

Mechanisms of Neural Cell Death: Implications for Development of Neuroprotective Treatment Strategies

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Summary: It has been increasingly recognized that cell death phenotypes and their molecular mechanisms are highly diverse. Necrosis is no longer considered a single entity, passively mediated by energy failure. Moreover, caspase-dependent apoptosis is not the only pathway involved in programmed cell death or even the only apoptotic mechanism. Recent experimental work emphasizes the diverse and interrelated nature of cell death mechanisms. Thus, there are both caspase-dependent and caspase-independent forms of apoptosis, which may differ morphologically as well as mechanistically. There are also necrotic-like phenotypes that require *de novo* protein synthesis and are, therefore, forms of programmed cell death. In addition,

forms of cell death showing certain morphological features of both necrosis and apoptosis have been identified, leading to the term *aponecrosis*. Considerable experimental evidence also shows that modulation of one form of cell death may lead to another. Together, these observations underscore the need to substantially revise our conceptions about neuroprotection strategies. Use of multiple treatments that target different cell death cascades, or single agents that moderate multiple cell death pathways, is likely to lead to more effective neuroprotection for clinical disorders. **Key Words:** Apoptosis, necrosis, caspase, calpain, AIF.

INTRODUCTION

Neuronal loss occurs as an essential feature of normal CNS development, as well as in acute or chronic neurodegenerative disorders.¹⁻⁴ It has long been recognized that a variety of factors can trigger secondary auto-destructive reactions within the CNS, including free radicals, excitatory amino acids, eicosanoids, lipid degradation products, tissue cations, inflammation, and immune responses.^{5,6} These secondary injury factors are released or activated over a period from seconds (e.g., lipid, cation changes) to days (e.g., inflammatory, immune responses) after the primary insult and may act either sequentially or in parallel to cause delayed or expanding cell death. Detailed reviews of delayed injury processes/mechanisms can be found elsewhere.^{5,6} Depending on developmental age, injury severity, or a specific cell type, injury factors can trigger alternative mechanisms of cell death.⁷

The focus of research on secondary brain injury has historically been on mechanisms related to necrosis. This

has led to the evaluation of numerous pharmacological strategies, including calcium channel blockers, corticosteroids and antioxidants, glutamate receptor antagonists, opioid receptor antagonists, thyrotropin-releasing hormone analogs, and magnesium administration, as well as various anti-inflammatory and immune modulatory treatments.⁸ Although several of these strategies have shown modest benefits in the treatment of acute spinal cord injury in humans,^{9,10} neuroprotection studies in head injury and stroke have been disappointing. In part, this may reflect flaws in trial design or failure to more directly parallel pre-clinical and clinical studies^{11,12}; however, to a significant degree, such negative trials may result from conceptual difficulties, namely a failure to appreciate the complexity of cell death phenotypes and mechanisms.

CELL DEATH PHENOTYPES

Nomenclature relating to the cell death phenotypes has been confusing. One of the early attempts to classify cell death phenotypes, based upon morphological analyses in developmental models, was made by Schweichel and Merker,¹³ who identified three types of cell death (types 1, 2, and 3). Type 1 is manifested by nuclear condensa-

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tion and pyknosis, reduced cytoplasmic volume, late cell fragmentation, and phagocytosis. Type 2, or autophagic degeneration, is characterized by substantial autophagic vacuolization in the cytoplasm. Type 3, or cytoplasmic cell death, is characterized by general disintegration and deletion of organelles. Features of type 1 cell death appear identical to those classically identified by Kerr as apoptosis.¹⁴ Types 2 and 3 show features associated with necrosis¹⁵; in 1990, this classification was recapitulated and refined by Clarke,¹⁶ who distinguished two subtypes of cytoplasmic cell death, 3A non-lysosomal disintegration and 3B cytoplasmic. Additional morphological forms have been described, but it has been suggested that these may reflect different cell types or initiating stimuli rather than fundamentally different cellular mechanisms.¹⁷ Further details regarding these morphological classifications can be found in several excellent reviews.^{15,17,18}

More recent studies have focused on the roles of apoptosis *versus* necrosis in neuronal loss associated with acute or chronic neurodegenerative disorders. Apoptosis and necrosis have been distinguished by various histological and biochemical criteria. Thus, necrosis is characterized by loss of membrane integrity and cellular swelling, damage to organelles, lysosomal disruption, and uncontrolled cell lysis that often leads to tissue inflammation.¹⁹ In contrast, typical features of apoptotic cells include preserved membrane integrity and cell shrinkage, cytoplasmic and nuclear condensation, and plasma membrane blebbing. At later stages of apoptosis, cells may disintegrate into apoptotic bodies, which are engulfed by neighboring cells. Moreover, it does not lead to an inflammatory response.¹⁹

Although such morphological differences have been widely used to distinguish these two types of cell death, some pathologists have suggested a modified nomenclature. Thus, the Society of Toxicologic Pathologists proposed use of the term "necrosis" to describe cell death independent of a specific pathway, with "oncosis" used to reflect cell swelling as may occur with acute ischemia and "apoptotic" as a modifier of cell death ("necrosis") showing apoptotic features as described above.²⁰ However, apoptosis and necrosis may, in fact, share certain common signal transduction pathways and cooperate in cell death.²¹ Indeed, apoptosis and necrosis may be induced by the same insult, with the magnitude of the insult determining the decision of the cell to undergo one of the alternative cell death pathways.²² Intracellular ATP levels appear to be an important factor in the initiation of apoptotic or necrotic execution programs.^{23,24} Moreover, inhibition of one of these cell death pathways may increase cell loss through the other.^{7,25}

Recent studies have further complicated the classification of cell death phenotypes, as well as the mechanisms involved.²¹ Careful morphological analyses of dy-

ing neurons in mouse models of Huntington's disease and amyotrophic lateral sclerosis failed to identify either classical necrosis or apoptosis^{24,26} but rather revealed features more consistent with an alternative type of neuronal cell death that has been termed paratosis.²⁷ This type of neuronal death requires gene expression, displays non-apoptotic morphology with vacuolization, and is independent of caspase activation. Whether this form of cell death should be characterized within the existing nomenclature as type 2 or 3B is uncertain,²⁸ but many descriptions of non-apoptotic cell death showing cytoplasmic vacuolization appear to be most closely related to type 3B.²⁷ In addition, forms of apoptosis with non-classical morphological features and different biochemical markers have been identified.²⁹ Thus, there appear to be multiple forms of programmed cell death, with different morphological features and likely distinct molecular mechanisms (FIG. 1). Recognition of this diversity has substantial implications for the development of more effective neuroprotective strategies.

CASPASE-DEPENDENT APOPTOSIS

The apoptotic machinery initially identified in *Caenorhabditis elegans* is evolutionarily conserved in higher organisms.³⁰ Programmed cell death in this nematode is largely controlled by two pro-apoptotic genes (*ced-3* and *ced-4*) and one anti-apoptotic gene (*ced-9*). Although molecular mechanisms controlling apoptosis in mammals are far more complex, homologs for *ced-3* (caspases), *ced-4* [apoptosis protease activating factor-1 (*apaf-1*)], and *ced-9* (*bcl-2*) have been identified.²⁹ The term "caspase" refers to cysteinyl aspartic acid-proteases.³¹ Caspases share considerable sequence identity around the putative active site cysteine: QAC(R/A/G)G.³¹ These enzymes are translated as zymogen proforms that include an N-terminal prodomain, a large subunit, and a C-terminal small subunit, which are separated by specific caspase recognition sites. Processing of caspase precursors at these sites leads to their activation. Active caspases are heterotetramers consisting of two large and two small subunits. Upon activation, caspases may cleave their own precursors or other procaspases, resulting in a caspase activation cascade.

Most caspase substrates are cleaved by these proteases after aspartate at P1; however, some exceptions to the rule have also been reported.³² Variability in the P2-P4 region determines substrate specificity for individual members of the family. Based on their substrate specificity, caspases have been divided into group I (caspase-1, -4, and -5, which prefer the tetrapeptide sequence WEHD), group II (caspase-2, -3, and -7, which preferentially cleave DEXD), and group III (caspase-6, -8, and -9, which prefer (L/V)EXD).³¹

Based on mechanisms of activation, caspases have

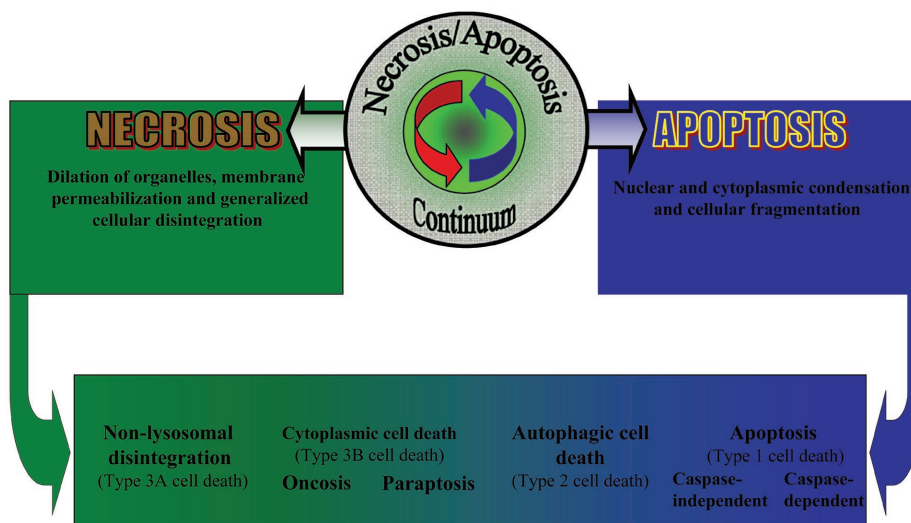


FIG. 1. Apoptosis-necrosis continuum. Cell death includes morphologically distinct types that have been characterized in various ways. No single classification encompasses all of the variant descriptions in the literature. Clearly, there is a continuum of cell death variants, with multiple variants coexisting in both clinical disorders and clinically relevant experimental models. Here we depict an apoptosis-necrosis continuum leading to cell death with different morphological features.

been divided into initiators and effectors. The initiator caspases, such as caspase-8 and -9, begin the disassembly process and activate downstream effector caspase-3, -6, and -7, leading to an amplified caspase cascade.³³ Active effector caspases contribute to the morphological and biochemical manifestation of apoptotic death, such as membrane blebbing, condensation or margination of chromatin and nuclear fragmentation, as well as alterations in activity of numerous nuclear and cytosolic enzymes. Unlike effector caspases, initiator caspases have a long N-terminal prodomain, do not undergo autoactivation, and do not cleave one another. For activation they require additional proteins, such as death domain-containing receptors (type I apoptosis) or functional apoptosomes (type II apoptosis).¹

A third group of mammalian caspases, which includes caspase-1, -4, -5, -11, -12, and -13, has been defined in terms of their function as pro-inflammatory enzymes. In contrast to the effector caspases that are involved in the execution of the apoptotic process, pro-inflammatory caspases are poor substrates for other caspases, and their apical activation pathways are poorly understood.

Pathways of caspase-3 activation

Of the 14 caspases identified in mammals, caspase-3 appears to be the major effector in neuronal apoptosis triggered by various stimuli. Strong evidence supporting the specific role for this protease comes from studies on caspase-3 knockout mice, in which brain development is profoundly altered.³⁴ A role for caspase-3 in injury-induced neuronal loss was subsequently established using semi-specific peptide caspase inhibitors *in vivo* and *in vitro*.³⁵

Two major caspase-3 activating pathways have been

identified: an extrinsic pathway involving cell-surface receptors, and an intrinsic pathway resulting from alterations at the level of the mitochondrion and activation of the apoptosome.¹ The extrinsic pathway requires oligomerization of “death” receptors and the formation of death-inducing signaling complex.³⁵ This process is regulated by members of the tumor necrosis factor (TNF) family of cytokines that include Fas ligand (Apo-1/CD95 ligand), TNF- α , TNF-related apoptosis-inducing ligand, and TNF-related weak inducer of apoptosis.^{35,36} Oligomerization of a death receptor leads to binding of its intracellular domain to the adaptor molecule Fas-associated death domain or caspase and RIP adaptor with death domain, which recruits initiator procaspase-8 and triggers its activation. The released active caspase-8 activates downstream executioner caspases-3 and -7. In addition, activated caspase-3 may cleave procaspase-8,³⁷ thereby amplifying the death process. Initiation of apoptosis through the extrinsic pathway does not usually require new mRNA or protein synthesis.

In some cell types caspase-8 triggers the intrinsic apoptotic pathway by cleaving cytosolic Bid, a pro-apoptotic member of the Bcl-2 family. The C-terminal portion of Bid translocates to the outer mitochondrial membrane and triggers the release of cytochrome *c* from mitochondria to the cytoplasm.³⁸ In the presence of ATP or dATP, cytochrome *c* binds to Apaf-1 and allows the recruitment and activation of procaspase-9 within the apoptosome.³⁵ Caspase-9 cleaves and activates executioner caspases-3 and -7. Active caspase-3 can cleave and activate at least four other caspases (-2, -6, -8, and -10) and also participates in a feedback amplification loop involving caspase-9.³⁷

In contrast to the extrinsic pathway, initiation of the intrinsic apoptotic cascade in specific cell types often requires gene induction and protein synthesis, where an essential role has been attributed to p53 and pro-apoptotic members of Bcl-2 family.^{35,39} However, precise mechanisms by which gene activation signals Bcl-2 family proteins remain to be elucidated.

Numerous reports support an essential role for the intrinsic caspase pathway in neuronal apoptosis after cerebral ischemia, brain trauma, spinal cord injury, Huntington's disease, Alzheimer's disease, and seizures.^{1,4,35,40} Moreover, similar to caspase-3 knockout mice, null mutants of caspase-9 show severe defects of programmed cell death in the nervous system.⁴¹ The majority of these mutants are embryonic lethal or die shortly after birth. A general reduction of neuronal death is found in their embryonic brain tissue; however, the phenotype of caspase-9^{-/-} mice is more severe than that of caspase-3^{-/-}, which suggests that caspases other than caspase-3 (such as caspase-7) may also function downstream from caspase-9.⁴¹ Furthermore, insertional mutagenesis or targeted disruption of *apaf-1* gene also produces perinatal lethality that is associated with marked developmental abnormalities. Apaf-1^{-/-} cells fail to activate caspase-3 through the intrinsic pathway of apoptosis *in vivo* or *in vitro*, which presumably accounts for similar developmental abnormalities seen in Apaf-1-, caspase-9-, and caspase-3-deficient embryos.^{34,41} This suggests that activation of procaspase-3 by the apoptosome-mediated pathway plays a critical role in neuronal apoptosis at early stages of brain development.

Modulation of caspase activation

Caspase activation can be modulated at many critical steps. For example, the anti-apoptotic Bcl-xL protein⁴² acts as a major inhibitor of cytochrome *c*-mediated caspase activation; transgenic mice lacking *bcl-x* die in embryonic development and show increased death of post-mitotic neurons.⁴³

Initially, it has been suggested that Bcl-xL directly binds to Apaf-1, thus preventing activation of caspase-9. However, recent studies demonstrate that intracellular localization of Apaf-1⁴⁴ differs from that of Bcl-xL⁴⁵ and indicate that Bcl-xL, like other anti-apoptotic members of the Bcl-2 family, is involved in the control of permeability of mitochondrial membranes and the release of cytochrome *c* via interaction with Bax.⁴⁶ On the other hand, Bcl-xL can be cleaved by caspases, and the resulting C-terminal fragment of Bcl-xL potentially induces apoptosis.⁴⁷

Bax is another member of the Bcl-2 family found to promote apoptosis. Under normal conditions, Bax predominantly localizes in the cytosol but translocates to mitochondrial and other membranes early in apoptosis.⁴⁸ Once translocated to mitochondria, Bax forms homo-

oligomers, leading to loss of mitochondrial membrane potential, cytochrome *c* release, formation of the apoptosome complex, and caspase activation.⁴⁹ Bax deficiency does not cause hyperplasia or malformations of the nervous system, but it decreases apoptosis in the developing CNS and reduces neuronal death caused by Bcl-x disruption.⁵⁰ Recent findings by Scorrano and co-workers⁵¹ suggest that Bax and another "multidomain" pro-apoptotic protein, Bak, are required to maintain homeostatic concentrations of Ca²⁺ in the endoplasmic reticulum (ER). The authors found that mouse embryonic fibroblasts deficient for both these proteins had a reduced resting concentration of calcium in the ER that results in decreased uptake of Ca²⁺ by mitochondria after Ca²⁺ release from the ER. Hence, this function of Bax and Bak may control the apoptotic fate of cells responding to Ca²⁺-dependent stimuli, such as oxidative stress.

Cytochrome *c* release can be induced by members of the caspase family.^{52,53} Of particular interest are recent reports that suggest involvement of caspase-2 in mitochondria-dependent apoptosis by inducing the release of cytochrome *c* and other mitochondrial apoptogenic factors into the cell cytoplasm.^{53,54} However, other studies show that initiation of the intrinsic pathway is mediated before caspase activation.⁵⁵

Apoptosome-mediated apoptosis may additionally be regulated by more complicated mechanisms. Thus, numerous alternatively spliced isoforms of certain apoptosis regulators, such as Bcl-x,⁵⁶ caspase-9,⁵⁷ and Apaf-1⁵⁸ have been shown to play opposing roles in regulating apoptosis.⁵⁹ Furthermore, growth factors can promote cell survival by activating the phosphatidylinositol-3'-OH kinase and its downstream target, the serine-threonine kinase Akt. Cardone and collaborators⁶⁰ found that active Akt can phosphorylate recombinant human caspase-9 on serine-196 and inhibit its activity. However, the corresponding Akt phosphorylation site was not found in the cloned mouse ortholog.⁶¹ Akt may inhibit activation of caspases-9 and -3 by posttranslational modification of a cytosolic factor downstream of cytochrome *c* and before activation of caspase-9.⁶² Activation of the PI3'/K/Akt signaling pathway by growth factors leads to phosphorylation of pro-apoptotic Bad⁶³, thereby altering its function. On the other hand, an increase in intracellular Ca²⁺ concentration results in activation of the protein phosphatase calcineurin, which can dephosphorylate Bad.⁶⁴ In other models, dephosphorylation of Bad can be achieved by Ras-dependent activation of the protein phosphatase 1 α .⁶⁵ Dephosphorylated Bad forms a heterodimer with Bcl-xL, displacing Bax; this leads to the release of cytochrome *c* and activation of downstream caspases, promoting apoptosis.⁶⁶ Active caspase-3 can specifically cleave Akt and, thus, amplify the death process.⁶⁷

Other regulatory mechanisms that parallel the pro-

Mitochondrial Factors and Apoptosis

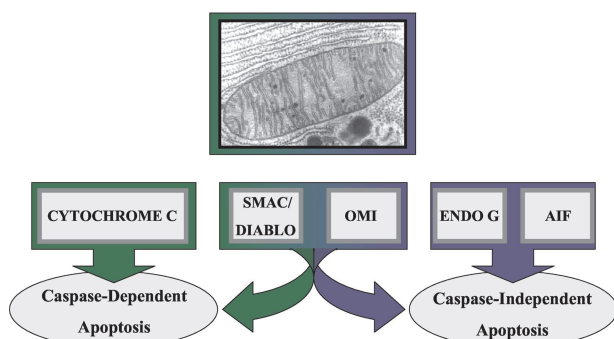


FIG. 2. A variety of mitochondrial proteins have been identified, whose translocation precipitates or contributes to apoptotic cell death. These include those leading primarily to caspase-dependent apoptosis (cytochrome *c*), to caspase-independent cell death (AIF, endonuclease G), or to both (Smac/DIABLO, Omi/HtrA₂).

apoptotic action of cytochrome *c* involve the mitochondrial proteins Smac/DIABLO and Omi/HtrA₂, which are also released into the cytosol when cells undergo apoptosis (FIG. 2). These proteins promote caspase-9 activation by modulating inhibitors of apoptosis proteins (IAPs),⁶⁸ removing their inhibitory activity.^{69,70} Initially identified as baculoviral suppressors of apoptosis in host cells, seven IAP genes have been found in mammalian genomes.⁷¹ Anti-apoptotic activity of corresponding proteins has been attributed to the conserved baculovirus IAP repeat (BIR) domain.⁷² Members of this protein family, XIAP, c-IAP-1, and c-IAP-2 bind procaspase-9 and prevent its activation within the apoptosome and can also inhibit active caspases-3 and -9.⁷³

Smac seems to function as a general neutralizer of IAPs by binding to the BIR2 and BIR3 domains of c-IAP1, c-IAP2, and XIAP.⁶⁹ This could presumably prevent interactions of the IAPs with caspases, therefore releasing their inhibition of caspase activation and caspase activities.⁶⁹ The serine protease Omi/HtrA₂ is also released from mitochondria and inhibits the function of XIAP by direct binding in a way similar to Smac/DIABLO.⁷⁴

Recently, Wang and co-workers⁷⁵ identified two additional proteins that regulate mitochondrial cell death: the oncoprotein prothymosin- α , which inhibits assembly of the apoptosome, and tumor suppressor putative HLA-DR-associated proteins (PHAPs), which stimulate activation of apoptosome. Previous studies have demonstrated that PHAPs can interact with the protein ataxin-1, suggesting that these proteins may contribute to a polyglutamine repeat disorder such as spinocerebellar ataxia type I.⁷⁶

The release of cytochrome *c* from mitochondria to cytosol does not necessarily determine cell fate, since activation of the apoptosome is controlled by other factors. Thus, the cellular stress response can lead to cellular

protection by inducing heat-shock proteins (Hsp). Two members of this family, Hsp70 and Hsp90, prevent cytochrome *c*/dATP-mediated caspase activation by direct association with Apaf-1, thereby preventing recruitment of caspases to the apoptosome complex.^{77,78} A third member of the family, Hsp27, also inhibits cytochrome *c*-mediated activation of caspases by binding to cytochrome *c* and thus prevents the interaction of Apaf-1 with procaspase-9.⁷⁹

Other caspases

Nakagawa et al.⁸⁰ showed that the caspase cascade can also be initiated by caspase-12. Caspase-12-knockout mice are resistant to apoptosis induced by cellular stress, and caspase-12-deficient cortical neurons are resistant to apoptosis induced by β -amyloid. In this stress-induced pathway caspase-12 activates caspase-3, and this process can be reversed by antisense-mediated inhibition of caspase-12.⁸¹ It has been proposed that increased intracellular Ca²⁺, resulting from ischemic injury or β -amyloid cytotoxicity, may induce apoptosis through simultaneous activation of caspase-12 and inactivation of Bcl-xL by calpain.⁸²

Furthermore, in other models, an upstream activator of caspase-1,⁸³ caspase-11 is able to cleave and activate caspase-3. Caspase-11-deficient mice demonstrate reduced caspase-3 activity and cell death caused by brain ischemia.⁸⁴ This caspase has also been shown to be activated by cathepsin B⁸⁵ that, in turn, can be activated by calpains.⁸⁶

Results of several studies suggest that activation of caspase-2 plays a critical role in initiation and progression of apoptosis. Thus, diminished expression of this caspase in PC12 cells and sympathetic neurons mediated by antisense oligonucleotides rescues them from death caused by trophic factor deprivation.⁸⁷ More recently, Lassus and collaborators⁵³ demonstrated that caspase-2 is required for the permeabilization of mitochondria, the release of cytochrome *c* and Smac, and translocation of Bax from the cytoplasm to mitochondria in response to cytotoxic stress.

In contrast, results of knockout studies show that death of motor neurons during development is accelerated in caspase-2-deficient mice, and that caspase-2-deficient sympathetic neurons undergo apoptosis more effectively than wild-type neurons when deprived of nerve growth factor.⁸⁸ Thus, caspase-2 may serve either as a positive or negative regulator of apoptosis.

Developmental changes

Clinical data suggest that outcomes and mortality after acute brain injury are age-dependent, with more severe responses in infants than in adults.⁸⁹ Such differences in response to injury may be explained, in part, by differential susceptibility to apoptosis and associated caspase-3 activity in brain as a function of developmen-

tal age.^{90,92} During brain maturation, the potential of the intrinsic apoptotic pathway is progressively reduced; such repression is associated with down-regulation of Apaf-1 and caspase-3 gene expression.⁴⁰ Injury-induced cytochrome *c*-specific cleavage of procaspase-9, followed by activation of caspase-3 in mature brain, correlated with marked increases in Apaf-1 and caspase-3 mRNA and protein expression.⁴⁰ These results suggest that differential expression of Apaf-1 and caspase-3 genes may underlie regulation of apoptotic susceptibility during brain development, as well as after acute injury to mature brain, through the intrinsic pathway of caspase activation.

CASPASE-INDEPENDENT APOPTOSIS

Although caspases have been recognized as important mediators of apoptosis, there is accumulating evidence suggesting the existence of caspase-independent mechanisms of neuronal death (FIG. 2).^{29,93} Blockade of the caspase execution machinery may only temporarily rescue damaged neurons, and classical apoptotic features can still appear in caspase-inhibited neurons and other cell types.⁹⁴ In experimental models of stroke, caspase inhibition affords protection in certain neuronal populations but not in others.⁹⁵ These results are consistent with the idea that the involvement of specific caspase-dependent and caspase-independent mechanisms of cell death may depend on brain region, cell type, and age.⁹⁶ Although there has been extensive investigation of caspase-mediated cell death processes, much less is known about molecular mechanisms involved in the regulation of caspase-independent apoptosis and their role in brain development.

Specific mitochondrial factors appear to play a central role in both caspase-dependent and caspase-independent apoptosis. Thus, cytochrome *c*, Smac/DIABLO, and HtrA2/Omi are released from mitochondria during initiation of apoptosis and control activation or activity of caspases.^{29,69,74,93} In contrast, apoptosis-inducing factor (AIF) and endonuclease G promote caspase-independent cell death.^{29,93} However, Smac/DIABLO and HtrA2/Omi may also contribute to caspase-independent apoptosis.⁹³

AIF

AIF normally localizes in mitochondria; however, after an apoptotic insult, it translocates to the cytosol and the nucleus, where it induces peripheral chromatin condensation and high molecular weight DNA fragmentation.^{93,97} Nuclear translocation of AIF has been observed under a wide variety of cell death inducing conditions such as protein kinase inhibition, *c*-Myc overexpression, ceramide exposure, or *in vitro* chemotherapy.^{93,97} Importantly, AIF translocation also occurs after ATP depletion that leads to inhibition of apoptosis and promotion of necrotic cell death.⁹⁷ Intracellular redistribution of AIF

correlates with large-scale DNA fragmentation and chromatin condensation, which occurs even in cells lacking Apaf-1 or caspase-3.⁹⁸ This suggests that AIF may be responsible for the caspase-independent features of apoptosis. Kinetic analyses revealed that release of AIF from mitochondria does not correspond to that of cytochrome *c* that has been explained by differential localization of these proteins within mitochondria.⁹⁷

AIF possesses a second, oxidoreductase, enzymatic activity different from its apoptotic potential.⁹⁹ The cellular targets of AIF remain elusive. However, heat shock protein 70 (Hsp70) physically interacts with AIF and inhibits its apoptotic effects both *in vitro* and in intact cells.¹⁰⁰ Overexpression of Bcl-2 also blocks the AIF redistribution.²⁹

A recent study on Harlequin (*Hq*) mice has suggested a vital role for AIF in neuron survival in the aging mouse brain.¹⁰¹ In this report, Klein and co-authors¹⁰¹ found that *Hq* mutation caused an approximately 80% reduction in AIF expression and associated progressive degeneration of terminally differentiated cerebellar and retinal neurons in mice after 3 months of age. Reduced levels of AIF in mutant cells increased their susceptibility to exogenous and endogenous peroxide-mediated apoptosis that was inhibited by AIF overexpression. Furthermore, terminally differentiated neurons in the cerebellum and retina (but not in other neural tissues) undergo unscheduled cell cycle reentry, providing another molecular mechanism by which free-radical damage can lead to neuronal death.¹⁰¹ A pathway involving redistribution of AIF has been reported after acute brain injury in rats.¹⁰²

In our preliminary studies, we demonstrated that in contrast to Apaf-1 and procaspase-3 proteins, AIF is up-regulated during brain development in rats (Yakovlev, unpublished observation). This observation suggests that developmentally orchestrated regulation of Apaf-1, caspase-3, and AIF expression may determine the relative roles of caspase-dependent and caspase-independent mechanisms of neuronal cell loss in the course of brain maturation and brain injury.

Endonuclease G

Endonuclease G was identified as the mitochondrial nuclease involved in mitochondrial DNA replication.¹⁰³ It can be specifically activated by apoptotic stimuli, such as TNF- α , anti-Fas antibodies, and UV irradiation, and, as for many other nucleases, induces internucleosomal fragmentation of nuclear DNA.¹⁰⁴ Once released into the cytosol and translocated to the nucleus, this nuclease generates oligonucleosomal DNA fragmentation even in the presence of caspase inhibitors or absence of DNA fragmentation factor (DFF40),¹⁰⁴ another apoptotic endonuclease that is activated by caspase-3 and digests genomic DNA into oligonucleosomal fragments.² DFF40 is a 40-kDa protein that is translated in the pres-

ence of its inhibitor (DFF45), which functions as a specific chaperone and forms a heterodimer with DFF40 in proliferating cells. Unlike endonuclease G, DFF45 is cleaved by caspase-3, releasing the nuclease activity of DFF40 from inhibitory control and triggering DNA fragmentation.¹⁰⁵

In contrast to Li et al.,¹⁰⁴ who reported DNA laddering resulting from endonuclease G activity, Van Loo et al.¹⁰⁶ only observed higher-order (~50 kb) DNA degradation. Furthermore, in comparison to DFF40, much higher concentrations of endonuclease G are required for DNA degradation,⁹³ suggesting a requirement for other cofactors, such as DNase I,¹⁰⁷ to induce effective DNA fragmentation.

Omi/HtrA2

Protein analysis of supernatants isolated from mitochondria exposed to recombinant tBid, a pro-apoptotic Bcl-2 member, revealed the presence of the serine protease Omi, also called HtrA2.⁷⁰ The autoprocessed amino-terminal domain of HtrA2/Omi demonstrates homology to Smac/DIABLO and Reaper family proteins.²⁹ Processed Omi/HtrA2 is released to the cytoplasm upon induction of apoptosis where it binds XIAP resulting in caspase-9 activation.^{29,70,93} This function of Omi is similar to that of Smac/DIABLO, which also binds IAPs and activates caspase-9¹⁰⁸; however, HtrA2/Omi can also induce apoptosis in a caspase-independent manner.^{29,93} When overexpressed extramitochondrially, it induces cell death, which is neither accompanied by significant caspase activation nor prevented by caspase inhibitors. This effect of HtrA2/Omi appears to depend on its serine protease activity, since its catalytically inactive mutant does not induce cell death.^{70,74}

Smac/DIABLO

Smac/DIABLO is also able to induce cell death independently of its binding to IAPs. Thus, Roberts and coworkers¹⁰⁹ have demonstrated that a short splice variant of Smac/DIABLO (Smac β) that lacks the mitochondria targeting and IAPs binding domains potentiates apoptosis induced by death receptor and chemical stimuli. Furthermore, expression of a NH(2)-terminally truncated Smac mutant (Delta75), which lacks the entire IAP-interacting domain, potentiates apoptosis to the same extent as Smac/DIABLO and Smac β .¹⁰⁹

Although expression of Smac/DIABLO is undetectable in adult mouse brain under normal conditions, Shibata and collaborators¹¹⁰ reported its induction after transient focal ischemia in mice. However, it is still unclear whether this mitochondrial protein plays an essential role in neuronal death *in vivo*, since the knockout mice are viable and do not demonstrate any obvious phenotypic abnormality; moreover, Smac/DIABLO^{-/-} cells undergo typical apoptosis induced by a variety of stimuli.¹¹¹

MOLECULAR PATHWAYS OF CELL NECROSIS

Neuronal necrosis is often observed under severe pathophysiological conditions, such as hypoxia, ischemia, hypoglycemia, exposure to reactive oxygen metabolites and toxins, among others. This type of cell death has also been described in such neurodegenerative disorders as Alzheimer's, Huntington's, Parkinson's diseases, amyotrophic lateral sclerosis, and epilepsy.^{112,113} Moreover, autophagic and cytoplasmic types represent alternative necrosis-like forms of programmed cell death that normally occur during development.¹¹⁴

In contrast to apoptosis, necrosis has been considered a passive and disordered process leading to accidental and unavoidable death of cells exposed to extreme insults. However, new findings have revised this concept. Thus, execution of necrotic death depends on activation of Ca²⁺-dependent proteolysis that employs proteases different from caspases. Moreover, similar morphological and ultrastructural features of necrotic cells of different origin suggests existence of a conserved execution program. Recent studies on genetic mechanisms of excitotoxic degeneration of the six touch receptor neurons in *C. elegans* established a role for CLP-1 and TRA-3 calpain-like proteases and aspartyl proteases ASP-3 and ASP-4, two orthologs of mammalian cathepsins.¹¹⁵ The authors demonstrated that overexpression of ASP-3 and -4 can induce spontaneous vacuolization and death of neurons in the ventral nerve cord. RNAi-mediated inhibition of ASP-3 and -4 expression resulted in neuroprotection.

According to the "calpain-cathepsin" hypothesis, activation of cathepsins occurs as a result of Ca²⁺-dependent activation of calpains. In agreement with this hypothesis, inhibition of calpain activity in worms by z-Val-Phe-CHO markedly reduced the number of degenerating neurons. RNAi-mediated knockdown of six of 17 *C. elegans* calpain homologs revealed an essential role for *clp-1* and *tra-3* genes in neuronal loss. Simultaneous repression of both calpain or both cathepsin genes by RNAi resulted in an enhanced suppression of neurodegeneration. However, no synergy was observed between aspartyl proteases and calpain, suggesting their contribution to the same pathway of neurodegeneration in *C. elegans*. Overall, results of this study indicate that diverse death-initiating conditions lead to Ca²⁺-dependent activation of calpains and a subsequent increase in cathepsins activity, leading to cell destruction.¹¹⁵

Calpains

Mammalian calpains compose a large family of constitutively expressed intracellular cysteine proteases.⁸⁶ Two major groups of these enzymes are known as μ - and m-calpains (also called calpains I and II, respectively). The enzymes of both groups require Ca²⁺ for their ac-

tivation. While μ -calpains are active at micromolar Ca^{2+} concentrations *in vitro*, active m-calpains require millimolar Ca^{2+} . Although a large number of calpain substrates have been demonstrated, including cytoskeletal proteins, growth factor receptors, and transcription- and cell-cycle-related proteins, the precise function of calpains *in vivo* remains unclear.⁸⁶

Calpains have been proposed to play a role in regulating normal signal transduction processes by cleaving cytoskeletal proteins such as talin and α -actinin, membrane proteins such as the epidermal growth factor receptor, integrin, N-CAM, and cadherin, and enzymes such as protein kinase C and calmodulin-dependent kinase.¹¹⁶ Constitutive expression of calpains in mammalian CNS and the property of activation by Ca^{2+} indicate that these proteases can also contribute to neurodegeneration. Activation of calpains has been proposed to contribute to neuronal cell death in ischemic brain injury,¹¹⁷ brain trauma,¹¹⁸ and Alzheimer's disease.¹¹⁹ Moreover, calpain inhibitors show neuroprotective effects in animal models of cerebral ischemia¹¹⁷ and traumatic brain injury.¹¹⁸

Cathepsins

Like calpains, cathepsins also belong to the papain superfamily of cysteine proteases. They are synthesized as inactive precursors and undergo proteolytic activation. Cathepsins are localized predominantly to the lysosomes, but have also been found in cell nuclei and cytosol.⁸⁶ Neuronal lysosomes are known to contain at least cathepsins B, D, and L; activity of these enzymes has been implicated neuronal injury,⁸⁶ and cathepsin inhibitors such as CA-074 and E-64c have been demonstrated to significantly inhibit neuronal death.¹²⁰

The cytosolic activity of cathepsin D increases significantly from 2 to 6 months in the rat brain, and by 36 months it shows twice the activity of the lysosomal/mitochondrial fraction.¹²¹ Our preliminary data, on the other hand, suggest that in contrast to the pro-apoptotic genes, such as procaspase-3 and Apaf-1, m-calpain protein expression is up-regulated during rat brain development (unpublished observations). Taken together, regulatory mechanisms that control expression of key apoptotic and necrotic genes during brain development appear to predetermine different types of injury-induced neuronal death.

COOPERATION OF CELL DEATH PROGRAMS

As indicated previously, morphological descriptions of apoptosis and necrosis do not describe all variations of cell death. There are many examples when biochemical and morphological features of more than one type of programmed death can be found in the same cell,¹¹³ and inhibition of one form of cell death can lead to domina-

tion of another.⁷ These experimental studies support the existence of a spectrum of death programs in cells.²¹ Thus, Formigli and collaborators²¹ found that antimycin A, a metabolic poison that affects mitochondrial respiratory chain complex III, induced a type of cell death that shared dynamic, molecular, and morphological features with both apoptosis and necrosis, which they called aponecrosis. Inhibition of caspase-3 caused a shift from aponecrosis to necrosis, and higher doses of antimycin A induced necrosis. The analysis of the cellular energetic stores showed that the levels of ATP were a primary determinant in directing toward active cell death (apoptosis), aponecrosis, or necrosis. These and other recent data indicate that apoptosis and necrosis may not necessarily be two independent pathways, but rather may share some common events, at least with regard to signal transduction pathways and early phases of the cell death process. This idea is consistent with observations that anti-apoptotic genes, such as *bcl-2*, are able to inhibit both apoptotic and necrotic cell death.¹²²

Furthermore, it is becoming increasingly clear that various types of cell death can share pathways of their execution. For example, recent data suggest a cross-talk between calpains and caspases. Thus, m-calpain can cleave and activate procaspase-12, leading to the caspase cascade.⁸² In addition, calpain may be responsible for cleaving the loop region in Bcl-xL, thereby transforming an anti-apoptotic molecule into a pro-apoptotic one.⁸²

CELL DEATH MECHANISMS: IMPLICATIONS FOR TREATMENT

The existence of multiple cell death pathways with both overlapping and distinct molecular mechanisms, combined with the observation that inhibition of one such pathway may enhance alternative ones, suggest that treatment strategies should optimally be directed at multiple targets/mechanisms. Traditionally, neuroprotection treatment approaches have focused on single receptors or injury factors, and have been primarily aimed at reducing necrotic cell death.¹¹ Because necrosis is an early event, and it is often not possible to treat patients until many hours after acute injuries, such strategies are not likely to be effective. Indeed, virtually all neuroprotective treatment trials in stroke or traumatic brain injury have failed.^{11,123} Alternatively, many recent experimental studies have attempted to modulate caspase-mediated apoptosis. Such studies may make more conceptual sense, as apoptosis is a more delayed event, and use of caspase inhibitors has proved effective in experimental stroke, head injury, and spinal cord injury.³⁵ However, this approach is potentially problematic for several reasons. First, there is evidence that inhibition of apoptosis may enhance necrotic cell death.²¹ Second, the effectiveness of peptide caspase inhibitors may relate to their ability to reduce necrotic cell

death, possibly mediated by calpains (Knoblach and Faden, unpublished observations).

These observations suggest that optimal neuroprotective approaches should include either combination treatment strategies directed toward multiple cell death pathways or the use of single compounds that may inhibit more than one cell death mechanism (multipotential agents). We have previously shown in a cell culture model of traumatic neuronal cell death, which induces both apoptotic and necrotic cell death, that combination treatment with an NMDA antagonist and a caspase inhibitor has additive, if not synergistic, neuroprotective actions.¹²⁴

A prototypic multipotential agent is thyrotropin-releasing hormone (TRH). TRH serves as a physiologic antagonist for endogenous opioids, peptidyl leukotrienes, and platelet-activating factor, all of which have been implicated in secondary tissue injury; it also improves cerebral blood flow, cellular bioenergetic state, and ionic homeostasis.¹²⁵ TRH has substantial neuroprotective actions in brain and spinal cord injury models,¹²⁶ and has shown effectiveness in human spinal cord injury.⁹

During the past several years, a number of other neuroprotective agents have been evaluated that have multipotential actions. Dexanabol (HU-211) is an NMDA antagonist that also has antioxidant and anti-TNF properties; it has good neuroprotective activity in experimental models of stroke and head injury.¹²⁷ AM36 has actions as a sodium channel blocker, NMDA antagonist and free radical scavenger.¹²⁸ It has been shown to have neuroprotective actions *in vivo* and *in vitro*, as well as anti-apoptotic effects.^{128,129} 35b is a cyclized dipeptide, somewhat related to a metabolic product of TRH, which blocks both apoptotic (caspase-dependent) and necrotic cell death in primary neuronal cell cultures, and shows striking neuroprotective actions following traumatic brain injury in rats and mice.^{130,131}

As molecular mechanisms of non-caspase-dependent programmed cell death are defined, other types of combined treatment or multipotential treatment approaches should be developed. Because multiple cell death pathways may have common upstream initiators, or share signal transduction cascades, new drug development strategies directed at such common targets should be considered for the treatment of both acute and chronic neurodegenerative disorders.

Acknowledgments: This work was supported by Department of Defense Cooperative Agreement DAMD17-99-2-9007, NIH Grants R01 NS36537 and R01 NS41119 to A. I. F., NIH Grant P30 HD40677 Core Subcontract to A. I. F., and NIH Grant R01 NS38941 to A.G.Y.

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