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ORIGINAL ARTICLE

Matrix Metalloproteinase Inhibition Enhances the Rate of Nerve Regeneration In Vivo by Promoting Dedifferentiation and Mitosis of Supporting Schwann Cells

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Abstract

After peripheral nerve injury, Schwann cells (SCs) vigorously divide to survive and produce a sufficient number of cells to accompany regenerating axons. Matrix metalloproteinases (MMPs) have emerged as modulators of SC signaling and mitosis. Using a 5-bromo-2-deoxyuridine (BrdU) incorporation assay, we previously found that a broad-spectrum MMP inhibitor (MMPi), GM6001 (or ilomastat), enhanced division of cultured primary SCs. Here, we tested the hypothesis that the ability of MMPi to stimulate SC mitosis may advance nerve regeneration in vivo. GM6001 administration immediately after rat sciatic nerve crush and daily thereafter produced increased nerve regeneration as determined by nerve pinch test and growth-associated protein 43 expression. The MMPi promoted endoneurial BrdU incorporation relative to vehicle control. The dividing cells were mainly SCs and were associated with growth-associated protein 43–positive regenerating axons. After MMP inhibition, myelin basic protein mRNA expression (determined by Taqman real-time quantitative polymerase chain reaction) and active mitosis of myelin-forming SCs were reduced, indicating that MMPs may suppress their dedifferentiation preceding mitosis. Intrasciatic injection of mitomycin, the inhibitor of SC mitosis, suppressed nerve regrowth, which was reversed by MMPi, suggesting that its effect on axonal growth promotion depends on its promitogenic action in SCs. These studies establish novel roles for MMPs in peripheral nerve repair via control of SC mitosis, differentiation, and myelin protein mRNA expression.

Key Words: Axonal regeneration, Matrix metalloproteinases, Myelin, Myelin basic protein, Nerve growth, Schwann cell.

INTRODUCTION

When damaged peripheral nerve fibers undergo Wallerian degeneration (WD) (i.e. axonal degeneration, myelin breakdown and removal, facilitated by infiltrating macrophages distal to the site of injury [1]), they leave behind dedifferentiating Schwann cells (SCs) inside basal lamina tubes to reenter the cell cycle (2). Schwann cell proliferation ensures the formation and survival of sufficient SC numbers to support axonal regeneration (3). The fate of postmitotic SCs is determined by the phenotype of axons with which they associate during the process of radial sorting (4); myelin-forming SCs (mSCs) pair with axons designated to myelinate at a 1:1 ratio, whereas non-mSCs (nmSCs) support multiple unmyelinated axons (up to 10 in humans). Continuous and dynamic axonal to SC signaling interactions are involved in molecular regulation of SC mitosis, which are induced in WD through the action of macrophage-released mitogens (5) and myelin degradation products (3, 6).

The onset of axonal growth manifests within hours of peripheral nerve injury as regenerating sprouts grow down the endoneurial tubes (2). Axon growth cones interface closely with the SC membrane and basal lamina on either side, providing both an adhesive substratum and a plethora of molecular guidance cues (4). The success of nerve regeneration after rat sciatic nerve crush injury is caused in part by preserved integrity of SC basal lamina and endoneurial tubes (2). Because sciatic nerve crush injury is characterized by a 30-hour lag before the axons enter the degenerating distal stump (2, 7), identifying modulators of WD, SCs, and their basal lamina function during this lag period may lead to the development of new therapeutic strategies for stimulating nerve regeneration in this critical early period. In a model of nerve regeneration that occurs independent of WD, SCs initiate axonal guidance in part by deposition of laminin, a growth-promoting component of SC basal lamina (8) and a substrate of matrix metalloproteinases (MMPs) (9).

Matrix metalloproteinases are members of an extracellular protease family of collagenases, gelatinases, stromelysins, and membrane-type MMPs (9) that have emerged as early modulators of WD, SC function, and SC basal lamina remodeling (10–13). Matrix metalloproteinase 9 (gelatinase B) is among the earliest MMPs to respond to peripheral nerve damage; it is expressed in SCs within 1 hour and peaks at 24 hours after injury and promotes axonal degeneration, macrophage infiltration, and myelin degradation in damaged nerves (14–17). We have recently reported that robust MMP-9 induction within 24 hours occurs in both degenerating (distal)

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and regenerating (proximal) nerve segments of axotomized sciatic nerves (15), suggesting a potential role for MMP-9 in the early events of peripheral nerve regeneration. Proximal stumps of axotomized nerves of MMP-9–knockout mice display an increase in 5-bromo-2-deoxyuridine (BrdU) incorporation in SCs, indicating that MMP-9 suppresses SC mitosis during nerve regeneration (15). In primary cultured SCs, MMP-9 inhibits mitosis via ErbB-mediated activation of the extracellular receptor kinase (ERK) mitogen-activated protein kinase pathway. Furthermore, treatment with the MMP inhibitor (MMPi), GM6001, reverses the effect of MMP-9 and further promotes SC proliferation (15). Although GM6001 treatment stimulates dorsal root ganglion (DRG) neurite outgrowth in vitro (18), the effects of MMPi therapy on SC mitosis or nerve regeneration in vivo are unidentified.

The present study determined the effects of MMPi treatment initiated immediately after rat sciatic nerve crush injury. Overall, the data indicated that MMPs are potent modulators of SC proliferation, differentiation, and myelin protein expression after peripheral nerve injury and therefore are potential therapeutic targets in the early postinjury period.

MATERIALS AND METHODS

Animals and Surgeries

Adult female Sprague-Dawley rats (200–225 g, n = 102) were obtained from Harlan Labs (San Diego, CA). Animals were housed at 22°C under a 12-hour light/dark cycle with ad libitum access to food and water. Anesthesia was achieved using 4% isoflurane (Baxter, Deerfield, IL) in 55% oxygen by inhalation, or rodent anesthesia cocktail of Nembutal (50 mg/mL; Abbott Labs, North Chicago, IL), diazepam (5 mg/mL; Steris Labs, Phoenix, AZ), and saline (0.9%; Steris Labs) by intraperitoneal (i.p.) injection. Sciatic nerves were exposed unilaterally at the midthigh level and crushed using fine forceps twice for 5 seconds each. Animals were killed using an overdose of injection of rodent anesthesia cocktail (previously specified) i.p., followed by lethal intracardiac injection of Euthasol (100–150 mg/kg; Virbac, Fort Worth, TX). Nerve sections proximal and distal to crush were collected for analysis. Sham operations included unilateral sciatic nerve exposure. All procedures were performed according to National Institutes of Health Guidelines for Animal Use and protocols approved by the San Diego VA Healthcare System Institutional Animal Care and Use Committee.

MMPi and Mitomycin Treatments

The broad-spectrum MMPi GM6001 (ilomastat or *N*-[(2R)-2-(hydroxamido carbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide; Chemicon, Temecula, CA) has a reported Ki of 0.4 nmol/L for MMP-1, 27 nmol/L for MMP-3, 0.5 nmol/L for MMP-2, 0.1 nmol/L for MMP-8, and 0.2 nmol/L for MMP-9. GM6001 and vehicle (dimethyl sulfoxide in ethanol) were prepared according to the manufacturer's instructions and administered at 10 mg/kg per day by i.p. injection, as shown effective in other models of neuronal damage (14, 19–22). The MMPi and vehicle were administered immediately after sham or nerve crush surgery and once daily for 5 days thereafter. Mitomycin (Sigma, St Louis, MO; at 10 μg in 6 μL of Ringer's lactate solution [Sigma]) was administered by intrasciatic injection using a 33-gauge needle once immediately before nerve crush surgery to inhibit endoneurial cell mitosis (23–25).

Nerve Pinch Test

The rate of axonal regeneration was evaluated using nerve pinch test (7, 26–28), as illustrated in Figure 1A. Based on the anticipated speed of sciatic nerve regrowth, the test was performed between 3 and 7 days after nerve crush (7), although it can be successful as early as 2 days after crush (26). The sciatic nerve and its tibial nerve branch were exposed in lightly anesthetized rats. Consecutive 1-mm-long segments of the tibial nerve were pinched with a pair of fine forceps starting from the distal end of the nerve and proceeding in the proximal direction until a reflex response, consisting of a contraction of the muscles of the back, was observed. The distance between the most distal point of the nerve that produced a reflex withdrawal response and the stitch marking the original crush site was measured under a dissecting microscope and identified as the *regeneration distance*. The test was performed by an experimenter blinded to the experimental groups of 4 to 6 per group. Animals were killed as previously described, and sciatic nerves were isolated for Western blotting.

Western Blotting

Three-millimeter-long sciatic nerve segments (Fig. 1C) were collected in n = 4/group, snap frozen in liquid N_2, and stored at −80°C. Protein extraction was performed in lysis buffer consisting of 50 mmol/L Tris-HCl, pH 7.4, 1% NP 40, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin and leupeptin, and 1 mmol/L sodium orthovanadate. Equal amounts (30 μg) of protein, determined with BCA Protein Assay (Pierce, Rockford, IL), were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using Laemmli buffer system and transferred to nitrocellulose membranes using an iBlot dry blotting system (Invitrogen, Carlsbad, CA) at 20 V for 7 minutes. The membranes were blocked with 5% nonfat milk (Bio-Rad Laboratories, Hercules, CA), incubated with rabbit anti–growth-associated protein 43 (anti–GAP-43) or mouse anti–β-actin antibody (see later) in 5% bovine serum albumin overnight at 4°C, washed in Tris-buffered saline containing 0.05% Tween, and incubated for 1 hour at room temperature (RT) with horseradish peroxidase–conjugated anti-rabbit or anti-mouse secondary antibody (1:5000; Cell Signaling Technology, Danvers, MA). The blots were developed using enhanced chemiluminescence (ECL Elite; Amersham Biosciences, Pittsburgh, PA). Densitometry of mean GAP-43–to–β-actin ratios was performed in each group (n = 4) using National Institutes of Health ImageJ 1.38 software.

In Vivo BrdU Labeling and Detection

Endoneurial BrdU incorporation was studied as described (15, 29). Rats (n = 5/group) received i.p. BrdU (100 mg/kg; Calbiochem, San Diego, CA) or vehicle (1 mmol/L Tris, 0.8% NaCl, 0.25 mmol/L EDTA, pH 7.4)

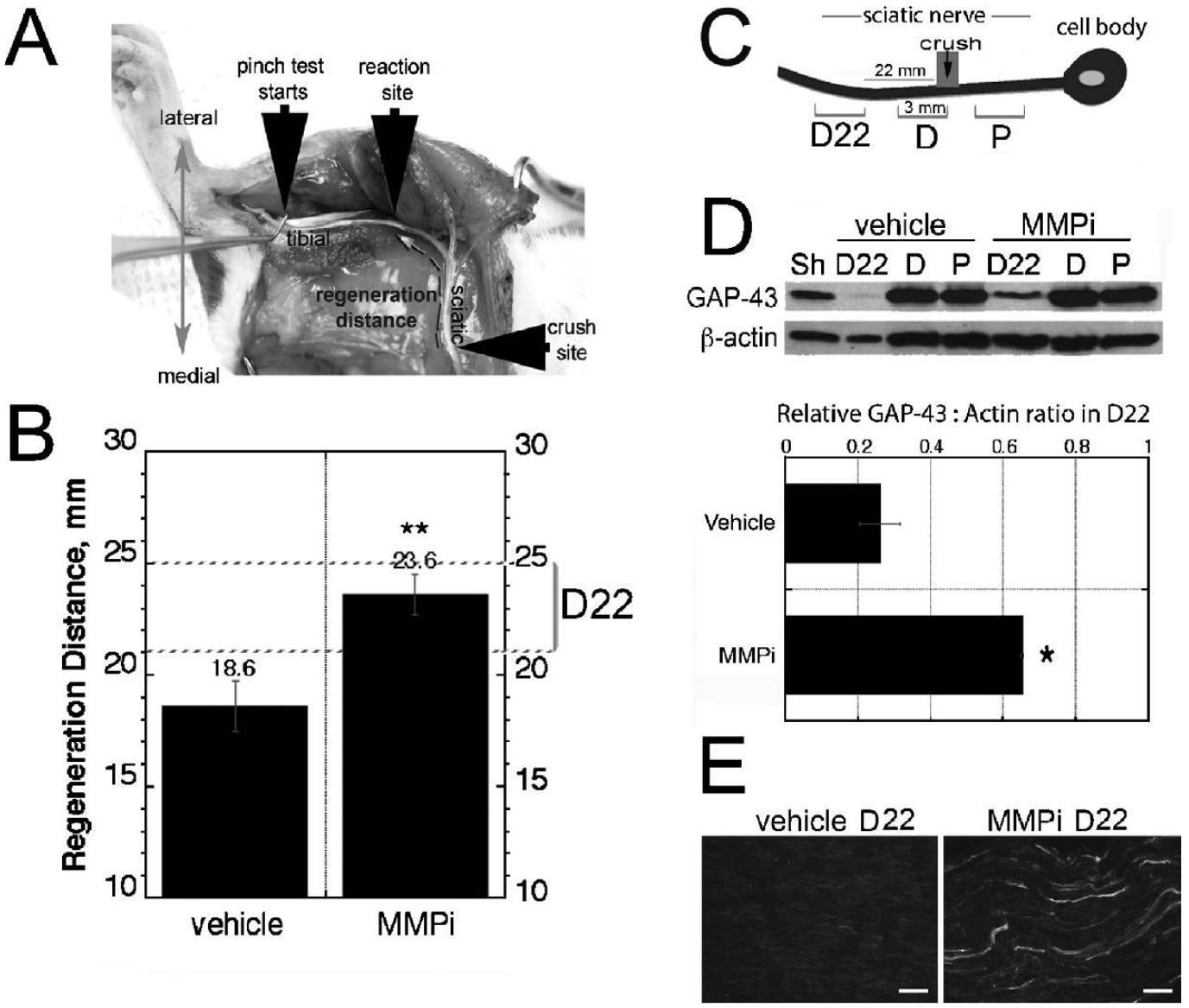

FIGURE 1. Matrix metalloproteinase inhibitor (MMPi) GM6001 treatment promotes peripheral nerve regeneration. **(A)** Nerve pinch test in a rat viewed from the dorsal plane. The exposed sciatic nerve and its tibial branch are pinched with forceps in 1-mm-long consecutive segments starting from the distal end of the tibial nerve and proceeding in the proximal direction until a reflex response is observed (reaction site). The distance between this site and the crush site was identified as the *regeneration distance* (broken arrow). **(B)** Nerve pinch test after immediate GM6001 therapy in crushed sciatic nerve compared with vehicle, expressed as mean regeneration distance (mm) ± SD (n = 6–8/group, ** $p < 0.01$). **(C)** Schematic representation of the analyzed 3-mm crushed nerve segments (not to scale), immediately proximal (P), immediately distal (D), and 22 to 25 mm distal (D22) to the crush site. **(D)** Western blot for growth-associated protein 43 (GAP-43) in the segments described in **(C)**. A representative of 4 blots is shown. The graph represents the mean of GAP-43–to–β-actin ratios in D22 segments in n = 4/group ± SEM, * $p < 0.05$. Sham-operated (Sh) nerves are used as controls. **(E)** The GAP-43 immunofluorescence in longitudinal D22 segments after vehicle or MMPi therapy, representative micrograph of n = 3/group. Scale bars = 25 μm.

injection 2 and 4 days after sham or sciatic nerve crush surgery and MMPi or vehicle treatment. In a separate set of experiments, rats (n = 4/group) received intrasciatic mitomycin, as previously described, followed by nerve crush surgery and MMPi or vehicle. At 5 days after nerve crush, animals were anesthetized and perfused with 4% paraformaldehyde and killed, as previously described. Sciatic nerves were isolated, postfixed in 4% paraformaldehyde overnight, rinsed, cryoprotected in graded sucrose, embedded into OCT compound in liquid N_2, and cut into 10-μm-thick longitudinal sections. The sections were rinsed in PBS, hydrolyzed in 2N HCl in PBS for 30 minutes, digested with 0.01% trypsin for 30 minutes at 37°C, and washed with PBS. Nonspecific binding was blocked with 10% normal goat serum, followed by mouse or rat anti-BrdU antibody (see later) for 2 hours at 37°C, PBS rinse, and goat anti-mouse Alexa 564 (red)

antibody (Invitrogen) for 1 hour at RT. Imaging was performed using a Leica DMR fluorescent microscope.

Antibodies

For immunofluorescence and Western blotting, the following antibodies were used: polyclonal rabbit anti-rat GAP-43 (1:1000; Chemicon), mouse anti–β-actin (1:10,000; Sigma), polyclonal anti-S100 (1:400; Dako, Carpinteria, CA), polyclonal rabbit anti-CD68 (1:100; ED1 subclone, Abcam, Cambridge, MA), polyclonal rabbit anti-von Willebrand factor (1:1000; Abcam), rabbit anti-glial fibrillary acidic protein ([GFAP] 1:500; Dako), rabbit anti-myelin protein zero (1:150; P0, Protein Tech Group, IL), mouse anti-BrdU (1:1000; Sigma), and rat anti-BrdU antibody (1:250; Abcam). All primary antibodies were diluted in Tris-buffered saline containing 1% bovine serum albumin.

Immunofluorescence and Quantitative Morphometry

Cryoprotected and OCT-embedded sciatic nerve sections were rehydrated in graded ethanol and PBS. Five percent sodium borohydride in 1% dibasic sodium phosphate buffer was applied for 5 minutes to block endogenous aldehyde groups. Antigen Retrieval Solution (Dako) was applied for 5 minutes at 95°C, then for 20 minutes at RT. Nonspecific binding was blocked with 10% normal rabbit or goat serum followed by primary antibody (as previously described) incubation overnight at 4°C. After BrdU labeling, 10% rabbit or goat serum was reapplied, followed by the second primary antibody for 1 hour at RT and a Tris-buffered saline rinse containing 0.1% Tween 20 and second secondary antibody for 1 hour at RT or overnight at 4°C. For secondary antibodies, goat anti-rabbit or anti-mouse antibody conjugated to Alexa 564 (red) or Alexa 488 (green) were used from Molecular Probes (1:200; Invitrogen). Tris-buffered saline was used for all washes. Sections were mounted using Slowfade gold antifade reagent (Molecular Probes). Signal specificity was controlled by omitting the primary antibody or replacing it with the appropriate normal immunoglobulin G. Imaging was performed using a Leica DMR fluorescent microscope and Openlab 4.0 software (Improvision, Inc, Waltham, MA).

Immunoreactive cell profiles for BrdU (red), CD68-positive macrophages (green), or for colocalization (yellow) of BrdU with CD68 or with S100 were quantified in 4 animals per group, 3 sections per animal, 4 randomly selected areas per section in 10-mm longitudinal distal and proximal sciatic nerve segments, using Openlab 4.0 software (Improvision). All quantifications were done at 40× objective magnification by an experimenter blinded to the experimental groups.

Real-Time Quantitative Polymerase Chain Reaction

Taqman primers and probes for rat myelin basic protein ([MBP] NM_017026.2), P0 (NM_017027.1), and GFAP (NM_017009.2) were obtained from Applied Biosystems Inc (Foster City, CA). Distal and proximal crush nerve segments were collected and stored in RNA-later (Ambion, Austin, TX) at −20°C. RNA was extracted with Trizol (Invitrogen) and treated with RNAse-free DNAse (Qiagen, Valencia, CA). The RNA purity was verified by OD260/280 absorption ratio of approximately 2.0. A SuperScript first-strand real-time quantitative polymerase chain reaction (RT-qPCR) kit (Invitrogen) was used to synthesize cDNA. Gene expression was measured by RT-qPCR (MX4000; Stratagene, La Jolla, CA) using 50 ng of cDNA and 2× Taqman Universal PCR Master Mix (Applied Biosystems) with a 1-step program (95°C for 10 minutes, 95°C for 30 seconds, and 60°C for 1 minute for 50 cycles). Duplicate samples without cDNA (no-template control) showed no contaminating DNA. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as a normalizer gene. Relative mRNA levels were quantified in 4 to 8 animals per group and calibrated to sham-operated nerves using the comparative Ct method (30). A fold change was determined by the MX4000 software as described (31).

Statistical Analyses

Statistical analyses were performed using KaleidaGraph 4.03 or SPSS 16.0 software by unpaired Student *t*-test or 1-way analysis of variance for repeated measures, followed by Tukey-Kramer post hoc test. Values of $p < 0.05$ were considered significant.

RESULTS

Because spatiotemporal activity of various MMP family members spans from hours to days after sciatic nerve damage (11, 12, 14, 16, 17), the specific broad-spectrum MMPi GM6001 treatment was administered immediately after sciatic nerve crush and daily thereafter for the duration of the study.

MMP Inhibition Enhances the Rate of Axonal Regeneration

Nerve pinch test determines the extent of axonal regrowth by measuring the distance between crush injury and the most distal point on the nerve that produces a reflex withdrawal response when pinched with forceps (7, 26, 27) (Fig. 1A). Based on the anticipated speed of sciatic regrowth, the test was performed between 3 and 7 days after nerve crush (7). Nerve pinch test demonstrated the significant increase in regeneration distance after MMPi treatment (23.6 ± 0.87 mm) compared with vehicle (18.6 ± 1.12 mm) at 5 days after crush (Fig. 1B).

Growth-associated protein 43, an axonally transported phosphoprotein found on the inside membrane of regenerating axons (2), was quantified by Western blotting and immunofluorescence in the following 3-mm nerve segments: immediately proximal (P), immediately distal (D), and 22 to 25 mm distal (or D22) to the crush site (Fig. 1C). The D22 segments were selected for MMPi- and vehicle-treated groups based on the anticipated difference in the content of regenerating fibers. No significant difference in GAP-43 levels was observed between the treatments in the segments immediately distal and proximal to crush injury (Fig. 1D), consistent with the conclusion that MMPi is not likely to

affect the number of regenerative fibers or their collateral branches. A statistically significant increase in GAP-43 expression was seen in the D22 segments of MMPi-treated nerves compared with that of vehicle (Figs. 1D, E), corresponding to their accelerated rate of regeneration determined by pinch testing.

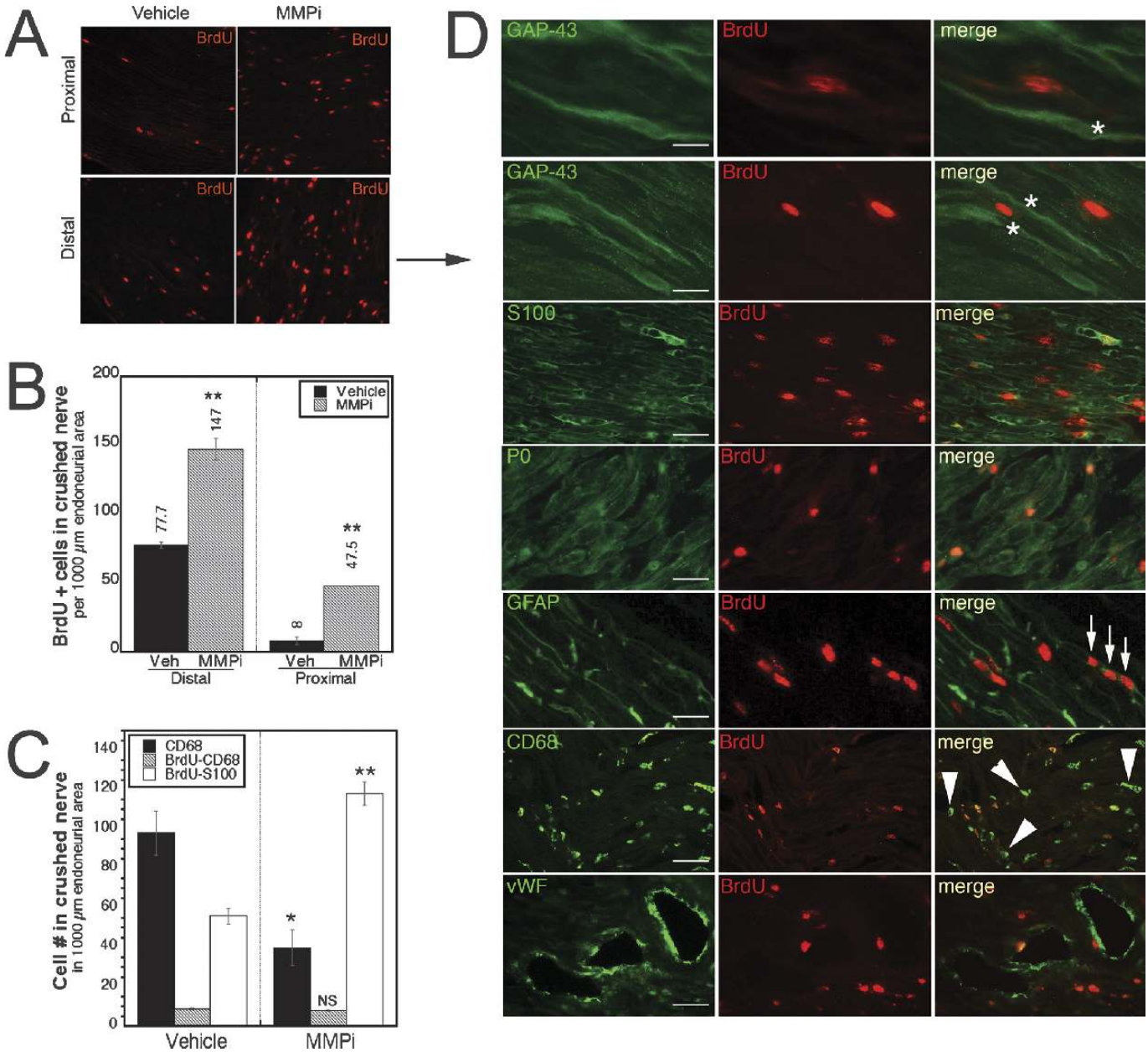

FIGURE 2. Matrix metalloproteinase inhibitor (MMPi) GM6001 treatment promotes Schwann cell (SC) mitosis in regenerating nerves. **(A)** Bromo-2-deoxyuridine (BrdU) incorporation (red) in distal and proximal crushed sciatic nerves after immediate GM6001 or vehicle (Veh) treatment. BrdU was administered at 2 and 4 days after crush. **(B)** Quantitative morphometry of the mean BrdU-positive cells ± SEM. **(C)** Mean CD68-positive, CD68/BrdU-positive, or S100/BrdU-positive profiles ± SEM in 1,000 μm^2 of endoneurial area. Panels **(B)** and **(C)** were based on n = 4/group, 2 sections/n, 3 randomly selected areas per section at 400× magnification. *$p < 0.05$, **$p < 0.01$ (1-way analysis of variance, Tukey-Kramer post hoc test). **(D)** Dual immunofluorescence for BrdU (red) with phenotypic cell markers (green) in distal segments of MMPi-treated nerves. Dividing cells are identified in association with regenerating axons (growth-associated protein 43 [GAP-43] asterisks), SCs (S100), myelinating SCs (mSCs) (P0) and dedifferentiating/non-mSCs (glial fibrillary acidic protein [GFAP]), macrophages (CD68), and endothelial cells (von Willebrand factor [vWF]). Arrows indicate 3 mitotic cells in a row. Arrowheads point to macrophages/monocytes that have not taken up BrdU. Representative micrographs of n = 4/group. Scale bars = 40 μm (P0, CD68, vWF); 25 μm (GAP-43 [top micrograph], S100, GFAP); 10 μm (GAP-43 [second micrograph]).

Increased Incidence of Mitotic SCs After MMP Inhibition

The BrdU-positive profiles were significantly greater after MMPi compared with those of vehicle treatment in both distal and proximal nerve segments (Figs. 2A, B). The dividing cells were identified by colocalization of BrdU with GAP-43 for regenerating axons (26), S100 protein for SCs of all phenotypes, GFAP for dedifferentiating and nmSCs, P0 for mSCs (4) (both nmSCs and mSCs undergo mitosis after sciatic nerve injury [3, 32]), and CD68 for macrophages and von Willebrand factor for endothelial cells that undergo mitosis during revascularization (33). Many BrdU-positive mitotic cells were associated with GAP-43–positive regenerating axons (Fig. 2D). The dividing cells were identified as S100-, P0-, and GFAP-positive SCs. Occasional endothelial cells and macrophages colocalized with BrdU. As indicated by the arrowheads in Figure 2D, many CD68-positive cells were not BrdU positive. Quantification of BrdU-positive SCs and macrophages indicated a substantial increase of dividing SCs but not macrophages in MMPi-treated compared with vehicle-treated nerves (Fig. 2C). It is important to note that total counts of CD68-positive macrophages were reduced with MMPi treatment (Fig. 2C), consistent with earlier reports (14, 17, 34).

MMP Inhibition Promotes Dedifferentiation of mSCs

Sciatic nerve injury stimulates dedifferentiation of mSCs before mitosis (3, 32). Because many P0-reactive mSCs were BrdU positive in MMPi-treated nerves, we analyzed the effect of MMPi therapy on their dedifferentiation. After vehicle treatment, MBP and P0 mRNA levels sharply declined in distal and only slightly in proximal nerve segments, consistent with the reported observations at 5 days of sciatic nerve injury (35, 36) (Fig. 3). Increased GFAP mRNA was observed distal and proximal to injury, as anticipated from the earlier reports (3, 32). Matrix metalloproteinase inhibitor therapy further reduced MBP mRNA only in the proximal segment, producing no change in P0 or GFAP mRNA levels in either segment at the analyzed time point. These data indicate that MMPs regulate MBP mRNA expression during peripheral nerve injury in vivo in addition to their control of MBP proteolysis (14, 37).

Reversal of the Effect of Antimitogenic Mitomycin in Inhibition of Nerve Regrowth

Intrasciatic mitomycin treatment was used to inhibit endoneurial cell proliferation (23–25) (Fig. 4A). As anticipated, intrasciatic mitomycin significantly reduced the numbers of BrdU-positive cells in the distal segment of crushed nerve relative to Ringer's lactate solution control (Fig. 4B). Daily MMPi treatment reversed this action of mitomycin, indicating an increase in BrdU-reactive cells compared with mitomycin alone (Fig. 4B). Mitomycin drastically reduced the regeneration distance (3.75 ± 0.75 mm) compared with the control (12.2 ± 0.75 mm), and MMPi treatment administered immediately after injury reversed this mitomycin-induced suppression of nerve regrowth relative to vehicle (9.8 ± 1.0 mm) (Fig. 4C).

DISCUSSION

Peripheral nerve regeneration relies on SCs to undergo rapid proliferation between 2 and 4 days after injury and to produce a sufficient number of cells that can pair with and provide molecular guidance to growing axons. We assessed whether MMP inhibition, which promotes SC proliferation in vitro (15), contributes to peripheral nerve regeneration in vivo. We show that systemic daily i.p. MMPi treatment for 5 days initiated immediately after rat sciatic nerve crush (*a*) enhanced the rate of nerve fiber regrowth by promoting SC mitosis; (*b*) reversed the action of antimitogenic mitomycin to inhibit axonal regeneration, indicating that promitotic

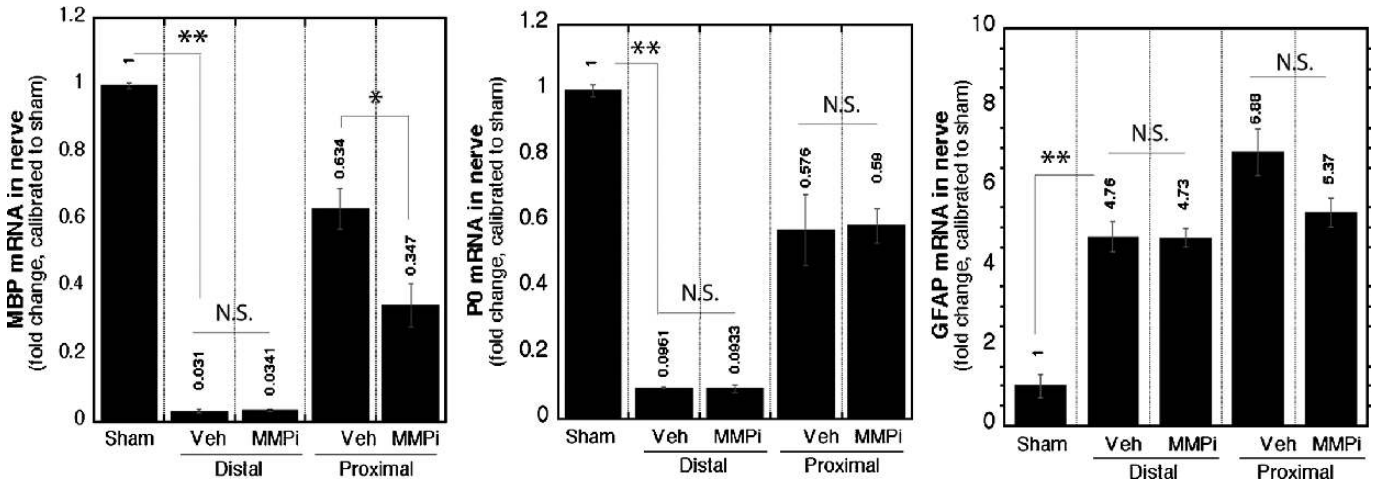

FIGURE 3. Matrix metalloproteinases (MMPs) control myelin basic protein (MBP) mRNA expression in regenerating nerves. Real-time Taqman real-time quantitative polymerase chain reaction for MBP, P0, and glial fibrillary acidic protein (GFAP) in crushed sciatic nerves after immediate MMP inhibitor ([MMPi] GM6001) or vehicle (Veh) treatment normalized to glyceraldehyde 3-phosphate dehydrogenase and calibrated to sham. The mean ± SEM of n = 4 to 6/group; *$p < 0.05$, **$p < 0.01$ (1-way analysis of variance and Tukey-Kramer post hoc test). N.S., not significant.

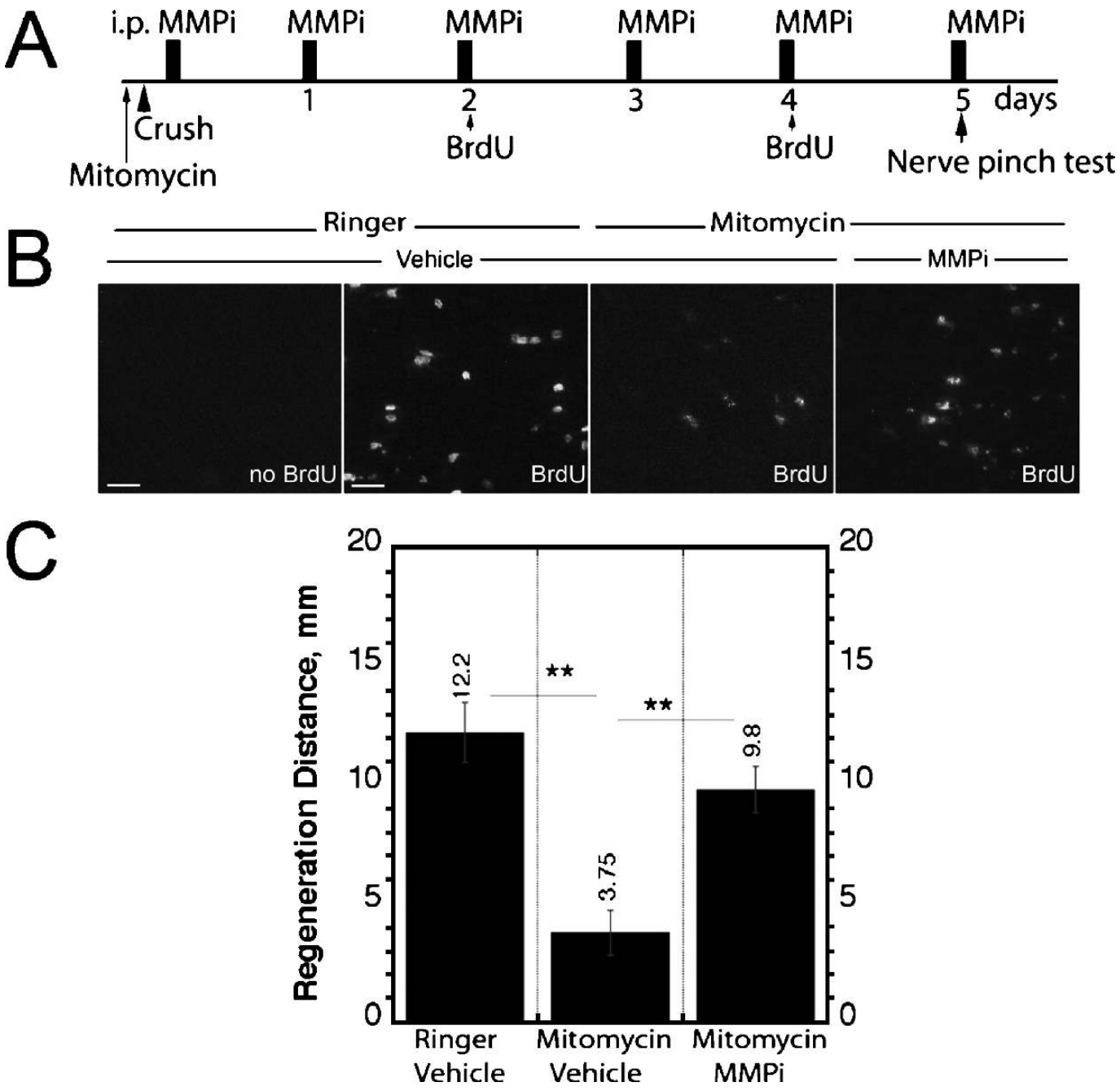

FIGURE 4. Matrix metalloproteinase inhibition (MMPi) reverses antimitotic and growth-inhibitory actions of mitomycin in vivo. **(A)** Experimental schedule for intrasciatic mitomycin administration immediately before sciatic nerve crush surgery, followed by daily experimental intraperitoneal (i.p.) GM6001 therapy, bromo-2-deoxyuridine (BrdU) injection (at Day 2 and Day 4) and nerve pinch test at Day 5 after crush injury. **(B)** BrdU incorporation in distal crushed nerve segments that received BrdU, mitomycin, MMPi, or respective vehicle treatments. Representative micrographs of n = 4/group. Scale bars = 25 μm. **(C)** Nerve pinch test after intrasciatic mitomycin and immediate and daily MMPi or respective vehicle treatments. The mean regeneration distance (mm) ± SEM of n = 4/group, analyzed by 1-way analysis of variance (**$p < 0.01$).

MMPi action contributes to its growth-promoting ability, and (*c*) contributed to SC dedifferentiation via selective inhibition of MBP mRNA expression in vivo. The latter finding adds to the established role of MMPs in myelin proteolysis (14, 37, 38) and regulation of ErbB and other trophic signaling pathways in SCs (15, 39).

Early Events of Peripheral Nerve Injury Under MMP Control

The enhanced sensory nerve fiber regrowth after GM6001 therapy established by nerve pinch test in vivo is consistent with its reported promotion of DRG neurite outgrowth in vitro (18). The effect of MMPi on regeneration

of motor fibers that do not elicit a withdrawal reflex during nerve pinch test (26), however, remains to be determined. Because GM6001 therapy facilitates motor recovery after spinal cord injury (21), this is a likely action in injured peripheral nerve.

After spinal cord injury, immediate GM6001 treatment inhibits vascular permeability and neuroinflammation associated with early peak of MMP-9, whereas extended treatment was not effective because of MMP-2 action in wound healing (21, 40). Likewise, MMPs act differentially in peripheral nerve, and distinct mechanisms of MMP induction may relate to their differential actions in nerve repair. For example, predegeneration of cultured sciatic nerve explants with MMP-2, but not MMP-9, is beneficial to growth of DRG neurons by degradation of inhibitory chondroitin sulfate proteoglycan (41, 42). For example, MMP-9, but not MMP-2, is upregulated immediately after peripheral nerve injury (11, 14, 16, 17), and its expression in SCs is induced by proinflammatory (tumor necrosis factor, lipopolysaccharide) but not trophic (e.g. nerve growth factor, neuregulin 1 stimuli in vitro (15). Early inhibition of neuroimmune activity by anti–tumor necrosis factor therapy enhances the rate of sciatic nerve regeneration (28).

The promotion of axonal growth by MMPi may relate to protection from MMP-9–mediated neuroinflammation and macrophage infiltration (14, 17, 34). Indeed, macrophages can suppress dystrophic DRG neurite outgrowth by physical attack and retraction of axonal sprouts (43); inhibition of MMP-9, but not of MMP-2, results in resumption of neurite growth by reversing this macrophage action (44). After MMPi treatment, reduced numbers of macrophages (even in the absence of its effect on CD68-positive cell mitosis) is thought to be caused by the ability of MMPi to prevent breakdown of the blood-nerve barrier (11, 17, 34). Minocycline, an emerging neuroprotective tetracycline (45) and nonspecific MMPi (46), reduces MMP-9 expression in injured nerves and improves nerve regeneration by its antiischemic effect on SCs despite impaired revascularization (33). Mitosis of endothelial cells indicate ongoing revascularization in MMPi-inhibited nerves, so the potentially negative effects of GM6001 on angiogenesis (47) during nerve repair deserve future consideration. Overall, establishing the spatiotemporal activities and specific roles of MMP family members is essential for the development of successful selective MMPi therapies to stimulate nerve repair.

MMPs and Regulation of SC Signaling and Function

The MMPi-mediated increases in SC mitosis in vivo are consistent with earlier findings in cultured SCs (15, 48). We found that the numbers of BrdU-positive cells were substantially higher in the distal nerve stump where SC proliferation occurs and spread only a few millimeters proximal to a lesion (49). Nevertheless, MMPi was relatively more effective in promoting cell mitosis in proximal (5-fold) compared with distal (2-fold) stumps. Matrix metalloproteinases control proteolysis of myelin proteins (18, 37), and the lesser numbers of mitotic cells in the distal stumps of MMPi-treated nerves might be caused by the reduced content of promitogenic myelin degradation products and macrophages (14). An increased number of SCs in the proximal stump may suggest that more collateral branching exists after MMPi treatment. Inasmuch as the nerve pinch test measures the regeneration distance after injury, we conclude that MMPi promotes the rate of axonal regrowth rather than the intensity of axonal branching. This conclusion is supported by the finding that GAP-43 expression was elevated only in the distal D22 segments of the MMPi-treated nerves, corresponding to their enhanced regeneration distance.

Matrix metalloproteinases may stimulate SC migration during myelin formation of DRG neurons (50). Because during nerve regeneration SCs typically migrate from the injury site in the retrograde direction toward the proximal stump (49), the proximal accumulation of mitotic SCs in MMPi-treated and MMP-9–null nerves (15) may represent a compensatory response to the reduced SC migration. Migrating SCs guide axons by deposition of growth-promoting laminin (8), which suggests that MMPi may continue to support neurite outgrowth by protecting laminin from degradation (51), despite its potentially negative regulation of SC migration (52). Matrix metalloproteinase inhibitors can also stimulate growth of DRG neurons by preventing the formation of growth-inhibitory fragments of myelin-associated glycoprotein (18).

Matrix metalloproteinases control a plethora of signal transduction pathways through proteolytic cell surface and extracellular ligand or receptor processing (51, 53), but understanding of their mechanisms of action in SCs is only starting to emerge. Matrix metalloproteinase 3 suppresses SC mitosis through the formation of antimitogenic fibronectin fragments (48); MMP-9 and MMP-28 can stimulate ERK mitogen-activated protein kinase signaling in SCs by activation of the NRG-1/ErbB ligand receptor system (15, 39), and ErbB inhibitor pretreatment reverses MMP-9–mediated suppression of SC mitosis (15). In the CNS, MMP-12 and MMP-9 regulate myelination through release of insulin-like growth factor 1 from IGFBP6 binding protein (54), and MMP-9 can use the insulin-like growth factor 1 receptor to activate SC ERK signaling (15). Both catalytically active MMP-9 and its catalytically inept substrate-binding hemopexin domain (MMP-9-PEX) activate ERK in SCs by low-density lipoprotein receptor–related protein 1 receptor agonist action (52). Because the MMP-9 hemopexin domain also acts as an antagonist to endogenous MMP-9 (55), cautious interpretation of the action of the MMP-9 hemopexin domain in MMP-9–rich environments of injured nerve and denervated SCs is warranted. Matrix metalloproteinase 9 induces 2 distinct phases of ERK activation in both differentiated and undifferentiated SCs (15). Thus, the functional outcome of MMP-mediated ERK activation may depend on SC phenotype or microenvironment. For example, MMP-28 is thought to use NRG-1/ErbB-ERK signaling for myelination of DRG neurons (39).

Multifactorial Regulation of Myelination and Neuron Survival

Dedifferentiation of mSCs is a prerequisite for their division (4), as indicated by a characteristically sharp decline

in MBP and P0 mRNA in distal nerve with only a slight reduction in the proximal segments (Fig. 3) (35, 36). Matrix metalloproteinase inhibitors reduced MBP mRNA only in the proximal stump, where its level remained high or possibly specifically in association with regenerating nerve. Both MBP and P0 are late components of myelination (4), but MMPi influenced the expression of MBP, but not of P0. Similarly, MMPi protects MBP, but not P0, from proteolysis (14). Thus, it is possible that an MMPi-mediated decline in MBP mRNA is the result of accumulated levels of its protein; it may also reflect selective roles of MMPs in regulation of NRG-1/ErbB and other pathways (15) of SC-driven myelination (56).

The increase in GFAP in both distal and proximal stumps of injured nerve (Fig. 3) also indicates dedifferentiation of both mSCs and nmSCs (3, 32). Matrix metalloproteinase inhibitors had no effect on GFAP mRNA expression at 5 days after crush. The present study focused on the early events of nerve repair, but because MMPs contribute to the formation of CNS myelin (54), MMPi effects on redifferentiation of postmitotic SCs and axonal remyelination at later time points should be determined in the future. The increased numbers of mitotic SCs along regenerating fibers we observed may explain the formation of shorter myelin internodes of myelinating DRG neurons after MMPi treatment in vitro (50).

Matrix metalloproteinases promote neuronal apoptosis (19, 57, 58), including that of DRG neurons (14, 59). Thus, the effects of MMPi on neuronal survival or other effects of MMPs on neuronal soma during nerve repair also remain to be investigated in the future.

Overall, the present study provides the first evidence that immediate MMPi therapy supports axonal regeneration in vivo, and that it acts by stimulation of SC division. Whether or not MMP-mediated suppression of SC mitosis and dedifferentiation at the early stages of nerve damage leads to reduced survival of SCs, it does contribute to phenotypic remodeling of SCs, myelin protein expression, and proteolysis.

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