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Matrix Metalloproteinase-9 Facilitates Remyelination in Part by Processing the Inhibitory NG2 Proteoglycan

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Remyelination is a critical repair process that is initiated after a demyelinating insult. The failure to remyelinate contributes to neurological diseases such as multiple sclerosis. Here, we test the hypothesis that proteinase activity is required for the extensive remodeling of the extracellular matrix that occurs during remyelination. We show that mice lacking matrix metalloproteinase (MMP)-9 are impaired in myelin reformation after lysolecithin-induced demyelination. This deficiency may be explained at least in part by the failure to clear the accumulation of NG2, an inhibitory proteoglycan that retards the maturation and differentiation of oligodendrocytes that are needed for remyelination. These results emphasize for the first time that upregulation of MMP activity can be important for facilitating regeneration from some types of CNS injury.

Key words: **myelin formation; metalloproteinase; extracellular matrix; oligodendrocyte; differentiation; proteoglycan**

Introduction

Various types of CNS insults lead to demyelination and axon loss, resulting in disorders such as multiple sclerosis (MS). The CNS does attempt recovery from such insults, and remyelination occurs in MS lesions, albeit to a limited extent (Prineas et al., 1993; Chang et al., 2002). Thus, if the intrinsic CNS repair process could be enhanced, remyelination might be facilitated. Although several factors contribute to remyelination, of paramount importance are the recruitment and maturation of OL precursors and the ensheathment of axons by their processes (Franklin, 2002). In this regard, proteases that are capable of remodeling the CNS matrix to allow for precursor cell migration and for the elongation of oligodendrocyte (OL) processes are likely to be critical.

The matrix metalloproteinases (MMPs) should be considered as candidate molecules for enabling remyelination, because they can degrade all protein components of the extracellular matrix (ECM) (McCawley and Matrisian, 2001). Although MMPs are known to have detrimental roles after injury (Yong et al., 2001), their consistent upregulation in the damaged CNS invites the hypothesis that these proteases have important functions in the repair process, particularly in remyelination. We have shown previously that an increase in MMP-9 levels occurs during the period of developmental myelination in both the corpus callosum and the optic nerve (Uhm et al., 1998; Oh et al., 1999): the *in vitro* inhibition of MMP-9 decreases OL process extension. Moreover, reformation of processes is diminished in OLs cultured from MMP-9 null mice compared with wild-type controls (Oh et al., 1999). These data indicate that MMPs, especially MMP-9, could play a central role in myelin formation during development.

After injury to the CNS, various ECM components such as proteoglycans are upregulated at the lesion sites. For example, the NG2 chondroitin sulfate proteoglycan accumulates as a result of both axonal injury (Zhang et al., 2001; Jones et al., 2002) and demyelinating insults, including MS (Chang et al., 2000; Watanabe et al., 2002). Although the role of NG2 in remyelination has not been investigated, this proteoglycan has previously been found to have an inhibitory effect on axon elongation (Dou and Levine, 1994; Fidler et al., 1999).

In this paper, we address the role of MMP-9 in myelin reformation after a lysolecithin-induced demyelinating injury to the spinal cord of adult mice. We find that although the initial demyelinating injury is equivalent between MMP-9 null and wild-type mice, the former exhibits a significant deficit in remyelination as a result of the decreased number of mature OLs found in the lesion area of the MMP-9 null mice. The mechanism underlying these phenomena may be explained in part by the failure of MMP-9 null mice to remove injury-induced deposits of NG2 proteoglycan. *In vitro* experiments indicate that NG2-rich substrata create an unfavorable environment for the maturation of progenitor cells into mature OLs. These results show for the first time that some of the MMPs that are expressed after CNS injury have reparative functions, suggesting that the selective enhancement of these MMP activities may improve recovery from CNS insults.

Materials and Methods

Lysolecithin microinjection and tissue preparation. Two- to 3-month-old female wild-type and MMP-9 null mice (129/SvEv) (Vu et al., 1998; Oh et al., 1999) were used in these experiments. Mice were anesthetized with a

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mixture of ketamine (200 mg/kg) and xylazine (10 mg/kg), and the spinal cord was then exposed. A 1.5 μl solution of 1% D-lysophosphatidylcholine (lysolecithin; Sigma, St. Louis, MO) was injected slowly (0.5 μl/min) into the dorsal column at the T3–T4 level using a 33 ga needle attached to a 5 μl Hamilton syringe. The needle was left for an additional 2 min to avoid backflow of the lysolecithin. Mice were allowed to recover for defined periods, after which they were killed and the spinal cord was removed. Subsequent processing of tissue for various analyses is described in the respective experimental sections.

Luxol fast blue stain for volumetric analysis and Epon embedding. For histological characterization of lesions, the spinal cord was fixed in 10% buffered formalin. One millimeter blocks covering 2 mm on either side of the lesion were embedded in paraffin, and 10 μm sections were cut at 100 μm intervals (thus, 10 sections per millimeter block were analyzed). Sections were stained for myelin using Luxol fast blue (Solvent blue 38; Sigma). Briefly, sections were deparaffinized, incubated in the solvent blue solution for 3 hr at 60°C, destained with 0.05% lithium carbonate, and counterstained with hematoxylin/eosin. The volume of demyelination was measured following protocols for infarct volumes in the stroke literature (Buchan et al., 1992). Basically, the demyelinated area in the dorsal column of each section was traced using an image processing system (ImagePro 4.5; Media Cybernetics, Silver Spring, MD). The percentage of lesion area was plotted graphically relative to its location, and the total lesion volume was then calculated by measuring the area under the curve.

For electron microscopy, spinal cords from lysolecithin-treated mice were fixed in 2.5% glutaraldehyde and processed for embedding in plastic. Semithin cross sections of the spinal cord were stained with toluidine blue and analyzed by electron microscopy to monitor remyelination in more detail.

Gelatin zymography. Extracts of 5 mm spinal cord segments (2.5 mm on either side of the lesion) were analyzed for MMP-9 activity at 12 hr, 24 hr, 3d, 7d, and 12 d after injury. Tissues were flash frozen in liquid nitrogen, and MMP-9 protein was extracted using gelatin-Sepharose 4B (Amersham Biosciences, Piscataway, NJ) described previously by Zhang and Gottschall (1997). Briefly, after homogenization of tissue in a buffer containing 10 mM $CaCl_2$ and 2.5% Triton X-100, the homogenates were centrifuged at 12000 × *g* for 30 min at 4°C. The supernatant was recovered and incubated with gelatin-Sepharose 4B for 1 hr, and the pellet was resuspended in buffer containing 50 mM Tris, pH 7.4, and 0.1 M $CaCl_2$ in water. This fraction was incubated 15 min at 60°C to release ECM-bound MMP-9 that is Triton X-100 insoluble. Proteins were collected by centrifugation and incubated with gelatin-Sepharose 4B. Finally, the two fractions were pooled together, and the gelatin-Sepharose pellet was resuspended in 100 μl elution buffer (containing 10% DMSO). The entire sample was loaded onto a 8% SDS gel containing 1 mg/ml gelatin. The gel was washed in buffer (2.5% Triton X-100, 50 mM Tris, 5 mM $CaCl_2$) for 3–4 hr to remove SDS and allow renaturation of MMPs in gel. The gel was then left for 48 hr in incubation buffer (50 mM Tris, 5 mM $CaCl_2$, and 2 μM $ZnCl_2$) to allow MMPs to degrade the gelatin in their immediate vicinity. Finally, zones of gelatin degradation representing proteolytic activity were identified by staining the gel with Coomassie blue and destaining with methanol and acetic acid in water (3:1:6). The identity of MMP-9 in Figure 1*D* has been confirmed previously by molecular sizing, Western blotting, and immunodepletion experiments (Uhm et al., 1998; Oh et al., 1999).

Immunohistochemistry. Spinal cord tissue was removed 1 or 2 weeks after injury, and immunohistochemistry was performed to examine the number of immature or mature OLs and the extent of myelination in the dorsal column of wild-type and MMP-9 null mice. Four millimeter spinal cord blocks with the injection site in the center were fixed in 4% paraformaldehyde, sucrose protected, and frozen. Thirty micrometer transverse sections were cut on a cryostat through the entire 4 mm block. Sections were treated with Triton X-100 for 30 min before immunoblocking and then incubated overnight at 4°C with anti-glutathione *S*-transferase pi (GSTpi) (1:500; Biotrim, Dublin, Ireland) to label mature OLs. Adjacent sections were stained with anti-NG2 (Ozerdem et al., 2001) and double-labeled with cyclic nucleotide phosphodiesterase (CNPase) antibody (Sternberger Monoclonals, Lutherville, MD) to visualize myelin. We incubated the sections with secondary antibodies, rabbit anti-Alexa 546 and mouse anti-Alexa 488 (1:500; Molecular Probes, Eugene, OR), for 1 hr at room temperature.

The number of mature OLs (GSTpi) present in the dorsal column after lesion was quantified by cell count throughout 4 mm of tissue surrounding the lesion. The average number of OLs per section was plotted for each millimeter and compared between +/+ and −/− injured mice.

The ionized calcium-binding adapter molecule 1 (Iba1) antibody (kindly provided by Dr. Y. Imai, Tokyo, Japan) was used to visualize the presence of macrophages and microglia in and around the lesion (Ito et al., 1998). Twenty micrometer frozen sections from lysolecithin-injured wild-type and MMP-9 null mice were used for this experiment. The positive staining was visualized by DAB histochemistry (Sigma). MMP-9 immunohistochemistry to localize MMP-9 expression after injury was performed on 10-μm-thick frozen sections with a mouse monoclonal antibody against mouse MMP-9 (CDEM-ABA). CDEM-ABA was prepared by immunizing MMP-9 knockout mice (Dubois et al., 1999) with recombinant mouse MMP-9 and by classical hybridoma technology (Kohler and Milstein, 1975). Western blot analysis of complex biological samples showed that the monoclonal antibody reacted exclusively with MMP-9 (Descamps et al., 2002). Sections were incubated overnight at 4°C and visualized by DAB. Some sections were visualized by fluorescence probes and double labeled with either NG2 or Iba1 antibodies as described above.

To document the extent of macrophage- and microglia-positive cells in the dorsal column (see Fig. 4*E*), tissue sections were analyzed using a semiquantitative scoring system dependent on the proportion of the dorsal column occupied by Iba1-immunoreactive cells. A score of zero denotes no positive cells, whereas increasing numerical scores represent the area of the dorsal column in a given section being occupied by Iba1-positive cells: 1 = 1–24%, 2 = 25–49%, 3 = 50–74%, and 4 = 75–100%. Blinded analysis was performed by three observers, and their results were 90% congruent. Three sections spaced 150 μm apart per tissue block from three animals each were graded at the lesion site and rostral and caudal to the lesion.

NG2 degradation assay. Assays to investigate whether NG2 is a substrate for MMP-9 degradation were performed using the full-length NG2 ectodomain (NG2-EC) (Tillet et al., 1997). Recombinant human MMP-9 (Calbiochem, La Jolla, CA; 100 ng) and mouse MMP-9 (Chemicon, Temecula, CA; 100 ng) were activated by incubation with 1 mM amino phenyl mercuric acetate as described by the manufacturer and then incubated with 1 μg of NG2-EC for 3 hr at 37°C. The solution was then resolved on a 8% SDS gel, transferred to polyvinylidene difluoride membrane (Millipore Inc. Bedford, MA), and probed using a rabbit anti-NG2 antibody. As control, 1 μg non-MMP-treated NG2 protein was loaded. Bands were detected with ECL illuminescence (Amersham Biosciences).

OL culture experiments. Rat OL progenitor cells were purified by immunomagnetic purification using the A2B5 antibody and magnetic beads coated with a secondary antibody (MACS; Miltenyi Biotech, Auburn, CA). Briefly, brains were removed from 4-d-old Sprague Dawley rats, and the cells were dissociated by 0.25% trypsin treatment (15 min). The cells were then separated from debris using Percoll density centrifugation (Oh et al., 1999) and incubated with the A2B5 monoclonal antibody (hybridoma supernatant) (Eisenbarth et al., 1979) for 1 hr at 4°C, followed by incubation with bead-coated anti-mouse IgM for 30 min at 4°C. Cells were then purified by magnetic sorting according to the manufacturer's instructions. The OL precursor cells were plated onto 16-well Labtek slides (Nalge Nunc International, Naperville, IL) at a density of 50,000 cells per well.

To prepare substrata for cell culture, Labtek slides were first coated with poly-ornithine (PO) (10 μg/ml). Some PO surfaces were then coated with NG2 (10 μg/ml) with or without GAG side chains for 30 min at room temperature (Tillet et al., 1997). Parallel batches of NG2 underwent MMP-9 pretreatment as described above before coating of PO surfaces. A nonspecific peptidase (clostridiopeptidase A; Sigma) was also used in some experiments to investigate whether further reversion of NG2 inhibition on OL maturation was observed when NG2 had been fully degraded before the cells were plated.

After cell plating, the A2B5 precursors were allowed to mature for 48 hr at 37°C in a 5% CO_2 environment. Feeding medium was an OL dif-

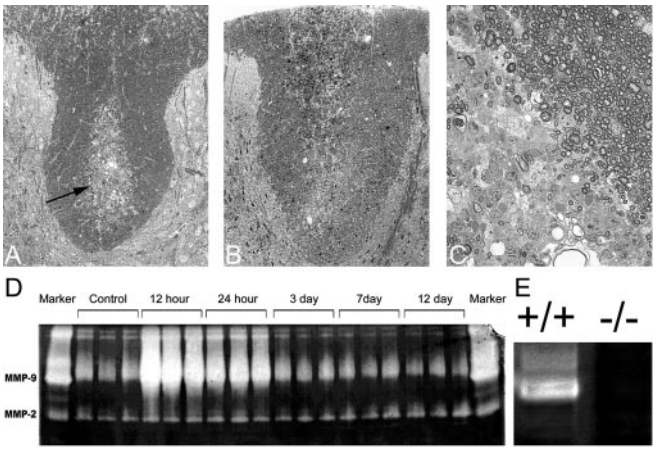

Figure 1. Lysolecithin-induced demyelination leads to subsequent remyelination; MMP-9 expression is elevated shortly after injury. The injection of lysolecithin into the dorsal funiculus results in a prominent demyelination 1 week after injury (*A*, arrow). The boundary between the demyelinated lesion and normal CNS is shown in more detail in *C*. At 2 weeks after injury, remyelination is extensive (*B*), and only minimal disruption is seen in the white matter of the dorsal funiculus. All panels are toluidine blue-stained semithin sections. By zymography (*D*), MMP-9 protein level is increased at 12 and 24 hr after injury, but not at later time points, compared with noninjured controls; three mice (wild type) per time point were analyzed. To confirm the specificity of the zymographic reactions, there was no MMP activity present in CNS extracts of MMP-9 null mice (−/−), whereas this was evident in wild-type (+/+) animals (*E*).

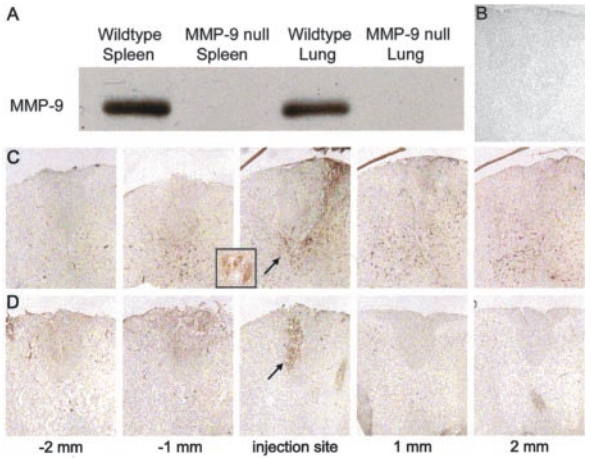

Figure 2. Immunolocalization of MMP-9 in the spinal cord after lysolecithin-induced injury. To localize MMP-9, a monoclonal antibody against mouse MMP-9 was used. The specificity of this antibody was confirmed by Western blot of wild-type and null tissues (*A*). Sections from normal wild-type spinal cord did not label for MMP-9-immunoreactive cells (*B*), but positive elements were readily apparent by 1 d of injury (*C*, all panels) and were still apparent, although reduced, at day 7 (*D*, all panels). At 1 d of injury, MMP-9 expression was most pronounced in the gray matter surrounding the dorsal column (*C*, arrow); inset shows the morphology of immunoreactive cells, many of which were neuronal-like. MMP-9-positive cells at 1 d were detected at the injury site and in sections from tissue blocks 1–2 mm on either side of the injury. In contrast, at day 7 (*D*, all panels), MMP-9 immunoreactivity was mostly confined only to the injection site, within the dorsal column (*D*, arrow). At both 1 and 7 d, some MMP-9 labeling was present on rod-like structures suggestive of blood vessel labeling. In the absence of the primary antibody to MMP-9, no structures are reactive (data not shown). All tissues in this figure are from wild-type animals.

ferentiation medium (DMEM) containing 100 μg/ml BSA, 5 μg/ml insulin, 100 μg/ml tranferrin, 60 ng/ml progesterone, 16 μg/ml putresine, 30 nM sodium selenite, 30 ng/ml 3,3′,5-triiodo-L-thyronine, 30 ng/ml L-thyroxine, 10 nM D-biotin, 10 ng/ml PDGF, and 0.5% FCS. The number of galactocerebroside-positive OLs (Oh et al., 1999) present after 48 hr in each condition was obtained by counting the total number of labeled cells within each well (three wells per condition, and experiments were repeated three times).

To evaluate OL process extension, purified mouse OLs (Oh et al., 1999) were plated on 16-well Labtek slides coated either with PO alone or on PO plus intact NG2 substrate as described above.

Statistical analysis. Statistical evaluation for the extent of remaining demyelination at 2 weeks after injury was performed using unpaired *t* test (two-tailed) to compare the means between the two groups (see Fig. 3). Comparison between monocytoid infiltrates in Figure 4 was performed using Mann–Whitney *U* test. ANOVA with Tukey–Kramer multiple comparisons was used to compare the number of mature OLs between wild-type and MMP-9 null mice after injury (see Fig. 5) and to compare the degree of OL maturation after NG2 exposure *in vitro* (see Fig. 8).

Results

Lysolecithin induces demyelination with ensuing remyelination

The experimental model in these studies uses local application of lysolecithin, which produces a demyelinating injury followed by remyelination (Blakemore et al., 1977). Figure 1*A* is a representative picture of demyelination in the dorsal column of the spinal cord 1 week after the microinjection of 1% lysolecithin into this area. After 2 weeks, much of the demyelinated area has been repaired by remyelination (Fig. 1*B*), in accordance with reports that remyelination initiates ~1 week after lysolecithin injury (Jeffery and Blakemore, 1995). Altogether, the quick onset of demyelination and the ensuing remyelination make the lysolecithin model a useful one for investigating the role of MMPs in remyelination.

Expression of MMP-9 protein in the mouse spinal cord after lysolecithin-induced demyelination of the dorsal column

We first investigated the MMP-9 profile by gelatin zymography. Homogenized tissue from around the injection site showed increased MMP-9 levels at 12 and 24 hr after injury, but not at subsequent time points (Fig. 1*D*). Because there is the potential of spatial and differential cellular localization of MMP-9 after injury, we subjected tissue sections to MMP-9 immunohistochemistry using a monoclonal antibody to MMP-9 (Fig. 2*A*). Although uninjured spinal cord did not stain positively for MMP-9 (Fig. 2*B*), MMP-9-immunoreactive cells were readily detected at 24 hr in the gray matter surrounding the dorsal column (Fig. 2*C*). MMP-9-stained elements had the morphology of neurons (Fig. 2*C*, inset), but some were rod-shaped blood vessels. MMP-9 immunolabeling was present throughout the 2 mm of tissue that bordered the injection site on either side, thus accounting for the increase in MMP-9 levels detected by zymography.

In contrast to the 24 hr time point, MMP-9 immunoreactivity at 7 d after lysolecithin treatment was mostly confined to the dorsal column of sections containing the injection site (Fig. 2*D*); the identity of these cells will be described further below. Some rod-shaped blood vessels outside of the injection site were also labeled for MMP-9, but these profiles were few. The localized MMP-9 staining at 7 d that is present mainly at the injection site likely accounts for the failure of zymograms of whole tissue block to demonstrate an absolute increase of this protease at 7 d compared with control (Fig. 1*D*). Overall, these results place MMP-9 at the site of injury at time points correlating to the initiation of remyelination.

MMP-9 null mice have an impaired capacity for remyelination

To investigate whether the expression of MMP-9 in the lesion area after lysolecithin administration is beneficial or detrimental to recovery, we used MMP-9 null mice developed by Vu et al.

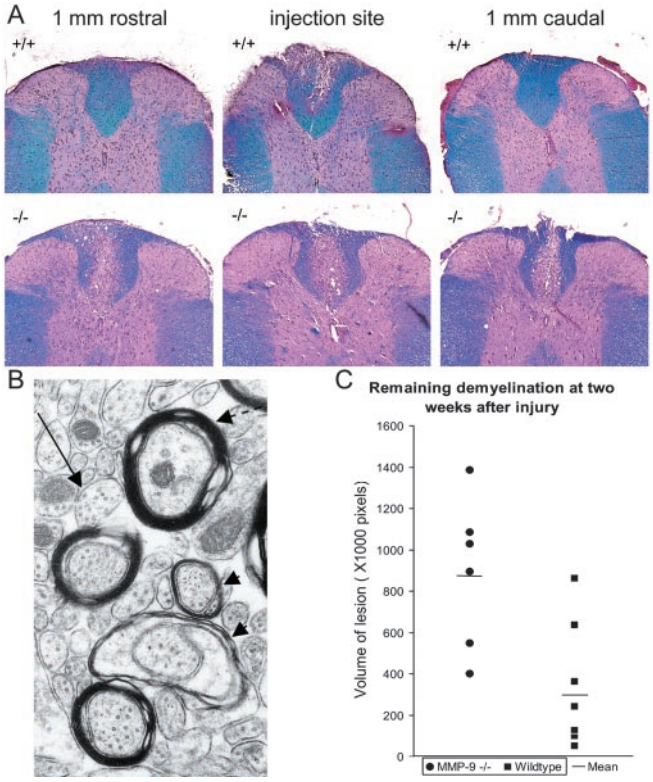

Figure 3. MMP-9 null mice are deficient in myelin reformation 2 weeks after injury. *A* shows Luxol fast blue and hematoxylin/eosin-stained specimens of wild-type (top three sections) and MMP-9 null (bottom three sections) spinal cords 2 weeks after lysolecithin injury. Although the injection site in wild-type mice still shows evidence of residual demyelination, likely related to physical trauma at this epicenter, sections 1 mm rostral or caudal to the injection site have remyelinated. In contrast, demyelination is still widespread in corresponding sections from MMP-9 null mice. Electron microscopy of an MMP-9 null animal (*B*) 2 weeks after injury reveals that some axons have remyelinated (arrowhead; thin myelin sheaths), but most are still demyelinated (an example is indicated by the long arrow). An axon that presumably did not undergo demyelination (myelin sheath of normal thickness) is indicated by the dashed arrow. This electron micrograph shows that axons are left intact by the lysolecithin injury. Analysis in individual animals of the total demyelinated volume at 2 weeks shows a significant difference in long-term demyelination in MMP-9 null mice compared with wild-type mice (*C*) ($p = 0.013$; two-tailed unpaired *t* test).

(1998). The absence of MMP-9 expression in the knock-out mice was shown previously by zymography (Oh et al., 1999) and confirmed by Western blot analysis here (Fig. 2*A*). Light microscopy of Luxol fast blue-stained spinal cord sections was used to evaluate the extent of remyelination. Two weeks after injury, a clear difference was found between lesions in wild-type and null mice. Although remyelination was substantial in wild-type animals except for residual demyelination at the injection site, the dorsal column of MMP-9 null mice exhibited areas of continued demyelination (Fig. 3*A*).

The lack of remyelination in MMP-9 null mice could be attributable to axonal defects; however, electron microscopic examination of lesions in MMP-9 null mice suggests that axons are intact (Fig. 3*B*) and indistinguishable from the axons of wild-type mice (data not shown). Other research has shown that axons are not disrupted in the lysolecithin injury model (Woodruff and Franklin, 1999). Furthermore, there are attempts at remyelination in the MMP-9 null mice, as evidenced by the presence of some thin myelin sheaths that are typical of remyelination. Such examples of remyelination are scarce, however, and many demyelinated axons are still present in the vicinity of the lesion (Fig. 3*B*).

The difference in remyelination at 2 weeks after injury was quantified by volumetric measurements of demyelinated zones throughout the spinal cord. Figure 3*C* shows that MMP-9 null mice have significantly larger demyelinated volumes compared with wild-type mice ($p = 0.013$). Thus, MMP-9 is important for the normal remyelination of the spinal cord that occurs after a lysolecithin injury.

Deficient remyelination in MMP-9 null mice is not caused by defective leukocyte recruitment

Detailed investigations by others (Hinks and Franklin, 2000; Kotter et al., 2001) have demonstrated that myelin clearance after lysolecithin injection is a prerequisite for remyelination. This clearance requires macrophage entry into the injured spinal cord. Because MMPs are implicated in the transmigration of leukocytes, including macrophages (Shipley et al., 1996; D'Haese et al., 2000; Lanone et al., 2002), we addressed the possibility that macrophage and microglia numbers in the lesion might be decreased as a result of the MMP-9 deficiency, thus delaying the myelin clearance necessary for remyelination. Several factors argue against this possibility. First, at 1 week after the lysolecithin injury, the extent of the demyelinated lesions was comparable between wild-type and MMP-9 null mice (Fig. 4*A*,*B*). This would not be the case if macrophage invasion was impaired and myelin clearance thus attenuated. Second, immunohistochemistry for Iba1, a marker for microglia and macrophages (Ito et al., 1998), revealed qualitatively similar densities of these monocytoid cells in and around the lesions in both groups of mice at 7 d after injury (Fig. 4*C*,*D*). This was also the case at 2 d after injury (data not shown). To confirm that the amount of macrophages and microglia was not altered as a result of the MMP-9 deficiency, we subjected tissue sections to blinded analyses using a semiquantitative scoring system dependent on the proportion of the dorsal column occupied by Iba1-immunoreactive cells. Three observers graded the sections, and results were 90% congruent. Figure 4*E* demonstrates that the density of monocytoid infiltrates was not significantly different between wild-type and MMP-9 null mice when sections were evaluated either at the injection site ($p = 0.5$) or 150 μm rostral ($p = 0.82$) and caudal ($p = 0.82$) to the lesion. Finally, RNase protection assays for cytokines (mCK-2b set; PharMingen, San Diego, CA) and chemokines (mCK-5c set, PharMingen) that are vital for leukocyte entry to the dorsal column revealed no apparent differences in these mediators of inflammation between wild-type and MMP-9 null mice (see supplemental data; available at www.jneurosci.org). Collectively, these results indicate that the lack of remyelination in MMP-9 null mice is not accounted for by an impairment in primary demyelinating and inflammatory responses after lysolecithin injury.

Mature OLs are found in reduced numbers around the lesion site in MMP-9 null mice

The number of mature myelin-forming OLs was analyzed to determine whether OL numbers could account for the deficiency of remyelination in MMP-9 null mice. At 1 week after lysolecithin injection, an antibody to GSTpi, a marker for mature OLs (Tansey and Cammer, 1991; Mason et al., 2001), detected significantly lower numbers of OLs in the dorsal column of MMP-9 null mice compared with wild-type mice (Fig. 5*A*–*C*). A similar lack of OLs in the MMP-9 null mice was also detected at 2 weeks after injury (data not shown). This difference was not caused by an intrinsic lack of OLs in MMP-9 null mice, because sections of spinal cord between 4 and 5 mm remote from the injection site exhibited

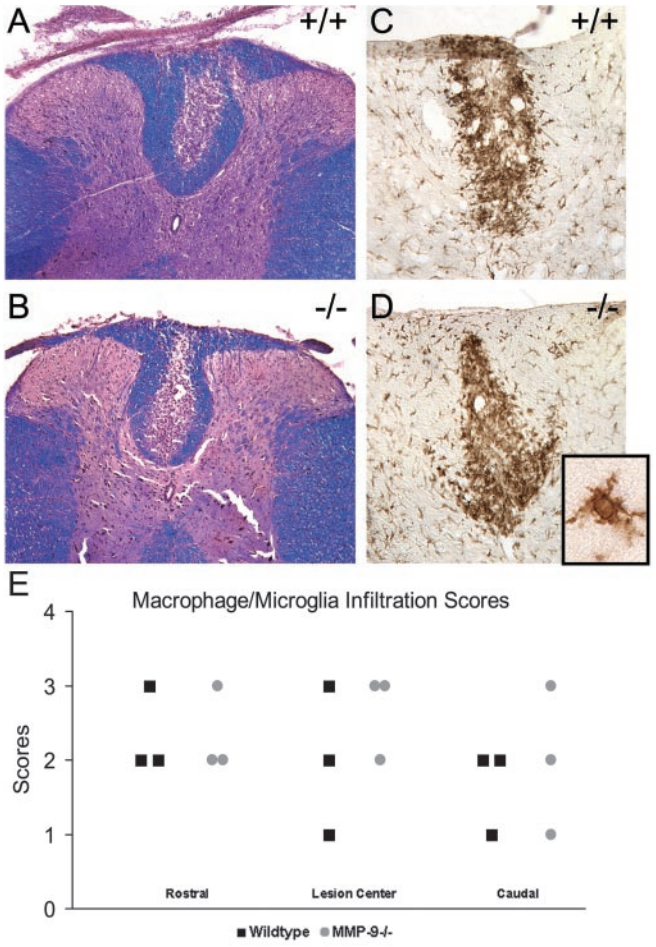

Figure 4. The lesion size and density of macrophages and microglia at 1 week after injury is comparable between wild-type and MMP-9 null animals. *A* and *B* show representative areas of demyelination 7 d after lysolecithin administration to wild-type (*A*) and MMP-9 null (*B*) mice. Monocytoid infiltration and activation indicated by Iba1 staining are not inhibited in MMP-9 null mice (*D*) compared with wild-type mice (*C*). A blowup (inset) of one Iba1 positive cell is shown in *D*, bottom right. To verify that the extent of macrophage and microglia accumulation is not different between the wild-type and MMP-9 null animals, sections were subjected to scoring by three blinded observers using a scale described in Materials and Methods. *E* demonstrates that the density of Iba1-positive cells is not significantly different between wild-type (+/+) and null (−/−) mice, either at the lesion center or 150 μm rostral and caudal to the lesion.

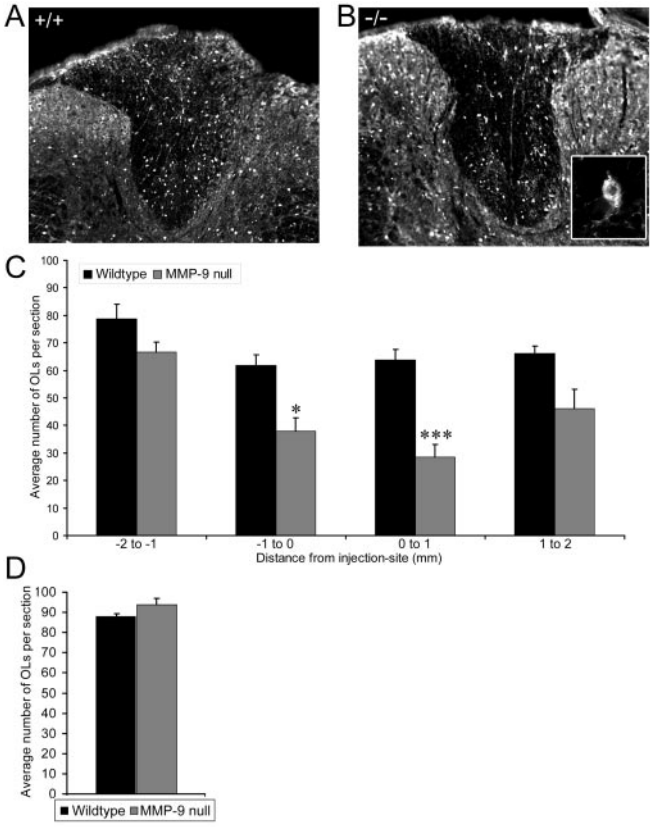

Figure 5. MMP-9 null mice have reduced numbers of mature OLs at the lesion sites after lysolecithin treatment. *A* (wild type) and *B* (MMP-9 null) show mature OLs (GSTpi+) present in the dorsal column 1 week after injury. A blowup (inset) of a GSTpi+ cell is also shown. The total number of OLs in the dorsal column was quantified, and the average number per section from each 1 mm spinal cord block was plotted (C) (from 4 wild-type and 3 MMP-9 −/− mice). A significant difference is seen between wild-type (black bars) and null (gray bars) mice in the number of mature OLs within 1 mm of the injury site on either side, i.e., in the −1–0 mm and 0–1 mm blocks, where 0 represents the injection site. Values displayed are mean ± SEM; $*p <$ 0.05 and $***p < 0.001$. Finally, nine sections spaced 100 μm apart from a 1 mm spinal cord block remote (4–5 mm rostral) from the lesion were sampled from wild-type and MMP-9 null mice ($n = 3$ each). *D* shows that the number of OLs in both groups was similar, thus indicating that there is no intrinsic lack of OLs in the MMP-9 null mice under basal conditions.

comparable OL numbers between the two groups (Fig. 5*D*). These results suggest that impaired remyelination in MMP-9 null mice is caused by the reduced numbers of myelinating OLs during the critical period of remyelination.

The NG2 proteoglycan is densely deposited in lesions and is not cleared in MMP-9 null mice

The deficiency of OLs in MMP-9 null mice could be the result of an inability of OL progenitor cells to differentiate in a timely manner into OLs. We used an antibody against the NG2 proteoglycan in an attempt to address this hypothesis. NG2 is a membrane-spanning chondroitin sulfate proteoglycan (CSPG) expressed on the cell bodies and processes of oligodendrocyte progenitor cells in both the neonatal and adult CNS (Levine and Nishiyama, 1996; Nishiyama et al., 1996; Watanabe et al., 2002). In the uninjured spinal cord, NG2-positive cells are readily detected in both the gray and white matter and are distinct from microglia (Fig. 6*A*) or astrocytes (data not shown).

At 7 d after lysolecithin injection, a dense area of NG2 immunoreactivity is evident in lysolecithin lesions (Fig. 6*B*) in both wild-type and MMP-9 null mice. This dense accumulation of NG2, not seen in the uninjured spinal cord, is not all cellular associated and thus was presumed to be in the extracellular matrix (Fig. 6*C*) (see supplemental movie). Although NG2 is normally an integral membrane component, after CNS injury substantial deposition of the proteoglycan into the ECM has been observed (Jones et al., 2002). This is most likely caused by proteolytic release of the NG2 extracellular domain from the cell surface (Nishiyama et al., 1995). The intensity of the ECM staining in our sections made it impossible for us to accomplish our goal of quantifying oligodendrocyte progenitors in the area of the lesion; however, the presence of the ECM-bound NG2 opened an additional line of inquiry. When the fate of the NG2 accumulation was examined at 2 weeks after injury, NG2 immunoreactivity in wild-type mice was no longer densely accumulated but instead was seen clearly mainly on cellular structures (Fig. 6*D*,*E*). Much of this NG2 immunoreactivity in wild-type mice was in close proximity to that for CNPase (Fig. 6*F*), an early marker of myelinating OLs and myelination. In contrast to wild-type mice,

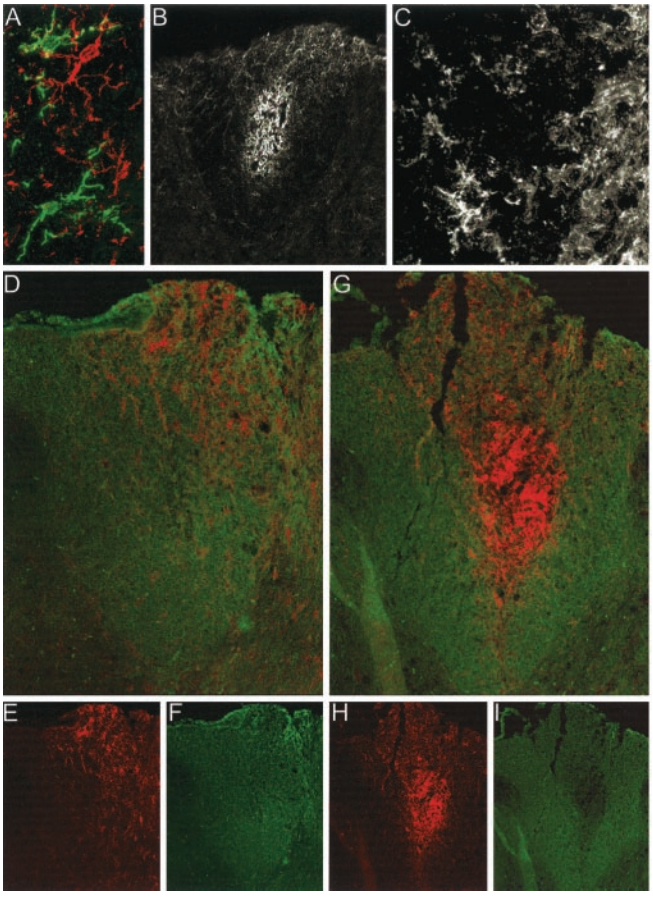

Figure 6. Immunoreactivity for NG2 proteoglycan reveals the persistence of a dense NG2 accumulation in MMP-9 null but not wild-type mice. *A* represents NG2 immunoreactivity (red) in normal mice on presumed OL progenitor cells. NG2 staining was not seen on microglia (green, Iba1 staining) or astrocytes (data not shown). At 1 week after a lysolecithin injury, NG2 immunoreactivity appears as a dense deposit at the lesion site (*B*). This dense accumulation is replaced by a more diffuse NG2 staining (*D*, *E*, red) at 2 weeks after injury corresponding to areas of remyelination as denoted by CNPase staining (*D*, *F*, green). In contrast, MMP-9 null mice at 2 weeks continue to have a dense matrix of NG2 (*G*, *H*, red). In this dense NG2-containing area, remyelination as inferred from CNPase staining (*G*, *I*, green) was not evident. Much of the dense NG2 matrix seen at 2 weeks in MMP-9 null mice cannot be clearly resolved on cell structures (*C*, arrows) (see supplemental movie).

sections from MMP-9 null mice at 2 weeks still exhibited a pattern of dense NG2 immunoreactivity that did not overlap with CNPase staining (Fig. 6*G–I*). In fact, CNPase staining is essentially absent from the null mouse lesions, in keeping with the deficiency of remyelination.

In summary, the deposition of NG2 after lysolecithin injury is cleared in wild-type mice but remains dense in MMP-9 null mice, suggesting that the presence of MMP-9 is required for breakdown of the matrix-associated NG2. Correspondingly, remyelination proceeds in the wild-type but is deficient in MMP-9 null mice. These results suggest that NG2 is a biological substrate for MMP-9 activity and that NG2 accumulation in the absence of MMP-9 inhibits remyelination by interfering with maturation and differentiation of OL precursors.

Sources of MMP-9 after lysolecithin injury

The expression of MMP-9 protein in the lesioned dorsal column (Fig. 2*D*) prompted us to examine its cellular sources. By double immunohistochemistry and confocal imaging, the main source of MMP-9 at 7 d after injury was determined to be Iba1-positive microglia and macrophages (Fig. 7*A*). Most NG2-positive cells did not overlap with the MMP-9 immunoreactivity (Fig. 7*B*). Of the few cells that showed colocalization, we were unable to determine whether these were oligodendrocyte progenitor cells or a subset of microglia and macrophages that are immunoreactive for NG2 in injurious conditions. In summary, in wild-type mice 1 week after lysolecithin administration, the main source of MMP-9 is microglia or macrophages.

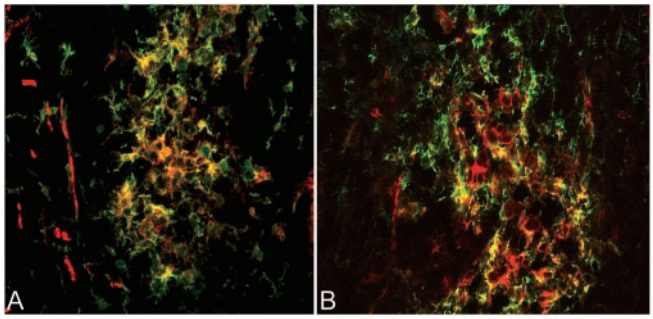

Figure 7. Immunolocalization of MMP-9. *A* shows MMP-9 (red) immunoreactivity colocalizing to Iba1 (green)-positive cells in the injured (7 d) dorsal column of wild-type mice; the overlap is depicted by the yellow label. In *B*, most of the MMP-9 (red)-immunoreactive cells are not NG2 (green) positive, although colocalization was found in some cells (yellow).

Processing of NG2 by MMP-9 facilitates OL maturation *in vitro*

Because of the close apposition of NG2 and MMP-9 immunoreactivity after injury and the failure of NG2 clearance in MMP-9 null mice, we tested the hypothesis that NG2 is a substrate for MMP-9 and that MMP-9 processing of NG2 alters the functional properties of the proteoglycan. The purified recombinant NG2 ectodomain was incubated with recombinant MMP-9, and the mixture was then subjected to gel electrophoresis. Immunoblot analysis was used to detect the appearance of proteolytically cleaved NG2 fragments. Figure 8 shows that the NG2 preparation contains principally two high molecular bands and less intense lower molecular bands (Fig. 8*A*, lane 1). Treatment with active MMP-9 resulted in increases in the intensity of at least five bands spanning from 200 to 80 kDa; this increase was inhibited by BB94, a broad-spectrum MMP inhibitor. Figure 8*B* focused on the formation of two lower molecular weight bands in response to active MMP-9. Incubation with recombinant murine or human MMP-9 generated these two products in a manner that was inhibited by BB94 (Fig. 8*B*). The proteolysis of NG2 by MMP-9 is fairly restricted compared with that produced by another protease, clostridiopeptidase A, which completely degrades NG2 into low molecular weight forms (Fig. 8*C*).

The proteolysis of NG2 by MMP-9 coupled with the accumulation of NG2 and the scarcity of mature OLs in MMP-9 null mice after lysolecithin treatment suggested to us that the failure to degrade matrix-bound NG2 in MMP-9 null mice might affect the maturation of OLs after injury. Similarly, NG2 could be inhibitory for process extension by OLs. To test the latter, we plated mature mouse OLs on slides coated with intact NG2 to observe process extension. We found no inhibitory effects of the NG2 substrate compared with control conditions (plated without NG2) on the overall capacity of cells to extend processes (Fig. 8*D*,*E*). Finally, to test whether NG2 affected maturation of OLs, A2B5-purified rat OL progenitor cells (Barres et al., 1996) were plated on an intact NG2 matrix or on an NG2 matrix that had been preincubated with activated MMP-9. Figure 8*F* shows that

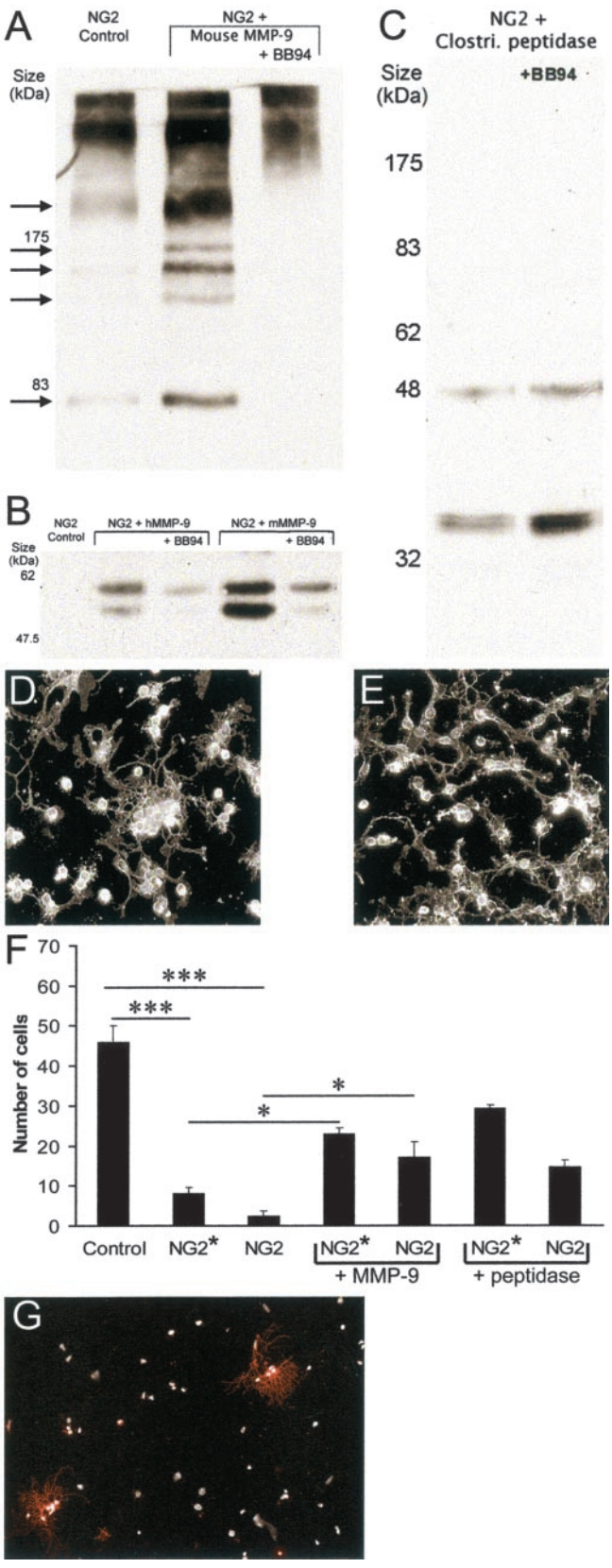

Figure 8. NG2 is a substrate for MMP-9, and the NG2 inhibition of OL precursor maturation is overcome by previous incubation of NG2 with MMP-9. *A* and *B* show that the incubation of NG2 with activated MMP-9 generates new fragments or increases the amount of existing ones (*A*, arrows). This proteolysis is inhibited by 100 nM of BB94 (*A*, lane 3; *B*, lanes 3, 5). In *B*, both human MMP-9 and mouse MMP-9 are able to generate the bands at 50 and 60 kDa in a manner that is inhibited by BB94. *C* shows that clostridiopeptidase degraded NG2 almost completely, and this was not inhibited by BB94. These results of MMP-9 processing of NG2 were seen in three experiments. *D* and *E* demonstrate OL process extension on PO alone (*D*) or on PO plus NG2 (*E*). NG2 did not inhibit OLs from extending elaborate processes in culture. In *F*, purified A2B5-positive OL progenitor cells were allowed to mature for 48 hr on either PO alone (control) or on PO coated with NG2. The number of galactocerebroside-positive OLs across the entire well was then counted. There was a significant reduction in the number of mature OLs (*y*-axis) when the OL precursors were plated on an NG2 substrate; however, when the NG2 substrate was pretreated with activated MMP-9, significantly more OLs were generated. Complete degradation of NG2 by clostridiopeptidase (NG2 + peptidase) resulted in no further increase in OL numbers compared with that elicited by activated MMP-9. The effect of MMP-9 was not different whether the NG2 contained GAG side chains (NG2*) or not. *$p < 0.05$; ***$p < 0.001$. These results were produced in three experiments. *G* shows a representative picture of the mature OLs with their elaborate processes.

the progenitor cells were arrested in their immature state when plated on NG2 *in vitro*, but that this arrest was partially overcome when NG2 had been subjected to MMP-9 cleavage. The NG2 core protein was responsible at least in part for the inhibitory effect on OL progenitor maturation, because NG2 preparations with (NG2*) or without (NG2) the chondroitin sulfate side chain had similar inhibitory potencies. The restricted processing of NG2 by MMP-9 (Fig. 8*A*,*B*) was sufficient to partially abrogate the ability of the proteoglycan to block OL maturation. Complete digestion of NG2 by clostridiopeptidase A had an equivalent effect in overcoming NG2 inhibition of OL maturation (Fig. 8*F*).

Overall, these experiments suggest that the expression of MMP-9 after injury is required for the proteolytic cleavage of nonpermissive NG2, thus allowing OL maturation and differentiation and subsequent remyelination of axons.

Discussion

Role of MMP-9 in facilitating remyelination

The ability to remyelinate after a demyelinating injury depends on various factors, including the influx of mononuclear phagocytes to clear myelin debris, the responsiveness of axons, a favorable extracellular environment including the matrix, and the availability of progenitor cells that differentiate into mature OLs to initiate the remyelinating program. We now add to this list the presence of proteolytic activity, because the absence of MMP-9 impairs the remyelinating program after lysolecithin-induced demyelination. MMP-9 does not appear to be required for the initial entry of phagocytes into the CNS, because equivalent numbers of mononuclear phagocytes are present in the lesions of both wild-type and MMP-9 null mice and because the extent of demyelination is comparable between both groups at 1 week after injury (Fig. 4). In other models, macrophage infiltration is more dependent on MMP-12 than on MMP-9 (Shipley et al., 1996; Lanone et al., 2002). The similar cytokine and chemokine profiles (see supplemental data) seen in wild-type and MMP-9 null mice further argue against a differential inflammatory response as a determinant of impaired remyelination in MMP-9 deficiency. Although the role of axons was not systematically addressed in this study, the apparently normal morphological profiles of axons in electron micrographs from wild-type and MMP-9 null animals suggest that the state of axons does not explain the differences in remyelinating capacity seen in this study.

We noted a decline in the numbers of mature OLs in lesion areas in the MMP-9 null mice compared with wild-type controls (Fig. 5). This does not appear to be attributable to an intrinsic deficiency of OLs in MMP-9 null mice, because tissue away from the lesion in MMP-9-deficient animals exhibits OL densities similar to those observed in wild-type counterparts (Fig. 5*D*). Al-

though it is possible that the deficiency of mature OLs is caused by a scarcity of oligodendrocyte progenitors in the lesioned area, we were not able to verify this because of the intense ECM-associated NG2 immunoreactivity in the injury site. The dense NG2 accumulation seen in the dorsal column of both wild-type and MMP-9 null mice at 1 week after injury was resolved in wild-type mice by 2 weeks after injury, leaving NG2 associated principally with progenitor cells; however, dense NG2 deposits remained in the lesions of MMP-9 null mice (Fig. 6).

The persistence of NG2 immunoreactivity in MMP-9-deficient mice prompted the investigation of NG2 as a substrate for MMP-9 proteolysis. Here it is interesting to note that MMP-9 expression becomes downregulated in neurons and elevated in microglia and macrophages in the lesion area at 1 week after injury in close proximity to NG2-positive cells. Previously, MMP-9 was found capable of binding to the core protein of CSPGs (Winberg et al., 2000). We extended this observation by demonstrating that the incubation of NG2 with MMP-9 resulted in new fragments (Fig. 8*A*,*B*), the formation of which could be blocked by a metalloproteinase inhibitor. The precise sequence of NG2 that is processed by MMP-9 is not known, but cleavage is rather selective when compared with that produced by clostridiopeptidase A, which causes substantial degradation of NG2 (Fig. 8*C*). Furthermore, the subtle processing of NG2 by MMP-9 is sufficient to alter its inhibitory effect on OL maturation *in vitro* (Fig. 8*F*). Another potential explanation for the lack of remyelination could be that NG2 is inhibiting OL process extension because NG2 is known to inhibit neuronal process extension. We investigated this potential mechanism and found that NG2 did not affect OL process extension *in vitro*. Our finding from Figure 5, where we find less mature OLs present in the dorsal column of MMP-9 null mice at 7 d after injury, also argues for a deficiency in maturation of OLs rather than a failure in their ability to extend processes. Altogether, these results demonstrate that NG2 accumulates after injury, that MMP-9 activity is required to clear the NG2 deposition, and that MMP-9 processing overcomes the negative impact of NG2 on OL maturation and remyelination.

The current result that NG2 is a substrate for MMP-9 is of relevance not only to remyelination but also to the recovery from various types of CNS insults in which proteoglycans are deposited in lesions. Our work suggests that when MMP-9 is upregulated after CNS injury, some of its activity may be aimed at degrading inhibitory ECM molecules to enable axonal regeneration and remyelination. The OL maturation in our study is influenced primarily by the NG2 core protein, because NG2 with or without the chondroitin sulfate side chain inhibited OL maturation *in vitro* (Fig. 8).

MMPs: detrimental or beneficial after injury?

The overexpression of MMPs in the injured adult CNS has primarily been considered to be harmful to the tissue (Yong et al., 2001); however, several lines of investigation have suggested that MMPs may have beneficial functions when expressed in the CNS. Metalloproteinase activity has been suggested to regulate axon elongation (Zuo et al., 1998; Hayashita-Kinoh et al., 2001) or to play a role in modulating axon guidance cues (Galko and Tessier-Lavigne, 2000; Hattori et al., 2000) *in vitro*. During development, application of MMP inhibitors results in a decrease in axon elongation and misguidance of the retinotectal tract in *Xenopus* (Webber et al., 2002). Furthermore, MMPs are elevated during myelination of the mouse corpus callosum and optic nerve, where they may play a role in facilitating OL process outgrowth (Uhm et al., 1998; Oh et al., 1999; P. H. Larsen and V. W. Yong, unpublished data). The current results, however, are the first to show *in vivo* that MMP activity is beneficial for recovery from an insult to the adult CNS.

Is the clearance of NG2 the only mechanism by which MMP-9 can affect remyelination? We think this unlikely and favor the view that there are likely to be additional roles of MMP-9 in repair and recovery of the CNS. Thus, MMP-9 and other MMPs may facilitate cell migration to injured areas that require replenishment or may promote process extension as a prelude to interaction with and remyelination of axons. Other beneficial aspects could include a role in angiogenesis, in the release of growth factors sequestered by the ECM (Whitelock et al., 1996), or in the subtle processing of cell–cell recognition molecules (e.g., notch or neuregulins) that allow repair (for review, see Yong et al., 2001). It is possible that MMP-9 is important for several individual mechanisms that work in concert to allow remyelination and repair of the injury. These potential additional mechanisms of MMP-9 in remyelination deserve investigation in future studies.

It is clear that several MMPs are elevated simultaneously after various forms of CNS insults (Yong et al., 2001). We are currently examining the expression of other MMPs after lysolecithin demyelination and have found that MMP-12 transcript is also elevated after injury, but at later time points (3 d). Furthermore, MMP-12 can also degrade NG2 *in vitro* (our unpublished observations). Thus, why MMP-12 did not compensate for MMP-9 to clear NG2 is unclear, but differential temporal and spatial expression could be contributing factors. Indeed, it remains unclear whether the impaired remyelination in the MMP-9 null mice is a permanent fixture or whether this represents a delay in remyelination. In the original description of the MMP-9 null mice, the deficiency in the growth of long bones was found to be a transient phenomenon (Vu et al., 1998).

Against the beneficial aspects of MMPs must be balanced their reported detrimental effects. MMPs can be neurotoxic (Gu et al., 2002), or they can produce axonal injury (Newman et al., 2001) or demyelination (Anthony et al., 1998). In MMP-9 null mice, brain (Wang et al., 2000) or spinal cord (Noble et al., 2002) injuries have resulted in deficits in behavioral recovery when compared with wild-type animals. Clearly, the complex roles of MMPs in the developing or injured CNS require further attention. The spatial and temporal expression of specific MMP family members by identified cell types and the interaction of MMPs with other molecules present at that specific place and time are critical factors that could determine the beneficial or detrimental functions of MMPs.

In conclusion, we have defined for the first time a useful property of an MMP member after CNS insult. Our results show that MMP-9 produced predominantly by microglia and macrophages plays a significant role in clearing the NG2 chondroitin sulfate proteoglycan, and this may allow OL precursor cells to mature and differentiate into myelin-forming OLs at the site of injury.

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