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Review

Apoptotic Cell Death Following Traumatic Injury to the Central Nervous System

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Apoptotic cell death is a fundamental and highly regulated biological process in which a cell is instructed to actively participate in its own demise. This process of cellular suicide is activated by developmental and environmental cues and normally plays an essential role in eliminating superfluous, damaged, and senescent cells of many tissue types. In recent years, a number of experimental studies have provided evidence of widespread neuronal and glial apoptosis following injury to the central nervous system (CNS). These studies indicate that injury-induced apoptosis can be detected from hours to days following injury and may contribute to neurological dysfunction. Given these findings, understanding the biochemical signaling events controlling apoptosis is a first step towards developing therapeutic agents that target this cell death process. This review will focus on molecular cell death pathways that are responsible for generating the apoptotic phenotype. It will also summarize what is currently known about the apoptotic signals that are activated in the injured CNS, and what potential strategies might be pursued to reduce this cell death process as a means to promote functional recovery.

Keywords: Caspases, Apoptosis, Ischemia, Spinal cord trauma, Brain trauma, Adenovirus gene delivery

Traumatic and ischemic injury to the central nervous system (CNS) is an important clinical problem affecting millions worldwide. Therapeutic strategies that target the biochemical consequences of CNS injury are a major focus of research in the biomedical community. As such, it is essential that we gain a better understanding of the significant biochemical steps that contribute to cell death following CNS injury. Following the initial insult in most CNS injury types, there is a delayed and prolonged period of secondary damage that involves a number of destructive pathophysiological and biochemical events (Tator and Fehlings, 1991; Anderson and Hall, 1993; Young, 1993; Lynch and Dawson, 1994; Jakeman *et al.,* 2001). The observation that many of these secondary events occur relatively late following injury makes them amenable to therapeutic interventions.

Over the last several years, numerous studies have suggested that apoptotic cell death is emerging as a critical factor that contributes to ongoing cell loss following CNS injury. Apoptotic cell death is a fundamental and highly regulated cellular process in which a cell is instructed to actively participate in its own demise. This process of cellular suicide plays an essential role in the regulation of cell numbers during development, and the maintenance of tissue homeostasis throughout adult life. Cells undergoing apoptosis exhibit distinct morphological and biochemical features, which include shrinkage of the cytoplasm, chromatin condensation, membrane blebbing, internucleosomal DNA fragmentation, and formation of apoptotic bodies. In contrast to apoptosis, cells undergoing necrotic cell death exhibit rapid cytoplasmic and mitochondrial swelling, disruption of the plasma membrane, and activation of a significant inflammatory response. The unique morphological features of apoptosis occur in the same stereotypic pattern in all of the multicellular organisms that have been examined to date. These provide the first indication that the intrinsic molecular machinery is highly conserved throughout evolution.

In many types of CNS injury, evidence of apoptotic cell death can be detected hours to weeks following injury and occurs in numerous cell types. Depending upon the type of injury, these cells include neurons, astrocytes, oligodendroglia, and inflammatory cells such as neutrophils, microglia, and macrophages. However, at the present time, it is not known whether the loss of cells through apoptosis contributes significantly to neurological dysfunction. Given these findings, understanding the biochemical signaling events

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controlling apoptosis is a first step towards developing therapeutic agents that target this cell death process. This review will summarize what is currently known about the apoptotic signals that are activated in different types of CNS injuries, and what strategies might be pursued to minimize this cell death process as a potential means for promoting functional recovery.

The caspase-3 apoptotic cascade

The extracellular signaling events that lead to apoptosis following traumatic CNS injury are not entirely clear. However, it has been demonstrated that intracellular apoptotic signals leading to a common endpoint, i.e., caspase-3 activation, occur in both neurons and glia following spinal cord injury (Emery *et al*., 1998; Hayashi *et al*., 1998; Springer *et al*., 1999; Beattie *et al*., 2000; Citron *et al*., 2000; Li *et al*., 2000; Springer *et al*., 2000). Similar studies have documented evidence of caspase-3 activation in other types of CNS injury models, including head trauma and ischemia/stroke (Gottron *et al*., 1997; Hara *et al*., 1997; Yakovlev *et al*., 1997; Chen *et al*., 1998; Endres *et al*., 1998; Ni *et al*., 1998; Pike *et al*., 1998; Posmantur *et al*., 1998; Velier *et al*., 1999; Zhang *et al*., 1999; Clark *et al*., 2000; Eldadah and Faden, 2000; Shibata *et al*., 2000; Zhu *et al*., 2000). Numerous *in vitro* studies have demonstrated that caspase-3 activation is initiated, in part, by upstream events that result in the release of cytochrome c and Smac/DIABLO from the mitochondria (Liu *et al*., 1996; Kluck *et al*., 1997; Bossy-Wetzel *et al*., 1998; Skulachev, 1998; Yang and Cortopassi 1998; Slee *et al*., 1999; Du *et al*., 2000; Verhagen *et al*., 2000). Cytochrome c then interacts with Apaf-1 (apoptosis protease activating factor-1) to promote the activation of caspase-9, an upstream activator of caspase-3 (Liu *et al*., 1996; Kluck *et al*., 1997; Kluck *et al*., 1997; Li *et al*., 1997; Yang *et al*., 1997; Zou *et al*., 1997; Bossy-Wetzel *et al*., 1998; Pan *et al*., 1998; Skulachev, 1998; Slee *et al*., 1999).

Smac/DIABLO promotes capsase-9 and caspase-3 activation by removing them from inhibition by members of the inhibitor of apoptotic proteins (IAPs) family (see below). Cytochrome c release and caspase-3 activation occur in SCI and other traumatic injuries to the CNS (Yakovlev *et al*., 1997; Emery *et al*., 1998; Pike *et al*., 1998; Clark *et al*., 1999; Springer *et al*., 1999; Citron *et al*., 2000; Clark *et al*., 2000; Li *et al*., 2000; Springer *et al*., 2000). There is evidence that the release of cytochrome c from the mitochondria can be controlled, in part, by the anti-apoptotic proteins bcl-2 and Bcl-XL through their interaction with the pro-apoptotic protein bax (Manon *et al*., 1997; Jurgensmeier *et al*., 1998; Narita *et al*., 1998; Reed *et al*., 1998; Skulachev, 1998; Finucane *et al*., 1999; Finucane *et al*., 1999; Korsmeyer, 1999). For a better understanding of the involvement of the bcl-2 gene family in cerebral ischemia and brain trauma, there is an excellent review article by Graham *et al*. (2000).

The first IAP family member identified was p35, a baculoviral protein that suppresses apoptosis in a number of cell types (including cells of the CNS) and under a variety of conditions (Rabizadeh *et al*., 1993; Hisahara *et al*., 2000). Subsequent studies demonstrated that several IAPs exist and function by binding to the inactive pro-caspase and therefore, inhibiting its processing to the active form (Hay *et al*., 1994; Hay *et al*., 1995; Hay, 2000). Mammalian IAPs include XIAP, c-IAP1, c-IAP2, NAIP, and Survivin, although XIAP inhibits caspase-3 activation at a K_i that is much lower compared to the other IAPs. Recent studies suggest that the removal of IAP inhibition is dependent upon Smac/DIABLO, the second cell death protein (along with cytochrome c) that is released by the mitochondria during apoptosis (Du *et al*., 2000; Verhagen *et al*., 2000). Smac/DIABLO displaces XIAP from caspase-9, allowing the latter to be activated through its interactions with Apaf-1, and can also remove XIAP directly from caspase-3.

Given what is currently known about the players involved in the intracellular pathways leading to apoptosis, it is important to know whether similar steps occur in different types of CNS injury. The remainder of this review will focus on the evidence that supports the presence of apoptosis in different types of CNS injuries, including ischemia and stroke, traumatic brain injury, and traumatic spinal cord injury. There is clear evidence supporting a role for caspase-3 in all types of CNS injury. However, the temporal and spatial expression pattern that leads to caspase-3 activation is different across injury types and injury severity. This review also will discuss some of the evidence documenting what steps have been taken to reduce apoptosis, and whether this has functional consequences. Finally, we will elaborate on an experimental strategy that we are pursuing for reducing apoptosis in the injured spinal cord.

Ischemia/reperfusion injury

Ischemic CNS injury was one of the first models to provide evidence of delayed apoptotic cell death. There is also a large body of literature that examines the involvement of caspase-3 and other members of this cysteine protease family. Many of these studies are summarized well in a review by Eldadah and Faden (Eldadah and Faden, 2000). However, as pointed out in a review by MacManus and Buchan, although many of the underlying biochemical signaling events that are associated with caspase-3 activation occur in ischemia/stroke, the delayed cell death following ischemic injury may not fall into the classic definition that is usually associated with apoptosis (MacManus and Buchan, 2000). This author clearly agrees with some of the comments raised in this review. The authors raise an important question about generalizing apoptotic signaling events and mechanisms across injury types. This has clearly stimulated our thinking of defining cell death as being absolute in terms of following an apoptotic versus necrotic pathway to destruction. In fact, it can be argued that many components of the caspase-3 apoptotic cascade can be activated in a cell following stimuli that lead to rapid necrosis.

This small digression is only important as a reminder that exhaustive examination using complimentary approaches is necessary to make clear delineations between cells undergoing necrosis versus apoptosis. Regardless, the existing literature described below provides clear evidence that some of the key players (e.g., caspase-3) are involved in ischemia/stroke. Therefore therapeutic approaches that limit apoptosis may have beneficial neurological effects.

A study reported by Moskowitz and co-workers in 1997 was one of the first to examine the role of caspases in ischemia and excitotoxic injury to the CNS (Hara *et al*., 1997). In a series of experiments, the general caspase inhibitor, z-VAD-fmk or the caspase-3-like inhibitor z-DEVD-fmk, were injected using an intracerebroventricular approach. The authors report that there was a significant reduction in infarct volume, and an improvement in functional recovery following a 2-hour middle cerebral artery occlusion and an 18-hour reperfusion period. The effect of the caspase inhibitor treatments did not appear to be related to the altered hemodynamic function as physiological parameters, including blood flow, mean arterial blood pressure, and core body temperature, were unaffected by the drug treatment. Importantly, treatment with the caspase-3-like inhibitor (z-DEVD-fmk) is neuroprotective, even when administered 1 hour (but not later) following onset of reperfusion.

The excitotoxic actions of glutamate are known to contribute to neuronal cell loss and the neurological deficits associated with ischemic/reperfusion injury. Moreover, work from the laboratories of Lipton, Nicotera and co-workers have found that glutamate exposure leads to necrotic or apoptotic cell death, depending upon the response of the mitochondria to the excitotoxic insult (Ankarcrona *et al*., 1995; Bonfoco *et al*., 1995; Leist *et al*., 1997; Nicotera *et al*., 1997). To test whether glutamate leads to caspase-3 mediated apoptosis, Moskowitz and colleagues co-injected one of two glutamate receptor agonists (N-methyl-D-aspartate or NMDA and alpha-amino-3-hydroxy-5-methyl-4-isoxazole proprionate or AMPA) and a nonselective caspase inhibitor (z-VAD-fmk) directly into the uninjured striatum. As found in the ischemia injury model above, treatment with the caspase inhibitor significantly decreased the lesion volume that was induced by AMPA, and to a lesser extent, that induced by NMDA injections. In similar studies, it was found that the same tetrapeptide inhibitor of caspase-3-like, but not a caspase-1-like inhibitor, blocked glutamate-induced apoptosis in cerebellar granule neurons grown in culture (Du *et al*., 1997).

In a series of follow-up experiments, the same ischemia/reperfusion injury model was used to examine the activation and cellular localization of caspase-3, as well as the therapeutic potential of blocking caspase-3 activation (Endres *et al*., 1997; Hara *et al*., 1997; Endres *et al*., 1998; Ma *et al*., 1998; Namura *et al*., 1998). An increase in caspase-3-like enzyme activity was observed immediately following reperfusion; activity levels peaked over the next 30-60 minutes. At 1-12 hour after reperfusion, activated caspase-3 was detected in neurons that were restricted to brain regions that were supplied by the middle cerebral artery. At later times (12-24 hours), activated caspase-3 was localized to TUNEL-positive neurons, indicating the presence of fragmented DNA in these cells. This latter observation is important as it provides clear evidence that cells expressing caspase-3 are indeed destined to die via apoptosis. Specifically, the expression of activated caspase-3 in a cell may not necessarily mean that it is committed to apoptotic cell death. In fact, it has been difficult to observe evidence of DNA fragmentation in cells expressing activated forms of caspase-3. This may simply be a temporal problem, caspase-3 may be an early event relative to the appearance of DNA fragmentation.

In separate studies, two different groups of investigators found that transient global ischemia results in an increase in caspase-3 mRNA in neurons of the striatum and hippocampus that undergo apoptosis following this type of insult (Chen *et al*., 1998; Ni *et al*., 1998). Recent studies have also provided additional evidence of caspase-3 activation in other models of cerebral ischemia following strokes in adults and neonates. This includes the mitochondrial release of cytochrome c and activation of caspase-9, a proximal caspase in the caspase-3 apoptotic cascade (Gillardon *et al*., 1997; Chen *et al*., 1998; Cheng *et al*., 1998; Namura *et al*., 1998; Pulera *et al*., 1998; Kiprianova *et al*., 1999; Krajewski *et al*., 1999; Ouyang *et al*., 1999; Velier *et al*., 1999; Han *et al*., 2000; Han and Holtzman, 2000). Several recent studies using caspase inhibitors provide support of these original observations, and demonstrate that injury severity can affect the efficacy of caspase inhibitors in reducing cell death. For example, in a four-vessel occlusion model (severe injury), treatment with a caspase-3 inhibitor, but not a caspase-1 inhibitor (z-YVAD-fmk), increases cell survival in the CA1 region of the hippocampus, a group of cells that are highly susceptible to ischemic injury (Chen *et al*., 1998). In a less severe ischemia model that involves transient occlusion of the middle cerebral artery, both the caspase-3 and caspase-1 inhibitors reduced infarct volume and increased cell survival (Hara *et al*., 1997). This latter observation was confirmed using a similar transient MCA occlusion model (Li *et al*., 2000), although these investigators found no neuroprotective effects of a caspase-3 inhibitor when the more severe four-vessel occlusion model was used. However, other investigators report that treatment with the caspase-3 inhibitor z-DEVD-fmk was indeed effective in reducing cell loss in the CA1 region using the four-vessel occlusion (Gillardon *et al*., 1999). Taken together, these studies provide compelling evidence that caspase-3 activation is responsible, in part, for the widespread pattern of apoptotic cell death that is observed in ischemia/reperfusion injury. However, it is not entirely clear whether caspase inhibition is neuroprotective in more severe injury models. This may be due to differences in the model used, ischemia/reperfusion times, or the time of delivery of the caspase inhibitor. Regardless, the results of these studies echo the comments of MacManus and Buchan that we must be cautious in our

interpretations of evaluating apoptotic cell death in the CNS (MacManus and Buchan, 2000).

Traumatic brain injury

Studies from several laboratories have provided fairly conclusive evidence that apoptotic cell death in the rat brain occurs following traumatic injury (i.e., lateral fluid percussion and cortical contusion injury) (Rink *et al*., 1995; Clark *et al*., 1997; Newcomb *et al*., 1999). A recent review describes evidence that supports the involvement of apoptosis, and pro- and anti-apoptotic genes in traumatic brain injury (TBI) (Raghupathi *et al*., 2000). As described previously, the bcl-2 family of apoptotic proteins plays an important role in regulating this cell death process. Bcl-2, which functions to limit activation of the apoptotic cascade, increased in surviving cells following TBI in experimental animals and humans (Clark *et al*., 1997; Clark *et al*., 1999; Graham *et al*., 2000). In another study that examined TBI in rats, the pro-apoptotic protein bax was observed to translocate from the cytosolic compartment to the nucleus of cells undergoing apoptosis (Kaya *et al*., 1999). This latter observation is intriguing in light of the fact that bax is normally thought to function at the level of the mitochondria. Therefore, it is not entirely clear what role bax might play following translocation to the nucleus.

The involvement of caspase-3 in TBI has been observed, although the research examining this death effector has not been as extensive as in other models (i.e., ischemia and spinal cord injury). Regardless, early studies from Faden's laboratory presented the first evidence that apoptosis in TBI was dependent, in part, upon caspase-3 activation (Yakovlev *et al*., 1997). In this study, DNA fragmentation activity was observed in cytosolic extracts that were isolated from the ipsilateral cortex and hippocampal formation as early as 4 hours following injury. At this time point, these investigators observed a 4-5 fold increase in caspase-3 mRNA levels, and a 25-50% increase in caspase-3-like enzyme activity levels in these same regions. When the caspase-3-like tetrapeptide inhibitor z-DEVD-fmk was administered into the lateral ventricles 30 minutes before, and 6 and 24 hours after injury, DNA fragmentation and the number of apoptotic neurons in the injured cortex were dramatically reduced. Finally, the same treatment paradigm resulted in improvement of neurological function, as measured by a number of motor tasks that are sensitive to traumatic brain injury. In support of these findings, a recent study reported that post-injury treatment with z-DEVD-fmk also reduced both cell loss and lesion volume. However, these investigators found no evidence of improved neurological function as assessed on a motor skill task (Clark *et al*., 2000).

A hallmark of many types of traumatic CNS injuries is the rapid breakdown of certain cytoskeletal proteins that are critical for maintaining structural integrity. Interestingly, alpha-spectrin has specific amino acid sequences, which allows it to be processed by at least two different cysteine proteases, including calpain and caspase-3. In several studies by Hayes and co-workers (Pike *et al*., 1998; Pike *et al*., 1998; Posmantur *et al*., 1998), they report that following TBI, alpha-spectrin is processed primarily by calpain in the injured cortex, while a caspase-3 processed form predominates in the deeper structures (hippocampus and striatum) where tissue loss is less severe relative to the overlying cortex. In support of these findings, a study by McIntosh and co-workers describes the caspase-3-mediated cleavage of ICAD/DFF45 within 2 hours following TBI (Zhang *et al*., 1999). As a consequence, CAD/DFF40 would then be released from its heterodimer inhibitory complex and enter the nucleus to degrade chromosomal DNA. Taken together, the results of these studies provide additional support that caspase-3 activation is a major underlying factor that is responsible for apoptotic cell death following experimental traumatic injury to the CNS. The importance of these observations is exemplified by a study that reported caspase-3 activation and DNA fragmentation in the human brain following acute traumatic injury (Clark *et al*., 1999).

Traumatic spinal cord injury

A number of studies have provided compelling evidence of widespread apoptosis of neurons, oligodendroglia, and microglia following SCI (Katoh *et al*., 1996; Li *et al*., 1996; Crowe *et al*., 1997; Liu *et al*., 1997; Shuman *et al*., 1997; Emery *et al*., 1998; Hayashi *et al*., 1998; Lou *et al*., 1998; Yong *et al*., 1998). An excellent review by Beattie *et al.* provides a summary of current findings (Beattie *et al*., 2000). Many of the cells that exhibit an apoptotic phenotype were located several millimeters from the injury epicenter. This indicates that this cell death process contributes to the pattern of white and gray matter tissue loss that is commonly found in traumatic SCI. At least one group has put forth the hypothesis that apoptosis of oligodendroglia in areas distant to the injury epicenter may contribute to long-term neurological dysfunction (Crowe *et al*., 1997; Shuman *et al*., 1997). Specifically, the loss of oligodendroglia can result in demyelination and dysfunction of uninjured descending axonal tracts that control voluntary motor function (Blight 1983; Blight and Decrescito, 1986; Blight 1989). Therefore, understanding the intracellular signaling pathway(s) involved in this cell death process may prove critical for developing therapies that will reduce oligodendroglial cell loss following spinal cord injury.

In a study published in 1998, a group from the Miami Project to Cure Paralysis reported that cells exhibiting apoptotic morphologies in injured human spinal cord tissue also contained the activated form of caspase-3 (Emery *et al*., 1998). This was particularly evident in oligodendroglia located in white matter tracts adjacent to the lesion epicenter. At the same time, our laboratory was investigating the components of the caspase-3 apoptotic cascade using an

experimental model of traumatic spinal cord injury in rats (Springer *et al*., 1999). Initially, we used a DNA fragmentation assay to demonstrate that apoptotic-associated DNase activity is present in cytosolic fractions of injured (but not control) spinal cords that were obtained 4 and 24 hours following an incomplete contusion injury. We then used a fluorogenic substrate assay to measure caspase-3-like enzyme activity levels in cytosolic extracts of an injured spinal cord. Within 1 hour following injury, enzyme activity levels increased 6-fold and remained elevated up to 24 hours in cytosolic extracts from the injured spinal cords. These findings were supported by a recent study by Festoff and co-workers that document the increased caspase-3 expression and activity in the injured spinal cord (Citron *et al*., 2000). In this study, these investigators reported a rapid up-regulation of caspase-3 mRNA and protein following injury. These changes occurred in regions (mRNA) and cells (protein) of spinal cord exhibiting the highest level of apoptosis.

The results of these initial experiments led us to speculate that the upstream molecular machinery responsible for caspase-3 activation in vitro (i.e., cytochrome c release and caspase-9 activation) should be detectable in the injured spinal cord. Using immunoblotting and semi-quantitative densitometry, we found that cytochrome c levels are significantly elevated 30 min after injury, and remain elevated for at least 24 hours. As predicted, caspase-9 activation also occurred and followed a temporal pattern similar to that observed for the cytochrome c release. The observation that elevated cytochrome c levels and caspase-9 activation occur within 30 min following injury suggests that these events are indeed upstream to the caspase-3 activation, which is first detected at 1 hour following injury.

Similar to the study examining caspase-3 mediated cleavage of DFF45/ICAD following TBI (Zhang *et al*., 1999), immunoblotting was used to examine cytosolic fractions of the spinal cord for evidence of DFF45/ICAD cleavage following injury. In our study, there was clear evidence of DFF45/ICAD cleavage at 1 and 4 hours following injury, with levels decreasing in 24 hours. While the pattern of DFF45/ICAD proteolysis is consistent with a role for caspase-3, other possibilities cannot be ruled out. To address this, animals were given an intrathecal injection of the caspase-3-like inhibitor (z-DEVD-fmk) an hour before the spinal cord injury. The results of this experiment demonstrate that DFF45/ICAD cleavage is reduced in spinal cords of the animals that received the z-DEVD-fmk treatment. Interestingly, the caspase-3-like inhibitor had no affect on caspase-9 activation. Taken together, these results provide compelling evidence that DFF45/ICAD cleavage following spinal cord injury involves the proteolytic actions of caspase-3, and occurs downstream to caspase-9 activation.

These experimental findings suggest that rat spinal cord contains the molecular machinery that is necessary for activation and execution of the caspase-3 apoptotic pathway. Although recent studies have implicated a role for caspase-3 in apoptosis in acute spinal cord injury, it remains to be determined whether intervention with inhibitors of capsase-3 like activity (i.e., z-DEVD-fmk) reduce apoptotic cell loss and promote functional recovery. Moreover, the identity of the extracellular apoptotic death signal(s) that is generated following spinal cord trauma and other types of injury is unknown at this time, and is the focus of ongoing and future studies. The delayed onset of apoptosis in oligodendroglia distant to the site of injury appears to be unique to spinal cord injury and has important therapeutic implications. In particular, the death of these cells leads to axonal demyelination and dysfunction in areas distant to the injury epicenter, which may further contribute to the long-term neurological deficits.

Therapeutic interventions: delivery of anti-apoptotic genes to the injured CNS

As indicated previously, in a relatively short period of time we have been able to uncover a number of basic principles about apoptotic cell death that is associated with traumatic injury to the CNS. The rapid onset of caspase-3 activation in some injury models indicates that there is a relatively narrow therapeutic time-window for implementing treatments that target caspase-3 activation. However, apoptosis does persist for some time following injury. As demonstrated in some of the studies described, strategies targeting caspase-3 activation and enzyme activity may prove beneficial in minimizing the cleavage of substrates involved in generating the apoptotic phenotype. Given this approach, understanding the molecular components of the apoptotic program is an essential initial step in developing treatments that target this cell death process.

One approach that we are pursuing is to use adenoviral vectors to deliver anti-apoptotic genes to the injured spinal cord. A similar strategy could also be considered for other types of injuries. In fact, this strategy has been used to limit cell death following ischemic injury (Martinou *et al*., 1994; Linnik *et al*., 1995). Adenoviruses generally exhibit a short-term expression of the gene of interest (typically 4-6 weeks), due to non-integration into the host genome. However, the transient expression would be considered optimal in CNS injury, as apoptosis is thought to range from hours to weeks following the initial insult, as is the case in spinal cord injury (Crowe *et al*., 1997; Liu *et al*., 1997; Shuman *et al*., 1997). Therefore, the expression of the anti-apoptotic gene may not be required at extended time points when apoptosis is no longer a predominant event in the injured CNS.

The genes of interest to be delivered to the injured CNS are based on their potential to inhibit different steps in the caspase-3-mediated apoptotic cascade (Fig. 1). Akt (also known as protein kinase B) is a serine/threonine protein kinase that transduces intracellular anti-apoptotic signals that are mediated by serum or growth factors that activate phosphoinositide 3-kinase, or PI3-kinase (Franke *et al*., 1995;

Datta *et al*., 1997; Dudek *et al*., 1997; Kennedy *et al*., 1997; Coffer *et al*., 1998; Downward 1998; Nunez and del Peso, 1998; Goswami *et al*., 1999; Kennedy *et al*., 1999; Flores *et al*., 2000). Overexpression of Akt prevents apoptosis in certain cells, including neurons, following withdrawal of serum or growth factors (Yao and Cooper, 1995; Datta *et al*., 1997; Dudek *et al*., 1997; Chen *et al*., 1998; Crowder and Freeman, 1998; Eves *et al*., 1998; Ulrich *et al*., 1998; Bhave *et al*., 1999; Vaillant *et al*., 1999; Virdee *et al*., 1999; Flores *et al*., 2000; Kermer *et al*., 2000). The precise mechanisms by which Akt promotes cell survival are not entirely clear. However, one potential target is BAD, which is inhibited when phosphorylated by Akt (Datta *et al*., 1997; Cardone *et al*., 1998; Nunez and del Peso, 1998; Hayakawa *et al*., 2000; Zhou *et al*., 2000). The involvement of BAD is thought to be related to intracellular events upstream of caspase-3, such that this pro-apoptotic protein translocates to the mitochondria where it binds and inhibits the anti-apoptotic actions of bcl-2 and Bcl-X_L. A recent study from our laboratory suggests that BAD dephosphorylation is an early upstream event that leads to caspase-3 activation following SCI (Springer *et al*., 2000). The activation of Akt appears to require translocation to lipid membranes in order to interact with downstream lipid products of PI3-kinase. This observation has lead to the development of constitutively active Akt via N-terminal myristolation, which promotes translocation of Akt to membranes (Cardone *et al*., 1998; Gold *et al*., 1999). Therefore, the adenoviral-mediated transfer of activated Akt may prove beneficial in reducing some of the early stages of the apoptotic cascade.

The ability of Akt to inhibit apoptosis is most likely related to the events that are upstream to the mitochondrial release of cytochrome c (and possibly Smac/DIABLO), a critical step for the activation of caspase-3 in a number of cell types, including cells of the injured spinal cord. A similar argument can be made for overexpressing Bcl-X_L, an anti-apoptotic member of the bcl-2 gene family that binds to and inhibits the actions of other pro-apoptotic bcl-2 family members. A second potential step to target in the caspase-3 apoptotic cascade involves the events that are upstream of caspase-3, but downstream of the cytochrome c release, in particular the family of inhibitor of apoptosis proteins (Deveraux *et al*., 1998; Duckett *et al*., 1998; Ekert *et al*., 1999; Ekert *et al*., 2001). The first identified IAP family member was p35, a baculoviral protein that suppresses apoptosis in a number of cell types (including cells of the CNS) and under a variety of conditions (Rabizadeh *et al*., 1993; Simons *et al*., 1999; Xu *et al*., 1999; Eberhardt *et al*., 2000; Hisahara *et al*., 2000; Kugler *et al*., 2000; Korhonen *et al*., 2001). Subsequent studies demonstrated that several IAPs exist and function by binding to the inactive pro-caspase, therefore, inhibiting its processing to the active form (Hay *et al*., 1994; Hay *et al*., 1995; Hay, 2000). Mammalian IAPs include XIAP, c-IAP1, c-IAP2, NAIP, and Survivin, although XIAP inhibits caspase-3 activation at a K_i that is much lower compared to the other IAPs. Recent studies suggest that the removal of XIAP inhibition is dependent upon Smac/DIABLO, the second cell death protein (along with cytochrome c) that is released by the mitochondria during apoptosis (Du *et al*., 2000; Verhagen *et al*., 2000). Smac/DIABLO displaces XIAP from caspase-9, allowing the latter to be activated through its interactions with Apaf-1. It can also remove XIAP directly from caspase-3. Taken together, these studies suggest that the overexpression of IAPs, such as XIAP and p35 (a broad caspase inhibitor), should reduce caspase-3 activation in cells following CNS injury.

A third approach is to overexpress proteins that serve as competitive inhibitors of caspase-3, based on the presence of preferred amino acid cleavage sites. In the late 1990s, caspase-3 was found to cleave a novel cytoplasmic protein termed ICAD (for inhibitor of caspase-activated DNase), which is homologous to the DNA fragmentation factor-45 (DFF45), originally described in HeLa cells (Liu *et al*., 1997; Enari *et al*., 1998; Liu *et al*., 1998; Sakahira *et al*., 1998; Inohara *et al*., 1999; Sakahira *et al*., 1999). ICAD normally forms a heterodimer with and inhibits the actions of CAD (or DFF40), the first caspase-activated DNase that is responsible for the DNA fragmentation pattern that is a hallmark of apoptosis. The cleavage of ICAD by caspase-3 disassembles the heterodimer, resulting in the activation of CAD that then translocates to the nucleus to degrade genomic DNA. It is now known that ICAD exists in at least two forms, termed $ICAD_L$ (or DFF45) and the smaller alternately spliced form $ICAD_S$ (or DFF35), both of which contain two caspase-3 cleavage sites and inhibit the actions of CAD. Therefore, it could be argued that the overexpression of a naturally occurring caspase-3 substrate would serve as a competitive inhibitor of caspase-3 on other endogenous apoptotic substrates. The caspase-mediated cleavage of ICAD/DFF45 has been demonstrated in several models of CNS injury (Springer *et al*., 1999; Zhang *et al*., 1999; Springer *et al*., 2000; Cao *et al*., 2001). However, the choice of substrate is important as the larger form of ICAD (DFF45) also functions to promote the expression of CAD (DFF40), which might increase the expression of this DNase. In contrast, the smaller $ICAD_S$ polypeptide binds to and inhibits CAD (DFF40) with equal or greater potency, but plays no role in its synthesis or folding of the DNase component (Enari *et al*., 1998).

The ability to overexpress a competitive caspase inhibitor has potential for limiting apoptotic cell death. This is similar to using tetrapeptide-based cell permeable inhibitors (e.g., z-DEVD-fmk) that have been effective in reducing apoptosis in vitro and in vivo. However, the efficacy of these small peptides is somewhat limited given their relatively short half-life, the need for chronic injection protocols, and limited cellular penetration. Therefore, the use of the adenoviral gene transfer to deliver and overexpress anti-apoptotic proteins of interest to cells in vivo following CNS injury has great therapeutic potential. This approach alleviates problems associated with existing methods for inhibiting apoptosis,

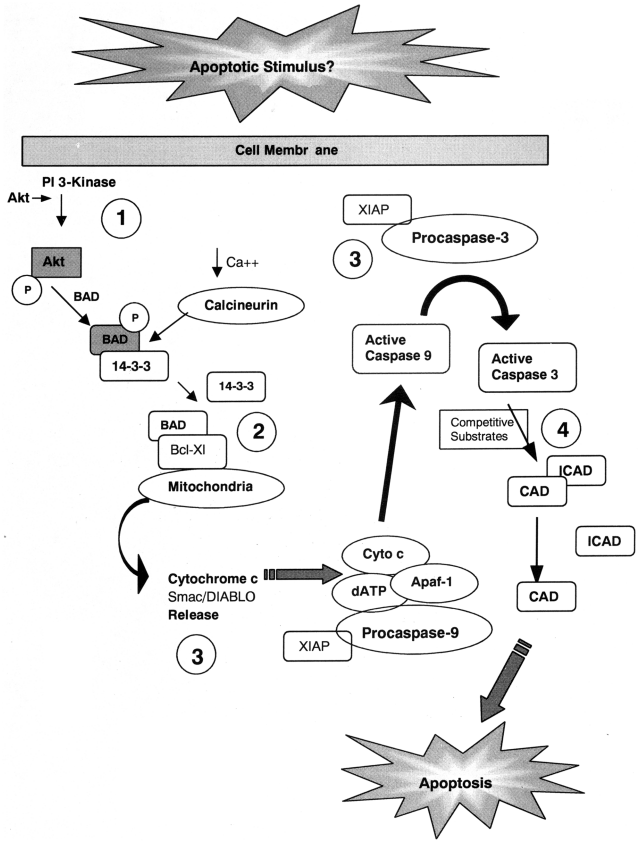

Fig. 1. Schematic representation of one mechanism leading to activation of the caspase-3 apoptotic cascade. Circled numbers represent targeted areas where the adenoviral-mediated transfer of anti-apoptotic genes would affect continued signaling through the cascade. Overexpression of activated Akt at level **1** would promote PI3-kinase activity and phosphorylation of BAD. The overexpression of Bcl-X_L at level **2** would interfere with the pro-apoptotic actions of BAD and bax. The release of Smac/DIABLO from the mitochondria results in the displacement of XIAP, and this could be inhibited by overexpressing XIAP at level **3**. Finally, overexpression of substrates at level **4** containing DXXD amino acid motifs recognized by caspase-3 would serve as competitive inhibitors once caspase-3 is activated in the cell. See text for detailed descriptions.

including limited delivery into the cell, repeated or chronic injection/infusion protocols, and lack of specificity to apoptotic pathways. Over the past two years, several studies reported the use of replication-deficient viral-mediated gene transfer to successfully overexpress a number of proteins in the CNS (Choi-Lundberg *et al.*, 1997; Bemelmans *et al.*, 1999; Franklin *et al.*, 1999; Kitagawa *et al.*, 1999; Bohn *et al.*, 2000; Eberhardt *et al.*, 2000; Fathallah-Shaykh *et al.*, 2000; Huber *et al.*, 2000; Kordower *et al.*, 2000; Watabe *et al.*, 2000; Yagi *et al.*, 2000; Yukawa *et al.*, 2000; Boer *et al.*, 2001; Watabe *et al.*, 2001). Recombinant adenovirus gene transfer has also been used to express functional proteins in the spinal cord (Smith *et al.*, 1996; Smith *et al.*, 1997; Romero and Smith, 1998; Smith and Romero, 1999; Romero *et al.*, 2000). Therefore, the use of this strategy to deliver anti-apoptotic genes that target different steps in the caspase-3 apoptotic cascade may prove beneficial in reducing apoptosis. This will support or refute the hypothesis that this cell death process contributes to neurological dysfunction following CNS injury.

Summary

The studies described in this review provide clear evidence that apoptotic cell death is prominent and widespread in many types of CNS injuries. As such, there are two important questions that need to be addressed. First, what is the role of apoptosis in the functional deficits observed following CNS trauma? In order to answer this question, it will be necessary to identify which steps in the caspase-3 cascade are the most effective for suppressing the apoptotic process. To accomplish this, it will be necessary to block these pathways using methods such as overexpressing different apoptotic genes that target different steps in the caspase-mediated apoptotic cascade. Second, will therapies designed to inhibit caspase-3-mediated apoptosis promote cell survival and improve functional recovery? At this point in time, there is evidence demonstrating that caspase inhibition can promote cell survival in some models of CNS injury. However, there is limited evidence that caspase inhibition is effective in promoting functional recovery. Regardless, as our understanding of the extracellular and intracellular apoptotic signaling events that occur following CNS injury continues to develop, it will be possible to identify therapeutic strategies that will test the functional role of apoptosis in CNS injury.

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