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Caspase Activation in Neuronal and Glial Apoptosis Following Spinal Cord Injury in Mice

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Abstract

The involvement of caspases in apoptosis after spinal cord injury (SCI) was investigated in adult mouse spinal cord after contusion. Sections of spinal cord were processed for staining 7 days after SCI with the fluorescent dye Hoechst 33342, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL), and immunostaining with an antibody (CM1) recognizing activated caspase-3. Caspase-3- and caspase-8-like enzyme activities were measured colorimetrically at 8 hours to 7 days after SCI using the specific substrates Asp-Glu-Val-Asp-p-nitroanilide and Ile-Glu-Thr-Asp-p-nitroanilide, respectively. Hoechst 33342 staining showed small, bright areas in fragmented nuclei. Double labeling with TUNEL plus immunostaining with cell type-specific markers identified TUNEL-positive neurons stained by anti-neuronal nuclear protein/neurons antibody, and TUNEL-positive oligodendrocytes stained by anti-cyclic nucleotide 3'-phosphohydrolase antibody. Double labeling with CM1 and cell-type specific markers similarly identified CM1-positive neurons and oligodendrocytes. Caspase-8-like enzyme activity was increased significantly on days 3 and 7 ($p < 0.01$), whereas caspase-3-like activity increased on day 7 ($p < 0.01$). Intraventricular injection of a nonspecific tetrapeptide caspase inhibitor or a specific tetrapeptide inhibitor of caspase-3 just after SCI reduced enzyme activity at 7 days. Apoptotic cells were identified with TUNEL staining in both neurons and oligodendrocytes in mice after SCI, which also showed activated caspase-3. Increased caspase-3- and caspase-8-like activity was detected in the injured spinal cord on days 3 and 7. Caspase protease activities may be involved in delayed neuronal and glial apoptosis after SCI.

Key words: spinal cord injury, mice, apoptosis, caspase-3, caspase-8, caspase enzyme activity

Introduction

Decades of investigations have greatly improved our understanding of the complex pathophysiology of spinal cord injury (SCI). The mechanism of neuronal impairment may involve primary damage due to external mechanical force and additional damage representing secondary degeneration initiated from the site of the primary damage.[4,6,17,21,42,43] Apoptotic cells are present in injured spinal cord, suggesting that degeneration at the mechanically damaged site and delayed demyelination of tracts at a distance from the injury might be partly due to apoptosis.[13,26,31,34,38,50]

Apoptosis is one of the two types of cell death: necrosis occurs in a matter of seconds; whereas apoptosis or programmed cell death is a much slower process, representing a series of events over a few hours to several days, depending on the initiating stimulus. Apoptosis is characterized ultrastructurally by degradation of nuclear chromatin, condensation of the cytoplasm and nucleus, and ultimately fragmentation of the cell into apoptotic bodies.[11,27] These distinct changes in cellular structure differ sharply from those seen in necrosis.

Apoptosis requires activation of a cysteine protease enzyme that cleaves peptides at a position next to an aspartyl residue.[3,14] This family of cysteine aspartyl proteases, now numbering 14, are called the caspases, and shows similarities to the product of ced-3, an essential cell death gene in *Caenorhabditis elegans*. Caspases can be divided into two main groups: initiators (caspase-1, -2, -8, and -9) and effectors such as caspase-3. Current evidence suggests that several distinct routes can lead to caspase activation depending upon the stimulus that cleaves the initiator enzyme. Such initiation activates caspase-3 and induces apoptosis. Caspases are all expressed as proenzymes including three domains: the N-termi-

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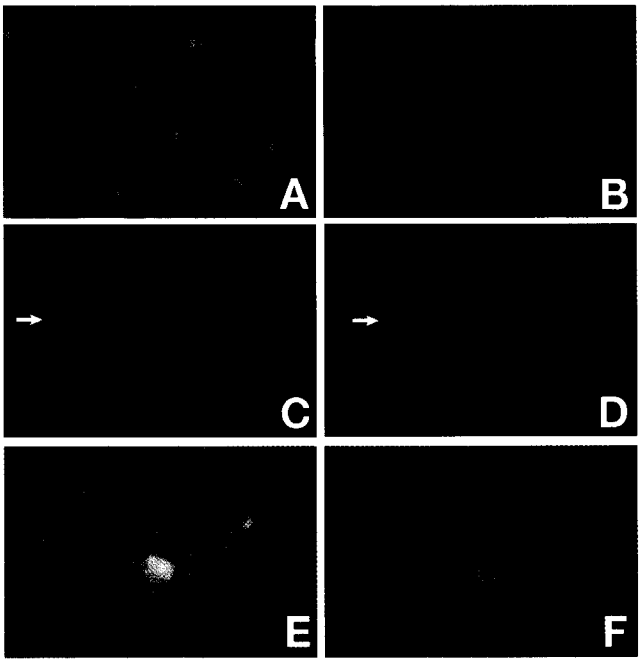

Fig. 1 **Photomicrographs showing immunostaining for apoptosis and double staining with cell-specific markers of transverse cord sections. Hoechst 33342 staining of uninjured naive spinal cord shows healthy-appearing, oval nuclei with a diffuse chromatin distribution (A), whereas various stages of apoptotic change including condensed chromatin, and irregularly shaped nuclei and formation of apoptotic bodies are detected at 7 days after spinal cord injury (B). Neuronal apoptosis in contused mouse spinal cord is demonstrated by double staining with terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) in red fluorescence (C) and a cell-specific marker of anti-neuronal nuclear protein/neuron in green fluorescence (D) at and near the lesion site (*arrow*). Apoptosis of oligodendrocytes is also demonstrated by double staining with TUNEL in red fluorescence (E) and a cell-specific marker of cyclic nucleotide 3'-phosphohydrolase antibody in green fluorescence (F) within the white matter near the ventral horn. ×400.**

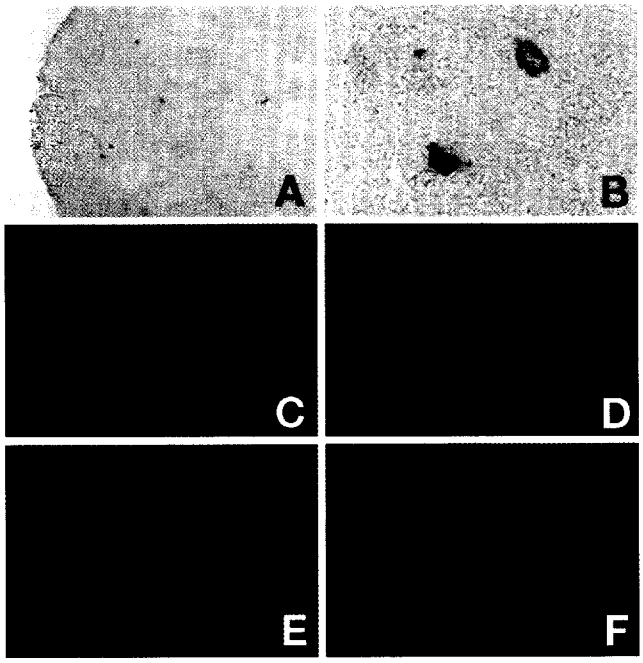

Fig. 2 **Photomicrographs showing CM1 immunostaining and double staining with cell-specific markers of transverse cord sections at 7 days after injury. CM1 immunostaining of the region near the ventral horn at low (A) and high magnification (B) demonstrates dark, shrunken cells containing clumped chromatin. Staining for activated caspase-3 is evident within the nucleus. Fluorescent double staining shows CM1-positive cells in red fluorescence (C), which are also stained with a neuronal marker, anti-neuronal nuclear protein/neuron in green fluorescence (D). In a different area, CM1-positive cells in red fluorescence (E) are marked with cyclic nucleotide 3'-phosphohydrolase antibody in green fluorescence (F). A: ×80, B–F: ×400.**

in the white matter (immunohistochemical staining for oligodendrocytes using CNP antibody), suggesting apoptosis of both neurons and oligodendrocytes (Fig. 1E, F). Neuronal apoptosis was mainly seen in small neuron in the intermediate gray matter. On the other hand, large motoneurons were preserved in the ventral horn. However, only a small number of large motoneurons were stained with TUNEL at the lesion site.

Caspase-3 (CM1) staining and immunohistochemistry double staining: Naive control spinal cord had no CM1-positive cells. CM1-positive cells were demonstrated as dark, shrunken cells containing clumped chromatin with staining for activated caspase-3 within the nucleus in the white matter near the ventral horn in injured spinal cord at 7 days after SCI (Fig. 2A, B). Double immunofluorescence also detected the presence of activated caspase-3 in neurons as anti-NeuN-positive cells (Fig. 2C, D), oligodendrocytes as CNP antibody-positive cells (Fig. 2E, F), and astrocytes (not illustrated).

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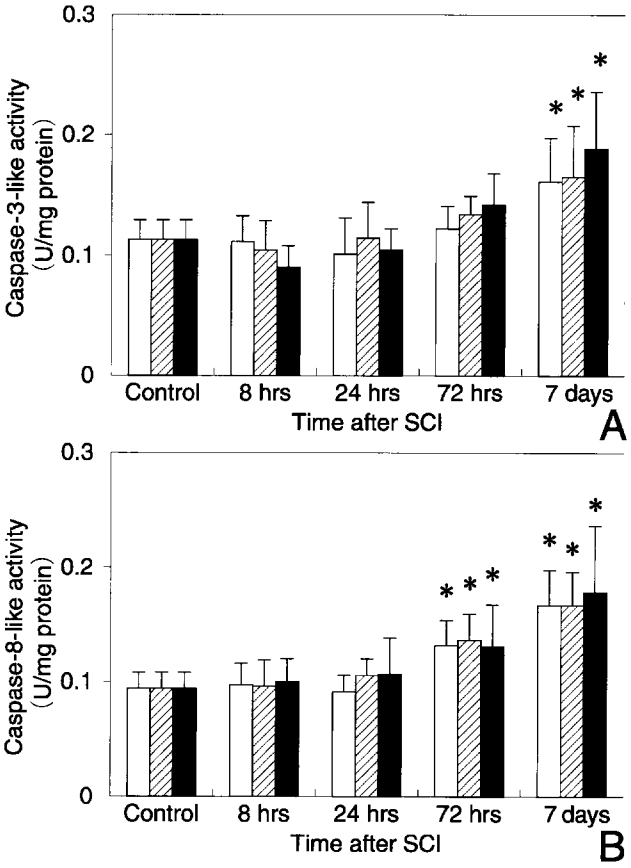

Fig. 3 Caspase-3-like (A) and caspase-8-like activities (B) measured in cytosolic protein extracts from traumatized mouse spinal cord in the rostral (*open bars*), center (*hatched bars*), and caudal sections (*filled bars*) from naive controls or spinal cord injury (SCI) mice 8, 24, or 72 hours or 7 days after injury. Values are mean ± SD (n = 4 for naive mice; n = 12 for 8, 24, and 72 hrs; n = 10 for 7 days). *p < 0.01 with one-way analysis of variance and post hoc Dunnett's test.

III. Changes in caspase-3 and caspase-8 protease activities after SCI

Lysates from naive spinal cords (n = 4) showed low peptide cleavage activities for both DEVD-pNA (caspase-3-like) and IETD-pNA (capase-8-like) substrates, and were considered to be nonspecific background. Lysates from the rostral, lesion site, and caudal tissue blocks from injured spinal cord exhibited significant increases in caspase-8-like enzyme activity on days 3 and 7 (n = 12 for 8, 24, and 72 hrs; n = 10 for 7 days, $p < 0.01$); and a significant increase in caspase-3-like enzyme on day 7 ($p < 0.01$) (Fig. 3). No significant regional differences in caspase activity were found between the three adjacent 1.5-mm tissue blocks. Lysates from the sham-operated animals showed low levels of enzyme activity at each time point (data not shown).

IV. Effects of inhibition of protease activity after SCI

Z-VAD-fmk and Z-DEVD-fmk injection produced decreases in DEVD-pNA and IETD-pNA substrate-cleaving activity in injured spinal cords at 7 days relative to activities in vehicle controls at 7 days (naive control mice [n = 4] and vehicle- [n = 12] or Z-VAD-fmk-treated mice [n = 5], and naive control mice [n = 4] and vehicle- [n = 12] or Z-DEVD-fmk-treated mice [n = 8]). Z-VAD-fmk inhibited both caspase-3 and -8 enzyme activity significantly ($p < 0.01$) (Fig. 4A, B). The inhibitory effect of Z-DEVD-fmk was relatively weak (Fig. 4C, D).

Discussion

The present study demonstrated by immunohistochemical double staining that apoptotic cell death occurred in both neurons and oligodendrocytes, and that activated caspase-3 was present in both cell types at 7 days after SCI. Colorimetric assay showed an increase in caspase-8-like activity in spinal cord homogenates after SCI preceded an increase in caspase-3-like activity. Intraventricular injection of a tetrapeptide inhibitor (Z-VAD-fmk or Z-DEVD-fmk) inhibited caspase activity at 7 days after SCI. These results strongly suggest that caspase proteases act as inducible cell death effectors in the spinal cord after traumatic injury.

Spinal cord cell death following injury results partly from apoptosis, as determined by morphological, electrophoretic, and TUNEL methods.[13,26,31,34,50] Apoptotic cell death occurs in both neurons and oligodendrocytes, and apoptosis is prominent in the white matter where wallerian degeneration occurs.[13,34] Loss of oligodendrocytes due to apoptosis in the damaged spinal cord is relatively widespread, occurring even at considerable distances from the initial compression injury.[32] The present study confirmed that apoptotic cell death occurred in both neurons and oligodendrocytes at 7 days after SCI. Moreover, expression of activated caspase-3 (CM1) was detected in both neurons and oligodendrocytes. These results suggest that neuronal and glial apoptotic cell death is caused by induction of the caspase cascade.

Apoptosis can be induced by a variety of stimuli, but execution of the apoptotic program involves a common mechanism that relies on activation of the caspases, which are cysteine proteases belonging to the interleukin-1-converting enzyme/CED-3 family. The roles of individual caspases and their relative

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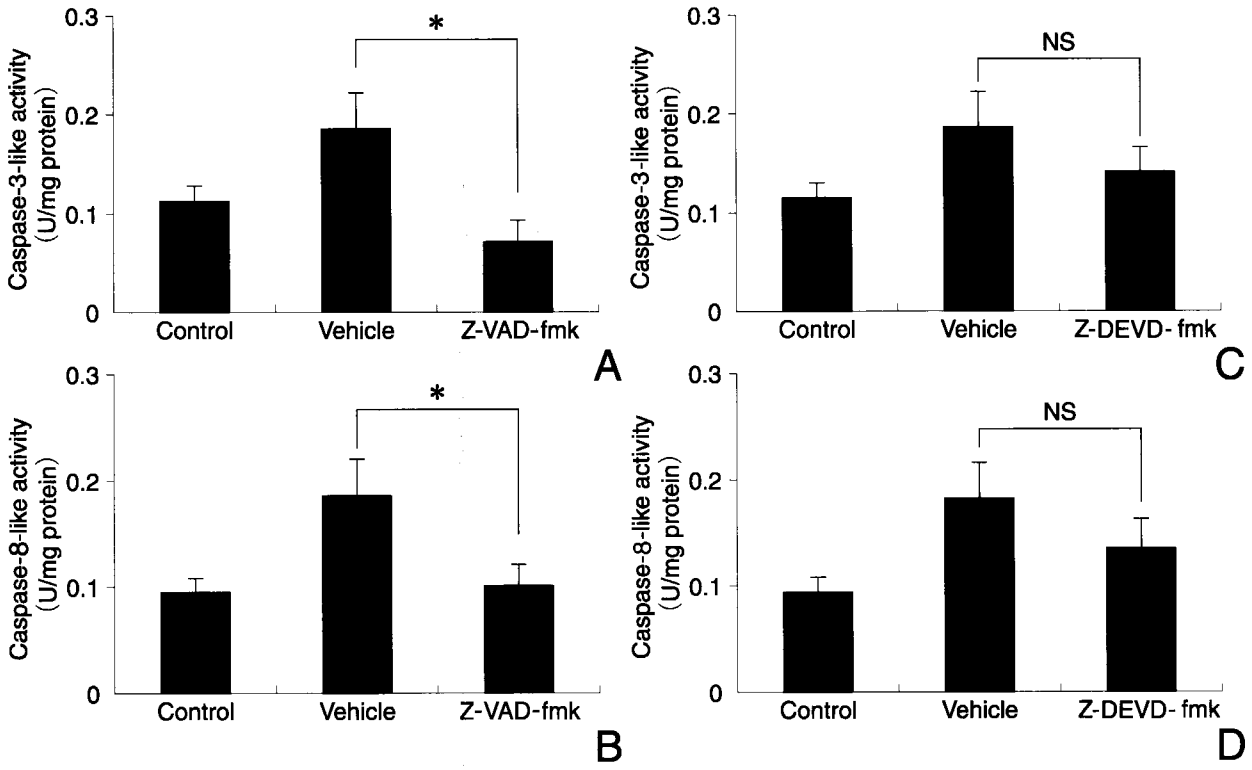

Fig. 4 Effect of in vivo caspase inhibitor treatment on caspase-3- (A, C) and caspase-8-like protease activities (B, D) in naive control mice (n = 4) and vehicle- (n = 12) or Z-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-fmk)-treated mice (n = 5) at 7 days after spinal cord injury (A, B), and in naive control mice (n = 4) and vehicle- (n = 12) or Z-Asp-Glu-Val-Asp-fluoromethyl ketone (Z-DEVD-fmk)-treated mice (n = 8) at 7 days after spinal cord injury (C, D). Values are mean ± SD. *$p < 0.01$ with one-way analysis of variance and post hoc Dunnett's test.

importance in apoptosis have recently been clarified.[42] Caspase-8 and caspase-10 are activated early in the apoptotic process and are considered initiators, whereas caspase-3 and caspase-7 are activated at a later phase of apoptosis and function as effectors that act upon a large number of substrates. Cell death receptor-induced pathways of apoptosis require activation of caspase-8 and caspase-3, whereas apoptosis following growth factor deprivation and stress-induced cell injury appears to occur via mitochondrial dysfunction with release of cytochrome c, which triggers activation of caspase-9 and then caspase-3.[1,20,23,29,30,44,46] In addition, apoptosis may be mediated through poorly understood caspase-independent pathways.[48]

Little is known about upstream initiating steps promoting caspase activation in the traumatized spinal cord, either receptor- or non-receptor-driven. SCI in rats causes an increase in the cytosolic level of cytochrome c within 30 minutes after injury which persists for at least 24 hours, indicating rapid transduction of cell-death signals to the mitochondria.[39] Caspase-9 activation was detected within 30 minutes after injury, and caspase-3 enzyme activity increased within 1 hour after injury, and both remained high for up to 24 hours. A 17-fold increase in caspase-1 activation occurs 24 hours after SCI in a mouse model.[33] In our study, no increase in caspase-8-like activity was noted until day 3. Caspase-8 activation might occur upstream of caspase-3 activation via pathways induced by cell-death receptors such as tumor necrosis factor (TNF) receptors. The present experiments demonstrated the temporal profile of caspase-8 and caspase-3 activity in the mouse SCI model. Delay of caspase-3 activation until 7 days after SCI indicates delayed transduction of cell death signals mediated through receptors as previously suggested.[13] The intracellular and receptor-mediated programmed cell death pathways contribute to a long time course of secondary injury processes after SCI. The time courses of caspase enzyme activities in our study differed from those reported previously, possibly reflecting differences in severity of SCI.[33,39] In a severe transient focal

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The authors have written a wonderful paper on the role of caspase in the process of apoptosis after injury of spinal cord in mouse spinal cord injury (SCI) model. I think it was interesting that CM1-positive cells were demonstrated as dark, shrunken cells containing clumped chromatin with staining for activated caspase-3 within the nucleus in the white matter near the ventral born in injured spinal cord at 7 days after SCI. They discussed the regional profile of caspase activity after SCI. However, the reason and process were unclear. The inhibition of caspase activity will result in prevention of neuronal and glial damage after SCI. I strongly hope that a pharmacological approach to this issue will be developed in the near future to treat SCI.

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This is a very interesting study evaluating the cascade of molecular events that accompanies spinal cord injuries. The hypothesis of the authors is that apoptosis represents an important and irreversible change that occurs in the spinal cord following spinal cord injury. The mediation of apoptosis is not fully understood, and it is known that caspases play a role in other tissues and in other mechanisms of apoptosis in the central nervous system. After a standard weight drop injury to the spinal cord in mice, significant increases in caspase-8-like enzyme activity were noted as long as seven days following injury. This enzyme activity and its downstream effects were inhibited by an intraventricular injection of a non-specific caspase inhibitor and also by a specific caspase-3 inhibitor injected very shortly after the spinal cord injury. These results suggest the possibility that caspase activity may play a significant role in delayed neuronal and glial apoptosis following spinal cord injury and that these mechanisms of injury may be capable of manipulation using appropriate enzyme inhibitors.

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In this study, the authors demonstrated the involvement of caspase-3- and caspase-8-dependent apoptosis in neuronal cell death after spinal cord injury (SCI). By using double labeling with CM1 and cell-type specific markers, the authors clearly showed that both neurons and oligodendrocytes undergo apoptosis after SCI. The most important finding in this study is that intraventricular injection of Z-VAD-fmk, a caspase inhibitor, significantly reduced activation of both caspase-3 and caspase-8. It is therefore extremely interesting to examine whether intraventricular administration of the inhibitor can prevent the delayed neurological deterioration after various CNS injuries. Recently, it has been reported that the transplantation of in vitro-expanded fetus-derived neurosphere cells into the rat SCI model revealed functional recovery.[2] Furthermore, activation of endogenous neural progenitors by growth factors has been reported to lead to massive regeneration of hippocampal pyramidal neurons after ischemic brain injury.[1] These findings together with the data presented in this paper suggest that the combination of anti-apoptotic therapy and regeneration therapy will be a powerful approach to treat delayed neuronal damage caused by CNS injuries and ischemic brain diseases.

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