Apoptosis and Expression of Bcl-2 after Compression Trauma to Rat Spinal Cord

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Abstract. We have evaluated by in situ nick-end labeling the presence of apoptotic cells in the spinal cord of rats with compression injury at the level of Thm, mild, moderate, and severe degrees resulting in no neurologic deficit, reversible paraparesis, and paraplegia, respectively. Rats with compression injury surviving 4 or 9 days showed apoptotic glial cells in the longitudinal tracts of the Thm, the cranial Thm, and the caudal Thm segments. The apoptotic cells were most frequently observed in Thm. They did not express glial fibrillary acidic protein (GFAP) and their morphology was compatible with that of oligodendrocytes. Neurons of the gray matter did not present signs of apoptosis. In addition, we studied the immunohistochemical expression of Bcl-2, an endogenous inhibitor of apoptosis. Compression induced Bcl-2 immunoreactivity in axons of the long tracts, particularly after moderate and severe compression and 1 day survival. Neurons of dorsal root ganglia were immunoreactive but the neurons of the spinal cord were unstained. The accumulation, presumably caused by arrested axonal transport in sensory pathways, was absent in rats surviving 9 days. In conclusion, compression trauma to rat spinal cord induces signs of apoptosis in glial cells, presumably oligodendrocytes of the long tracts. This may induce delayed myelin degeneration after trauma to the spinal cord. Bcl-2 does not seem to be upregulated in oligodendrocytes.

Key words: Apoptosis; Bcl-2; Rat; Spinal cord; Trauma.

INTRODUCTION

The pathophysiology of spinal cord trauma is complex. The primary physical injury will disrupt membranes of neurons and glial cells, destroy axons and myelin in the longitudinal tracts, and disrupt microvessels (1, 2). However, the final lesions will be influenced by a number of injury-promoting factors leading to secondary injuries (1, 2). They include formation of edema, death of neurons and glial cells, as well as delayed changes of the longitudinal tracts (1, 2).

Cell death is one important ingredient of secondary injuries. Previously, most experiments on trauma to the cord have been focused on necrotic cell death characterized by plasma membrane damage and leakage of cell constituents into the extracellular fluid (1, 2). However, death of neurons and glial cells may be the result of programmed cell death (also called apoptosis) which is a specific mode of death controlled by intrinsic suicide program of cells (3–5). This can be activated by exogenous factors including glutamate, calcium ions, and free radicals (3). Cells undergoing apoptosis will be autodigested by proteases and they will be cleared without inducing inflammation (6). The importance of apoptosis for the pathogenesis of secondary injuries after trauma to the cord has not been dealt with in previous publications.

One key event in apoptosis is protease activation, which will induce nuclear and cytoplasmic lesions (6). Experiments carried out in tumor biology have shown that some types of cells have intrinsic protective programs which repress apoptosis (6). Bcl-2 is one compound with suppressing effects on apoptosis; its action has recently received considerable attention (7). The production of the protein can be upregulated in many types of cells (8), particularly in various neurodegenerative disorders (9). Whether this happens in diseases of the spinal cord is unknown.

Systematic studies regarding the expression of Bcl-2 in the nervous system do not seem to have been carried out. However, Bcl-2 has been demonstrated in neurons of the human fetal spinal cord (10), sensory ganglia (11), and the temporal lobe of autopsy cases (12). Normal glial cells in vivo do not appear to express Bcl-2 (10, 12), but reactive astrocytes around intracerebral lesions exhibit a moderate Bcl-2 immunostaining (13).

We have used a recently developed molecular biological technique to label dying cells in situ (14) to find out if apoptosis occurs after graded compression trauma to rat spinal cord. In addition, we studied whether trauma will induce any changes in the immunohistochemical expression of Bcl-2, one of the modifiers of programmed cell death.

MATERIALS AND METHODS

Animals

Tissue samples were derived from 56 rats (male, average weight 370 g) that were used in parallel investigation on axonal and dendritic changes following compression trauma to the spinal cord using antibodies to β-amyloid precursor protein.
Fig. 1. The position of the spinal cord injury at Th₅₋₆ is marked with an arrow. A. Low power view. B. High power view showing the position of the Th₅₋₆ segment.

(βAPP) and microtubule-associated protein 2 (MAP2) (15, 16). Five rats not included in our previous studies were used to investigate the distribution of Bel-2 immunoreactivity in various parts of the nervous system. Food and water were provided ad libitum before and after the experiments. The rats were kept at a temperature of 20°C, controlled thermostatically and exposed to alternate light and dark periods of 12 hours (h).

**Spinal Cord Injury and Physiological Parameters**

We used a rat model with compression injury of the spinal cord (17, 18). The animals were anesthetised with a mixture of fentanyl 2.5 mg/ml and midazolam 1.25 mg/ml in distilled water in a total volume of 1.5 to 2 ml/kg of body weight, given subcutaneously. A catheter (PE 50) was inserted into the tail artery for continuous recording of the mean arterial blood pressure and sampling of blood. The rats were placed in a prone position on a heating pad and the body temperature was kept constant by the use of a rectal thermometer.

The laminae of Th₅ and Th₆ vertebrae, which overlie the Th₅ and Th₆ segments of the spinal cord, were removed, leaving the dura intact. The animals were placed in a stereotactic frame with 2 adjustable forceps applied to the spinous processes of vertebrae cranial and caudal to the laminectomy in order to stabilize the spinal cord. A predetermined weight was applied on the exposed dura for 5 minutes (min). The position of the injury to the cord is presented in Figure 1.

The values of the physiological parameters of animals required to be included in this study were as follows: P₀₂ > 9.5 kPa, PCO₂ 4.5–6 kPa, pH 7.35–7.45 and the mean arterial blood pressure > 100 mm Hg. There was a transient short-lasting increase of blood pressure during compression.

The 56 rats were randomly divided into 14 experimental groups (Table 1). One control group consisted of normal non-operated rats; another control group was composed of rats with laminectomy but without compression and 24 h survival period. The other groups contained rats subjected to a load of 9, 35, and 50 g respectively, resulting in no neurological deficit, transient paraparesis, or paraplegia of the hind limbs tested by the inclined plane method (15, 16, 19). The injured rats were allowed to survive for 4 h, 7, 1, or 9 days.

**Morphological Techniques**

When sacrificed, the animals were sedated with the same anesthetic used during operation and perfused through the heart with 200 ml of a phosphate buffer solution (PBS, pH 7.4) followed by 200 ml of a 4% formaldehyde solution in the same buffer at 100 mm Hg. The spinal cord was removed, samples from the Th₅, Th₆, and Th₇ segments were put in fixative overnight, dehydrated, and transverse samples were embedded in paraffin. Five-micron-thick sections were cut.

**In situ nick-end labeling:** Sections to be processed for apoptosis recognition by the in situ nick-end labeling technique (14) were cut from the Th₀ segments of all the animals and from Th₅ and Th₆ segments of rats subjected to compression injury. From each paraffin blocks 3 randomly chosen sections were used; altogether 492 sections were stained. These sections were treated in the following sequences: deparaffination, incubation in 20 μg/ml proteinase K for 15 min at room temperature, wash in double distilled water 4 times for 20 min, rinse in 2% hydrogen peroxide for 15 min, wash in double distilled water. Incubation was then carried out in a reaction solution for 60 min at 37°C. This solution contained 0.005 nM/μl biotin-16-2'-deoxy-uridine-5'-triphosphate (biotin-16-dUTP) and 0.2 e.u./μl
TDT in a buffer (30 mM Trizma base, 140 mM sodium cacodylate, 1 mM cobalt chloride, pH 7.2). TDT (catalog number 220 582) and dUTP (catalog number 1093 070) were obtained from Boehringer Mannheim, Germany. The reaction was terminated by rinsing in a buffer (300 mM sodium chloride, 30 mM sodium citrate, pH 7.6) for 15 min at room temperature; thereafter wash in double distilled water, incubation in 2% bovine serum albumin for 15 min, wash in double distilled water and immersion in PBS buffer for 5 min.

The reaction product was visualized by the avidin-biotin-peroxidase complex method using 3,3-diamino-benzidine tetra-hydrochloride as the chromogen (Vectastain Elite Kit, Vector Laboratories, Burlingame, CA). The sections were counterstained with hematoxylin. For control purpose sections were treated as the test sections but TDT or dUTP were omitted.

Sections from animals showing apoptotic nuclei were double stained by the in situ nick-end labeling method (ended with nickel enhancement) and gial fibrillary acidic protein (GFAP) immunohistochemistry. After the nick-end labeling procedure, sections were soaked in 1% bovine serum albumin, then incubated in rabbit anti-GFAP serum (code no. Z334, Dakopatts, Glostrup, Denmark) at a dilution of 1:200 for 30 min, then exposed to goat anti-rabbit IgG for 30 min. The reaction product was visualized by the avidin-biotin-peroxidase complex method using ethylcarbazol as the chromogen.

Bcl-2 immunohistochemistry: In order to investigate the expression of Bcl-2, sections were cut from the same paraflin blocks as we used for the nick-end labeling. From some rats with spinal cord compression we also cut sections from the Th5 and Th10 segments of the cord. Finally, formalin-fixed, paraffin-embedded tissue was obtained from 5 normal adult rats to study the Bcl-2 expression in various regions of the nervous system. Sections were taken from the fronto-parietal region, the spinal cord, the lumbar and cervical dorsal root ganglia and the trigeminal ganglion.

The sections were treated in the following manner: microwaved for 10 min in citrate buffer (pH 6.0), submerged in 1% hydrogen peroxide in methanol for 30 min, in 20% normal rabbit serum in PBS buffer for 30 min, and then incubated overnight with a monoclonal antibody against Bcl-2 (code M 887, Dakopatts, Glostrup, Denmark) at a dilution of 1:80. Preliminary tests with different dilutions from 1:40, 1:80, to 1:200 had shown that the dilution 1:80 was optimal, providing a clear signal and no background staining. Thereafter the sections were exposed to rabbit anti-mouse IgG for 30 min and the reaction product was visualized by the same method as used for the in situ nick-end labeling technique.

To intensify the reaction product we applied the nickel enhancement procedure combined with the glucose on some sections—the glucose oxidase method (20–22). For control purpose the primary antibody was omitted and thereafter the sections were treated as those in which the Bcl-2 antiseraum had been applied.

Other techniques: Immunostaining of myelin basic protein (MBP) was used to visualize injuries of myelinated tracts of the cord resulting from trauma. We used a monoclonal antibody against MBP (code MCA 70, Serotec, UK) at a dilution of 1:100 and the same visualization procedure as for Bcl-2 immunohistochemistry. Finally, we had hematoxylin- and eosin-stained sections available from our previous studies.

Cell Counts and Statistics

We counted the number of labeled cells in each of the 492 cross-sections of the spinal cord stained with the nick-end labeling technique. Cells located in regions with hematomas were avoided since their nature was hard to define in such lesions and some of them may have been of hematogenous origin.

For comparison of multiple means between groups factorial analysis of variance was used. Fisher’s protected least squares difference (PLSD) test was performed for post hoc testing (Statview 4.01, Abacus Concepts). Values in figures are given as the mean ± SEM. Differences with a p-value <0.05 were considered significant.

RESULTS

Structural Changes

The structural changes induced by mild, moderate, and severe compression (Fig. 2) of the spinal cord from the rats included in the present study have been presented elsewhere (15, 16). Therefore, only a summary of the most important changes is provided.

Mild compression: There were no macroscopical changes of the compressed Th5-12 segment. Microscopically, a few nerve cell bodies were condensed. The white matter contained a few swollen axons and vacuoles but MBP immunostaining was normal. The cranial Th5 segment was unchanged but the caudal Th10 segment showed some vacuolation of the tissue.

Moderate compression: The compressed Th5-9 segment from rats with moderate injury displayed multiple small bleedings in the gray matter at 4 h and 1 day. Some nerve cell bodies were condensed and MBP immunostaining revealed a moderate loss of myelinated fibers in the dorsal and ventral horns. The longitudinal tracts of the injured Th5-12 segment contained expanded axons and vacuoles; MBP immunostaining showed a patchy loss of myelinated fibers, particularly 1 to 9 days after injury.

The cranial Th5 and particularly the caudal Th10 segments showed some condensed neurons and vacuolation of the white matter. There was a mild reduction of MBP immunoreactivity in the white matter of the Th5 segment and somewhat more pronounced loss of staining in the Th10 segment.

Severe compression: This degree of spinal cord injury induced changes of the same quality as in the preceding group, but the lesions were more pronounced. The compressed Th8-9 segment was swollen and had many bleedings. One day after compression a large necrosis occupied almost the entire cross section of the cord. The subpial region was better preserved with a few remaining MBP immunostained fibers.

The cranial Th7 and particularly the caudal Th10 segment presented many condensed neurons and vacuolation of the white matter. The caudal segment had in addition many petechial hemorrhages, chiefly located in the gray
matter. The cranial Th₁ segment presented mild loss of MBP immunoreactivity of the longitudinal tracts and a more marked reduction in the caudal Th₁₀ segment.

Apoptosis

Morphology of Labeled Cells: Sections of the spinal cord stained by the nick-end labeling method did not show any background staining. Seven of the 8 control rats (Fig. 4) showed only occasional labeled cells in cross sections of the cord; one control rat did not present any labeled cells at all. Neurons in the gray matter of the cord of rats with or without trauma were not labeled.

Rats with compression injury of the spinal cord and a survival period of 4 or 9 days presented labeled glial cells at the site of compression and in the segments taken cranial and caudal to the injury. The labeled glial cells stained dark yellow (DAB) or deep brown (nickel enhancement). The staining of the nucleus was very intense (Fig. 3); the nucleus usually appeared to be compact with a rounded configuration sometimes associated with a clear perinuclear space. Double staining showed lack of GFAP immunoreactivity.

Distribution of Labeled Cells: Rats with compression injury of the spinal cord presented labeled glial cells in the injured Th₁₀, the cranial Th₁, and the caudal Th₁₀ segments. Most of the cells were randomly distributed in the ventral, lateral, and dorsal columns of the white matter. Very few of them were located in the gray matter.

Cell counts showed a statistically significant increase in the number of labeled cells of the cranial Th₁ segment compared with the compressed Th₁₀ segment 9 days after mild to severe injury and 4 days after moderate and severe compression (Fig. 4). The number of labeled cells in the caudal Th₁₀ segment showed a statistically significant increase in rats with mild to severe compression and a survival period of 9 days compared with those of the compressed segment. Labeled glial cells were more abundant in the cranial Th₁ segment than in the Th₁₀

Fig. 2. Structural changes in the Th₁₀ segment of the spinal cord of rats subjected to mild, moderate, and severe injury. Mild compression induced minor structural alterations (A), moderate compression caused small multifocal bleedings particularly of the gray matter and vacuolation of the longitudinal tracts (B, D). Rats with severe compression showed more pronounced bleedings and destruction of the longitudinal tracts (C). A, B, C stained with hematoxylin and eosin, D immunostained for myelin basic protein.
Fig. 3. Sections stained with the nick-end labeling method showing several labeled glial nuclei (arrows) in the white matter from a rat with moderate compression and 4 days of survival (A, B, C). In Figure 3A two labeled glial cells are seen in the white matter. Note absence of labeling of neurons. Sample obtained from the Th segment.

segment of rats with moderate and severe compression. This difference was statistically significant 4 days after moderate compression and 9 days after severe injury.

Time Course: Labeled glial cells increased in number 4 and 9 days after compression, but no statistically significant change was seen 4 h and 1 day after injury (Fig. 4). Labeled glial cells appeared to be more frequent in rats examined 9 days after mild to severe injury compared with rats surviving only 4 days.

Relation to Degree of Compression: The number of labeled glial cells varied between rats with mild, moderate, and severe injury (Fig. 4). Cell counts showed that they were more frequent in the cranial Th segment after moderate and severe injury compared with mild compression. Such a difference was not obvious in the compressed Th8-9 segment, presumably due to the more severe destruction of the tissue with hemorrhages.

Bcl-2 Immunohistochemistry

Normal Controls and Laminectomized Rats: Bcl-2 immunoreactivity was present in a few glial cells in the subpial region of the cord but was not seen in nerve cell bodies. A few Bcl-2 immunoreactive rounded profiles of unknown origin were present in the longitudinal pathways of the dorsal columns (Fig. 5A).

Most neurons of dorsal root ganglia showed immunoreactive cytoplasm (Fig. 5B). This phenomenon was present in the trigeminal ganglion as well. The sciatic
Fig. 4. Graph showing the number of labeled glial cells at different time intervals after mild, moderate and severe compression of rat spinal cord at the Th₆₋₇ segment. Stars indicate statistically significant increase (p < 0.05) in the number of apoptotic cells compared with controls.
nerve did not contain any immunoreactivity, but in the spinal nerve roots some axons had immunoreactive material presumably located in the cytoplasm of Schwann cells. Sections from the brain did not contain any immunoreactive material.

**Spinal Cord Compression:** Trauma to the spinal cord did not result in any staining of the neurons of the gray matter in the Th₁, Th₂, Th₃, Th₄, and Th₅ segments of rats with moderate compression, but induced a remarkable change in the expression of Bcl-2 in axons of the cord. All the rats with compression showed expanded, very intensely immunoreactive axons in the dorsal, ventral, and lateral tracts 1 day after injury (Fig. 5C, D). They were never seen in the corticospinal tracts (Fig. 5D). Four days after compression, such Bcl-2 immunoreactive axons had decreased in number and had a reduced staining intensity. Nine days after compression, Bcl-2 immunoreactivity was no longer present in the expanded axons.

The immunoreactive axons of rats with mild compression were confined to the injured Th₁₀ segment. Animals with moderate and severe compression showed such labeling in the compressed Th₁₀ and in the caudal Th₁₀ segment. The immunoreactive axons were more frequent in rats with moderate injury than after mild compression. Rats with severe compression showed immunoreactive axons in the spared subpial region of the cord.

Dorsal root ganglia from the compressed segment were available in sections of 4 rats with moderate and severe injury. The pattern of Bcl-2 immunoreactivity in neurons of the ganglia was the same as in the control rats.

**Discussion**

The hallmark of programmed cell death is endonucleolysis (23, 24) by enzymes cleaving DNA into oligonucleosomal fragments (25). Morphologically, programmed cell death is characterized by apoptosis, including nuclear pyknosis and formation of membrane-
bound apoptotic bodies, chromatin margination, membrane blebbing, cell condensation with preservation of organelles, and detachment from adjacent cells (26, 27). However, apoptotic cells are not easy to identify in routinely stained tissue sections. Therefore, a new technique, called in situ nick-end labeling, has been developed to facilitate recognition of apoptotic cells in tissue sections by using terminal deoxynucleotidyl transferase (TdT) to incorporate biotinylated deoxyuridine to the 3'-hydroxyl termini of DNA breaks (14).

Compression trauma to the spinal cord is associated with death of neurons, glial cells and cells of the microvessels (1, 2). Previously, most interest has been directed towards the necrotic form of cell death and we have not found any previous investigation on apoptosis following trauma to the cord. By using the in situ nick-end labeling technique in the present study we detected glial cells undergoing apoptosis preferentially in the longitudinal tracts of the white matter. These cells had the morphological appearance and position of oligodendrocytes and did not express GFAP. We therefore believe that the glial cells we observed are oligodendrocytes. However, in order to prove their oligodendrocyte nature further studies with electron microscopy and the DNA ladder technique should be applied.

Death of neurons and oligodendrocytes after trauma to the spinal cord is important since this will interfere with connectivity and induce myelin degeneration. Previous studies have shown that both cell types can die by programmed cell death, for instance, hippocampal and cortical neurons after ischemia (28–31) and retinal ganglion cells after optic nerve axotomy (32). Apoptosis may be involved in neurodegenerative disorders (6, 33), ischemic brain lesions (28, 29, 34), tumors (6) and is of particular interest in oncology (27). In vitro, serum and growth factor withdrawal, calcium ionophores, glucose withdrawal, membrane peroxidation, and free-radical-induced damage can induce programmed cell death of central neural cells (35).

About 50% of oligodendrocytes in the developing rat optic nerve normally die by programmed cell death. These cells require proper amounts of survival factors from neighboring cells (36). Axons probably play a crucial role in controlling oligodendrocyte survival (37). If rat optic nerve is cut behind the eye most oligodendrocytes in the nerve selectively die (37). DNA fragmentation of oligodendrocytes has been observed in the temporal lobe white matter of patients with Alzheimer's disease (33, 38).

Compression thus induced a delayed change indicating apoptosis of glial cells—presumably oligodendrocytes. Four and 9 days after trauma cells with evidence of apoptosis were markedly elevated in number, especially in the Th and Th, segments, i.e. cranial and caudal to the compression. Delayed death of oligodendrocytes may be one additional and presumably significant alteration after trauma to the cord. Loss of such cells will reduce the possibility of remyelination after axonal injury and promote myelin degeneration taking place after compression injury of the cord.

Our model of spinal cord injury enables us to induce different degrees of axonal injuries at the compressed segment of the cord (16). Mild injury, which does not produce any signs of neurologic deficits, is associated with rather few axonal injuries indicated by the β-amyloid precursor protein immunohistochemical technique (16). Moderate and severe compression, which cause transient paraparesis and paraplegia, respectively, are associated with a substantial axonal damage in the compressed segments (16). The time course, distribution, and frequency of apoptosis in the present experiments are consistent with the axonal hypothesis of oligodendrocyte dependence of intact axons. The possibility exists that after spinal cord trauma the injured axons fail to produce signals crucial for oligodendrocyte survival.

Four and 9 days after compression most apoptotic glial cells were identified in the segments cranial and caudal to the injury compared with the injured segment. This phenomenon was most marked in rats with moderate and severe trauma. We do not know why there is such a difference but it may be that oligodendrocytes are injured in quite different ways in the segment directly exposed to the compression, e.g. direct trauma or ischemia, compared with those of the perifocal segments and that the injury processes have different time courses.

Compression trauma to the rat spinal cord of moderate and severe degrees is associated with loss of neurons in the gray matter of the cord (16, 17, 39). This loss of cells is most likely the consequence of injuries to the cell body or the processes of neurons caused by the physical trauma itself or by secondary circulatory failure. Our present study did not show any evidence of apoptosis in the spinal cord neurons at 4 h, 1, 4, and 9 days after trauma. We can not exclude the possibility that this mode of cell death occurs at any other time point after compression of the cord.

We included observations on the immunohistochemical expression of Bcl-2 after trauma to the spinal cord since this protein is thought to be an endogenous inhibitor of programmed cell death. There was no evidence of upregulation of Bcl-2 in nerve cell bodies of the gray matter after compression, nor did we find evidence of programmed cell death in these neurons after trauma.

An unexpected transient expression of Bcl-2 immunoreactivity was found in injured axons of the white matter following compression injury of the cord. Detectable amounts of Bcl-2 immunoreactivity were not present in ventral motor neurons of the cord, but previous investigations have shown that neurons of dorsal root ganglia express this protein (11). Axonal accumulation of Bcl-2...
immunoreactive material at the site of compression may thus be caused by arrested anterograde axonal transport in ascending pathways originating from dorsal root ganglia. Bel-2 protein may well influence pathological processes going on after spinal cord trauma since Bel-2 has proved to reduce necrotic neural cell death by interfering with reactive oxygen molecules (40). The protein is expressed in the aged human brain and in various neurodegenerative disorders (9). In such conditions Bel-2 is enriched in lipofuscin and autophagic vacuoles and appears to be a general cell reaction which accompanies an increased formation of lipofuscin (9).

ACKNOWLEDGMENTS

The authors would like to thank Madeleine Jarlid, Gunilla Tibbling, Ulla Carlson, and Lena Norman for their technical help.

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Received August 10, 1995
Revision received November 10, 1995
Accepted November 14, 1995