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Diversity in the Mechanisms of Neuronal Cell Death

Review

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Neurons may die as a normal physiological process during development or as a pathological process in diseases. The best-understood mechanism of neuronal cell death is apoptosis, which is regulated by an evolutionarily conserved cellular pathway that consists of the caspase family, the Bcl-2 family, and the adaptor protein Apaf-1. Apoptosis, however, may not be the only cellular mechanism that regulates neuronal cell death. Neuronal cell death may exhibit morphological features of autophagy or necrosis, which differ from that of the canonical apoptosis. This review evaluates the evidence supporting the existence of alternative mechanisms of neuronal cell death and proposes the possible existence of an evolutionarily conserved pathway of necrosis.

Mammalian neurons are among the most long-lived cell types. Although the recent discovery of neuronal stem cells raised the possibility of neurogenesis in adult brain, it is a well-accepted dogma that most neurons in our nervous system live as long as we do. Our neurons, however, are not invulnerable. During embryonic development, the nervous system is sculpted by neuronal cell death: excess neurons are removed to ensure proper and precise pre- and postsynaptic connection. In addition, neurons may also die prematurely during adult life when subject to acute or chronic neurotoxic conditions caused by accidental or genetic factors.

Neuronal cell death may occur through diverse mechanisms. In a classic study of cell death during development, Schweichel and Merker (1973) divided cell death into three types based on the differences in the ultrastructural morphological features. The type 1 cell death, which was previously termed apoptosis by Kerr, Wyllie, and Currie (Kerr et al., 1972), is characterized by cytoplasmic condensation, nuclear pyknosis, chromatin condensation, DNA fragmentation, cell rounding, membrane blebbing, cytoskeletal collapse, and the formation of membrane bound apoptotic bodies that are rapidly phagocytosed and digested by macrophages or neighboring cells. A large and growing body of evidence suggests that neuronal cell death during development is executed at least in part through apoptosis. The studies in the past decade have revealed a framework and key players in this pathway, which include a family of cysteine proteases termed caspases, adaptor proteins such as Apaf-1 (required for the activation of caspases), and a family of mitochondria-associated proteins termed the Bcl-2 family (Yuan and Yankner, 2000). These factors control apoptosis in a wide range of systems, including neurons.

The type 2 cell death described by Schweichel and Merker is characterized by the appearance of numerous cytoplasmic autophagic vacuoles of the lysosomal origin, followed by mitochondrial dilation and enlargement of the ER and the Golgi apparatus. Other hallmarks of apoptosis such as nuclear pyknosis and membrane blebbing may also occur later in autophagic death but are less prevalent. Autophagic cell death has been described in neurons during neuronal development (Schweichel and Merker, 1973) and in association with neurodegenerative diseases (Petersen et al., 2001). Although autophagy plays an important role in cellular homeostasis, i.e., in the turnover of intracellular organelles and long-lived proteins, excessive autophagy has been proposed to cause cellular destruction. Increased lysosomal activity, which may or may not accompany an increase in autophagy, has also been observed during neuronal cell death associated with neurodegenerative diseases (Nixon and Cataldo, 1995). Although autophagic cell death has received increasing attention in recent years, its molecular mechanism has been studied primarily in lower eukaryotes such as yeast (Ohsumi, 2001), and details in the mammalian systems remain sketchy (Mizushima et al., 2002).

The type 3 cell death is distinguished from the type 2 cell death by its lack of lysosomal involvement. Instead, the type 3 cell death begins with swelling of intracellular organelles, followed by the formation of empty spaces in the cytoplasm, which eventually fuse with each other. Clarke (1990) further subdivided the type 3 cell death into types 3A and 3B. Although both type 3A and 3B show dilation of intracellular organelles and formation of nonlysosomal-derived vacuoles, they differ in the apparent manner of cell destruction. Both the nucleus and the cytoplasmic membrane of a cell undergoing type 3A death are destroyed by fragmentation, whereas the nucleus of type 3B shows karylosis or edema, and its cytoplasmic membrane rounds up. The type 3 cell death is similar to what is now known as necrosis, which is characterized by early swelling of intracellular organelles followed by swelling and loss of the plasma and nuclear membrane integrity. Although necrosis has been frequently associated with pathological neuronal cell death, certain developmental neuronal cell death has also been found to exhibit features of necrosis (Clarke, 1990).

Apoptosis research in the past decade has provided an enormous amount of mechanistic information. In comparison, very little is known about the mechanisms of type 2 and type 3 cell death. Fortunately, the mechanisms of cell death appear to be highly conserved during evolution. Much of what we have learned about mammalian apoptosis can be traced back to the elegant studies on programmed cell death in *C. elegans* (Metzstein et al., 1998), which was honored by the 2002 Nobel Prize. Similarly, there are interesting studies exploring the mechanisms of cell death in invertebrates, which show features similar to mammalian type 2 and type 3 cell

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death. This review will attempt a critical overview on the current understanding of mammalian neuronal cell death and will draw upon the mechanistic comparisons of both apoptotic and nonapoptotic cell death in invertebrate organisms whenever appropriate.

Caspase-Dependent Neuronal Apoptosis—the Canonical Programmed Cell Death

Apoptosis is an active and highly orderly process displaying characteristic morphologic changes including nuclear condensation and fragmentation, cytoplasmic shrinkage, plasma membrane blebbing, and exposure of phosphatidylserine. The dead cells eventually fragment into membrane bound apoptotic bodies, which are phagocytosed by macrophages and surrounding cells without inducing inflammatory response. Hallmarks of apoptosis have been observed during neuronal development and neuronal cell death caused by acute and chronic injury (Yuan and Yankner, 2000). Due to the highly conserved and uniform nature of apoptotic cell death, the presence of its characteristic morphologic features described above has been universally accepted as evidence for the involvement of the apoptotic mechanism in diverse neuronal cell death paradigms.

The Bcl-2, caspase, and Apaf-1/ced-4 families constitute the core apoptotic machinery in neurons as well as in many other cell types (Figure 1). Although Ced-9, the anti-apoptotic member of the Bcl-2 family in *C. elegans*, is only involved in regulating caspase Ced-3 activation, the mammalian Bcl-2 family may regulate noncaspase-dependent cell death (Tsujimoto et al., 1997). To avoid confusion, we will define apoptosis here as caspase-dependent cell death. The apoptotic machinery is tightly controlled by the balance of survival and death signals. A prototypic example is nerve growth factor (NGF)-regulated neuronal survival pathways. NGF binding to its receptor tyrosine kinase, TrkA, activates a host of prosurvival proteins such as PI-3 kinase, Akt, and MEK/MAPK, which regulate apoptosis by inhibiting proapoptotic proteins such as Bad and Forkhead and by activating prosurvival proteins such as CREB and NF-κB (Yuan and Yankner, 2000), which directly or indirectly regulate the core apoptotic machinery. In the absence of NGF, however, these events are reversed so that pro-apoptotic proteins are activated and anti-apoptotic proteins are suppressed.

At least one of the key events promoting neuronal apoptosis in the absence of NGF is the activation of BH3-domain-only members of Bcl-2 family. This is an evolutionarily conserved regulatory mechanism as the induction of the expression of *egl-1*, a BH3-domain-only protein, is a key event in the initiation of programmed cell death in *C. elegans* (Conradt and Horvitz, 1998). Regulation of neuronal cell death in mammals, however, is more complex than that of *C. elegans* as multiple BH3-domain-only proteins and additional control mechanisms, such as phosphorylation, also participate to set the rheostat of life and death. One of the known examples is the phosphorylation of BAD by Akt and MAPK, which inhibits its pro-apoptotic activity (Datta et al., 1999).

In *C. elegans*, BH3-only protein Egl-1 turns on the cellular death switch by heterodimerizing with Ced-9 and thereby preventing the latter from interacting with Ced-4, the proapoptotic adaptor and activator for Ced-3. Released Ced-4 in turn interacts with and activates Ced-3, a caspase critical for execution of programmed cell death in *C. elegans* (Figure 1; Metzstein et al., 1998). The essence of this regulatory step is conserved in mammalian apoptosis. Bim and Dp5/Hrk are two BH3-only members of the Bcl-2 family that have been shown to play roles in mammalian neuronal cell death similar to that of Egl-1 in *C. elegans*. Expression of Dp5 and Bim is induced in cerebellar granule neurons induced to undergo apoptosis by membrane depolarization in low-potassium media (Harris and Johnson, 2001). The levels of Bim are also elevated in NGF-deprived sympathetic neurons and axotomized sciatic nerves (Putcha et al., 2001; Whitfield et al., 2001). Furthermore, the loss of Bim was shown to delay neuronal cell death, confirming that Bim is one of the contributing factors in the initiation of neuronal apoptosis.

Similar to that of Egl-1 in *C. elegans*, mammalian BH3-only proteins such as Bim and Dp5 also act by heterodimerizing with anti-apoptotic members of Bcl-2 family such as Bcl-2 and Bcl-xL. *bcl-xL*-deficient mice die around embryonic day 13 with extensive excessive neuronal apoptosis in the central and peripheral nervous systems (Motoyama et al., 1995), suggesting that Bcl-xL is critical for the survival of immature neurons during the development of the nervous system. Although the nervous system development of *bcl-2*$^{-/-}$ mice is normal, substantial loss of motor, sensory, and sympathetic neurons is observed in the postnatal period, suggesting a role for Bcl-2 in the survival of some neuronal subgroups (Michaelidis et al., 1996).

While the predominant function of *C. elegans* Ced-9 is to inhibit and prevent Ced-4 from activating Ced-3, mammalian anti-apoptotic members of Bcl-2 family appear to have developed an alternative mechanism of action by preventing apoptosis-associated mitochondrial changes (Figure 1). This may be accomplished by transient interactions of BH3-only pro-apoptotic members of the Bcl-2 family, such as tBid and Bim, with the multidomain pro-apoptotic members of the Bcl-2 family, such as Bax and Bak, resulting in the oligomerization of Bax and Bak and the permeabilization of the outer mitochondrial membrane and the efflux of cytochrome c (Letai et al., 2002). Release of cytochrome c from mitochondria has been recognized as a key event in initiating the activation of caspases through the formation of the cytochrome c/Apaf-1/caspase-9 complex. *apaf-1* deficiency results in severe craniofacial malformation with extensive hyperplasia of diencephalon and midbrain, more prominent in the mantal layer where differentiated neurons are localized than in the ventricular zone containing mitotic neuronal stem cells (Cecconi et al., 1998). Consistent with its role in mediating caspase activation downstream of mitochondria, *apaf-1*$^{-/-}$ mouse embryos show a severe defect in the activation of caspase-3 in the brain.

Activation of caspase-9 by Apaf-1/cytochrome c complex is a key event in the initiation of caspase activation downstream of mitochondria (Wang, 2001). *caspase-9* deficiency also causes embryonic lethality and a gross perturbation of brain development exhibited as a significant expansion of ventricular zone and a lack of cas-

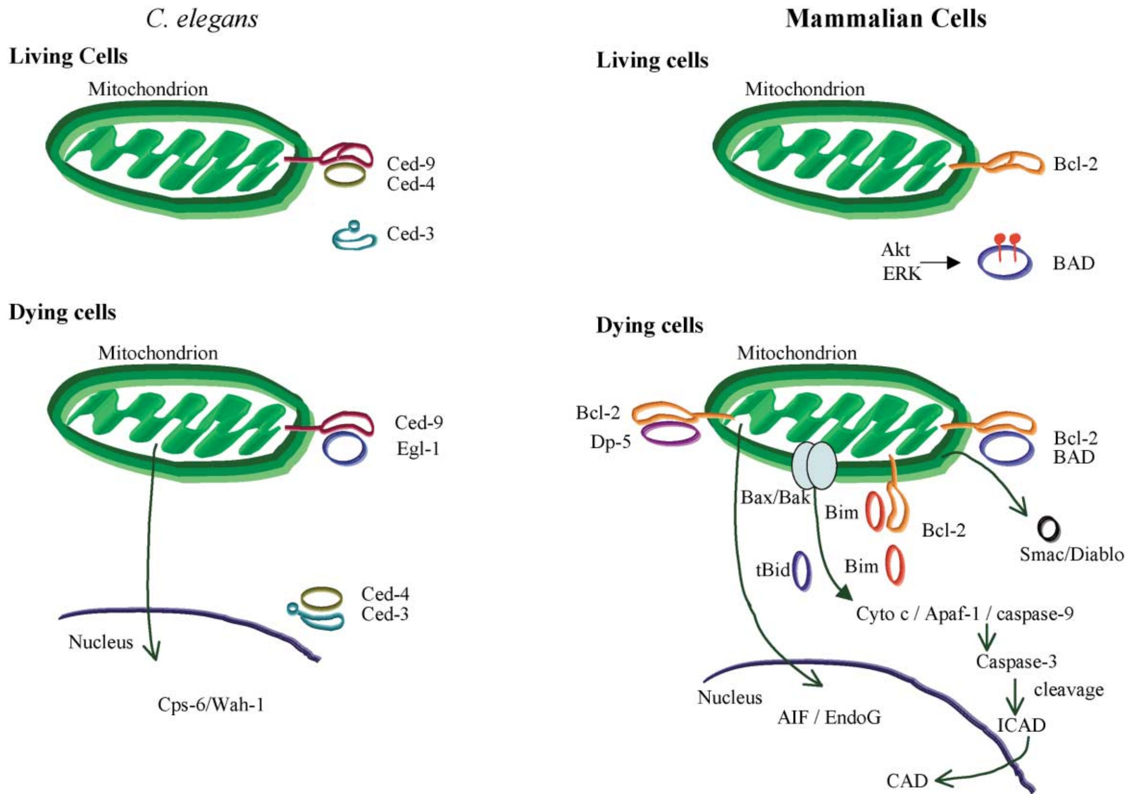

Figure 1. Apoptosis Pathways of *C. elegans* and Mammalian Cells

In living cells, mitochondrially localized Ced-9 binds to Ced-4, which prevents Ced-4 from activating Ced-3. In dying cells, the expression of *egl-1* is induced. Egl-1 binds to Ced-9 to alleviate its inhibition of Ced-4. Ced-4 then translocates to the area around nucleus and activates Ced-3. The activation of Ced-3 is at least partially responsible for the mitochondrial release of Cps-6 and Wah-1, which may cooperate to induce DNA fragmentation. In mammalian cells, anti-apoptotic Bcl-2 family proteins and anti-apoptotic kinase Akt and ERK protect the mitochondrial integrity by inhibiting pro-apoptotic Bcl-2 family members. In dying cells, several pro-apoptotic members of Bcl-2 family (tBid, Dp-5, Bim, Bax, Bak, and BAD) may antagonize the anti-apoptotic Bcl-2 family proteins to induce mitochondrial damage. The subsequent release of cytochrome c from damaged mitochondria induces the formation of apoptosome by recruiting caspase-9 and Apaf-1. Active caspase-9 cleaves and activates caspase-3, which in turn cleaves a variety of cellular substrates including ICAD and allows CAD to induce DNA laddering. Apoptotically damaged mitochondria also release other factors, such as AIF and EndoG, to facilitate DNA fragmentation and Smac/Diablo to facilitate caspase activation.

pase-3 activation (Hakem et al., 1998). Although the lack of Apaf-1 and caspase-9 both results in brain developmental defects, it is not yet clear whether the impacted areas are exactly identical, as a deficiency in Apaf-1 has been described to preferentially affect apoptosis in the mantal layer where differentiated neurons are localized (Cecconi et al., 1998), whereas caspase-9 deficiency has been shown to have a major effect in the numbers of immature neurons in the ventricular zone (Hakem et al., 1998). Clearly it will be beneficial to study these mutant mice side-and-side and with double mutants.

Active caspase-9 cleaves and activates short prodomain caspases such as caspase-3 and caspase-7, which carry out the execution steps of apoptosis by cleaving many downstream substrates. For example, the cleavage of ICAD (inhibitor of caspase-activated DNAase) by caspase-3 results in the activation of CAD (caspase-activated DNAase), which leads to the formation of DNA ladder (Enari et al., 1998). *caspase-3*$^{-/-}$ mice exhibit a genetic background-dependent perinatal lethality associated with excess neurons in the brain (Kuida et al., 1996). Since the genetic defects of *caspase-3*$^{-/-}$ mice are less severe than those observed in *apaf-1*$^{-/-}$ or *caspase-9*$^{-/-}$ mice, it is generally assumed that additional caspases, such as caspase-7, may carry out the execution of apoptosis in parallel with caspase-3 or that a compensatory mechanism is activated in *caspase-3*$^{-/-}$ mice.

In sum, apoptosis is a critical mechanism regulating neuronal cell number and proper connectivity in the developing nervous system. This is highlighted by the dramatic alterations in the central nervous system as a result of genetic deficiency in the core apoptotic machinery. These results suggest that apoptosis is crucial in regulating brain development.

Although it is unequivocal that caspase-dependent apoptosis plays an important role in regulating neuronal cell numbers during development, evidence is accumulating that neurons are also intrinsically capable of dying even when the canonical apoptotic mechanisms fail. For example, *apaf-1* deficiency was found insufficient to prevent death of embryonic peripheral neurons caused by the lack of trophic factor signaling in the *TrkA*$^{-/-}$ mice (Honarpour et al., 2001). This observation suggests that

Apaf-1-mediated caspase activation may not be involved in trophic factor deprivation-induced peripheral neuronal cell death and raises the possibility that peripheral neurons may be able to die through an alternative caspase-dependent or caspase-independent mechanism. Consistent with this proposal, Rakic and colleagues reported that apoptosis of spinal and cranial motoneurons and DRG sensory neurons was normal in *apaf-1*$^{-/-}$ mice; furthermore, brainstem, spinal cord, and peripheral ganglia in *caspase-9*$^{-/-}$ and *caspase-3*$^{-/-}$ embryos and mice were also found to be remarkably normal, with no apparent increases in cell numbers (Oppenheim et al., 2001). Although there is a prominent defect in caspase activation and an obvious lack of apoptotic cells in the tissues of *caspase-9*$^{-/-}$ and *caspase-3*$^{-/-}$ mice, the total number of dying neurons remain the same as that in wild-type embryos. Histological and electron microscopy studies revealed that dying *caspase-3*$^{-/-}$ or *caspase-9*$^{-/-}$ neurons exhibit extensive cytoplasmic vacuoles including dilation of mitochondria and rough endoplasmic reticulum, which morphologically resemble the type 3 cell death (Oppenheim et al., 2001). It is currently unclear whether these cell death events reflect somewhat redundant role of caspases in certain types of neuronal activation downstream from the irreversible neuronal commitment to death associated with mitochondrial collapse or from engagement of an alternative signaling/execution mechanism altogether. However, mouse knockout data clearly points to the existence of multiple alternative genetically predefined mechanisms of developmental cell death with respect to the roles of caspases, since only a subset of events during cell death critically requires caspase activity, whereas others may be mediated by caspase-independent mechanisms.

AIF—a Double Agent of Life and Death

AIF (apoptosis-inducing factor), a mitochondrial flavoprotein with an oxidoreductase domain, was identified in a screen for mitochondria-released pro-apoptotic factors (Susin et al., 1999). AIF is localized to the mitochondrial intermembrane space in living cells and translocates to the cytoplasm and nucleus under certain apoptotic conditions. In mammalian cells, AIF was shown to induce nuclear condensation and large-scale DNA fragmentation to ~50 kb fragments in a caspase-independent fashion. A possible role of AIF in mediating neuronal cell death was highlighted by a report from the laboratory of Valina Dawson, demonstrating that AIF is a downstream mediator of poly(ADP-ribose) polymerase-1 (PARP-1)-induced neuronal cell death (Yu et al., 2002). PARP-1 mediates protein ADP ribosylation in response to DNA damage, and its activation has been found to contribute to a variety of cell death paradigms. PARP has been shown to play a role in neuronal cell death induced by ischemia-reperfusion injury (Eliasson et al., 1997; Endres et al., 1997) and in NMDA-induced excitatory neuronal cell death (Yu et al., 2002). Consistent with the role of AIF in mediating PARP-1-dependent neuronal cell death, nuclear translocation of AIF was observed in wild-type, but not in *PARP-1*$^{-/-}$, cortical neurons (Yu et al., 2002). Furthermore, a neutralizing antibody to AIF prevented nuclear condensation and delayed neuronal cell death induced by the DNA damaging agent. Since multiple reports suggested that NMDA excitatory neuronal cell death is at least partially caspase independent and AIF nuclear translocation was observed in the presence of caspase inhibitors, these data suggest that AIF may be one of the mediators of caspase-independent neuronal cell death.

While the data from cultured neurons are consistent with a prodeath role of AIF, genetic analysis of a hypomorphic allele of AIF in mice provided an unexpected twist to this story. Ackerman's laboratory reported that Harlequin (*Hq*), a mouse mutant with progressive age-related degeneration of cerebellar and retinal neurons, is caused by a proviral insertion in the first intron of the *Aif* gene, leading to an 80% reduction in AIF expression (Klein et al., 2002). While the development of *Hq* mice is normal, a significant number of degenerating cerebellar granule cells were observed by 4 months of age. Purkinje cells in *Hq* mutant mice also degenerate, but only at a later time point than that of granule cells. Interestingly, while degenerating granule neurons exhibit typical signs of apoptosis including nuclear condensation and membrane blebbing, degenerating Purkinje cells show necrotic features with swollen mitochondria and membrane fragmentation (Klein et al., 2002). The earliest detectable abnormality in *Hq* cerebellum is the increased level of glutathione and catalase activity at 1 month of age, when the mutant cerebellum is still morphologically and functionally normal. Increased levels of lipid hydroperoxides, a sign of free radical damage, were found in the whole brain including cerebellum. Another hint of elevated oxidative stress in degenerating *Hq* cerebellar granule cells and retinal neurons was an increase in cytoplasmic immunoreactivity to 8-hydroxydeoxyguanosine (8-OhdG), suggesting oxidatively damaged mitochondrial DNA.

A parsimonious explanation of these potentially conflicting results is that AIF is a dual agent, which promotes DNA fragmentation under apoptotic conditions and maintains normal mitochondrial functions in living cells in a fashion similar to that of cytochrome c. A support for this hypothesis came from genetic knockout of *Aif*, which showed that ES cells hemizygous for *Aif*$^{-/Y}$ were unable to differentiate properly in vivo, suggesting that AIF performs a function that is vital for animal development (Joza et al., 2001).

Structurally, AIF contains a N-terminal FAD binding domain, a central NADH binding domain homologous to bacterial nicotinamide adenine dinucleotide (NAD)-dependent oxidoreductase, and a unique C-terminal domain (Mate et al., 2002; Ye et al., 2002). In vitro AIF exhibits NADH oxidase activity, but this activity is not required for its pro-apoptotic activity (Miramar et al., 2001). Increased oxidative stress detected in *Hq* animals suggests an intriguing possibility that the oxidoreductase activity of AIF may be related to its antiapoptotic function under normal conditions, aimed at elimination of ROS.

Structural analysis of AIF also revealed the presence of a strong positive electrostatic potential at the AIF surface, which may allow it to bind DNA in a sequence-independent manner and thus contribute to its activity in mediating DNA degradation (Ye et al., 2002). The DNA degradation activity appears to be an evolutionarily ancient activity of AIF, as RNAi-mediated inhibition of

Wah-1, the *C. elegans* ortholog of mammalian AIF, was shown to delay the normal progress of apoptosis and DNA degradation (Wang et al., 2002). *Wah-1* RNAi-treated worms were viable but exhibited slower growth rate and a smaller brood size than control worms.

AIF was shown to induce chromatin condensation and DNA fragmentation independent of caspase activity in cortical neurons stimulated by high doses of NMDA (Yu et al., 2002). However, since AIF may play a key role in serum deprivation-induced neuronal cell death (Joza et al., 2001; Klein et al., 2002), which is a caspase-dependent process regulated at least in part by BH3-only proteins such as Bim and Dp5/Hrk, it is reasonable to propose that AIF is regulated by caspases under certain apoptotic conditions. In *C. elegans*, the WAH-1 function is regulated by ced-3 caspase activity, as both its release from mitochondria and its DNA degradation activity are at least partially dependent upon *ced-3*. Consistent with this proposal, the release of mammalian AIF has also been proposed to be regulated in part by caspases (Arnoult et al., 2003).

In addition to AIF and cytochrome c, mitochondria also release other factors, such as EndoG (Li et al., 2001) and Smac/Diablo (Du et al., 2000; Verhagen et al., 2000), to facilitate the downstream events in apoptosis. The release of Smac has been proposed to play a role in facilitating trophic factor deprivation and amyloid β peptide-induced neuronal apoptosis downstream from mitochondrial damage (Deshmukh et al., 2002; Yin et al., 2002).

The Role of Autophagy in Neuronal Cell Death: Prodeath, Prosurvival, or a Self-Clearance Mechanism?

Autophagy is an intracellular lysosome-mediated catabolic mechanism that is responsible for the bulk degradation and recycling of damaged or dysfunctional cytoplasmic components and intracellular organelles (Klionsky and Emr, 2000). At the ultrastructural level, the main criterion for recognizing autophagy is the appearance of intracellular double membrane vacuoles containing cytoplasmic components such as fragments of endoplasmic reticulum or mitochondria and lysosomal hydrolases. Autophagy is an evolutionarily ancient cellular response to both extracellular stress conditions (nutrient deprivation, hypoxia) and intracellular stress conditions (accumulation of damaged organelles and cytoplasmic components) that allows lower eukaryotic organisms such as yeast to survive nutrient starvation conditions by recycling. Autophagy can be divided into microautophagy, macroautophagy, and chaperone-mediated autophagy, which differ in their mechanisms of vacuole formation and delivery of materials. Macroautophagy involves de novo formation of a sequestering vesicle in the cytosol and is the main mechanism involved in the degradation and recycling constituent of intracellular organelles. Microautophagy operates by protruding or invaginating a portion of pre-existing vacuolar membrane to engulf cytosol or organelles, whereas chaperone-mediated autophagy results from the delivery of the proteins with the signature sequence of KFERQ to lysosomes through designated lysosomal transporters and thus may be primarily involved in the degradation of specific proteins (Dice et al., 1990). All autophagic vacuoles eventually fuse with lysosomes, which provide hydrolases as degradating enzymes. We will limit our discussion to macroautophagy, which is the most-studied form and is associated with autophagic cell death, and we will refer to it simply as autophagy from now on.

A subset of physiological cell death in higher organisms associated with extensive formation of intracellular vacuoles was originally termed by Schweichel and Merker (1973) as type 2 cell death. The most dramatic example of autophagic-like cell death is found during the metamorphosis of insects. In *Drosophila*, it was found that the destruction of obsolete larval tissues such as midgets and salivary glands induced by pulses of the steroid hormone 20-hydroxyecdysone (ecdysone) is accompanied by massive accumulation of lysosomes, as well as hallmarks of apoptosis, including DNA fragmentation and caspase activation. Induction of key mediators of apoptosis in *Drosophila*, including the key regulators *rpr* and *hid*, caspases *dronc* and *dcp-1*, and the homolog of ced-4/Apaf-1, *dark/ark*, immediately precedes programmed cell death (Gorski et al., 2003; Lee and Baehrecke, 2001). Expression of p35, a general caspase inhibitor encoded by baculovirus, inhibits the death of midgets and salivary glands, indicating the requirement of caspases in this process (Jiang et al., 1997). Interestingly, the expression of p35 also reduces the prevalence of autophagic vacuoles, suggesting a role of caspases in initiating the autophagic process (Lee and Baehrecke, 2001). Thus, although the metamorphic cell death exhibits morphological features of autophagy, distinguishable from the canonical apoptosis described above, it may nevertheless share many of the same molecular players as apoptosis. The activation of autophagy during metamorphosis may be necessitated by the need to eliminate large amounts of larval tissue in the absence of enough professional engulfment cells and thus might represent a clearance mechanism for dead cells rather than being directly involved in cell death.

Neuronal cell death with features of autophagy has also been described during vertebrate development. One such example is the naturally occurring developmental death of neurons in chick isthmo-optic nucleus (ION) (Clarke, 1990). The survival of developing chick isthmo-optic neurons requires both anterograde signals from tectum and retrograde signals from retina. The neurons of ION that make inappropriate projections are eliminated through a cell death mechanism involving formation of autophagic vacuoles. Furthermore, NGF deprivation-induced sympathetic neuronal cell death and serum deprivation-induced death of PC12 cells have also been found to exhibit autophagic features (Ohsawa et al., 1998; Xue et al., 1999).

Although the mechanism of autophagy in mammalian cells remains sketchy (Mizushima et al., 2002), genetic studies in yeast have revealed a complex pathway and have identified the key factors required for the activation and execution of autophagy (Klionsky and Emr, 2000; Ohsumi, 2001). Activation of autophagy allows yeast to survive nutrient starvation, and yeasts lacking apg6/vps30 are defective in both their ability to undergo nitrogen deprivation-induced autophagy and to properly sort selective vacuolar proteins. Beclin1, the human ortholog

of yeast Apg6/Vps30p, provides an intriguing link between autophagy and neuronal cell death. Beclin1, which was initially isolated as a Bcl-2-interacting protein, can complement yeast Apg6 function in yeast autophagy and can promote autophagy when overexpressed in cultured MCF7 cells (Liang et al., 1999). Further studies suggested that Beclin1 forms a complex with mammalian PI3-kinase and regulates membrane dynamics during autophagy (Kihara et al., 2001). Since Beclin1 overexpression also offers protection against Sindbis virus-induced neuronal cell death (Liang et al., 1998), it is possible that Beclin1-induced autophagy acts as a defense mechanism that results in lysosomal capture and removal of Sindbis virus. It is not clear whether the interaction of Beclin1 with Bcl-2 regulates its autophagic activity or its ability to protect from Sinbis virus, but it is unlikely that Beclin1 acts by directly inhibiting neuronal cell death.

Analysis of the mouse mutant *Lurcher* provided another possible example of autophagic neuronal cell death (Yue et al., 2002). Heterozygous *Lurcher* mice are ataxic due to the degeneration of the cerebellar Purkinje cells followed by the secondary death of granule cells and inferior olivary neurons during the first four postnatal weeks. Death of *Lurcher* Purkinje cells is due to a constitutively activating mutation in GluRδ2, one of the glutamate receptor (GluR) channel subunits. Yue et al. (2002) showed that GluRδ2 interacts with the complex of Beclin1 and nPIST, a PDZ domain-containing protein. Although the functional consequence of this interaction is not clear, nPIST and Beclin1 can synergize to induce autophagy. Furthermore, mutant GluRδ2^{Lc}, but not the wild-type GluRδ2^{wt}, can also induce autophagy, and dying *Lurcher* Purkinje cells contain an excess of autophagic vacuoles. This evidence suggests, but does not prove, that Purkinje cell death induced by GluRδ2^{Lc} may be related to autophagy.

A direct conclusive demonstration of involvement of autophagy in neuronal cell death impinges on the demonstration that a loss-of-function mutation(s) in one of the genes required for autophagy can also block neuronal cell death. Unfortunately, we do not yet have such evidence, primarily due to the lack of understanding of the mechanism of autophagy in mammalian cells. A functional role of autophagy in neuronal cell death has been suggested through the inhibition of autophagy and delay of neuronal cell death by 3-methyladenine (3-MA). 3-MA has been shown to inhibit in vitro class III phosphatidylinositol-kinase activity, which had been previously found to be required for the sequestration step of autophagy in yeast (Klionsky and Emr, 2000). The ability of 3-MA to delay apoptosis in various systems, such as NGF deprivation-induced death of sympathetic neurons (Xue et al., 1999), has been taken as evidence supporting the involvement of autophagy in neuronal cell death. However, the poor specificity of 3-MA puts this conclusion on considerably shaky ground. Furthermore, the potentially contrasting roles of different phosphatidylinositol species in regulating autophagy and cellular vesicular trafficking and the multiple roles of PI3 kinases in regulating cell survival urge extra caution in drawing any quick conclusions (Cantley, 2002; Klionsky and Emr, 2000).

Thus, despite the extensive morphological evidence of autophagy during neuronal cell death and cell death in other systems, the conclusive evidence for a prodeath role of autophagy during neuronal cell death is still missing. Although it is not hard to imagine that wholesale degradation of intracellular organelles would adversely affect cell survival, the present evidence is not sufficient to conclude whether the autophagy per se can constitute an independent pathway of programmed cell death or merely one of the downstream manifestations of a much more complex process. The ability of caspase inhibitor p35 to halt metamorphic cell death without complete inhibition of autophagy in *Drosophila* suggests that it is caspase-mediated apoptosis, rather than autophagy, that plays the key role in the process. In mammalian cells, overexpression of Beclin1 has been shown to induce extensive autophagy in nutrient-deprived MCF7 cells without affecting cell viability (Liang et al., 1999), indicating that high levels of autophagic activity can be compatible with life.

One of the most obvious differences between canonical apoptosis and apoptosis with autophagic features may be the mechanism of dead cell degradation: most apoptotic cells are engulfed by professional or nonprofessional phagocytes, and the lysosomes of phagocytes are responsible for the final degradation of dead cell bodies; cells undergoing apoptosis with concomitant activation of the autophagic pathway may not only be committing suicide through the caspase-mediated mechanism, but also carrying out their own body cremation through autophagy. The latter process may be necessary for metamorphosis or large-scale tissue histolysis where the entire tissue needs to be eliminated and available engulfment cells would be insufficient to remove all of the dead cell bodies. The current data actually is not incompatible with the hypothesis that autophagy during neuronal cell death serves as a clearance mechanism and does not participate critically or directly in the execution process of cell death.

On the other hand, it is clear that autophagy is a prosurvival mechanism in yeast (Ohsumi, 2001). It is also formally possible that the activation of autophagy in mammalian cells in the event of nutrient and trophic factor deprivation is a cellular strategy to survive through perhaps temporary hardship through recycling intracellular components, rather than a death mechanism. A direct proof or disproof for this notion has to come from a better characterization of the mammalian autophagic pathways.

Why might some, but not other, apoptotic processes be associated with the activation of autophagy? It is interesting to speculate that perhaps the relative levels of activity of different classes of PI3 kinases play a role. Increasing levels of the class III PI3 kinase product phosphatidylinositol 3-phosphate (PI3P) stimulates autophagy, whereas increases in the class I PI3-kinase products, phosphatidylinositol 3,4-bisphosphate (PI3,4P) and phosphatidylinositol 3,4,5-trisphosphate (PIP3), inhibit autophagy (Petiot et al., 2000). The activity of class I PI3 kinase is also critical for the membrane recruitment and activation of Akt, a Ser/Thr kinase that was shown to mediate PI3K-dependent trophic factor-dependent survival (Crowder and Freeman, 1998; Dudek et al., 1997). Perhaps when apoptosis is activated through inhibition of class I PI3 kinase pathway, such as in NGF-

deprived sympathetic neurons, autophagy may be automatically activated as a result of imbalance between the activities of class I and class III PI3 kinases. In areas where many cells are being eliminated, a severe lack of prosurvival signals may result in an extreme imbalance between the class I and class III PI3 kinases, which may account for the massive activation of autophagy observed during development or metamorphosis.

The evidence of activation of autophagy has been found in neurodegenerative diseases. Increased levels of autophagy were observed in neuronal cell lines expressing mutant proteins associated with such diseases. Expression of Parkinson's disease (PD)-associated A53T, but not wild-type, α-synuclein in PC12 cells induced massive autophagic vacuole formation (Stefanis et al., 2001). In addition, dopamingeric neurons with autophagic vacuoles have been observed in the substantia nigra of PD patients, along with neurons displaying apoptotic features (Anglade et al., 1997). Excess amount of multivesicular bodies, endosomes, and lysosomes was also observed in biopsy tissue from Huntington's disease (HD) patients (Bahr and Bendiske, 2002). Overexpression of wild-type or mutant huntingtin, the protein associated with the development of HD, was found to result in the formation of cathepsin D-positive autophagic vacuoles, which accumulated huntingtin (Kegel et al., 2000). The mechanisms by which mutant huntingtin and α-synuclein induce formation of autophagic vacuoles are not clear. One possibility is that autophagy is activated as a compensatory mechanism for a defect or an insufficiency in the proteasome pathway, which may be impaired in chronic neuronal degenerative diseases (Kopito, 2000). The functional consequence of increased autophagic activity for neurodegeneration is also not clear. It has been suggested that excessive autophagy may directly lead to neuronal cell death; however, as discussed above, this hypothesis still awaits a convincing proof. Alternatively, since autophagy is a cellular catabolic mechanism involved in the removal of large protein aggregates that may be undegradable through the proteasome pathway, it is possible that autophagy might actually facilitate the removal of mutant or otherwise misfolded proteins. In this vein, Ravikumar et al. (2002) has recently shown that rapamycin, an inhibitor of TOR (the target of rapamycin), which is a member of PI3 kinase family known to stimulate autophagy by inhibiting the activity of p70S6 kinase, can reduce the formation of insoluble polyglutamine aggregates under certain cell culture conditions. Further study of the involvement of autophagy in the neurodegenerative diseases is clearly a very interesting research topic.

Lysosome—a "Suicide Bag"?

In addition to autophagy, elevated lysosomal activity per se and cytoplasmic release of lysosomal hydrolases have been proposed to induce cell death. Lysosomes contain more than 80 hydrolytic enzymes, including the cathepsins, a group of a dozen or more proteases of varying peptide bond specificities. The potential danger of the accidental release of lysosomal hydrolases was recognized by the discover of the lysosome, De Duve, who called it a "suicide bag" (De Duve, 1955). Lysosome plays a major role in degradation of long-lived and membrane-associated proteins and large protein complexes. The proper function of lysosomes is critical for cell and organism survival, and deficiencies in lysosomal function have been associated with multiple human ailments, including a large class of lysosomal storage diseases, some of which are characterized by massive neuronal cell death (Walkley, 1998). On the other hand, disruption of lysosomes, which can be induced by high doses of lysosomotrophic agents (Wilson et al., 1987), leading to release of lysosomal hydrolases, is also highly cytotoxic. Cell death induced by lysosomal disruption is distinct from apoptosis, as apoptotic cells preserve their lysosomal integrity until the last moment. Thus, complete lysosomal breakdown does not play a role in the execution of canonical apoptosis. Furthermore, although lysosomal hydrolases such as cathepsin B have been proposed to mediate both caspase-dependent and caspase-independent cell death in transformed cells (Foghsgaard et al., 2001), their role in the death of primary cells, including neurons, may be minor.

Although there is no strong evidence supporting the role of lysosomes in the downstream common execution pathway of neuronal cell death, induction of lysosomal degradative capacity as a cellular response to stress and neurotoxic proteins may inadvertently compromise the integrity of the "suicide bag" and lead to cell death. Along those lines, activation of lysosomal pathways has been recognized as an early feature of Alzheimer's disease (AD) (Nixon et al., 2000). The brains of AD patients showed a massive increase in cathepsin B and D levels as well as in the number of lysosomes in the vulnerable neurons of the CA1 region. We still do not know, however, if such increases in lysosomal activity in AD play a role in the induction of the neuronal cell death or actually reflect an effort by the neuronal cells to reduce or remove neurotoxic agents.

The neurotoxicity of Aβ1-42 peptide has been recognized as an important factor in the pathogenesis of Alzheimer's disease (Yuan and Yankner, 2000). At least a part of the extracellular pool of Aβ peptide is taken up by the low-density lipoprotein receptor-related protein in the presence of a ligand such as apoE. The newly up-taken E4 isoform of apolipoprotein E (apoE4), a genetic risk factor for late-onset AD, was found more frequently than apoE3 to colocalize with cathepsin D in neurons, suggesting that the apoE4 isoform may preferentially track through lysosomes in neurons (DeKroon and Armati, 2001). Although lysosomal degradation of Aβ may represent an important cellular pathway for reducing its neurotoxicity, accumulation of aggregated Aβ1-42 in late endosomes or secondary lysosomes may also eventually damage the integrity of lysosomal membrane and result in leakage of lysosomal hydrolases into cytosol, enhancing cell death (Yang et al., 1998). A neural cell line expressing apoE4 was found to be more sensitive to cytotoxicity of Aβ peptide than a line expressing apoE3 (Ji et al., 2002). These observations have led to the proposal that the insertion of apoE4 into the lysosomal membrane may facilitate the neurotoxicity of Aβ peptide by destabilizing lysosomal membrane.

Similar to AD, lysosomal dysfunction has been proposed to play a role in other neurodegenerative diseases such as PD and HD. We must point out, however, that although the evidence supporting a role of lysosomal

damage in neuronal cell death is tantalizing, it is far from conclusive. Our ability to obtain an unequivocal proof is hampered by the dual role of lysosomes in clearance of neurotoxic proteins as well as their ability to deliver a lethal insult once they are damaged. Nevertheless, a contribution of the lysosomal pathway to neuronal cell death in human neurodegenerative diseases cannot be discounted.

Regulated Cellular Necrotic Program

Although our discussion above points out the lack of sufficient proof that autophagy, lysosomes, and AIF can act as bona fide programmed cell death mechanisms independent of caspases, the presence of developmental cell death in *caspase* and *apaf-1* mutant mice supports the existence of a genetically controlled alternative cell death pathway(s). It is important to keep in mind that morphological evidence of neuronal cell death similar to that of type 3B necrosis was not only observed in *caspase-3*$^{-/-}$ and *caspase-9*$^{-/-}$ mice (Oppenheim et al., 2001), but also in classical studies of developmental neuronal cell death in normal developing embryos. In an elegant series of ultrastructural studies of developmental neuronal cell death, Chu-Wang and Oppenheim (1978) showed that naturally occurring motoneuron death may exhibit typical necrotic features, including dilation of ER, Golgi apparatus, and nuclear membrane without condensation of chromatin.

It is important, however, to differentiate such naturally occurring necrosis from the type of cell death observed in vitro under nonphysiological conditions, also commonly referred as "necrosis," which may include rapid swelling and bursting of entire cellular content. Cells dying in the physiological context would certainly be eliminated quickly through engulfment by professional or nonprofessional engulfment cells and therefore unlikely to burst as they may in tissue culture dishes. While necrosis has been traditionally referred to as unregulated pathological cell death, increasing examples of cell death with necrotic features described under normal physiological and/or certain pathological conditions suggest that at least in some instances necrosis may also be a regulated cellular mechanism. Since we know very little about the mechanism of necrosis, it is hard to come up with a precise definition of necrosis. We will define necrosis here operationally as caspase-independent cell death that occurs under certain normal physiological and pathological conditions.

Fas and TNF family of death receptors mediate the canonical apoptosis pathway that includes the recruitment of adaptor protein FADD and caspase-8 into the receptor complex to induce activation of caspase-8, which in turn stimulates the downstream caspases, such as caspase-3 (Cryns and Yuan, 1998). A surprising conclusion emerging in the last few years is that when caspases are inhibited in certain cell types, Fas and TNF can induce cell death with necrotic features including swelling of the mitochondria and ER, intracellular vacuolization, and dilation of the nuclear membrane (Matsumura et al., 2000; Vercammen et al., 1998). This process is accompanied by the loss of the mitochondrial transmembrane potential, but not by the release of cytochrome c, indicating that mitochondrial damage inflicted by necrosis may be fundamentally different from that caused by apoptosis. Consistent with this proposal, the treatment with an antioxidant, pyrrolidine dithiocarbamate (PDTC), blocks the mitochondrial swelling, necrosis-induced mitochondrial membrane potential loss, and cell death, but had no effect on apoptosis (Matsumura et al., 2000). The mechanism of receptor-mediated necrosis remains sketchy; however, it was shown to require both adaptor protein FADD (Fas-associated death domain), which is also necessary for apoptosis, and the Ser/Thr kinase RIP (Holler et al., 2000), which is dispensable in apoptotic cell death (Stanger et al., 1995). Thus, apoptosis and necrosis induced by Fas share some common mediators, which then bifurcate downstream to induce distinct apoptotic and necrotic signaling cascades.

RIP has been shown to mediate TNFα-induced NF-κB activation by recruiting IKK complex to TNFR in a kinase-independent fashion in apoptosis; however, the kinase activity of RIP is required for activation of necrosis (Holler et al., 2000) but not NF-κB activation in apoptosis (Ting et al., 1996). Thus, it appears that the induction of necrosis may represent a novel function of RIP, possibly operating through phosphorylation and regulation of key player(s) of necrosis. The role of RIP in neuronal cell death is not yet clear, although the formation of Fas signaling complex including RIP has been observed in ischemic brains (Qiu et al., 2002).

Early loss of ATP synthesis, which might be the result of a failure in homeostatic mechanism, is a salient feature of necrosis. This is in contrast to the initiation of an energy-dependent suicide mechanism such as apoptosis, in which a certain level of ATP synthesis is maintained until late in the process. Although a failure in homeostatic mechanism can be brought about by external accidental events, specific internal self-destruct mechanisms may also be able to trigger it. Since necrosis is characterized by swelling of intracellular organelles, it is possible that the loss of ATP synthesis is a direct consequence of mitochondrial swelling.

What may be the mechanism of the mammalian necrotic cell death? An interesting parallel can be drawn from the elegant work on necrotic cell death in *C. elegans*. Members of the degenerin gene family of *C. elegans* encode the subunits of the amiloride-sensitive epithelial membrane sodium channel, in which some mutations can cause necrotic neuronal degeneration including intracellular vacuolation and swelling. Most degenerating mutations in this family are dominant gain-of-function mutations that result in altered and/or increased channel activity (Hall et al., 1997). Driscoll's laboratory reported that loss-of-function mutations in calreticulin, a major Ca^{2+} binding (storage) chaperone in the endoplasmic reticulum, and its partner calnexin, an ER membrane protein, or double mutations in both *unc-68* (ryanodine receptor) and *itr-1* (inositol 1,4,5 triphosphate receptor) that block the ER Ca^{2+} release channels, or pharmacological manipulations that block ER Ca^{2+} release can all suppress the necrotic degeneration (Xu et al., 2001). Thus, an increase in the concentrations of cytoplasmic Ca^{2+}, elicited by the release of endoplasmic reticulum stores, may play a major role in initiating the downstream necrotic cell death events

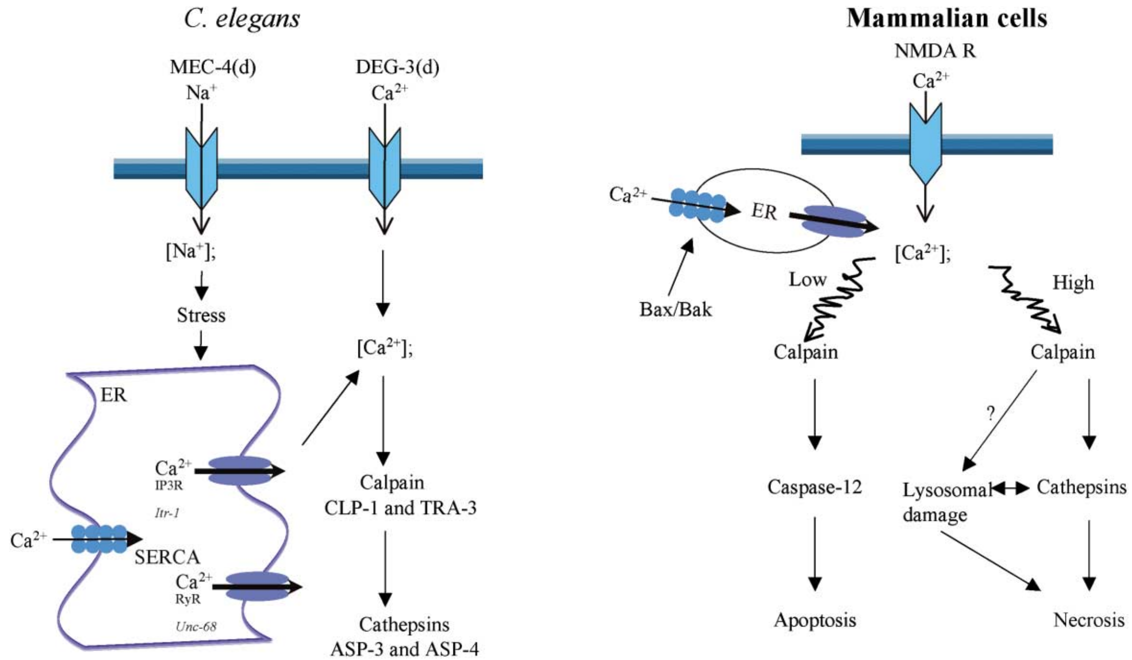

Figure 2. The Mechanism of Necrosis in *C. elegans* and a Possible Mechanism of Rises of $[Ca^{2+}]_i$ in Mammalian Cells Controls the Alternative Cell Death Pathways of Apoptosis or Necrosis

In *C. elegans*, specific dominant gain-of-function mutations in the cytoplasmic membrane Na^+ channel protein MEC-4 or Ca^{2+} channel protein DEG-3 result in the abnormal rises in $[Ca^{2+}]_i$ through either release of ER Ca^{2+} storage or increased entry of extracellular Ca^{2+}. Elevated $[Ca^{2+}]_i$ results in the activation of calpains, CLP-1 and TRA-3, which in turn activate the cytosolic cathepsins, ASP-3 and ASP-4, and necrosis. In mammalian cells, low levels of $[Ca^{2+}]_i$ rises from releases of ER Ca^{2+} storage may induce apoptosis through localized calpain activation, which in turn activates caspase-12. High levels of $[Ca^{2+}]_i$ may induce necrosis by extensive activation of calpains, which subsequently activate cathepsins.

induced by abnormal functioning of the cytoplasmic membrane Na^+ channels.

Excessive increases in intracellular Ca^{2+} were proposed to trigger the activation of calpains, the cytosolic Ca^{2+}-activated cysteine proteases, which may in turn degrade cytoplasmic proteins to generate the "translucent" appearance of cytoplasm often found in necrotic cell death. Consistent with this proposal, two calpains, CLP-1 and TRA-3, and two aspartyl proteases, ASP-3 and ASP-4, were found to be essential for MED-4(d)- and DEG-1-induced necrosis in *C. elegans* (Syntichaki et al., 2002). Furthermore, mutations in the genes encoding calpains and aspartyl proteases blocked not only necrotic cell death induced by abnormal activation of membrane Na^+ channels, but also necrotic cell death induced by mutations in *deg-3*, which encodes a cytoplasmic membrane Ca^{2+} channel, a part of the nicotinic acetylcholine receptor subunit. On the other hand, necrotic cell death induced by mutations in *deg-3* cannot be blocked by inhibiting ER Ca^{2+} release (Xu et al., 2001). Thus, the activation of calpains and aspartyl proteases may be a common downstream step activated by the elevated intracellular Ca^{2+} that results from disruptions of the intracellular ER Ca^{2+} homeostasis or abnormal membrane Ca^{2+} channel activity (Figure 2).

Interestingly, although ASP-3 and ASP-4 may be present in both cytoplasm as well as lysosomes, they do not contain the conserved lysosome-targeting, *N*-glycosylation site (Asp71) that is typical of cathepsin D lysosomal proteases. Instead, both ASP-3 and ASP-4 contain a different potential *N*-glycosylation site that is common in nonlysosomal cathepsin E proteases. Thus, ASP-3 and ASP-4 may represent cytoplasmic cathepsins. Furthermore, although both calpains, encoded by *Clp-1* and *Tra-3*, and cathepsins, ASP-3 and ASP-4, are essential, the lack of calpains cannot be complemented by overexpressing *Asp-3* or *Asp-4*, suggesting that calpains act upstream of the aspartyl proteases. These results support the model in which necrotic cell death induced by MEC-4(d) and DEG-1 is the result of elevated cytoplasmic Ca^{2+} leading to the activation of calpains, which in turn initiate a proteolytic cascade by activating aspartyl proteases. No observable effect of the loss of lysosome-specific cathepsins on necrotic cell death suggests that necrosis may be mediated through cytoplasmic protease activity, rather than through lysosome-mediated proteolysis.

Elevated cytoplasmic Ca^{2+} has long been implicated in the induction of the mammalian neuronal cell death. However, its significance and mechanism has been controversial and confusing due to the multiple roles of Ca^{2+} in the regulation of different cellular processes. It is clear that only when intracellular Ca^{2+} concentrations exceed the normal physiological levels does cell death occur. Such excessive rises of intracellular Ca^{2+} may be induced by excitotoxicity caused by the accumulation of glutamate as a result of ischemic brain injury and subsequent overstimulation of postsynaptic glutamate receptors (Bonfoco et al., 1995). Of these glutamate-gated channels, kainite and AMPA receptor channel

subtypes conduct Na^+. NMDA receptor channels, which play a key role in excitotoxicity, conduct both Na^+ and Ca^{2+}. In this regard, it is interesting to note that small molecule intracellular Ca^{2+} release blocker dantrolene was shown to be able to block most of the Ca^{2+} rise resulting from NMDA receptor activation and to protect against excitotoxicity in vitro and in vivo (Frandsen and Schousboe, 1991; Zhang et al., 1993). These data implicate intracellular Ca^{2+} stores in mammalian neuronal cell death and support the existence of the necrotic neuronal cell death mechanism analogous to the one induced by mutations in the degenerin family of *C. elegans*.

Calpains and cathepsins have been proposed to mediate neuronal cell death under a variety of neurotoxic conditions. For example, the "calpain-cathepsin" hypothesis was proposed to explain necrotic cell death after ischemic brain injury (Yamashima, 2000). According to this hypothesis, ischemic brain injury-induced NMDA activation leads to intracellular Ca^{2+} overload, which activates calpain. Activated calpain may in turn activate lysosomal cathepsin and induce necrosis (Figure 2). However, the definitive proof of such a hypothesis was hampered by the lack of specific inhibitors of calpain and cathepsins as well as the multiple roles of calpains and cathepsins in normal cellular physiology. The two ubiquitous forms of calpain expressed in mammalian tissues are known as μ-calpain and m-calpain, which are cytosolic Ca^{2+}-dependent heterodimeric thiol proteases consisting of a unique 80 kDa large subunit (from the genes *Capn1* and *Capn2*, respectively) and a common 28 kDa small subunit (*Capn4*). Although *Capn4*$^{-/-}$ ES cells are viable, *Capn4*$^{-/-}$ embryos die in midgestation due to development defects, thus preventing a definitive demonstration of a role for calpains in necrosis (Arthur et al., 2000). Furthermore, since the identities of cathepsins involved in executing mammalian necrosis are unclear, a direct involvement of lysosomes in necrosis must also be viewed as tentative. However, taking an analogy from *C. elegans*, we hypothesize that perhaps cytosolic cathepsin analogs are the targets of calpains in regulating mammalian necrosis.

Intracellular Ca^{2+} is implicated in regulating not only necrosis, but also apoptosis. Bax and Bak, two pro-apoptotic members of the Bcl-2 family controlling mitochondrial integrity, also regulate the resting concentration of Ca^{2+} in the ER (Scorrano et al., 2003). Reduction of the ER Ca^{2+} levels in Bax/Bak double knockout cells offers strong resistance to a variety of pro-apoptotic stimuli that release the ER Ca^{2+} from intracellular stores (such as arachidonic acid, C2-ceramide, and oxidative stress) by reducing mitochondrial Ca^{2+} uptake. Although the mechanisms by which Bax/Bak regulate the ER Ca^{2+} remain obscure, expression of SERCA (sarcoplasmic-endoplasmic reticulum Ca^{2+} adenosine triphosphatase) corrected both the ER Ca^{2+} levels and induction of apoptosis in response to certain specific signals in Bax/Bak-deficient cells, demonstrating a key role of the ER Ca^{2+} in regulating cellular sensitivity to apoptosis. Since the ER Ca^{2+} release was also shown to play a key role in regulating necrosis, it is possible that Bak/Bak double knockout cells may be protected not only from apoptosis but also from necrosis induced by certain signals. Since Bax/Bak act as mitochondrial receptors for BH3-only protein such as BAD and tBid, we speculate that some unidentified BH3-only factor(s) may be specifically targeting the ER Bax/Bak. Therefore, induction of necrotic cell death, such as the type 3B developmental cell death, could be the result of activation of Bax/Bak checkpoint, which could promote necrotic cell death by releasing the ER Ca^{2+} (Figure 2).

We should also point out while excessive elevation of intracellular Ca^{2+} is detrimental to neuronal survival, intracellular Ca^{2+} plays important roles in regulating multiple aspects of neuronal survival, differentiation, and activity that are not the subjects of this review.

Apoptosis or Necrosis

Although apoptosis and necrosis are mediated through distinct pathways, the same insult can lead to either apoptosis or necrosis depending on its intensity, the neuronal subpopulation involved, and the species, age, and genotype of the organism involved. For example, low doses and short duration treatment with thapsigargin and tunicamycin induces the activation of caspase-12, and this cell death is inhibited by caspase-12 deficiency and pancaspase inhibitor zVAD.fmk (Nakagawa et al., 2000). On the other hand, when cells are treated with high doses of thapsigargin and tunicamycin for longer periods of time, cell death is no longer inhibitable by caspase-12 deficiency or zVAD.fmk, indicating the activation of additional caspase-independent cell death mechanism(s) (T. Nakagawa and J.Y., unpublished data).

Such transition from a caspase-dependent apoptosis to caspase-independent cell death is by no means a rare phenomenon. In vitro, excitotoxic glutamate-induced cell death has been previously shown to cause a mixture of apoptosis and necrosis, and in vivo, ischemic brain damage induces a mix of apoptosis and necrosis. Caspase inhibitors have shown significant efficacy against apoptotic neuronal cell death induced by brain ischemia; however, the majority of injured neurons in ischemic brains exhibit necrotic rather than apoptotic features and are resistant to caspase inhibitors (Moskowitz and Lo, 2003). Apoptotic neurons are generally localized to the penumbra of the stroke injury, which is preferentially affected by caspase inhibitors, whereas necrotic neurons are localized to the core. We speculate here that perhaps the extent of intracellular Ca^{2+} rise determines whether neurons undergo apoptosis or necrosis. It is possible that localized ER damage releases sufficient amount of Ca^{2+} to cause the activation of limited amount of m-calpain, which has been shown to efficiently cleave and activate caspase-12 (Figure 2; Nakagawa and Yuan, 2000). Such a limited amount of active calpain may be sufficient to activate the caspase pathway but not enough to activate other proteases, such as the mammalian homologs of *C. elegans* ASP-3 and ASP-4. Catastrophic increases in the intracellular Ca^{2+} levels, such as those induced during ischemic brain damage or severe ER stress, may induce sufficient amount of active calpain to mediate necrotic cell death through aspartyl protease pathways. A testable hypothesis here would be that caspase-12 may be a better substrate for calpain than the mammalian homologs of ASP-3 and ASP-4.

The absolute requirement for caspase inhibitors or

other means of caspase inactivation for the induction of necrosis by Fas or TNFα in certain cell lines suggests that perhaps caspases may actively inhibit necrosis. It has been shown that FasL-induced apoptosis results in rapid cleavage of RIP (Martinon et al., 2000). Since RIP is a critical mediator of FasL-induced necrosis, but not apoptosis, the cleavage of RIP in apoptosis may effectively block the activation of necrosis pathway at an early signaling step. In addition, since overactivation of PARP-1 may deplete intracellular ATP and by itself promote the switch from apoptosis to necrosis, PARP cleavage by caspases during apoptosis may prevent induction of necrosis during apoptosis and ensures appropriate execution of caspase-mediated apoptosis (Herceg and Wang, 1999).

On the other hand, conditions that activate necrosis may be incompatible with the execution of apoptotic pathway. The ability of high levels of Ca^{2+} to induce swelling and disruption of mitochondria, causing permeability transition and loss of energy metabolism, is likely a key event leading to necrosis. Since apoptosis is an energy-dependent mechanism, loss of mitochondrial energy metabolism will prevent cells from entering this mode of death. Indeed, conditions leading to the depletion of the cellular energy stores in vitro were shown to promote necrosis at the expense of apoptosis (Ankarcrona et al., 1995). High levels of calpain activity may also directly cleave and inactivate pro-apoptotic proteins, such as Apaf-1 (Reimertz et al., 2001) and caspase-7, -8, and -9 (Chua et al., 2000), despite the fact that m-calpain has been implicated in activating caspase-12 (Nakagawa et al., 2000). Such cross inhibition of alternative pathways of cell death may ensure the successful execution of cell death program under physiological and pathological conditions. On the other hand, since these reciprocal regulatory events may be influenced by a wide variety of factors in addition to caspase inhibitors, individual cells may differ in their decision to undergo apoptosis or necrosis, resulting in a mixture of apoptotic and necrotic death in neuronal cultures. The mutual suppression of apoptosis and necrosis underscores the highly regulated and programmed nature of these processes.

The Future

The apoptosis research in the past decade has provided an enormous amount of mechanistic insight into the role of apoptosis in regulating neuronal cell death. Although we do not yet have an anti-apoptotic drug in clinic, all the existing literature supports inhibition of apoptosis as a promising strategy for treatment of at least a subset of neurodegenerative diseases. However, apoptosis is clearly only part of the story. The existence of multiple programs of cell death is now well supported by the experimental evidence. This represents a new challenge, as we still understand very little about the mechanisms of nonapoptotic pathways in mammalian cells. The possible existence of a mammalian necrosis mechanism analogous to that caused by mutations in the degenerin family of *C. elegans* also represents a new opportunity, as the inhibition of necrosis may have less impact than apoptosis on homeotic regulation of cell numbers and thus may provide more specific targets for treatment of neurodegeneration.

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