by different tissues; others are tissue specific and are linked to particular functions. For example, neurons rely on complex synaptic activity to function, and substantial loss of synaptic connectivity can become lethal (Berliocchi *et al.*, 2005). Processed caspase-3 has been detected in the retinal growth cones. Here, its activation, confined to a specific compartment, does not trigger a full apoptotic response but rather results in transient, localized changes in specific proteins involved in cone collapse and chemotropic response (Campbell and Holt, 2003). In a similar way, the ubiquitin-proteasome system has been implicated in apoptosis (Sun *et al.*, 2004), and axonal degeneration (Zhai *et al.*, 2003), as well as in neurodegenerative disease. However, the same system is required for physiological axonal pruning (Watts *et al.*, 2003). This suggests that the cell death signaling pathways may either (1) be used locally to eliminate unnecessary or injured structures or

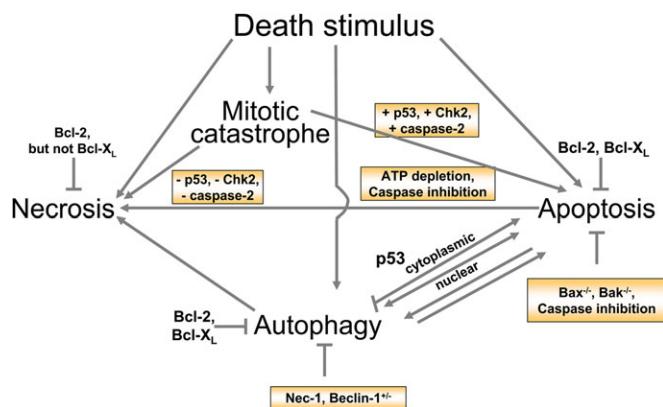


FIG. 4. Cross talk between different modes of cell death. Toxicants might trigger different modes of cell death depending on which pathways can be activated. Inhibition of one particular pathway might lead to cell death by a different mechanism. However, some inhibitors (Bcl-2) can block several cell death modalities. The p53 status of the cell is also important for the mode of cell death that is induced.

(2) mediate a physiological function independent of their role in cell death. Therefore, the activation of local apoptotic processes in differentiated neurons is not detrimental *per se* but may be part of a physiological response.

Regardless of the main execution pattern, death programs have at least two fundamental features in common: (1) they are evolutionarily conserved, which may be explained by a dual role of many components as both life and death signals, and (2) they involve the activation of one or more families of proteases and/or other degradative enzymes. In many instances, developmental cell death and death under pathological conditions share similar morphological features as well as signaling and execution systems (Fig. 4). This is true not only for apoptosis but also for other cell death modalities. For example, autophagic cell death, which is characterized by the presence of cytoplasmic vacuoles, occurs frequently in both neuronal development and neurodegenerative disease (Yuan *et al.*, 2003). The existence of conserved biochemical pathways for cell death signaling and execution has reduced the previous focus on the morphological characteristics of cell death. Despite efforts to define individual forms of cell death based on their appearance, it is clear that in all circumstances cell disassembly involves nuclear fragmentation/dissolution, organelle disruption (sooner or later), and, eventually, membrane lysis and phagocytosis. Thus, condensation and subsequent fragmentation of the nucleus occurs in apoptosis as well as in necrosis and autophagic cell death. In apoptosis, like in necrosis, the mitochondria can be partially or totally damaged and, again, in both apoptosis and necrosis, the cytoskeletal structure is compromised and molecules are exposed at the cell surface to promote recognition and scavenging of the dying cell. Therefore, a particular death program is not necessarily linked to morphological appearance. It seems more likely that several death executing routines may be activated concomi-

tantly within injured cells and that one or the other becomes predominant, depending on the stimulus and the metabolic state of the tissue (Fig. 4). For example, there is increasing evidence that apoptotic features can also be found when the main executioners of apoptosis, the caspases, are inhibited (Volbracht *et al.*, 2001), and conversely, that caspase-mediated cleavage of relevant proteins might be involved in cell lysis/necrosis (Schwab *et al.*, 2002). Thus, under pathological conditions, several protease families may cooperate to disassemble cells, targeting different organelles or subcellular structures. Although the predominance of one or the other death executing mechanism may be dictated by factors as different as energy requirement, signaling molecules or the intensity of a given insult, in many instances, the differentiation program within a given tissue dictates the way to die. This is particularly true of neurons, where the spatial selectivity of death signals and promiscuity of execution systems can result in the complex and relatively slow demise that occurs in neurodegenerative disease. The promiscuity of death subroutines is also evident in brain ischemia, where both caspase-dependent and caspase-independent death pathways are activated. The latter include Ca²⁺ activation of the calpain family of proteases. The predominance of one or the other death pathway can be explained in this case by the availability of ATP for the activation of the caspase-dependent routines (Orrenius *et al.*, 2003).

ROLE OF AUTOPHAGY IN TOXICITY

Many environmental contaminants, toxic drugs, and engineered nanomaterials can induce apoptosis, necrosis, or autophagy, depending on both cell type and dose (Orrenius and Zhivotovsky, 2006). As mentioned above, the widespread environmental pollutant, arsenic, might trigger either apoptosis or necrosis (depending on concentration) in various cell types. However, recently, it has been shown that autophagy is the predominant mode of cell death induced by sodium arsenite in human lymphoblastoid cell lines. "Autophagic cell death" is morphologically defined (by transmission electron microscopy) as a type of cell death that occurs in the absence of chromatin condensation but is accompanied by massive autophagic vacuolization of the cytoplasm (Klionsky and Emr, 2000). In contrast to apoptotic cells (whose clearance is ensured by engulfment and lysosomal degradation), cells that die with autophagic morphology do not seem to attract phagocytes, although this mode of cell death has not been characterized in sufficient detail *in vivo*. Autophagy was initially described as a survival mechanism, and some reports indicate that cells presenting features of "autophagic cell death" can still recover upon withdrawal of the death-inducing stimulus. Nevertheless, in some instances, autophagy may be responsible for the destruction of cells as a result of a protracted atrophy of the cytoplasm beyond a not yet clearly defined point-of-no-return.

With this in mind, one might question whether autophagy in lymphoblastoid cell lines is an effector mechanism of arsenite toxicity or, alternatively, a cellular compensatory mechanism (Bolt *et al.*, 2010). Another example is MCF-7 cells treated with the necrogenic agent, tamoxifen (Bursch *et al.*, 2008). Incubation of cells with 10 μM tamoxifen resulted in the lysis of almost all cells within 24 h. However, transient (1 h) incubation with the same dose allowed cells to recover, a process that involved autophagy. Similarly, treatment of HL60 cells with tamoxifen caused dose-dependent distinct responses: 1–5 μM tamoxifen resulted in autophagy, 7–9 μM triggered apoptosis, whereas 15 μM caused necrosis. Finally, it is of interest to note that autophagy induced by vitamin D, or its analogues, sensitizes some tumor cells to ionizing radiation, whereas the residual, surviving cell population remains in a senescent, growth-arrested state with minimal recovery of proliferative capacity (Gewirtz, 2007).

Depending on the type of lethal agent, the cell death process can be initiated in different intracellular compartments, and cross talk between these compartments appears essential for all cell death modalities. This interorganelle cross talk apparently involves several molecular “switches” within the signaling network (Fig. 4). Thus, p53 can be activated in response to DNA damage, or because of changes in the redox balance in the mitochondria, and Bcl-2 family proteins might act at the level of the mitochondria, ER, or nucleus. Nuclear p53 promotes the transcription of proapoptotic and cell cycle-arresting genes and also can act as an autophagy-inducing transcription factor. In contrast, cytoplasmic p53 might trigger apoptosis and/or inhibit autophagy, although the precise molecular mechanisms of this dual function are not known (Green and Kroemer, 2009).

Another example of cross talk between apoptosis and autophagy was described recently. Beclin-1 was originally identified as a Bcl-2-interacting protein, whose autophagic function can be inhibited by both Bcl-2 and Bcl-X_L (Shimizu *et al.*, 2004). Notably, although Beclin-1 possesses a BH3-only domain, and all BH3-only proteins of the Bcl-2 family are well-known inducers of apoptosis, Beclin-1 fails to trigger apoptosis. In fact, by stimulation of autophagy, it offers cytoprotection against various apoptogenic agents. However, in response to growth factor withdrawal, when autophagy precedes apoptosis, caspase-mediated cleavage of Beclin-1 inactivates autophagy and stimulates apoptosis by promoting the release of proapoptotic factors from the mitochondria (Fig. 4). In this model, a caspase-generated fragment of Beclin-1 seems to trigger an amplifying loop-enhancing apoptosis (Djavaheri-Mergny *et al.*, 2010).

Importantly, depending on the nature and severity of the stimulus, and on the cell type, the hierarchy of interorganelle cross talk might result in different cell death modalities. Moreover, in some cases, suppression of the function of a particular intracellular compartment might switch one mode of cell death to another. For example, inhibition of mitochon-

drial energy metabolism (lowering of ATP) can change the mode of cell death from apoptosis to necrosis. Similarly, inhibition of caspase activity might change apoptosis to necrosis or to autophagic cell death, whereas activation of calpain-mediated cleavage of autophagy-regulated protein, Atg-5, switches the mode of cell death from autophagy to apoptosis (Yousefi *et al.*, 2006) (Fig. 4).

There is accumulating evidence that the inhibition of autophagy is oncogenic. Indeed, loss of only one allele from either one of the two autophagy genes, beclin-1 or UVRAG, is sufficient to promote carcinogenesis. Mutation in beclin-1 is also common in human cancer. Further, Atg5 frameshift mutations were observed in gastric cancer, and mutations in Atg16 are often detected in Crohn's disease (Massey and Parkes, 2007). Mice deficient in Atg4C develop fibrosarcomas in response to carcinogen treatment. Multiple oncogenes, such as Bcl-2 and Akt, inhibit autophagy, supporting the hypothesis that autophagy contributes to tumor suppression. The discovery of damage-regulated autophagy modulator (DRAM) as a p53 target, which modulates both autophagy and apoptosis, as well as the finding that DRAM is inactivated in certain tumors are important steps forward in our understanding of how p53 controls autophagy and apoptosis, and how this relates to tumor suppression (Crighton *et al.*, 2006). Active autophagy can prevent tumor development by at least two mechanisms. It may switch the balance between protein synthesis and degradation toward degradation to reduce cell growth. Alternatively, it may act to remove defective mitochondria and other intracellular organelles that are potential sources of ROS, which can induce DNA damage and genome instability and thereby promote carcinogenesis. At the present time, however, it is too early to predict any possible effects of manipulation of autophagy on tumor growth, and whether this might become part of a therapeutic strategy in the future.

PERSPECTIVES

The discovery and characterization of genetically programmed modes of cell death, and their associated signaling networks, have opened new avenues for studies of the mechanisms by which foreign chemicals might perturb cell functions and cause tissue damage. A host of novel targets have been identified, which could be affected either directly or indirectly by toxicants. Of particular significance is the critical role of Ca²⁺ and ROS in the modulation of several cell death modalities because a wide variety of toxic chemicals are known to affect cellular Ca²⁺ signaling and/or redox balance. Hence, it appears that oxidative stress is a predominant mechanism of toxicity of the rapidly expanding group of engineered nanomaterials, although they might also interfere more directly with cell signaling. The same is true for low doses, or mixtures of the more traditional environmental pollutants, which have been found to trigger apoptotic cell death in various *in vitro* models,

although it remains questionable whether the same mechanisms are responsible for their *in vivo* toxicity. Arsenic compounds are good examples of dose-dependent toxicity in view of their ability to trigger either apoptosis or autophagy by activation of the appropriate signaling network at low doses, whereas they kill cells by mitochondria-mediated necrosis at high doses. There are several other examples of interference with physiological cell signaling by foreign chemicals, and more will probably follow. Hence, it is important to continue the work to unravel remaining secrets of cell death signaling in order to further our understanding of toxicity mechanisms and improve risk assessment.

In spite of the recent advances in our understanding of cell death mechanisms and associated signaling networks, much work remains to be done before we can fully appreciate the toxicological significance of these findings. Although it is clear that the activation of cell death pathways is responsible for acute toxicity of many drugs and chemical toxicants, their potential involvement in subacute or chronic toxicity caused by long-term exposure to other drugs or environmental pollutants remains to be investigated. An important question is then how we can translate observations from various *in vitro* models to *in vivo* toxicology? How can we compensate for differences in dose, tissue distribution, time of exposure, and complexity of the biological system? It is obvious that answers to these and similar questions require comparative studies under more *in vivo*-like conditions. However, the availability of novel technology and a host of suitably tailored biological models will hopefully help us overcome these difficulties and better understand the significance of cell death mechanisms for *in vivo* toxicology.

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