Immunohistochemical Study of Cathepsin D in the Spinal Cords of Rats with Clip Compression Injury

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ABSTRACT. This study examined the temporal expression of cathepsin D protein and its cellular localization in the spinal cords of rats after a clip compression injury to determine the involvement of cathepsin D in spinal cord injury (SCI). Western blot analysis showed a significant increase in the \(~31\)-kDa active form of cathepsin D on days 4 and 7 after the SCI, while the level of the \(~44\)-kDa inactive form remained relatively unchanged. Immunohistochemistry revealed cathepsin D with constitutive localization in most neurons and some astrocytes in the normal spinal cord to be intensely immunodetected primarily in CD68-positive activated macrophages/microglia in the SCI lesions. Overall, these findings suggest that cathepsin D plays an important role in the phagocytosis and lysosomal activation of macrophages/microglia during the central nervous system inflammation caused by trauma.

KEY WORDS: cathepsin D, clip compression injury, macrophages/microglia, spinal cord.

The mechanism of spinal cord injury (SCI) is thought to involve the primary mechanical injury as well as secondary damage induced by inflammatory responses. The neuropathological outcomes of SCI, in both clip compression injury [20] and weight-drop contusion models [2], are characterized by edema, axonal degeneration, the infiltration of inflammatory cells, and reactive astrogliosis.

The early response after SCI includes an increase in pro-inflammatory mediators, such as tumor necrosis factor alpha, nitric oxide, and interleukin-1 beta [15]. In addition, cellular infiltration and the proliferation of macrophages and astrogliosis occur at the site of the SCI [8, 10]. An increase occurs in the level of lysosomal proteinases in the lesion with central nervous system (CNS) inflammation, which is associated with activated macrophages/microglia and reactive gliocytes [3, 11].

Cathepsins are ubiquitous lysosomal enzymes that play important roles in protein turnover, housekeeping, and protein processing [22]. Cathepsin D is a major lysosomal aspartic acid protease and hydrolyzes select peptide bonds of the target proteins with high specificity [16, 19]. In the spinal cords of humans and rodents, cathepsin D is expressed constitutively in neurons and astrocytes, and this expression increases under pathological conditions, including amyotrophic lateral sclerosis [9, 21] and autoimmune encephalomyelitis (EAE) [11]. Recently, increased levels of cathepsin D mRNA were detected in the spinal cords of a mouse SCI model using microarray and real-time PCR analysis [5]. However, the pathological involvement of cathepsin D in SCI is not completely understood. This study examined the expression and cellular localization of cathepsin D in the spinal cords of rats with a clip compression injury to determine the involvement of cathepsin D during the course of SCI.

MATERIALS AND METHODS

Animals: Sprague-Dawley rats were purchased from Daehan Biolink (Chungbuk, Korea). The experiments used 8–12-weeks-old female rats weighing 160–200 g. All of the experiments were carried out in accordance with the guidelines for the care and use of laboratory animals of Cheju National University.

Spinal cord injury: The surgical procedure used to produce the clip compression injury was as published [12, 17]. The animals were anesthetized and subjected to laminectomy at T9/T10. Immediately following laminectomy, the spinal cord was compressed with a vascular clip (product# 52120: clip width, 3 mm; jaw length, 8 mm; occlusion pressure, 10–15 g; Stoelting Co., Wood Dale, IL, U.S.A.) that was applied vertically to the exposed spinal cord for 1 min. After compression, the muscles and skin layers were closed. In sham-operated control rats, only the laminectomy was performed. Thirty rats were used in this study. After inuring the spinal cord and at the time of tissue sampling, the animals lacked all hind-limb locomotion. Six animals each were killed on days 1, 4, 7, and 14 post-injury. Spinal cord tissues from the surgical sites were removed and fixed for histological and Western blot analyses, respectively.

Immunoblotting: The spinal cord tissue was homogenized in lysis buffer (40 mM Tris, 120 mM NaCl, 0.1% Nonidet

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40, 2 mM Na$_3$VO$_4$, 1 mM phenylmethylsulfonyl fluoride, 10 
µg/ml aprotinin, and 10 
µg/ml leupeptin) with 20 strokes in 
a homogenizer. The homogenate was transferred to micro-
tubes and centrifuged at 19,340 × g for 20 min.

For the immunoblot assay, the supernatant containing 20 
µg of protein was loaded into the individual lanes of 10% 
sodium dodecyl (lauryl) sulfate-polyacrylamide gels, elec-
trophoresed, and immunoblotted onto nitrocellulose mem-
branes (Schleicher and Schuell, Keene, NH, U.S.A.). The 
residual binding sites on the membrane were blocked by 
incubation with 5% nonfat milk in Tris-buffered saline 
(TBS; 10 mM Tris-HCl (pH 7.4), and 150 mM NaCl) for 1 
hr. Then, the membrane was incubated for 2 hr with goat-
polyclonal anti-cathepsin D antiserum (1:1,000 dilution; 
Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). The 
membranes were washed three times in TBS containing 
0.1% Tween 20 before being incubated for 1 hr with horse 
horseradish-peroxidase-conjugated anti-goat IgG (Vector 
Laboratories, Burlingame, CA, U.S.A.). The bound anti-
bodies were detected using enhanced chemiluminescence 
reagents (Amersham Corp., Arlington Heights, IL, U.S.A.) 
according to the manufacturer’s instructions. After imag-
ing, the membranes were stripped and re-probed with the 
mouse anti-beta actin antibody (1:20,000 dilution; Sigma-
Aldrich Corp., St. Louis, MO, U.S.A.). The membranes 
were washed three times in TBS containing 0.1% Tween 20 
before being incubated for 1 hr with goat horseradish-perox-
idase-conjugated anti-mouse IgG (Vector Laboratories). 
The bound antibodies were detected using enhanced chemi-
luminescence reagents (Amersham Corp.) according to the 
manufacturer’s instructions. 

RESULTS

Histopathological findings in the rat spinal cords following 
clip compression injury: While no lesions were observed 
in the normal controls (Fig. 1A), edema, neuron loss, and 
disorganization of the white and gray matter were noted on 
day 1 after the injury. On day 4 after the injury (Fig. 1B), 
the infiltration of large cells with granules in the cytoplasm 
such as phagocytes was observed, and some of the lesions 
contained red blood cells. On day 14, small round cysts, 
irregularly shaped spaces, phagocytes, and gliocytes were 
noted in the lesion (data not shown). All histological exam-
inations in this study gave similar findings, which were 
largely consistent with our previous reports [8, 10, 17].

Increased expression of cathepsin D in rat spinal cords 
after a clip compression injury: Western blot analysis was 
used to examine the temporal profile of cathepsin D expres-
sion in the spinal cord after a compression injury (Fig. 2). In 

![Fig. 1. Low-magnification images of the spinal cord sections of a normal control and spinal cord injury rats on day 4 post-injury. (A) No injury is observed in the normal control. (B) On day 4, a cavity containing infiltrating inflammatory cells was seen in the SCI lesion. GM, gray matter; WM, white matter. Hematoxylin and Eosin stain. Scale bars = 200 µm.](image-url)
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the spinal cords from the normal control rats, cathepsin D was detected constitutively with levels of the ~44-kDa inactive form and ~31-kDa active form similar to those reported elsewhere [21]. The expression of the ~31-kDa active form was significantly higher at days 4 and 7 post-injury (day 4: relative OD, 2.77 ± 0.58-fold, n=3, p<0.05; day 7: relative OD, 2.31 ± 0.32-fold, n=3, p<0.05; Fig. 2) compared to control levels, while the level of the ~44-kDa band remained unchanged in the spinal cords after the injury. The immunoreactivity of the ~31-kDa band declined slightly at 14 days post-injury.

Immunohistochemical localization of cathepsin D in rat SCI: Immunohistochemically, cathepsin D was detected in most neurons (especially, all motor neurons) of the gray matter (Fig. 3, A and B) and some long gliocytes of the white matter, for example, fibrous astrocytes (Fig. 3, A and C), in the spinal cords of the normal control rats. On day 1 after the injury, cathepsin D immunostaining was seen in some round cells and gliocytes in the white matter of the disorganized spinal cords (Fig. 3D). Four days after the injury, the spinal cord lesion contained massive round cells and large cells with granules in the cytoplasm such as phagocytes, which were positive for cathepsin D (Fig. 3E). Fourteen days after the injury, fewer round cells and large cells positive for cathepsin D were observed than at 4 and 7 days after the injury (Fig. 3F).

On day 4 after the injury, cathepsin D (Fig. 4A) was detected in most CD68-positive cells (Fig. 4B) in the spinal cords of the SCI-affected rats. This suggests that activated macrophages/microglia in SCI lesions are positive for cathepsin D.

DISCUSSION

This study demonstrated that the level of cathepsin D protein was significantly higher in the spinal cords with a clip compression injury than in normal controls. A transient increase in cathepsin D was seen 4 and 7 days post-injury. The level of cathepsin expression declined at day 14 post-injury. The increased expression of cathepsin D after SCI in the rat model was largely matched by the accumulation of macrophages and the activation of microglia [12], which may contain lysosomal proteinases, including cathepsin D. This suggests that cathepsin D is responsible for the progression of SCI.

Cathepsin D has two representative forms: a 44-47-kDa inactive form and a 30–31-kDa active form. It is an aspartic protease that is targeted to the phagosomes early during phagosome maturation in murine macrophages [18]. Our Western blot analysis showed that the 31-kDa active form of cathepsin D increased significantly at days 4–7 post-injury, while the inactive form did not change during progression of the SCI. This suggests that the increased expression of the active form of cathepsin D is caused by the infiltration and activation of macrophages/microglia in the SCI lesion.

Cathepsin D is essential for the proteolysis of different substrates within lysosomes [22]. Cathepsins often leak into the cytoplasm after the endocytosis of oxidizable substrates that destabilize the lysosomal membranes through lipid peroxidation [14]. The lysosomal proteinases increase in the lesion of CNS inflammation, and this is associated with infiltrating macrophages and reactive gliocytes [3, 11]. Moreover, the cellular infiltration and proliferation of macrophages and gliosis have been reported to occur at the injury site in rat spinal cords with a clip compression injury [8]. Cathepsin D expression increases in the macrophages observed in many inflammatory diseases, including inflammatory bowel disease [6], testicular ischemia/reperfusion injury [7] and chronic obstructive pulmonary disease [4]. In rat spinal cords with autoimmune inflammation, cathepsin D is expressed in the infiltrating macrophages [11]. Previously, we found that OPN/CD44 signaling, which may facilitate the cleaning of debris from damaged tissues and promote wound healing, was localized in activated mac-
Fig. 3. Immunohistochemical staining of cathepsin D in the spinal cords of normal rats (A-C) and rats 1 (D), 4 (E), and 14 (F) days after the injury (C). B and C show higher magnification images of the respective boxes in A and demonstrate that cathepsin D expression was detected in most neurons of the gray matter (B, arrows) and some gliocytes of the white matter (C, arrows) in the spinal cord of normal control rats. On day 1 (D), cathepsin D was observed in some round cells (arrows) and gliocytes (arrowheads) in the white matter of the mechanically damaged spinal cord, including extensive necrosis. Four days after the SCI (E), most of the large phagocytic cells with granules in the cytoplasm in the lesion showed increased immunoreactivity to cathepsin D (arrows). Fourteen days after the injury (F), fewer cathepsin-positive large cells were observed in the lesion (arrows). Counterstained with hematoxylin. Scale bars = 200 μm (A), 50 μm (B and C), and 30 μm (D-F).

Fig. 4. Immunofluorescence showing the co-localization of cathepsin D (A) with CD68 (B) in rat spinal cords 4 days after the injury. The arrows indicate cathepsin D immunoreactivity in the CD68-positive macrophages/microglia. Scale bars = 20 μm.
rophages/microglia in the lesion of SCI [12, 13]. This study showed that cathepsin D expression in the rat SCI lesion was localized primarily in most CD68-positive macrophages/microglia. Therefore, cathepsin D likely promotes the phagocytosis and lysosomal activity of macrophages/microglia in the SCI lesion.

The neuronal and glial expressions of cathepsin D remained unclear, although the expression of cathepsin D has been reported to increase in the neuronal cells of patients with neurodegenerative diseases, including Alzheimer’s disease [1]. In this study, constitutive cathepsin D expression in neurons and glial cells was observed in the spinal cords of the normal rats. Further study of neuronal and glial cathepsin D expression in the spinal cord is needed.

Overall, these results suggest that cathepsin D plays an important role in the activation of signal transduction of phagocytosis and lysosomal activity in activated macrophages/microglia in SCI.

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REFERENCES


