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# Forum Editorial

## Programmed Neuronal Death

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THE FORM OF CELL DEATH that we now call naturally occurring cell death, programmed cell death, or apoptosis was first observed over a century ago (3). About 100 articles published in the 19th century contain descriptions of naturally occurring cell death. Most of this early work focused on insects and amphibians undergoing metamorphosis. Even though these initial observations occurred long ago, little progress was made in understanding the underlying mechanisms of this form of cell death until the 1970s. Indeed, there was little recognition that naturally occurring cell death was different from other forms of cell death. This situation began to change when Kerr *et al.* (15) performed a detailed analysis of dying cells. Their studies demonstrated that this form of cell death has features that clearly distinguish it from another form of cell death, necrosis. In naturally occurring cell death, bits and pieces of plasma membrane pinch off from cells and fall away from them as small vesicles. In some cell types, particularly immune system cells, this membrane blebbing is so pronounced that Kerr *et al.* thought it looked like leaves falling off a tree. Because of this feature, they coined the word, apoptosis, to describe this form of death (from Greek meaning falling off or away from).

In addition to blebbing of the plasma membrane, another central feature of cells undergoing apoptosis is atrophy. In all cases of apoptotic death, cells shrink before dying. In necrotic death, on the other hand, cells rapidly swell and lyse, provoking an inflammatory response that injures nearby cells. Before an apoptotic cell can lyse and cause damage to adjacent tissues, phagocytic cells engulf it. Indeed, apoptotic cells provide signals to phagocytic cells that induce their engulfment. In general, necrotic cell death is a passive form of death that occurs in pathological conditions, whereas apoptosis is an active, physiologically appropriate death. In other words, necrosis is death by accident, whereas apoptosis is death by design (cell suicide).

In addition to membrane blebbing and atrophy, another prominent feature of apoptotic death is DNA fragmentation. DNA degrades in cells dying by nonapoptotic mechanisms as well. However, the pattern of apoptotic DNA fragmentation is very different from that occurring in other forms of cell death. During necrosis, DNA is cleaved into strands of many different lengths, whereas DNA in cells undergoing apoptosis is cleaved primarily between nucleosomes resulting in strands of DNA that are multiples of 200 bp pairs in length. Other cardinal features of an apoptotic type of death include chromatin condensation, translocation of phosphatidylserine from the cytoplasmic face of the plasma membrane to the outside of the membrane, and activation of a highly conserved molecular cascade of events that is responsible for the apoptotic changes.

After the initial characterization of apoptosis in the early 1970s, the field continued to languish for about 15 years before any progress was made in understanding the molecular processes underlying this form of cell death. The work of Robert Horvitz and his colleagues with cell death in the nematode, *Caenorhabditis elegans*, in the mid 1980s (7) initiated the modern study of apoptotic death. During the development of *C. elegans*, 131 cells normally die by apoptosis. Horvitz and his colleagues found several cell death mutants in which few, if any, of these cells died. Instead, the cells lived on into the adult animal and, for the most part, were physiologically normal. Using these mutants, they were able to clone and sequence a number of genes involved in different aspects of developmental cell death in these worms. Of these, three genes expressed by dying cells were found to be essential regulators of the apoptotic process. They called these genes *ced-3*, *ced-4*, and *ced-9* for *C. elegans* death genes 3, 4, and 9. Both *ced-3* and *ced-4* are proapoptotic, whereas *ced-9* is antiapoptotic. Most of the other *ced* genes are involved in phagocytosis of dying cells and are not parts of a cell-intrinsic death program. However, *ced-3*, *ced-4*, and *ced-9* are expressed by dying cells, and certain mutations block their apoptotic activities.

Several years after Horvitz's seminal work, other groups began finding mammalian homologues of *ced* genes. The first one discovered was *bcl-2*, a homologue of *ced-9*. Like *ced-9*, *bcl-2* is antiapoptotic. Since this initial discovery, many other *bcl-2* homologues have been found. Some of these have anti-

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apoptotic actions like *ced-9*, whereas others promote apoptosis. In general, members of the *bcl-2* family serve to regulate the apoptotic process. The next mammalian *ced* homologue discovered was a gene coding for interleukin-1β converting enzyme (ICE), a protease already known to convert the cytokine, prointerleukin-1, to the active form, interleukin-1. To date, about 15 members of this family of proteases have been discovered in mammals. They serve as the central executioners of apoptotic death. Collectively, the ICE family of proteases are called caspases, a name derived from the fact that they have a cysteine at their catalytically active site and cleave other proteins after an aspartate residue (cysteine aspartases). The last mammalian homologue of the *ced* genes found was apoptosis protease activating factor 1 (APAF-1), a homologue of *ced-4*.

There are two major apoptotic pathways in mammalian cells, an extrinsic and an intrinsic pathway. Activation of the extrinsic pathway occurs when a ligand, such as tumor necrosis factor, binds onto a cell surface receptor that is coupled to a cytoplasmic caspase, typically caspase 8. The ligand binding activates the caspase, which then cleaves and activates other downstream caspases that are responsible for cell death. There are many possible triggers for the intrinsic pathway. For example, the absence of a sufficient quantity of a required growth factor, viral infection, or exposure to a toxin can activate this pathway. Although there is still much that is not understood about how these triggers activate the intrinsic pathway, a great deal is known about the molecules involved. Typically, when the intrinsic pathway is triggered, a proapoptotic member of the Bcl-2 family, for example Bax, translocates from the cytoplasm to the outer mitochondrial membrane. Once at the membrane, Bax and similar Bcl-2 family members induce release of apoptogenic factors from the mitochondrial intermembrane space into the cytoplasm. How this release occurs is the subject of much investigation, and there is no clear consensus on the exact mechanism. Chief among the factors released from mitochondria is cytochrome *c*, a major component of the mitochondrial electron transport chain. Once in the cytoplasm, cytochrome *c* binds onto APAF-1 and induces it to oligomerize into a structure known as the apoptosome that consists of several molecules of APAF-1, cytochrome *c*, and caspase 9 (11). This oligomerization activates caspase 9, which then cleaves and activates other downstream caspases, causing death. The intrinsic and extrinsic apoptotic pathways are not mutually exclusive as the two can interact. For example, the extrinsic pathway can activate caspases that feed back onto mitochondria and cause cytochrome *c* redistribution that subsequently activates the intrinsic pathway. There are a number of other proteins known to be involved in apoptosis, some of which are discussed in review articles in this Forum.

Most cells in metazoan organisms constitutively express most or all of the molecular machinery necessary for rapidly killing themselves. Moreover, most of this machinery has no known function other than to eradicate cells. This seemingly precarious situation is important for many processes. For example, apoptosis aids in protecting against viral infection. It is beneficial to the organism as a whole if a cell infected by a virus activates the apoptotic cascade and kills itself before the virus has had a chance to replicate and infect surrounding tissues. Thus, apoptosis can serve as a backup for the immune system. Apoptosis is also important for tissue turnover. Many of the tissues in multicellular organisms undergo almost continuous replacement. Old cells die by apoptosis and are replaced by new cells. Apoptosis is very prominent during embryogenesis when it is involved in sculpting the developing organism. Other physiologically appropriate functions of apoptosis include negative selection in the immune system and tumor regression.

The subject of this Forum, programmed neuronal death, is a prominent feature of the developing vertebrate nervous system. Approximately 50% of the neurons produced during neurogenesis die an apoptotic death before birth or shortly thereafter. The purpose of this seemingly wasteful process is to sculpt the developing nervous system. It appears to be a means of assuring that there is an appropriate match between the innervation density of a neuronal target and the target size. In other words, larger targets have more neurons projecting to them in the adult animal because they suppress more apoptotic death during development than do smaller targets (24). Supply of a sufficient quantity of a neurotrophic substance provided by target or other tissues is the major determinant of which neurons survive apoptotic death during development. Those cells obtaining adequate amounts of neurotrophin live into adulthood, whereas those that receive too little die and are removed from the organism by phagocytosis.

Research into the molecular mechanisms of programmed neuronal death began in the late 1980s with the discovery that protein synthesis inhibitors block the apoptotic death of sympathetic neurons deprived of nerve growth factor (NGF) (22). This discovery led to the suggestion that NGF deprivation causes the induction of genes coding for proteins that are responsible for death. Consistent with this hypothesis, recent findings show that the proapoptotic BH3-only (*bcl-2* homology domain 3) genes, *dp5* and *bim*, are induced during the death of NGF-deprived sympathetic neurons (25). Many other components of the apoptotic cascade in these cells are constitutively expressed. It is unclear at present whether the protein products of *dp5* and *bim* are necessary for killing NGF-deprived sympathetic neurons or merely accelerate the apoptotic process. However, it is clear that *bax*, which is continuously expressed, is essential for the death of these and many other types of neurons (31). Sympathetic neurons from *bax*-deficient animals do not die when deprived of NGF because they do not release cytochrome *c* from their mitochondria. The protein products of *dp5* and *bim* act as cofactors for Bax and may be involved in aiding its association with mitochondria.

One of the earliest events occurring after withdrawal of NGF from sympathetic neurons is an increase in cellular levels of mitochodondrial-derived reactive oxygen species (ROS). Elevated ROS are also found in other types of neurons undergoing apoptosis (27). The ROS increase occurs within 3 h of NGF deprivation in rat sympathetic neurons. Greenlund *et al.* (8) presented evidence suggesting that these ROS are important for the apoptotic death of these cells. They found that microinjecting superoxide dismutase into sympathetic neurons or treating them with antioxidants slowed death caused by NGF withdrawal. They suggested that increased ROS in sympathetic and other neurons undergoing apoptosis contribute to death by acting as signaling molecules that cause the induction of genes coding for killer proteins. Although this may be true, recent work suggests that the ROS have a much more direct effect on the apoptotic apparatus. In this issue, Kirkland and

Franklin (17) review evidence suggesting that increased ROS after NGF withdrawal can rapidly induce release of cytochrome *c* from mitochondria (16, 19). They also discuss work from their laboratory showing that Bax is necessary for the increased ROS levels in both sympathetic and cerebellar granule neurons undergoing apoptosis. Their data suggest that the prooxidant state induced by Bax is an important component of the mechanism by which this protein causes release of apoptogenic factors from mitochondria into the cytoplasm.

Dugan and colleagues suggested that the ROS increase occurring in sympathetic neurons and other cells deprived of neurotrophic factors lies downstream of the mitogen-activated protein kinase kinase (MEK)/mitogen-activated protein kinase (MAPK) pathway (5). They found that this pathway becomes inactive after NGF withdrawal and that the MEK inhibitor, PD98059, causes increased ROS even in the presence of NGF. Their interpretation of this finding was that the MEK/MAPK pathway suppresses ROS production by mitochondria. However, Kirkland and Franklin report here that this interpretation is probably in need of revision (18). They find that PD98059 causes a profound suppression of reduced glutathione (GSH) levels in sympathetic neurons under all culture conditions tested and that this likely accounts for its ability to increase cellular ROS levels. Because GSH does not decrease during the apoptotic death of NGF-deprived sympathetic neurons (16, 19), it seems unlikely that the PD98059 findings are relevant to the mechanism by which NGF deprivation causes elevated ROS.

The survival-promoting effects of neurotrophins are well known and extensively investigated. Less well known and studied is that neurotrophins can, in some circumstances, exert toxic effects on cells. Gwag and Kim (9) discuss the ability of neurotrophic factors to augment neuronal injury. They review evidence showing that neurotrophins can increase necrotic cell death in some pathological situations, including those caused by oxygen/glucose deprivation and by free radicals. It appears that neurotrophins are only protective in certain contexts and that whether they support neuronal survival or cause damage depends on the type of neuron or the nature of the exposure.

It is becoming increasingly apparent that the apoptotic machinery is activated not only in physiologically appropriate situations, but also in pathological ones. An increasing body of evidence implicates apoptosis as, at least partially, responsible for the death of neurons in neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and others (32). Many of the neurons dying during the delayed phase of cell death after stroke also have apoptotic characteristics (2, 4, 30). Sugawara and Chan (29) discuss the well known role of ROS in stroke, focusing on mechanisms of ROS formation and clearance during blood flow reduction and reperfusion. They review evidence from experiments with mice in which superoxide dismutase and/or glutathione peroxidase has been deleted or overexpressed. Overexpression of these enzymes protects against ischemic insults, whereas deletion increases the damage incurred. The studies discussed provide strong evidence for the role of ROS in ischemic damage. Of particular interest to the topic of this Forum is the discussion of work showing release of cytochrome *c* and other apoptogenic factors in neurons receiving ischemic insults *in vivo*. This intriguing work suggests that the findings about ROS promotion of cytochrome *c* release in apoptosis caused by neurotrophin withdrawal (16, 19) may also apply to pathological conditions.

Adibhatla *et al.* (1) present work examining the effects of the compound, citicoline (CDP-choline), on phospholipase $A_2$ ($PLA_2$) activity and free radical formation in transient forebrain ischemia. Activation of $PLA_2$ during stroke can hydrolyze phospholipids and cause release of free fatty acids, including arachidonic acid, that can then cause formation of ROS, lipid peroxides, and downstream toxic aldehydes. Citicoline is protective in animal cerebral ischemia models and is currently in phase III clinical trials for treatment of stroke. Adibhatla *et al.* (1) report that this compound attenuates $PLA_2$ activity in both membrane and mitochondrial fractions during transient cerebral ischemia. It also attenuates loss of cardiolipin, arachidonic acid release, hydroxyl radical formation, and formation of the toxic aldehyde, malondialdehyde, possibly accounting for its neuroprotective effects.

Physical damage to neural tissue can also activate the apoptotic cascade. Leonard Levin's group provides two articles for the Forum concerned with the role of ROS in the apoptotic death of axotomized retinal ganglion neurons (20, 23). These cells undergo apoptosis when their centrally projecting axons are injured. The first article from the Levin group (20) investigates superoxide production in axotomized retinal ganglion cells in culture. They report that exposure to oxidative stress causes these cells to exhibit a secondary burst of superoxide production. Because this second burst is blocked by the protein synthesis inhibitor cycloheximide, they suggest that axotomy may induce the synthesis of a protein(s) that mediates the oxidative amplification. The second article from the Levin group (23) investigates the effect of oxidative stress on mitochondrial membrane potential in retinal ganglion cells and presents evidence that such stress activates the mitochondrial permeability transition pore in these neurons. This large macromolecular complex is composed of several outer and inner mitochondrial membrane proteins, including VDAC (voltage-dependent anion conductance), a possible redox-sensitive route for exit of mitochondrial apoptogenic substances (21).

Kanthasamy *et al.* (14) review the current state of knowledge about the role of protein kinase Cδ (PKCδ) in oxidative stress-induced apoptosis. This member of the protein kinase C family is redox-sensitive and is activated by oxidative stress. During apoptosis, caspases cleave PKCδ and cause it to become persistently active. Antioxidants can attenuate the caspase-dependent activation of PKCδ, suggesting that cellular redox status is important for its activation. Because many neurons enter prooxidant states during the apoptotic process, it seems likely that the redox-dependent activity of PKCδ is an important component of the apoptotic cascade.

Early in the modern study of apoptosis, an article from the laboratory of Stanley Korsmeyer (10) suggested a central role for cellular redox state in apoptotic death. Work from other laboratories at about the same time (6, 13) also pointed to an important role for the redox state of cells in apoptosis. However, this work was soon disputed by Jacobson and Raff (12) and Shimizu *et al.* (28) who showed that Bcl-2 blocks apoptosis in what was assumed to be anaerobic conditions where free radical production should be greatly reduced. These articles had the result of hampering investigation into the role of free radicals in apoptosis for many years. This is unfortunate as

subsequent findings revealed flaws in interpretation of the data in the articles (17). Because of this awareness and new findings concerning clear roles for ROS in apoptotic death (26), there has recently been a major resurgence of interest in the role of cellular redox state in apoptosis.

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## ABBREVIATIONS

APAF-1, apoptosis protease activating factor 1; GSH, reduced glutathione; ICE, interleukin-1β converting enzyme; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; NGF, nerve growth factor; PKCδ, protein kinase Cδ; $PLA_2$, phospholipase $A_2$; ROS, reactive oxygen species.

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