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Differential temporal expression of matrix metalloproteinases after spinal cord injury: relationship to revascularization and wound healing

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Abstract

Object—Matrix metalloproteinases (MMPs), particularly MMP-9/gelatinase B, promote early inflammation and barrier disruption after spinal cord injury (SCI). Early blockade of MMPs after injury provides neuroprotection and improves motor outcome. There is recent evidence, however, that MMP-9 and MMP-2/gelatinase A participate in later wound healing in the injured cord. The authors therefore examined the activity of these gelatinases during revascularization and glial scar formation in the contused murine spinal cord.

Methods—Gelatinase activity was evaluated using gelatin zymography 24 hours after a mild, moderate, or severe contusion injury. The active form of MMP-2 was not detected, whereas MMP-9 activity was evident in all SCI groups and rose with increasing injury severity. The temporal expression of gelatinases was then examined using gelatin zymography after a moderate SCI. The active form of MMP-9 was most prominent at 1 day, extended through the early period of revascularization, and returned to control by 14 days. The active form of MMP-2 appeared at 7 days postinjury and remained elevated compared with that documented in sham-treated mice for at least 21 days. Increased MMP-2 activity coincided with both revascularization and glial scar formation. Using in situ zymography, gelatinolytic activity was detected in the meninges, vascular elements, glia, and macrophage-like cells in the injured cord. Results of immunolabeling confirmed the presence of gelatinase in vessels during revascularization and in reactive astrocytes associated with glial scar formation.

Conclusions—These findings suggest that although MMP-9 and -2 exhibit overlapping expression during revascularization, the former is associated with acute injury responses and the latter with formation of a glial scar.

Keywords

matrix metalloproteinase-2; matrix metalloproteinase-9; angiogenesis; glial scar; spinal cord injury; mouse

Matrix metalloproteinases, a family of zinc- and calcium-dependent endopeptidases, are characterized by sequence homology and their ability to cleave ECM proteins.³⁷ There are at least 25 different MMPs, each with distinct but overlapping substrate specificities. The MMP-9 (105-kD mouse; 92-kD gelatinase human/gelatinase B) and MMP-2 (72-kD gelatinase/gelatinase A) are members of a subclass of MMPs that degrade gelatin (denatured collagens),

collagen types IV, V, and XI, elastin, and vitronectin, as well as numerous non-ECM proteins.^{38,41}

Matrix metalloproteinases are secreted in an inactive form and are activated in the ECM by a variety of mechanisms. The ECM forms a scaffold of proteins that surrounds cells and provides signals essential for cell migration, proliferation, differentiation, tissue remodeling, and cell death. Matrix metalloproteinases are not only able to degrade the ECM but also nonmatrix substrates, such as cell-surface and matrix-bound regulators of growth.⁵ They are regulated at three levels to prevent inappropriate tissue injury: transcriptional control, secretion as an inactive zymogen subject to proteolytic activation, and inhibition by endogenous tissue inhibitors of MMPs, referred to as TIMPs.⁴⁶ Both TIMP-1 and -2 are the dominant inhibitors of MMP-9 and -2, respectively.³⁷

Matrix metalloproteinase-9 is expressed at low levels in the CNS including anterior horn motor neurons²⁸ and is markedly upregulated in blood vessels, glia, and macrophages after injury.^{28,35,36} Matrix metalloproteinase-2 is constitutively expressed in certain populations of astrocytes and is upregulated in these cells after injury.³⁶ Although it is possible that other members of the MMP family may be involved in early tissue injury and wound healing in the injured spinal cord, we have focused on the roles of MMP-9 and -2 because of their established links to early barrier disruption, inflammation, angiogenesis, and glial scar formation.^{9,27,36}

Excessive activity of MMPs can be detrimental, leading to pathological conditions including barrier disruption, inflammation, and demyelination.⁴⁶ These proteases, however, are also integral to wound healing associated with angiogenesis.⁴⁴ Matrix metalloproteinase-9 modulates the release of vascular growth factors, as well as inhibitors to angiogenesis,^{3,6} and may be a critical angiogenic switch during carcinogenesis.³ In mice deficient in MMP-2 decreased angiogenesis and tumor progression have been shown.¹⁷ Moreover, in a model of inflammation-associated corneal neovascularization, MMP-2 and VEGF messenger RNA expression have been demonstrated to correlate with the appearance of new vessels, whereas MMP-9 has been implicated in remodeling of the endothelial basal lamina.²⁰

Gelatinases are also implicated in wound healing processes that include remodeling of the ECM and glial scar formation. Increased gelatinase activity is associated with the formation of scar tissue after SCI and is thought to clear pathways for neurite growth.⁹ Furthermore, there is evidence that MMP-2 can degrade CSPGs in the PNS as well as in cultured glia.^{26,48} Chondroitin sulfate proteoglycans are constituents of the glial scar and have been shown to impede axonal regrowth.⁷ Strategies to inhibit their expression after SCI have led to improved plasticity and functional recovery after spinal cord injury.⁴ Given these observations, it is of considerable interest to define better the interactions of MMPs, including MMP-2, in glial scar formation.

There is growing evidence that MMPs, including MMP-9 and -2, play very diverse roles in secondary pathogenesis and recovery after SCI. What remains unclear is the extent to which each of these gelatinases acts in the acutely injured spinal cord and during wound healing after SCI. To clarify their contributions to these events, we have examined their activity and have defined those cell types that express gelatinases in both the acutely injured spinal cord and during critical periods of wound healing associated with angiogenesis and formation of a glial scar. Analysis of our data suggests that, although these gelatinases are both active during the period of revascularization, there is a functional heterogeneity in their expression in the acutely injured spinal cord as well as during formation of a glial scar.

Materials and Methods

Experimental Model

Unless otherwise stated, adult male FVBn mice were anesthetized using 2.5% Avertin (0.02 ml/g body weight, intraperitoneally) and maintained at 37°C throughout the experiment by use of a warming blanket placed under the animal. A contusive injury was produced using modified procedures²⁸ originally described by Kuhn and Wrathall.¹⁹ Briefly, using aseptic techniques, the spinous process and T-8 laminae were removed, and a circular region of dura (~2.4 mm in diameter) was exposed. After stabilization of the vertebral column, a 1-, 2-, or 3-g weight was dropped 5 cm onto the exposed dura. After injury, the overlying skin was closed using wound clips. Postoperative care included manual bladder expression until recovery of emptying reflex. Surgical control mice (sham surgeries) were those that underwent laminectomy and were killed 1 day later.

Relationship of MMPs to Injury Severity

In the first type of experiment, we examined the relationship between injury severity and activity of MMP-9 and -2 at 24 hours after contusion injury produced by a 1-, 2-, or 3-g weight (five animals in each group). Mice were killed, and the lesion epicenter was prepared for examination by gelatin zymography.

Gelatin Zymography—Samples of frozen spinal cord, prepared from the epicenter, were weighed and homogenized by sonication (weight/volume ratio 1:10) in lysis buffer containing 50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, and 0.1% sodium dodecyl sulfate. Soluble and insoluble extracts were separated by centrifugation and the supernatant stored at -20°C. Equal amounts of supernatant were analyzed using gel zymography as described previously¹⁴ on 10% zymogram gelatin gels (Invitrogen, Carlsbad, CA). The proteins were renatured by washing in 2.5% Triton X-100. The gels were then incubated in substrate buffer (50 mM Tris-HCl, pH 7.5, 10 mM CaCl₂, and 0.01% sodium azide) for 4 days at 37°C to enable MMP-2, MMP-9, and other gelatinases to cleave the gelatin. Each gel was then stained with Coomassie blue for 1 hour and destained in a solution of 30% methanol and 10% acetic acid. Negative staining indicates the location of active protease bands. After exposure to sodium dodecyl sulfate during gel separation, proenzymes present in tissue extracts are activated without proteolytic cleavage.

Temporal Expression of MMPs After SCI

In the second type of experiment, we examined the temporal expression of MMPs by performing gelatin zymography. In addition, we determined the regional and cellular expression of gelatinase activity by in situ zymography combined with immunocytochemical analysis. It is known that other members of the MMP family can contribute to gelatinolytic activity, most notably, MMP-2.⁴⁶

Preparation of Tissue—Seven, two, three, three, three, and four mice were reanesthetized at 1, 3, 7, 14, 21, and 28 days, respectively, after receiving a moderate (2-g) SCI. A bilateral thoracotomy was performed, and the spinal cord was quickly removed and frozen at -70°C. At each time point, spinal cords were prepared for gelatin zymography.

In Situ Zymography—In situ zymography was conducted to detect and localize enzyme activity in tissue sections.³⁰ The uninjured and injured spinal cords were sectioned longitudinally (14 µm) on a cryostat and incubated in in situ zymography reaction buffer, consisting of 0.05 M Tris-HCl, 0.15 M NaCl, 5 mM CaCl₂, and 0.2 mM NaN₃ (pH 7.6), and 40 µg of fluorescein isothiocyanate-labeled gelatin (Molecular Probes, Eugene, OR) at 37°C for 1 hour in the dark. The gelatin with a fluorescent tag remains caged (that is, does not

fluoresce) until the gelatin is cleaved by gelatinase activity, such as that from MMP-2 or -9. This method detects regionally specific gelatinolytic activity but does not distinguish between gelatinases. The reaction product was visualized on fluorescent microscopy.

In Situ Zymography and Immunolocalization of Blood Vessels and Astrocytes

—Frozen sections, cut horizontally at 14 μ m, were incubated in in situ zymography reaction buffer at 37°C for 90 minutes. After a brief rinse in PBS, fixation was performed in a mixture of ice-cold acetone and alcohol (1:1) for 15 minutes, followed by immunohistochemistry. To localize PECAM-1, a vascular marker also known as CD31, sections were treated with a blocking and permeabilizing solution containing 2% normal rabbit serum with 0.2% Triton X-100 and 0.1% BSA in PBS for 30 minutes. Normal sheep serum was substituted for normal rabbit serum in the immunoreaction for detecting GFAP, a cell-specific intermediate filament protein in astrocytes. Primary antibody was incubated for 1 hour by using monoclonal rat anti-mouse PECAM-1 (1:100 in rabbit serum/Triton X/BSA; BD Biosciences Pharmingen, San Diego, CA), and Cy3-conjugated monoclonal mouse anti-porcine GFAP (1:400 in sheep serum/Triton X/BSA; Sigma, St. Louis, MO) antibodies at room temperature. After two 5-minute washes in PBS, Cy3-conjugated goat anti-rat immunoglobulin G (1:200 in rabbit serum/Triton X/BSA; Jackson Lab, Bar Harbor, ME) was added as the secondary antibody for PECAM-1 staining for 1 hour. Sections were washed twice with PBS for 5 minutes, subsequently placed on coverslips with Vectashield mounting medium (Vector Laboratories, Burlingame, CA), and observed under a fluorescence microscope. Frozen sections cut from cervical segments of the spinal cord were used as uninjured control. Specimens obtained in animals from each of the six time points were grouped as a batch and processed simultaneously under the same experimental conditions. In each time group, an additional section, serving as the negative control, was immunostained following the aforescribed procedure in the absence of primary antibody.

Results

Increased MMP-9 Activity Correlating With Injury Severity

We have previously shown that MMP-9 activity is up-regulated within the lesion's epicenter by 24 hours after SCI.²⁸ In the present study, we determined the extent to which this upregulation is influenced by injury severity. Spinal cord MMP activity was analyzed using gelatin zymography 24 hours after a mild, moderate, or severe spinal cord contusion injury (Fig. 1). Only the proMMP-9 and proMMP-2, the inactive zymogens, were evident in the sham-treated cord specimens. There was a marked upregulation of proMMP-9 and active MMP-9 after SCI in all injury groups. Active MMP-9 was detected at lower levels in the mild-SCI group compared with moderate-and severe-SCI groups. Robust and similar levels of active MMP-9 were found in spinal cords after moderate and severe injuries. Although there was no detectable active MMP-2, regardless of injury severity, proMMP-2 appeared modestly elevated in the moderately and severely injured groups relative to the mildly injured group.

Differential Temporal Expression of MMP-9 and MMP-2 After SCI

As assessed by gelatin zymography, MMP activity was evaluated from 1 to 28 days after SCI in moderately injured spinal cords (Fig. 2). At 24 hours after injury, MMP-9 activity was upregulated with bands corresponding to the MMP-9 active form and the inactive zymogen. This upregulation was reduced at 3 and 7 days, but remained elevated relative to levels observed in sham-treated mice. By Day 14 following injury, MMP-9 activity was similar to that seen in sham injury mice. The MMP-2 levels were similar to those detected in sham-treated animals at 24 hours after injury, but the inactive zymogen form increased at 3 days. The MMP-2 activity appeared most robust at 7 days postinjury, with bands that corresponded to both the active and

inactive forms. At 14 through 28 days after injury, MMP-2 activity decreased but remained elevated in relation to that in sham-injured mice.

Increased Gelatinolytic Activity After SCI

In situ zymography was used to define the regional and cellular expression of gelatinases in the spinal cord 1 to 21 days postinjury. Gelatinase activity was primarily restricted to the meninges and a limited number of blood vessels in the sham group (Fig. 3). At 1 day after injury (Figs. 3 and 4), more robust gelatinase activity was apparent in the meninges. In addition, there was evidence of more pronounced cellular staining, particularly in the vasculature.

There was a pronounced increase in gelatinolytic activity by Day 3 after injury throughout the lesion's epicenter. This increase corresponded to both vascular staining and staining in cells with a macrophage-like phenotype (Figs. 3 and 4). The most robust gelatinase activity was observed at 7 days postinjury and was localized to vessels, macrophage-like cells, and glia (Fig. 3). The perimeter of the epicenter was defined by collections of gelatinase-positive, macrophage-like cells. Between 14 and 21 days post-injury, several distinct changes occurred in the expression of gelatinase activity (Fig. 3). Activity appeared more consolidated within the epicenter. Moreover, the perimeter of the epicenter was defined by gelatinase-positive processes that segregated the lesion from the adjacent tissue.

In rats revascularization of the injured cord begins approximately 3 days postinjury and extends over several weeks.²⁴ Given the appearance of gelatinase-positive vessels during wound healing (Figs. 3 and 4), we focused on these structures during the period of revascularization (Fig. 5). As early as 3 days postinjury, small (10–20 μ m in length), intensely stained, truncated blood vessels were evident bordering the lesion's epicenter (Fig. 5). By 7 days after injury, gelatinase-positive vessels exhibited distinctly elongated profiles. Large diameter blood vessels (40 μ m in diameter) were apparent by 7 days and were most prominent bordering the epicenter between 14 and 28 days.

Gelatinase Activity Identified in Reactive Astrocytes Bordering the Epicenter

Astrocytic processes and cell bodies with typical stellate morphology were observed in both the white and gray matter by GFAP immunohistochemistry. At 1 and 3 days after SCI, GFAP immunostaining was absent in the lesion site, whereas delicate GFAP-positive processes were found in the intact tissue (Fig. 6). The intensity of the GFAP immunoreactivity surrounding the lesion area appeared to increase gradually during the 1st week after injury, while the lesion center remained negative for GFAP staining (Fig. 6). The gliotic scar, consisting of bundled astrocytic processes, became evident at the injured–intact interface at 14 days postinjury (Fig. 6). The formation of a compact glial scar by reactive astrocytes precluded the identification of individual GFAP-positive processes, particularly at 21 and 28 days following injury (Fig. 6). Nevertheless, the lesion's center exhibited negative GFAP immunostaining at all time points in the present study.

Gelatinase activity was mostly confined to the lesion area and appeared attenuated in regions more distant from the epicenter (Fig. 6). The coexpression of gelatinases and GFAP was confined to the injured–intact interface and was completely absent in the uninjured tissue (Fig. 6). On the formation of a glial scar beginning 7 days postinjury, gelatinase activity exhibited a complementary distribution relative to GFAP immunostaining. This finding suggests that the major source of gelatinases in the lesion area is unlikely to be reactive astrocytes.

Response of PECAM-1-Positive Blood Vessels After SCI

Sparsely distributed capillaries expressing PECAM-1 were observed in the lesion site as early as 24 hours after injury. At 3 days postinjury, these capillaries were generally short and

fragmented in appearance and larger in diameter (Fig. 7), whereas those in the intact tissue appeared to be long and slender. The number of PECAM-1-positive capillaries as well as their intensity of immunostaining in the lesion's center increased gradually within the 1st week after injury. At 14 days, PECAM-1-positive vessels in the epicenter were distinguished by their unusual convoluted morphology and larger diameter (data not shown). Smaller vessels, similar to those seen in uninjured tissue, were also identified in the lesion's center and the injured–intact interface at this time point. By 21 (Fig. 7) and 28 (data not shown) days postinjury, pronounced PECAM-1 immunostaining of vessels corresponded with the presence of numerous vessels that were distributed throughout the lesion's site.

Because of the changes in PECAM-1 staining during wound healing, we next determined whether these newly formed vessels could express gelatinases simultaneously. By 24 hours post-SCI, capillaries manifesting gelatinase activity were barely discernable within the lesion. Between 3 (Fig. 7) and 7 (data not shown) days postinjury, however, most PECAM-1-positive capillaries in the center of the lesion were found to coexpress gelatinases. At later periods, intense gelatinase activity obscured the cellular resolution, making it difficult to identify the colocalization of vascular structures (Fig. 7). At all time points, some PECAM-1-positive capillaries did not coexpress gelatinase, especially those capillaries located around the injured–intact interface.

Discussion

Relation of MMP-9 Activity Upregulation to Injury Severity: Early Inflammation

In our study of gelatinase activity in the murine spinal cord at 24 hours following a mild, moderate, or severe SCI, we demonstrated that the active form of MMP-9 increased with injury severity in the contused cord. In contrast, the active form of MMP-2 was not detected, regardless of injury severity. These findings support the work by others who have demonstrated increases in MMP-9 activity SCI,⁸ traumatic brain injury,⁴³ and ischemic brain injury.³² This increased MMP-9 activity may reflect upregulation within endogenous cells, such as glia and macrophages,²⁸ and/or increased numbers of infiltrating leukocytes. Notably, neutrophils express MMP-9,⁴¹ and their numbers increase in the traumatized cord with increasing injury severity.⁴⁰

Analysis of clinical data suggests that MMPs may be used as prognosticators of disease severity and progression in CNS diseases, particularly those characterized by inflammation. Although MMP-9 is not normally detected in the CSF in healthy individuals,^{13,31} it is found to be upregulated in CSF of patients with meningitis.¹⁸ Matrix metalloproteinase-9 is also elevated in the serum and CSF of patients with multiple sclerosis, and levels increase during periods of clinical relapse compared with periods of stability.^{21,22} Given these clinical findings, it will be of interest in future studies to determine the extent to which MMP activity in serum and CSF in patients with acute SCI is predictive of motor recovery.

Differential Temporal Pattern of Expression After SCI

Our study is the first to combine the methods of gelatin and in situ zymography to detail the expression of gelatinases after SCI. Gelatin zymography defines the relative activity of gelatinases MMP-2 and -9 (as well as other MMPs), whereas nonspecific gelatinase activity is demonstrated at the cellular level by in situ zymography. It is known that gelatin is a substrate for many MMPs, including the membrane MMPs and the collagenases.⁴⁶ In this study, however, gelatin zymography demonstrated that the predominant gelatinase activity in the injured spinal cord was due to MMP-2 and -9. These findings suggest MMP-2 and -9 were the primary MMPs responsible for driving the in situ zymographic reaction. We evaluated the temporal expression of MMPs within the acutely injured spinal cord—during

revascularization,²⁴ formation of a glial scar,²⁴ and after reestablishment of the blood–spinal cord barrier²⁹—and found a remarkable switch from the early expression of MMP-9 to MMP-2 by gelatin zymography at 7 days postinjury. Moreover, we found only modest gelatinase activity in blood vessels within the 1st day postinjury, whereas at 3 and 7 days increased gelatinase activity was noted in vascular elements and macrophage-like cells. In addition, gelatinase activity was localized in astrocytes bordering the lesion's epicenter. These findings suggest that MMP-9 is primarily expressed during the early initiation of angiogenesis, whereas MMP-2 is associated with ongoing angiogenesis and glial scar formation.

Both experimental and clinical evidence further support the involvement of MMP-9 in acute injury and MMP-2 during wound healing. In the murine brain subjected to traumatic injury, there is a marked increase in MMP-9 activity from 3 hours to 7 days, whereas MMP-2 activity is only modestly elevated.⁴³ Similarly, MMP-9 levels are increased as early as 3 hours and remain elevated for at least 4 days after ischemic brain injury.^{2,32} In contrast, MMP-2 levels do not increase until 4 days after an ischemic insult.³² Recent immunocytochemical studies of human ischemic brain injury also support this differential temporal expression of MMP-9 and -2. Matrix metalloproteinase-9 is primarily localized to neutrophils in the acutely injured ischemic brain, whereas MMP-2 is localized to macrophages of chronic lesions 1 week to 5 years following stroke.¹ A similar pattern is noted in the chronic demyelinating disease, multiple sclerosis, in which MMP-9 is localized in blood vessels, neutrophils, microglia, and macrophages of recent exacerbations, whereas MMP-2 is present in macrophages and active borders of more chronic lesions.¹ Together, these data suggest that such gelatinases may serve different functions in response to injury or disease.

Gelatinase Activity Coinciding With Revascularization After SCI

Angiogenesis has been well characterized in the rodent spinal cord after contusion injury.²⁴ Endothelial cell breakdown and death, and blood–spinal cord barrier breakdown are evident 1 to 3 days after injury. Between 3 and 7 days, the early appearance of vascular elements, associated with increased laminin staining, define the early angiogenic period. It is also in this period that macrophage-like cells and astrocytes express VEGF in injured murine spinal cord.⁴⁵ There is an increased number of vessels, including large-diameter vessels, by 14 days. No increases in blood vessels are noted between 14 to 28 days, suggesting completion of revascularization of the injured cord. This time course of revascularization coincides with the pattern of gelatinase activity we saw in the injured cord. We found maximal active expression of MMP-9 between 1 and 3 days postinjury. During this early time period, there appeared to be a gradual increase in the numbers of gelatinase-positive vascular structures in the epicenter. By 7 days postinjury, when MMP-2 activity was maximally expressed, there were numerous blood vessels expressing gelatinase throughout the epicenter. Although MMP-2 activity declined thereafter, it remained elevated relative to that observed in sham-treated animals up to 28 days postinjury. During this later time period, gelatinase activity decreased in vessels in the epicenter; however, robust activity was evident in large-diameter blood vessels within the penumbral zone. Together, these findings establish a close relationship between MMP-9 in the initiation of angiogenesis and MMP-2 during ongoing revascularization of the injured cord.

Gelatinases are known to be an integral part of angiogenic processes in a variety of organ systems. Both MMP-2 and -9 are released by endothelial cells to degrade the basement membrane surrounding vessels, thus clearing a pathway for new vessel sprouting.²⁷ In a model of corneal neovascularization in the rat, MMP-2 and VEGF RNA expression correlate with areas of development of new vessels. The MMP-9 messenger RNA, however, is found to be expressed mainly beneath the epithelial layer, suggesting a role for it in epithelial and basement membrane remodeling, rather than the actual extension of vessels.²⁰

We found that MMP-9 upregulation coincided with early revascularization of the injured cord. Whether or how MMP-9 contributes to the initiation of angiogenesis remains unclear. There is evidence that this protease can facilitate the release of VEGF, therefore fostering angiogenesis. Moreover, inhibitors of MMPs, as well as genetic deletion of MMP-9, prevent this angiogenic phenotype.³

Gelatinases and Physiological Angiogenesis

There are two distinct types of angiogenesis: physiological angiogenesis, which occurs in wound repair in short bursts and is self-limiting; and pathological angiogenesis (such as that in tumors), which persists indefinitely.^{23,27} Macrophages may play important roles in physiological angiogenesis, in part by secreting factors that degrade connective tissue matrix, such as MMPs.^{15,16} In this study, we identified macrophage-like cells that express gelatinases beginning at 3 days after injury. These cells peak at 7 days, and maintain a presence in the cord within the epicenter and surrounding area through 28 days. It is notable that these macrophage-like cells dominated in areas where vascular growth was evident. Similarly, Loy, et al.,²⁴ have reported dense macrophage/microglia infiltrates within areas of angiogenesis in the contused rodent cord. Thus, there is indirect evidence to support the involvement of macrophages in revascularization of the injured spinal cord.

Expression of MMPs at the Interface of Astroglial Scar

One of the well-known differences between the PNS and the CNS in mature mammals is that axons in the injured PNS are able to regenerate, whereas those in the CNS cannot.^{10,39} A vast array of research has been conducted to elucidate the possible mechanism underlying such a fundamental distinction. Inhibitory components in CNS myelin such as Nogo protein and myelin-associated glycoprotein, among others, have been implicated.¹² It has also been suggested that the intrinsic neuronal state, such as unfavorable cytosolic levels of cyclic nucleotides, may lower the capacity of mature neurons in the CNS to regenerate in response to various extracellular signals in the environment.³³ Historically, however, research has focused on the glial scar and how it may form a physical barrier to growing neurites.³⁴ Interestingly, increasing gelatinase activity caused by transient upregulation of MMP-9 and -2 has been shown to correlate with scar formation after a hemisection of the rat spinal cord.⁹ Furthermore, activated astrocytes within the glial scar express MMP-2 in the adult rat cortex after traumatic injury.²⁶ In a detailed study in which cryoculture assays were used, enzymatic pretreatment of adult peripheral nerve tissue with MMP-2 and -9 created a growth-permissive environment for neurite elongation.¹¹ Moreover, MMP-9 is necessary for the extension of oligodendrocytic processes in the mouse optic nerve, as TIMP-1 and anti-MMP-9 antibody treatment attenuate such outgrowth.³⁰ Taken together, these previous observations indicate that MMPs appear to play a growth-promoting role, possibly by active remodeling of ECM molecules in the CNS.

Injured CNS is, therefore, potentially able to support regeneration to a certain degree under experimental manipulations. When a glial scar forms, however, prevailing inhibitory factors are likely to counteract the healing process either mechanically or chemically, leading to the failure of axonal regeneration in adult CNS. Chondroitin sulfate proteoglycans, for example, are one of the major inhibitory components of the glial scar that forms after CNS injury.²⁵ Davies and colleagues⁷ demonstrated that axons of dorsal root ganglia neurons, implanted into white matter tracts in adult rats, can regenerate until they reach areas rich in proteoglycans, where they then stop or turn away. On the other hand, degradation of CSPGs causes injured spinal cord tissue to be more permissive to neurite outgrowth in vitro.⁴⁹ Likewise, in vivo treatment of the injured rat spinal cord with chondroitinase ABC, an enzyme that attenuates CSPG inhibitory activity, can promote axonal regeneration and thus improve functional recovery.⁴

The authors of a number of studies have shown that MMP-2 is able to degrade CSPGs in the PNS tissue and cultures of glia.^{26,48} It is thus of great importance to understand further the relationship between gelatinase expression and glial scar formation in the mouse injured spinal cord. We found that at all time points examined, no activated astrocytes, as distinguished by GFAP immunostaining, were present in the lesion's epicenter where gelatinase activity was the strongest. Activated astrocytes were mainly seen surrounding the perimeter of intense gelatinase activity that comprised the epicenter. A gliotic scar, consisting of massively entangled astrocytic processes with high GFAP immunoreactivity, became evident at the injured-intact interface at 14 days after injury. We found only those reactive astrocytes that immediately bordered the injury site colocalized with gelatinase activity. Despite its descendent gradation from the lesion epicenter outward, the intensity of gelatinase activity was still slightly higher at the injured-intact interface than elsewhere in the uninjured spinal cord. This finding is consistent with what is seen in the hemisected rat spinal cord, where only activated astrocytes closest to the epicenter produce gelatinases.⁹ It is our opinion that regenerative failure in the CNS is due, at least partially, to the fact that the expression of growth-promoting gelatinases are not extensive throughout the entire glial scar but, rather, are restricted to the perimeter of the lesion. The inadequate level of gelatinase activity may, therefore, be ineffective in counterbalancing the inhibitory molecules in the glial scar, which consequently arrest regenerating axons after SCI.

As previously mentioned, MMPs may facilitate axonal regeneration in the PNS. Results in the present study further define the upregulation of gelatinases within the lesion area for up to 28 days after SCI in the mouse. On the basis of their focalized distribution within the injured spinal cord, gelatinases are thus very likely associated with aggregation of macrophages and other inflammatory cells in the lesion's center. Nevertheless, further research will be needed to understand better how these gelatinases interact temporally and spatially with the glial scar, inhibitory molecules, and regenerating axons during wound healing.

Conclusions

We have previously shown that blockade of MMPs during the first 3 days after SCI is neuroprotective and promotes functional recovery.²⁸ Such findings demonstrate that MMPs are deleterious in the acutely injured spinal cord. In the present study, however, we demonstrated that the gelatinases MMP-9 and -2 are active during critical periods of wound healing including revascularization and glial scar formation. Unfortunately, how MMPs regulate these wound healing events remains unclear. As putative modulators of angiogenesis, the gelatinases may promote plasticity⁴⁷ and/or glial scar formation.⁴² Each of these processes would likely have opposing actions on recovery of function. Clearly, future studies will need to address the role of MMPs in the more chronically injured spinal cord, including the functional implications of MMP inhibition during wound healing.

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Abbreviations used in this paper

BSA	bovine serum albumin
CNS	central nervous system
CSF	cerebrospinal fluid

CSPG	chondroitin sulfate proteoglycan
ECM	extracellular matrix
GFAP	glial fibrillary acidic protein
MMP	matrix metalloproteinase
PBS	phosphate-buffered saline
PECAM	platelet endothelial cell adhesion molecule
PNS	peripheral nervous system
SCI	spinal cord injury
TIMP	tissue inhibitor of metalloproteinase
VEGF	vascular endothelial growth factor

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Fig. 1.

Representative zymograms of spinal cord demonstrating changes in gelatinase activity 24 hours after induction of a 1-, 2-, or 3-g-weight injury, corresponding to mild, moderate, and severe injury, respectively. Levels of proMMP-9 and proMMP-2 appeared elevated in the tissue after moderate and severe injury compared with mild injury. No active MMP-2 was identified in any of the injury groups. In contrast, active MMP-9 was apparent in the cord after injury and appeared more pronounced after moderate and severe injury compared with mild injury.

Fig. 2.

Time course of gelatinase activity after SCI. The positions of migration of zymogen and active forms of MMP-9 and MMP-2 are determined from standards of purified MMP-2 and -9. Representative zymograms of spinal cord show proMMP-9 activity increases relative to shams from 24 hours through 14 days postinjury. The active form of MMP-9 is moderately upregulated at acute time points and is not detected at later time points. The proMMP-2 activity is similar to that in sham-injured mice at 24 hours postinjury, and increases thereafter at all time points. The active form of MMP-2 is apparent at 7 to 21 days and returns to control levels by 21 days.

Fig. 3.

Time course of gelatinase activity by in situ zymography after SCI. Fluorescence is indicative of gelatinolytic activity. A: In sham injury, gelatinase activity is restricted to the meninges (*arrowhead*) and a few scattered blood vessels (*arrows*). B: At 24 hours, gelatinase is markedly upregulated within the meninges (*arrowheads*) and exhibits a more diffuse localization within the lesion epicenter. C: At 3 days, gelatinase activity appears more robust compared with the earlier time point and is most apparent in vascular structures (*arrows*). D: At 7 days, gelatinase activity is notably pronounced throughout the lesion epicenter. E–G: Gelatinase activity in the epicenter remains elevated by 21 days and is demonstrated in a complex network of cells and processes (F) as well as large-diameter blood vessels (G). Scale bars = 100 μ m (A–E), and 50 μ m (F and G).

Fig. 4.

Time course of cell types expressing gelatinase after SCI demonstrated in situ zymography. *Upper Left:* Small-diameter blood vessels express gelatinase in sham-injured cord (*arrows*). *Upper Right:* Similarly, gelatinase activity is identified within vascular structures at 24 hours postinjury (*arrows*). Macrophage-like cells, expressing gelatinase (*open arrows* [*center left*]), are first observed at 3 days postinjury, appear to peak at 7 days (*center right*), and persist at 14 days (*lower left*), and continue to at least 21 days postinjury (*lower right*). Scale bar = 50 μ m.

Fig. 5. Gelatinase is strongly expressed in blood vessels bordering the lesion epicenter. *Upper Left:* Within 3 days postinjury, truncated vascular structures express gelatinase activity (*arrows*). *Upper Right:* By 7 days postinjury, gelatinase-expressing blood vessels typically exhibit more elaborate, tubelike structures (*arrows*). *Lower Left and Right:* By 14 to 21 days postinjury, vessels of varying sizes likewise express gelatinase (*arrows*). Scale bar = 50 μ m.

Fig. 6.

Expression of gelatinases and glial scar formation in the injured–intact interface. A, D, G, and J: Gelatinase activity increases gradually in the lesion area. B, E, H, and K: In contrast, the formation of GFAP-positive glial scar is mostly found bordering the lesion area. C, F, I, and L: The complementary distribution of gelatinases and GFAP immunostaining suggests that reactive astrocytes are less likely to be the major source of gelatinase production at the lesion epicenter after SCI. Scale bar = 100 μ m.

Fig. 7.

Coexpression of PECAM-1 and gelatinases in newly formed blood vessels early after injury. A–C: In the lesion's center, gelatinase activity is colocalized with capillaries that express PECAM-1 early after SCI (*arrowheads*). D–F: Although the expression of gelatinases and the numbers of blood vessels appear to increase progressively over time (D and E), their close correlation gradually becomes less obvious at later time points (F). Scale bar = 100 μ m.