Apoptotic and Anti-Apoptotic Mechanisms Following Spinal Cord Injury

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Abstract. A number of studies have provided evidence that cell death from moderate traumatic spinal cord injury (SCI) is regulated, in part, by apoptosis that involves the caspase family of cysteine proteases. However, little or no information is available about anti-apoptotic mechanisms mediated by the inhibitors of apoptosis (IAP) family of proteins that inhibit cell death pathways. In the present study, we examined caspase and IAP expression in spinal cords of rats subjected to moderate traumatic injury. Within 6 h after injury, caspase-8 and -9 (2 initiators of apoptosis) were predominantly present in gray matter neurons within the lesion epicenter. By 3 days following spinal cord injury (SCI), caspase-8 and -9 immunoreactivity was localized to gray and white matter cells, and by 7 days following SCI, both upstream caspses were expressed in cells within white matter or within foamy macrophages in gray matter. Caspase-3, an effector caspase, was evident in a few fragmented cells in gray matter at 24 h following injury and then localized to white matter in later stages. Thus, distinct patterns of caspase expression can be found in the spinal cord following injury. XIAP, cIAP-1, and cIAP-2, members of the IAP family, were constitutively expressed in the cord. Immunoblots of spinal cord extracts revealed that the processed forms of caspases-8 and -9 and cleavage of PARP are present as early as 6 h following trauma. The expression of caspses corresponded with the detection of cleavage of XIAP into 2 fragments following injury. cIAP-1 and cIAP-2 expression remained constant during early periods following SCI but demonstrated alterations by 7 days following SCI. Our data are consistent with the idea that XIAP may have a protective role within the spinal cord, and that alteration in cleavage of XIAP may regulate cell death following SCI.

Key Words: Apoptosis; Apoptosis inhibitors; Caspases; Spinal cord injury.

INTRODUCTION

Recent reports have shown that apoptosis contributes to cellular damage occurring after spinal cord trauma (1–4). It is now firmly established that apoptosis is regulated by an intracellular proteolytic cascade, primarily mediated by members of the caspase family of cysteine proteases, which proteolytically cleave each other and also various key intracellular target proteins to destroy the cell. Three prototypal signaling pathways for the induction of apoptosis have been described (5, 6). One pathway involves the ligation of death receptors that activate pro-caspase-8 and possibly other initiator caspases (7–9). A second pathway is controlled by mitochondria and involves the apoptosis protease activating factor-1 (Apaf-1) (10–13) and caspase-9, located in mitochondria (14). Once activated by cytochrome c, Apaf-1 together with cofactor nucleotide triphosphates (dATP or ATP) bind and activate pro-caspase-9, which in turn cleaves and activates caspase-3 and other downstream caspses. Therefore, caspases-8 and -9 represent the pinnacle caspses in the death receptor and mitochondrial (cytochrome c/Apaf-1) pathways, respectively. More recently an endoplasmic reticulum apoptotic pathway mediated by caspase-12 has been described that may contribute to amyloid-β neurotoxicity (6).

The inhibitor of apoptosis protein (IAP) family of anti-apoptotic proteins, which are conserved across evolution with homologues found in vertebrate and invertebrate species, have a key function in the negative regulation of programmed cell death in a variety of organisms (15). Several mammalian homologues (XIAP, cIAP-1, cIAP-2, NAIP, Bruce, Survivin, and pIAP) have been identified, most of which have been demonstrated to inhibit cell death. Although the biochemical mechanism by which IAP-family proteins suppress apoptosis is controversial, at least some of the human IAPs (XIAP, cIAP-1, and cIAP-2) have been reported to directly bind and inhibit certain caspases, including caspases-3, -7 and -9 (16, 17). Thus, IAPs can inhibit caspses within both the death receptor and mitochondrial pathways (17).

The IAPs are characterized by a highly conserved ~70 amino-acid domain, termed the baculoviral inhibitory repeat (BIR), that can be present as many as 3 times in some IAPs (15). During apoptosis induced by the TNF family member Fas, XIAP is cleaved, separating the BIR1–2 domains from the BIR3-Ring domain. The BIR1–2 fragment is capable of inhibiting active caspses-3 and -7, but it is turned over rapidly in cells (18). Thus, cleavage of XIAP may be a mechanism for lowering the threshold of caspase activity necessary for inducing apoptosis.
Within the nervous system, IAPs have been shown to protect some types of neurons from insults often associated with ischemia (19, 20). Virus-mediated overexpression of NAIP (19) or XIAP (20) can prevent ischemic neuronal loss in the hippocampus. Moreover, the CA1 neurons protected in this manner appeared to function normally (20). Conversely, in severe spinal muscular atrophy the neuron-specific inhibitor of apoptosis, NAIP, is often dysfunctional due to missense and truncation mutations, suggesting that NAIP mutations may alter development of sensory and motor systems resulting in lethal muscular atrophy (21). Thus, anti-apoptotic strategies may be useful in treatment of acute and chronic neurodegenerative conditions.

In this report we investigated cellular expression and maturation of caspases-8, -9, -3, and IAP family members XIAP, cIAP-1, and cIAP-2 after spinal cord trauma. Our studies suggest that the death of spinal cord cells involves multiple caspases and that cleavage of XIAP following injury may also contribute to the apoptosis that occurs in this setting.

**MATERIALS AND METHODS**

**Animals and Spinal Cord Injury**

Sprague Dawley female rats (250–300 g) were anesthetized with 1% halothane and a mixture of 70% nitrous oxide and 30% oxygen. The tail and dorsal aspect of the back were shaved and scrubbed with Betadine solution. The corneal reflex and withdrawal to painful stimuli were monitored to determine whether an adequate amount of anesthesia was administered. Core temperature was measured in the rectal colon and maintained by a homeothermic blanket (Harvard® Kent, Harvard Apparatus, Hollister, MA) between 36°C and 37°C. The rats were intubated and implanted with a PE catheter (Intramedic®, BD Biosciences, Franklin Lakes, NJ) in the tail artery, and ventilated mechanically after infusion of pancuronium bromide (1 μg/g weight).

Traumatic injury was induced by the New York University weight drop device (22). A laminectomy was performed at vertebral levels T9–T10 and the cord was exposed without disrupting the dura. A moderate injury was induced by adjusting the height of the weight (10.0 g) to 12.5 mm above the exposed spinal cord. After injury, muscles were closed in layers, the incision was closed with wound clips, and the animals were allowed to survive for 6 h, 1, 3, or 7 days. Sham uninjured animals at all stages (n = 3) were included as controls.

**Antibodies**

Immunohistochemical and immunoblotting procedures employed antibodies from several sources to establish immunostaining and protein expression. Rabbit anti-caspase-3 (MF-393), anti-caspase-8 (MF-438), and anti-caspase-9 (MF-443) antibodies were kindly supplied by Dr. Donald W. Nicholson, Merck-Frosst Montreal, Canada and used at 1:1,000 dilution. Immunogens used to produce these antibodies were the large caspase subunit in each case. The antibodies recognize intact preproenzymes, the fully matured large subunits, and any processing intermediates that might occur during maturation. Rabbit anti-cleaved caspase-3 (17 kDa, 1:1,000) (cat. No. 9661) and anti-cleaved caspase-9 (37 kDa, 1:2,000) (cat. No. 9501S) were purchased from Cell Signaling Technology, Beverly, MA, and are specific for the processed forms of these enzymes. Rabbit anti-caspase-8 (p20, 1:500) (cat. No. SC-7890) was supplied by Santa Cruz Biotechnology, Santa Cruz, CA, and recognizes the p20 and proforms of caspase-8. Affinity purified rabbit anti-human/mouse cIAP-1 (cat. No. AF818, 1.0 μg/ml), anti-human/mouse cIAP-2 (cat. No AF817, 1.5 μg/ml), monoclonal anti-PARP clone C2–10 (cat. No. 4338-MC, 1:10,000) were purchased from R&D Systems, Inc., Minneapolis, MN. Specificity of binding of antibodies obtained from R&D Systems was evaluated on immunoblots of cellular extract controls that were provided by the manufacturer. Monoclonal anti-hILP/XIAP (cat. No. H62120, 1:250) was purchased from Transduction Laboratories, Lexington, KY, and rabbit anti-XIAP (1:1,000) was obtained from Apoptag, San Diego, CA. Mouse anti-actin monoclonal antibody (cat. No. MAB1501) was purchased from Chemicon International, Temecula, CA and used at 1:1,000 dilution.

**Immunohistochemistry**

Rats (n = 3 at each time point) were anesthetized and perfused transcardially with isotonic saline for 5 min, followed by fixative containing a mixture of 40% formaldehyde, glacial acetic acid, and methanol (FAM, 1:1:8 by volume) for 20 min. After perfusion, the vertebral columns containing the cord were immersed in FAM at 4°C for 24 h. The spinal cord was blocked and embedded in paraffin for tissue sectioning. Serial cross sections representing the traumatic epicenter and including white and gray matter areas were stained with anti-caspase-3, -8, -9, or anti-XIAP antibodies from several sources followed by biotinylated horse anti-rabbit immunoglobulin (1:1,000, Vector Elite ABC kit, Burlingame, CA) and streptavidin-horseradish peroxidase followed by 3,3’-diaminobenzidine (DAB) until a brown reaction product was observed. Negative controls without primary antibody and controls using an irrelevant antibody of the same isotope were included in each experiment.

**Immunoblotting**

For detection of caspases and IAPs, 3- to 4-mm segments of injured spinal cords (n = 5 at each time point) were homogenized in cell extraction buffer (100 mM HEPES, pH 7.5, 1% Triton X-100, 10 mM DTT, 1 mM PMSF, 5 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM EDTA). Proteins were resolved on 12% SDS-PAGE, transferred to PDVF membranes, and placed in blocking buffer (PBS, 0.1% Tween-20, 0.4% I-block [Tropix, Inc., Bedford, MA]) for 1 h and then incubated with anti-caspase or anti-IAPs monoclonal or polyclonal antibodies. Positive controls of cellular lysates provided by the manufacturer were run in parallel to determine antibody specificities. Visualization of the signal was by enhanced chemiluminescence. To control for protein loading, the immunoblots were stripped with Restore, Western blotting stripping buffer (Pierce, Rockford, IL) and probed for β-actin using monoclonal anti-actin.
RESULTS
Expression of Caspase-8 and Caspase-9 Following SCI

Antibodies to caspase-8 and -9 were used to assess the temporal pattern of expression of upstream caspases following SCI. In uninjured sham controls, no observable positive immunostaining was present in gray (Fig. 1A) or white (Fig. 1B) matter with the anti-caspase-8 antibodies. By 6 h after SCI, cytoplasmic staining was seen in small- and medium-sized neurons in the gray matter (Fig. 1C), and a few shrunken cells within the white matter demonstrated positive immunoreactivity, although more intense (Fig. 1D). By 24 h following SCI, a similar pattern of immunoreactivity was observed in gray matter (Fig. 1E), while small- and medium-sized cells in white matter showed diffuse cytoplasmic staining (Fig. 1F). This staining pattern was maintained at 3 days (not shown) following SCI. At 7 days after SCI, caspase-8 staining was confined to the gray matter in cells that appeared to be foamy macrophages, based on morphological criteria (Fig. 1G), whereas white matter demonstrated a few intensely stained apoptotic cell bodies (Fig. 1H).

The pattern of immunostaining obtained with the anti-caspase-9 antibodies is shown in Figure 2. No immunoreactivity for caspase-9 was observed in spinal

**Fig. 1.** Immunocytochemistry of caspase-8 (arrows) following SCI (A–H). Sham-operated control animals, gray (A) and white (B) matter; 6 h after injury, gray (C) and white (D) matter; 1 day after injury, gray (E) and white (F) matter; 7 days after injury, gray (G) and white (H) matter; magnification: ×630.
Fig. 2. Immunocytochemistry of caspase-9 (arrows) following SCI (A–H). Sham-operated control animals, gray (A) and white (B) matter; 6 h after injury, gray (C) and white (D) matter; 3 days after injury, gray (E) and white (F) matter; 7 days after injury, gray (G) and white (H) matter; magnification: ×630.

cords of sham-operated animals (Fig. 2A, B), whereas positive immunoreactivity for caspase-9 was detected within the epicenter of the injury as early as 6 h in cells throughout the gray (Fig. 2C) and white matter (Fig. 2D). Immunoreactive cells within the gray matter expressing caspase-9 were neuronal in appearance and demonstrated diffuse cytoplasmic staining (Fig. 2C), whereas white matter cells were shrunken, resembling a hallmark of apoptotic cells (Fig. 2D). A similar pattern of immunostaining was evident at 24 h post-injury in both gray and white matter (not shown). By 3 days, gray matter cells with pyknotic nuclei showed intense nuclear and cytoplasmic staining (Fig. 2E), while fewer cells in white matter demonstrated diffuse staining throughout the entire cell (Fig. 2F). By 7 days, immunopositive cells in the gray matter were present that resembled foamy macrophages (Fig. 2G), while the white matter contained shrunken pyknotic cells that intensely stained with anti-caspase-9 antibodies (Fig. 2H).

Caspase-3 expression was found within a small number of cells of the gray matter at 24 h after injury, a time at which we could clearly identify apoptotic bodies by basic histological analysis (Fig. 3A). Additionally, caspase-3 immunostaining appeared in white matter, but was not evident until 3 days following SCI (Fig. 3B).
XIAP Expression Following SCI

To assess the regulation of XIAP protein expression in damaged spinal cord, the temporal pattern of changes in XIAP immunostaining was determined. Figure 4 demonstrates that positive immunoreactivity was obtained within neurons (Fig. 4A) that was confined to the nucleus in uninjured sham animals, while positive immunoreactivity was seen in the nuclei of white matter cells (Fig. 4B). By 7 days after injury, diffuse immunostaining of the cytoplasm and nucleus staining was observed in neurons in gray matter (Fig. 4C), and robust XIAP immunostaining was present in white matter cells (Fig. 4D).

Immunoblot Analysis of Caspases

Immunoblot analysis was performed to determine the time-course of caspase processing following SCI and to establish antibody specificity. Figure 5A shows that the cleaved p17 subunit of caspase-8 and the cleaved p18 subunit of caspase-9 are generated within 6 hours after injury, supporting the hypothesis that multiple initiator caspases are expressed following SCI. These caspase subunits were also detected in spinal cord extracts at later stages after injury (Fig. 5A). However, multiple attempts to identify by immunoblot analysis the processed forms of caspase-3 in spinal cord extracts taken from injured animals with 2 anti-caspase-3 antibodies were unsuccessful. Moreover, experiments to detect active caspase-3 by the more sensitive hydrolysis of suitable fluorogenic substrates yielded negative results (not shown). Since PARP is a substrate for certain downstream caspases, including caspase-3, we performed immunoblot analysis on spinal cord extracts with monoclonal anti-PARP that recognizes both the full length (116 kDa) and the 85 kDa cleavage fragment. As shown in Figure 5A, the 116 kDa protein is cleaved to generate a 85 kDa fragment within 6 h after SCI, and PARP cleavage was detected in samples at time periods assessed following injury. The proteolytic cleavage of PARP in spinal cord extracts following SCI suggests the existence and activation of downstream caspases.

XIAP is Cleaved Following SCI

The time-course of XIAP expression and cleavage following SCI is shown in Figure 5B. Within 6 h following SCI, the full-length 53-kDa intact protein had been cleaved to generate a 30-kDa fragment that reacts with both monoclonal and polyclonal anti-XIAP antibodies. XIAP cleavage was also observed in extracts at later stages following injury. Since XIAP cleavage has been shown to correlate with caspase activation (18), it is possible that cleavage of XIAP may be a mechanism for lowering the threshold level of caspase activity necessary for inducing apoptosis.

Expression of cIAP-1 and cIAP-2 Following SCI

In order to determine whether other IAP family members may compensate for cleaved XIAP, we performed immunoblotting procedures on spinal cord extracts with anti-cIAP-1, anti-cIAP-2 (Fig. 5C). No measurable changes were detected in the levels of cIAP-1 or cIAP-2 between 6 h and 3 days following SCI. However, by 7 days there was a decrease in the expression of cIAP-1 and an increase in the level of cIAP-2.

DISCUSSION

Our results demonstrate that death of spinal cord cells in both gray and white matter following moderate spinal cord trauma involves processing of multiple caspases and cleavage of XIAP and PARP, thus contributing presumably to the apoptotic demise of cells in the cord. Activation of initiator caspases, such as caspase-8 and -9, was restricted to a subpopulation of cells and was first detected at 6 h after injury in the lesion epicenter. At later stages following SCI, these upstream caspases were present in white matter, indicating a distinct pattern of expression. Our results are consistent with those of Springer et al (4) who reported activated form of caspase-9 as early as 30 min following SCI and a 3-fold increase in the levels of caspase-3-cleaved DFF40/CAD within 1 h post-SCI. Together these observations provide in vivo evidence that key components of both the death receptor and mitochondrial apoptotic pathways are activated after SCI, but the identity of the death signals activating these pathways remains to be determined. In this regard, Bejina et al (23) have reported that SCI induces a rapid increase in expression of tumor necrosis factor-alpha (TNF-α) in spinal cord and activated peripheral monocytes as early as 1 h following injury, suggesting that TNF-α may serve as a putative death signal generated after spinal cord trauma. It should be noted however that caspase-8 activation has been reported to occur downstream of caspase-3 in some scenarios (24). Thus, further studies involving specific inhibitors of caspase-8 and -9 are required before a firm conclusion can be reached.

A small population of cells within the lesion epicenter immunostained for caspase-3 as early as 1 day after injury. The small percentage of cells expressing caspase-3 is probably the reason that this caspase was not detected by enzymatic assays or by immunoblot analysis. It is possible that CNS clearance could remove apoptotic cells early in the cell death sequence, thus accounting for the low percentage of cells expressing downstream caspases. However, immunoblots of injured spinal cords revealed that PARP, a substrate for downstream caspases, particularly caspase-3, is cleaved within 6 h after injury, supporting the hypothesis that caspase-3 is activated early.
following spinal cord trauma. Alternatively, a recent report has shown that caspase-3 plays a major role in cell death in immature neurons, but only a minor role in cell death in mature neurons after brain injury (25). Thus, it is possible that other caspases may primarily mediate neuronal death following SCI.

Previous investigations have stressed the role of oligodendrocytic apoptosis promoting the spread of spinal cord lesions following SCI. Crowe and colleagues (1) first identified oligodendrocytic changes following traumatic SCI. In human SCI tissue, Emery and colleagues (3) reported caspase-3 expression in oligodendrocytes at various times following injury. In the present experimental studies, cells resembling oligodendrocytes were observed to express caspase-8, -9, and -3. In addition, XIAP expression was also observed in white matter glial cells. Although double labeling strategies using confocal microscopy are required to critically evaluate the cell types that demonstrated caspase expression, nevertheless the present data support previous studies that emphasize white matter involvement in apoptotic mediated lesion progression after SCI.

The finding that caspase expression is associated with cleavage of the inhibitor of apoptosis XIAP is consistent with previous evidence that caspases cleave XIAP (18), but not cIAP-1 and cIAP-2 (16). In support of this observation, Johnson et al (26) have shown that XIAP is cleaved in apoptotic T lymphocytes, generating at least 1 prominent fragment of 29 kDa. This fragment was detected in T cells induced to undergo cell death through the death receptor and drug-mediated pathways, and remains associated with active subunits of caspase-3 and -7. These results indicate that XIAP, like CrmA and p35, may act as a suicide inactivator of caspases, undergoing cleavage to allow the death program to proceed when sufficient levels of caspase activation are achieved.

Fig. 3. Caspase-3 expression (arrows), 1 day after injury, gray matter (A); 3 days after injury, white (B) matter; magnification: \( \times 630 \).

Fig. 4. XIAP immunochemistry following SCI. Endogenous XIAP expression in sham-operated control animals; gray (A) and white (B) matter; 7 days following injury, gray (C) and white (D) matter; nuclear staining (arrows), staining of cell processes (arrow heads); magnification: \( \times 630 \).
Overexpression of XIAP, c-IAP-1, c-IAP-2, NAIP, or survivin suppresses apoptosis induced by TNF, Fas, staurosporin, VP16, Taxol, and growth factor withdrawal (27, 28–31). Although various IAP-family proteins are capable of suppressing apoptosis, their expression in vivo may be regulated differentially, thus providing regulatory mechanisms for cellular and environmental signaling. Our studies demonstrate that IAPs are differentially expressed within the spinal cord following injury. XIAP cleavage was associated with processing of upstream caspases, whereas c-IAP-1 and c-IAP-2 alterations were not detected until 7 days post-injury. These observations suggest that XIAP might function physiologically to inhibit small amounts of caspase activation that may occur inadvertently in the normal function of healthy cells. Dysregulation of the normal mechanisms that control XIAP protein cleavage could contribute to cell death induced by CNS injury. The mechanisms underlying changes in

**Fig. 5.** Activation of caspase-8 and -9 (A); cleavage of XIAP (B), and expression of cIAP-1 and cIAP-2 (C) following SCI. A: Activation of caspase-8 is indicated by detection of 17-kDa fragment, while activation of caspase-9 is indicated by detection of 18-kDa fragment. B: XIAP is cleaved within 6 hours following SCI, as indicated by detection of cleavage fragment of approximately 30 kDa. C: Expression of cIAP-1 decreases while cIAP-2 increase at 7 days following SCI. β-actin was used as an internal standard and control for protein loading.
cIAP-1 and cIAP-2 following SCI are currently under investigation. Understanding the molecular components of the apoptotic pathway induced after SCI may lead to therapeutic strategies preventing this cell death process.

ACKNOWLEDGMENTS

We thank Dr. Donald Nicholson, Merck-Frosst, Kirkland, Quebec, Canada for caspase antibodies. We also thank Dr. Alex Marcielo for technical support.

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Received October 13, 2000
Revision received January 24, 2001
Accepted January 30, 2001