Molecular Neurobiology

ISSN0893-7648/98/24(1–3): 131–144/$13.50

Caspase-Dependent Apoptotic Pathways in CNS Injury

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Abstract

Recent studies have suggested a role for neuronal apoptosis in cell loss following acute CNS injury as well as in chronic neurodegeneration. Caspases are a family of cysteine requiring aspartate proteases with sequence similarity to Ced-3 protein of Caenorhabditis elegans. These proteases have been found to contribute significantly to the morphological and biochemical manifestations of apoptotic cell death. Caspases are translated as inactive zymogens and become active after specific cleavage. Of the 14 identified caspases, caspase-3 appears to be the major effector of neuronal apoptosis induced by a variety of stimuli. A role for caspase-3 in injury-induced neuronal cell death has been established using semispecific peptide caspase inhibitors. This article reviews the current literature relating to pathways regulating caspase activation in apoptosis associated with acute and chronic neurodegeneration, and suggests that identification of critical upstream caspase regulatory mechanisms may permit more effective treatment of such disorders.

Index Entries: Caspases; apoptosis; Bcl-2; traumatic brain injury; spinal cord injury; cerebral ischemia; development.

Introduction

Tissue damage following brain injury results from both direct mechanical injury and secondary autodestructive reactions *(1)*. Secondary injury involves a cascade of biochemical changes that contribute to delayed tissue damage and cell death *(1)*. Although the focus of research on secondary brain injury has historically been on mechanisms related to necrosis *(2)*, recent studies have suggested a role for apoptosis in cell loss following stroke, spinal-cord injury or traumatic brain injury (TBI) *(3–8)*, as well in chronic degenerative conditions such as Alzheimer's disease (AD) *(9)*, Huntington's disease (HD) (10), Parkinson's disease (PD) *(11,12)*, and Amyotrophic Lateral Sclerosis (ALS) *(13–15)*.

Apoptosis and necrosis have been defined on histological criteria *(16)*. Tissue necrosis is

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typified by loss of membrane integrity, morphological signs of organelle damage, nuclear flocculation, loss of lyzosomal contents, cellular swelling, and uncontrolled cell lysis *(17).* Apoptosis is characterized by preservation of membrane integrity, cytoplasmic and nuclear condensation, diminution of cellular volume, plasma-membrane bleb formation, and morphological preservation of organeller structure. The cell eventually fragments into apoptotic bodies, which are engulfed by neighboring cells and degraded. During apoptosis, morphological changes are often accompanied by internucleosomal cleavage of genomic DNA *(18).* In contrast to necrosis, apoptosis does not result in a loss of cellular content and does not initiate an inflammatory response.

Apoptosis is a genetically controlled type of cell death. The key apoptotic genes were originally identified in *Caenorhabditis elegans.* Approximately 1 out of 10 cells that are generated during development of this nematode undergo apoptosis. Among 15 genes that control this process, at least three are critical: *ced-3* and *ced-4* are both required for cell death, whereas *ced-9* is inhibitory *(19).* It has been revealed that Ced-3 is a type of cysteine proteases (caspase) whose activation is the major cause of apoptosis in *C. elegans (20).* Activation of Ced-3 is mediated by the Ced-4 and inhibited in the presence of Ced-9 *(21,22).* These three proteins physically interact forming the apoptosome complex *(23).*

The apoptotic machinery identified in *C. elegans* is evolutionarily conserved. Thus, *ced* genes have their mammalian homologs, and the interactions described for nematode Ced proteins have also been detected in mammals *(23,24).* However, regulatory mechanisms of apoptosis in mammals appear much more complex than those in *C. elegans.*

Caspase Family of Cysteine Proteases

The term "caspase" refers for cysteine requiring aspartate proteases with sequence similarity to Ced-3 *(25).* These proteases share from about 30–50% sequence identity in a 115 residue segment around the putative active site cysteine: QAC(R/A/G)G. The protease activity of caspases is unique in that they all have an absolute substrate requirement for aspartate at the P1 (amino acid 1) and cleave following this residue (D-X). The requirement for D-X at P1 position in the recognition sequence for cleavage by caspases is absolute; however, there is some variability in the P2-4 region that determines substrate specificity for different caspases. Based on their preferential substrate specificity, caspases are divided into three distinct groups. Members of Group I (caspases -1, -4, and -5) all prefer the tetrapeptide sequence WEHD. In contrast, the optimal peptide recognition motif for Group II caspases (-2, -3, and -7) is DEXD. The caspases in Group III (-6, -8, and -9) prefer the sequence (L/V)EXD *(26).*

Caspases are translated as inactive zymogens and become active after specific cleavage. They are composed of an N-terminal prodomain, a large subunit, and a C-terminal small subunit, which are separated by specific caspase recognition sites. This means that active caspases can activate other caspases following an initial stimulus. Active caspases are heterotetramers consisting of two large and two small subunits from the cleaved pro-enzymes. Upon activation they may cleave their own precursors or other procaspases.

Based on their activation during progression of apoptosis, caspases can be divided into at least two subgroups: initiator and effector caspases *(27).* The initiator caspases, such as caspase-8 and -9, begin the disassembly process and activate the downstream effector caspases, such as caspase-3, -6, and -7, leading to an amplified caspase cascade *(28–30).* Activated effector caspases contribute most significantly 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 prodomane, do not undergo autoactivation, and do not cleave each other. For activation they require additional proteins such as death domain-contain-

ing receptors (type I apoptosis) or functional apoptosomes (type II apoptosis).

A third group of caspases, which includes caspases -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 less well-understood. Caspase-1 was the first identified member of the caspase family *(20).* Also known as, ICE (for interleukin-1β-converting enzyme), caspase-1 is responsible for processing of 31 kDa pre-interleukin-1β to its mature 17 kDa active form by cleaving the precursor protein at two target sites (FEAD and YVHD) *(31).*

Of the 14 caspases identified in mammals, caspase-3 appears to have the greatest degree of homology with Ced-3 *(32).* Caspase-3 is the major effector in neuronal apoptosis triggered by various stimuli. The first strong evidence supporting the specific role for this protease in neuronal apoptosis in the brain came from studies on mice deficient in caspase-3, in which brain development is profoundly altered, including cellular hyperplasia and disorganized cell deployment *(33).* A role for caspase-3 in injury-induced neuronal death was subsequently established using semispecific peptide caspase inhibitors in various models of apoptosis triggered by ischemic or traumatic injury in vivo and in vitro *(3–5,34–39).*

Because activation of caspases, and caspase-3 in particular, appears to be a major factor for execution of neuronal apoptosis in brain, evaluation of upstream modulatory mechanisms is important for understanding the regulation of the apoptotic process. Thus, caspase-3 can be activated by at least two mechanisms: an extrinsic pathway involving cell-surface receptors and an intrinsic pathway resulting from alterations at the level of the mitochondrion *(40–42).*

Pathways of Caspase Activation

Initiation and progression of apoptosis are often stimulus-specific. Thus, in a variety of cell types, apoptosis can be regulated by extracellular death factors. These factors are members of the tumor necrosis factor (TNF) family of cytokines and include Fas ligand (Apo-1/CD95 ligand), TNF-α, TNF related apoptosis-induced ligand (TRAIL)/Apo-2 ligand, and TNF-related weak inducer of apoptosis (TWEAK) *(43,44).* The pathway involves transduction mechanisms, where an important role has been attributed to a "death domain" sequence motif in the cytoplasmic segments of corresponding receptors *(45).* Caspase-8 appears to play the major role in the initiation of caspase cascade *(46–48).* Stimulation of death receptors by their respective ligands induces oligomerization of the receptors and the formation of a death-inducing signaling complex (DISC) *(49).* The intracellular domain of the death receptor binds to the adaptor molecule Fas-associating protein with death domain (FADD), which in turn recruits procaspase-8 allowing its autocleavage and activation. The released active caspase-8 activates downstream executioner caspases-3 and -7 *(45).* In addition, activated caspase-3 may cleave procaspase-8 *(42,50),* thereby amplifying the death process.

Active caspase-8 can initiate also downstream cleavage of executioner caspases by mitochondrial-dependent mechanisms. Thus, recent studies suggest that cell-death receptors may amplify their suicide signal by activating the apoptosome. In this pathway, caspase-8 cleaves Bid, a BH3 domain-containing proapoptotic Bc12 family member *(51).* C-terminal cleavage product of Bid translocates from cell cytosol to mitochondria and interacts with Bax, thus triggering its translocation from cytosol to mitochondial membranes followed by the release of cytochrome c, and activation of caspase-9 and downstream executioner caspases *(52,53).* In addition, Bid fragments can act as a membrane-destabilizing agent, increasing release of cytochrome c *(54).* The cytochrome c releasing activity of Bid is antagonized by Bcl2 *(53)* and effectively inhibited by the protein FLICE inhibitory protein (FLIP) (for FADD-like caspase, or caspase-8 [FLICE]-inhibitory protein): two alternatively spliced forms of FLIP interact with the adaptor protein

FADD and the caspase-8, thus potently inhibiting apoptosis induced by death receptors *(55).*

While studying the biochemical mechanism of caspase activation, Xiaodong Wang's group had discovered that cell extracts contained three major protein factors that worked together to activate caspase-3 and induce apoptosis *(41,56,57).* Identification of those factors revealed an intrinsic cell-death pathway that is initiated by release of cytochrome c from mitochondria to the cytosol *(41).* It is important to note that release of cytochrome c from mitochondria can be initiated by a variety of proapoptotic stimuli *(58–61).*

In the presence of ATP (or dATP), cytochrome c binds to the cytosolic adaptor protein Apaf-1 *(41).* The N-terminal 85 amino acids of Apaf-1 show 53% similarity to the N-terminal prodomain of the *C. elegans* Ced-3. This is followed by 320 amino acids that show 48% similarity to Ced-4. The C-terminal region of Apaf-1 comprises multiple WD repeats, which are proposed to mediate protein-protein interactions *(56).* Binding of cytochrome c to Apaf-1 allows the recruitment and activation of caspase-9 within the apoptosome *(41).* Capase-9 and Apaf-1 bind to each other via their respective N-terminal Ced-3 homologous domains. This event leads to caspase-9 activation *(41).* Active caspase-9, in turn, activates executioner caspases-3 and -7. Activated caspase-3 is required for the activation of four other caspases (-2, -6, -8, and -10) in this pathway and also participates in a feedback amplification loop involving caspase-9 *(41,42).*

Similarly to null mutants of caspase-3, both Apaf-1 and caspase-9 knockout mice demonstrate a variety of hyperplasias and disorganized cell deployment in the brain leading to death during embryonic development *(62–65).* This suggests that activation of caspase-3 by apoptosome-mediated caspase-9 activation has a critical role in the developing central nervous system (CNS) *(33,62,66).*

Modulation of Caspase Activation

The complexity of caspase-activation mechanisms is increased by the fact that they can be modulated in different ways at almost every critical step. Thus, Bcl-2 was the first identified mammalian homolog of the *ced* genes. It is homologous to Ced-9 and is involved in inhibiting apoptosis. Bcl-2 was first identified in B-cell lymphoma as a result of a chromosomal translocation, which leads to high expression of Bcl-2 in these tumors. Since the discovery of Bcl-2, an entire gene family has been identified. There are several different ways in which Bcl-2 members may function. Members of this protein family may serve as either positive or negative regulators of apoptosis. Among the anti-apoptotic proteins, Bcl-xL is predominantly expressed in mammalian brain *(67),* and acts as a major inhibitor of the cytochrome c-mediated pathway of caspase activation in neurons. Mice lacking *bcl-x* die as embryos, showing massive death of postmitotic neurons *(68).*

Initially, it was proposed that Bcl-xL directly binds to Apaf-1, thus preventing activation of caspase-9 *(69).* However, more recent studies have not confirmed this hypothesis *(70)* and have demonstrated that Apaf-1 has a cytoplasmic localization distinct from Bcl-xL *(71).* Thus, it appears that Bcl-xL, like other anti-apoptotic members of Bcl-2 family, controls mitochondrial-membrane permeability and redistribution of cytochrome c from the inter-membrane space to the cytosol during apoptosis via interaction with Bax *(72).* On the other hand, Bcl-xL can be cleaved by caspases, and the resulting C-terminal fragment of Bcl-xL potently induces apoptosis *(73).*

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 (74–76). Once been translocated to mitochondria, Bax forms homodimers leading to loss of mitochondrial membrane potential, cytochrome c release, formation of the apoptosome complex, and caspase activation *(77–80).* Although Bax deficiency does not cause hyperplasia or malformations of the nervous system, it decreases apoptosis in the developing CNS and prevents

increased neuronal death caused by Bcl-x disruption *(81–84).*

Apoptosome-mediated apoptosis also may be regulated by more complicated mechanisms. Thus, numerous alternatively spliced isoforms of certain apoptosis regulators, such as Bcl-x *(85,86),* caspase-9 *(87),* and Apaf-1 *(88),* have been shown to play opposing roles in regulating apoptosis *(89).* Furthermore, growth factors can promote cell survival by activating the phosphatidylinositide-3′-OH kinase and its downstream target, the serine-threonine kinase Akt. Cardone and collaborators recently reported that active Akt can phosphorylate recombinant human caspase-9 on serine-196 and inhibit its activity *(90).* However, the corresponding Akt phosphorylation site was not found in the cloned mouse ortholog *(91).* A more recent report suggests that Akt may inhibit activation of caspase-9 and -3 by post-translational modification of a cytosolic factor downstream of cytochrome c and before activation of caspase-9 *(92).* On the other hand, it has also been demonstrated that growth factor-induced activation of the PI3′K/Akt signaling pathway results in the phosphorylation of BAD, another member of BCL-2 family *(93),* thereby altering its pro-apoptotic function. Various proapoptotic stimuli lead to increased intracellular Ca^{2+} concentrations, resulting in activation of the protein phosphatase calcineurin, which can dephosphorylate BAD *(94).* In other models, dephosphorylation of BAD can be achieved by Ras-dependent activation of the protein phosphatase 1 alpha *(95).* Dephosphorylated BAD forms heterodimer with BCL-XL displacing BAX that lead to release of cytochrome c and activation of downstream caspases, promoting apoptosis *(96).* When caspase-3 is active, it can specifically cleave Akt, thereby amplifying the death process *(97).*

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 Hsp 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 *(98,99).* 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 *(100).*

Another regulatory mechanism that parallels the proapoptotic action of cytochrome c involves a novel mitochondrial protein, Smac, which is released into the cytosol when cells undergo apoptosis. Smac promotes caspase-9 activation by binding to inhibitor of apoptosis proteins, IAPs *(101),* removing their inhibitory activity *(102).*

Recently Nakagawa et al. *(103)* showed that the caspase cascade can also be initiated by caspase-12. Caspase-12-deficient mice were resistant to endoplasmic reticulum stress-induced apoptosis and caspase-12-deficient cortical neurons were resistant to apoptosis induced by β-amyloid (Aβ) *(103).* Caspase-12 is able to activate caspase-3 in the A549 cell line and antisense-mediated inhibition of caspase-12 reduces apoptosis *(104).* It has been proposed that disturbance to intracellular Ca^{2+} storage as a result of ischemic injury or Aβ cytotoxicity may induce apoptosis through calpain-mediated caspase-12 activation and Bcl-xL inactivation *(105).*

Caspase-11, another member of the murine caspase family, which serves as an upstream activator of caspase-1 *(106),* is a critical initiator caspase responsible for the activation of caspase-3. Caspase-11-deficient mice have a reduced number of apoptotic cells and a defect in caspase-3 activation after middle cerebral-artery occlusion *(107).* Caspase-11 has been shown to be activated by cathepsin B *(108),* which, in turn, can be activated by calpain *(8).*

Although it is unclear whether all discovered regulatory mechanisms of the apoptosome-mediated pathway (Fig. 1) are functional during neuronal apoptosis in the mammalian brain, mitochondrial alterations accompanied by release of cytochrome c and caspase-3 acti-

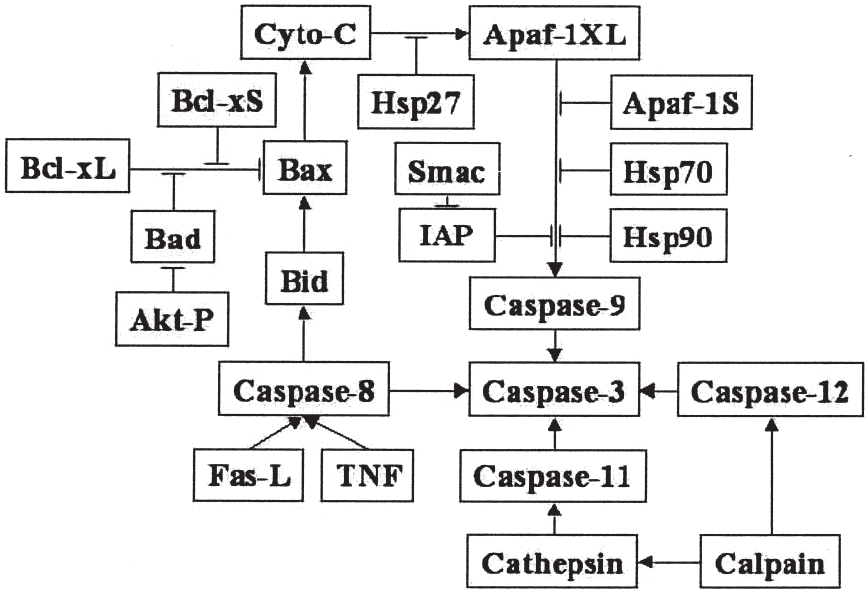

Fig. 1. Modulation of caspase activation.

vation have been almost universally seen in models of CNS injury *(109–115).*

Pathways of Caspase Activation in Acute Neuronal Injury

The ability of caspase inhibitors to block neuronal cell death has established a crucial role of caspases (caspase-3, in particular) in various models of apoptosis triggered by ischemic or traumatic injury in vivo and in vitro *(3–5,34–39).*

Thus, we have previously demonstrated that fluid percussion-induced TBI in rats results in induction of neuronal apoptosis with high frequency in both traumatized rat cortex and hippocampus. In this model, we have identified post-traumatic increase in mRNA for both caspase-1 and caspase-3 in affected brain regions; however, increased enzymatic activity was detected for caspase-3-like, but not caspase-1-like proteases. Intracerebroventricular administration of z-DEVD-fmk, a somewhat selective tetrapeptide inhibitor of caspase-3, before and after injury markedly reduced post-traumatic apoptosis, as demonstrated by DNA electrophoresis and terminal deoxynuclestidyltransferase d UTP Nick End Labeling (TUNEL) staining. Such treatment also significantly improved neurological recovery *(3).* The participation of caspase-3 activation in neuronal death after TBI in rats was also suggested by Clark et al. *(4).* These authors observed cleavage of procaspase-3 in cytosolic cellular fractions and an increase in caspase-3-like enzyme activity in injured brain, as well as neuroprotective effect of DEVD-fmk after TBI *(4).*

Preliminary data from Knoblach et al. *(116)* showed that activation of caspases-3 and -9 contributes to the secondary injury response after moderate lateral fluid-percussion TBI in rats. In contrast, caspase-8 cleavage fragments were not detected in this study at any time after injury. Immunohistochemistry for active forms of caspases-3 and -9 showed that they are expressed by both neurons and glia, and in TUNEL-positive cells that have morphologic features of apoptosis. Postinjury treatment with the pan-caspase inhibitor z-VAD-fmk sig-

nificantly improved both composite motor neuroscores and spatial learning in a Morris water-maze task *(116).*

Namura et al. *(37)* examined the expression, activation, and cellular localization of caspase-3 in adult mouse brain after temporary middle cerebral-artery occlusion. They found that procaspase-3, but not its cleavage products, was expressed in neurons throughout brain. Activation of caspase-3-like enzymes was detected in brain homogenate as early as 20 min after reperfusion *(37).*

In a similar murine model of brain ischemia, Endres and colleagues *(117)* examined the neuroprotective effects of two caspase family inhibitors, z-VAD-fmk and z-DEVD-fmk, and the N-methyl-D-aspartate (NMDA) receptor antagonist, MK-801, administered intracerebroventricularly. Both caspase inhibitors decreased infarct size, oligonucleosomal DNA laddering, and neurologic deficits when administered 6 after reperfusion. By contrast, the therapeutic window for MK-801 did not extend beyond the time of occlusion, probably because it does not inhibit apoptotic cell death *(117).* Our own preliminary studies showed that of z-DEVD-fmk has neuroprotective effects in a mouse model of brain trauma, even when administred *icv* as late as 8 h after injury (unpublished observations).

A role for caspase-3 in "ischemic" injury was further explored in vitro, using cultured neurons from embryonic rat forebrain subjected to 6 h hypoxia. Neuronal expression of the active cleavage product and proteolytic activity of caspase-3 was increased after hypoxia to nearly 10-fold over control values at 96 h postinsult. In this model, caspase-3 activity was also blocked dose-dependently by Asp-glu-val-Asp-aldehyde (DEVD-CHO); this peptide inhibitor reduced the number of apoptotic cells and prevented the hypoxia-induced decrease in cell viability, even when given 24 h postinsult *(118).*

Recent studies have implicated activation of caspase-3 in apoptotic processes in seizure-related neuronal injury. Using a model of status epilepticus in rats induced by kainic acid, Kondratyev and Gale *(119)* demonstrated expression of caspase-3 activity in rhinal cortex and amygdala at 24 h. Intracerebroventricular administration of z-DEVD-fmk caspase-3 inhibitor prior to and following status epilepticus substantially attenuated apoptotic cell death, both in hippocampus and cortex, as evaluated by analysis of internucleosomal DNA fragmentation and neuronal nuclear morphology *(119).*

Kermer and co-authors investigated whether caspase inhibition provides long-term neuroprotection of adult rat retinal ganglion cells following optic-nerve transection and demonstrated that treatment for 2 wk with z-DEVD-cmk increased the number of surviving cells 4 wk postlesion from 11 to 24%. However, treatment with this inhibitor over the entire experimental period of 4 wk had no additional effect *(120).*

Since activation of caspases, and caspase-3 in particular, appears to be a major event in the execution of neuronal apoptosis in the brain, analysis of upstream mechanisms in the injured neurons is important. The apoptosome-mediated pathway of neuronal apoptosis has been investigated in detail using a model of traumatic spinal-cord injury in rats. Springer and collaborators reported that the upstream and downstream components of the cytochrome c-dependent apoptotic pathway are activated in injured spinal cord, and are found in neurons and in oligodendroglia *(121).*

Krajewski et al. have reported that caspase-9 may be found in mitochondria of several cell types, including brain neurons, and that it is released by exposure to Ca^{2+} or Bax in vitro *(110).* These authors also observed that, in an animal model of transient global cerebral ischemia, caspase-9 was released early from mitochondria and accumulated in neuronal nuclei within hippocampus and other vulnerable regions of postischemic brain *(110).*

Despite the fact that Fas-ligand and TNF-α are widely expressed in the nervous system, the potential role of caspase-8-mediated apoptotic pathway in neurons has received limited attention. Recently, caspase-8 activation has been investigated using in vitro models of HD and AD *(122,123).* Sanchez and co-authors

demonstrated that death of primary rat neurons resulted after overexpression of polyglutamine repeat (Q79) was preceded by activation of caspase-8. Inhibition of this caspase blocked Q79-induced cell death. Co-expression of Q79 with the caspase inhibitor CrmA, a dominant-negative mutant of FADD, Bcl-2, or Bcl-xL prevented the recruitment of caspase-8 and inhibited cell death. In addition, this study reports the presence of activated caspase-8 in affected brain regions from HD patients but not in normal controls *(122).* Ivins et al. provided evidence that Aβ also might initiate apoptosis by cross-linking death receptors of the Fas/TNFR family *(123).* They showed that the caspase-8 inhibitor peptide, IETD-fmk, inhibited neuronal death induced by Aβ and that neurons expressing dominant-negative FADD were protected from apoptosis in this model of AD *(123).*

Recently, caspase-8 activity was also attributed to neuronal apoptosis following hypoxic-ischemic injury to the developing brain *(124)* and transient spinal-cord ischemia *(125).* On the other hand, Velier and collaborators reported that after permanent focal stroke in rats, active forms of caspase-8 and caspase-3 were found differentially expressed in lamina V and lamina II/III, respectively *(126).* The authors suggested that the molecular mechanisms of cell death differ among the populations of neurons comprising the cerebral cortex.

Developmental Changes in Caspase-3 Activation

Clinical observations suggest that age influences outcomes and mortality after brain injury; however, why the same insult results in different response to injury in adults and in infants remained unclear. Using different models of experimental brain injury, recent reports suggest age-dependent differences in susceptibility to apoptosis. Bittigau et al. *(127)* reported age dependency of apoptotic neurodegeneration in the developing rat brain after head trauma. In 7-d-old rats, mechanical trauma triggered widespread apoptosis in the hemisphere ipsilatral to the trauma site. This was accompanied by elevation of caspase-3 activity and DNA fragmentation in vulnerable brain regions. Severity of trauma-triggered apoptosis in the brains of 3- to 30-d-old rats was age-dependent, was highest in 3- and 7-d-old animals, and demonstrated a subsequent rapid decline.

Pohl et al. also reported that susceptibility to apoptotic death after TBI is dependent on age *(128),* with 3- and 7 d-old rats showing the highest vulnerability. By 10 and 14 d, the severity of apoptotic cell loss had markedly decreased; by 30 d of age, damage was minimal and limited to the site of impact.

These results may be explained, in part, by another report showing that the expression of caspase-3 mRNA in the brain is highly regulated during the postnatal period *(129).* De Bilbao and colleagues investigated the expression of caspase-3 mRNA by *in situ* hybridization in the mouse brain from birth to adulthood. From 1 postnatal d to 12 postnatal d, caspase-3 mRNA was expressed ubiquitously in all brain nuclei, including areas where neurogenesis occurred; this expression decreased substantially after day 12. The authors also found a positive correlation between areas displaying high levels of mRNA and apoptotic nuclei. In the adult brain, caspase-3 mRNA was restricted to the piriform and entorhinal cortices, to the neocortex, and to areas where neurogenesis is observed *(129).*

The involvement of caspase-3 in cell death after hypoxia-ischemia (HI) was also studied during brain maturation. Unilateral HI was produced in rats at postnatal days 7–60 by a combination of left carotid-artery ligation and systemic hypoxia. Activation of caspase-3 and cell death was examined *in situ* by confocal microscopy and by biochemical analysis. Active caspase-3 positive neurons was identified in more than 90% HI damaged striatal and neocortical neurons in 7-d-old pups; this number was reduced to approx 65% in striatum and 34% in the neocortex of 15-d-old pups, and to approx 26% in striatum and 2% in neocortex

of 26-d-old rats. In 60-d-old rats, less than 4% of the damaged neurons in striatum and less than 1% in neocortex were positive for active caspase-3. Western-blot analysis demonstrated that the level of inactive caspase-3 in normal forebrain tissue gradually declined from a high level in young pups to very low levels in adult rats. Concomitantly, HI-induced active caspase-3 was reduced from a relatively high level in young to a barely detectable level in mature rats *(130).*

Conclusion and Future Directions

Collectively, these studies suggest that apoptosis of CNS cells may play an important pathophysiological role in acute and chronic neurodegeneration. Both intrinsic and extrinsic caspase pathways appear to be activated in association with such apoptosis. Because apoptosis occurs later and over a longer postinjury time frame than necrosis, pharmacological inhibition of apoptosis may offer substantial therapeutic advantages over traditional therapies. Identification of critical upstream caspase regulatory pathways may permit even more effective interventions. Potential targets may include modulation of Akt, IAPs, or Smac.

Apoptotic pathways appear to play a greater role in perinatal brain injury than in adults. This may have important implications for understanding and treating such conditions as cerebral palsy.

Acknowledgments

This work was supported by NIH 1RO1NS-38941, NIH 1RO1NS36537, and DAMD 17-99-2-9007.

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