RESEARCH ARTICLE

Local inhibition of matrix metalloproteinases reduced M2 macrophage activity and impeded recovery in spinal cord transected rats after treatment with fibroblast growth factor-1 and nerve grafts

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Abstract

Alternatively activated macrophages (M2 macrophages) promote central nervous system regeneration. Our previous study demonstrated that treatment with peripheral nerve grafts and fibroblast growth factor-1 recruited more M2 macrophages and improved partial functional recovery in spinal cord transected rats. The migration of macrophages is matrix metalloproteinase (MMP) dependent. We used a general inhibitor of MMPs to influence macrophage migration, and we examined the migration of macrophage populations and changes in spinal function. Rat spinal cords were completely transected at T_8, and 5 mm of spinal cord was removed (group T). In group R, spinal cord-transected rats received treatment with fibroblast grow th factor-1 and peripheral nerve grafts. In group RG, rats received the same treatment as group R with the addition of 200 μM GM6001 (an MMP inhibitor) to the fibrin mix. We found that MMP-9, but not MMP-2, was upregulated in the graft area of rats in group R. Local application of the MMP inhibitor resulted in a reduction in the ratio of arginase-1 (M2 macrophage subset)/inducible nitric oxide synthase-postive cells. When the MMP inhibitor was applied at 8 weeks postoperation, the partial functional recovery observed in group R was lost. This effect was accompanied by a decrease in brain-derived neurotrophic factor levels in the nerve graft. These results suggested that the arginase-1 positive population in spinal cord transected rats is a migratory cell population rather than the phenotypic conversion of early $iNOS^+$ cells and that the migration of the arginase-1^+ population could be regulated locally. Simultaneous application of MMP inhibitors or promotion of MMP activity for spinal cord injury needs to be considered if the coadministered treatment involves M2 recruitment.

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Introduction

Nerve regeneration in the central nervous system (CNS) is difficult due to the excessive amount of inhibitory extracellular molecules and the weak neurite outgrowth of severed axons; however, recent progress has been made by enzymatic digestion of inhibitory molecules (Bartus et al., 2014), supplementation with stem cells, and promotion of neurite outgrowth (Ghannam et al., 2010). Macrophages are heterogeneous cell populations with distinct functions in tissue repair or injury. The polarization of macrophages occurs in response to the microenvironment *via* the expression of distinct functional programs. M1 (classically activated) macrophages can be induced by interferon-γ, lipopolysaccharide, tumor necrosis factor and granulocyte-macrophage colony-stimulating factor. M1 macrophages mediate resistance against bacterial infection, parasites, and tumor (Gordon, 2003; Mantovani et al., 2004). M2 (alternatively activated) macrophages can be induced by interleukin (IL)-4, IL-13, IL-10 and glucocorticoids (Franco and Fernandez-Suarez, 2015). Although M2 macrophages can be divided into subsets, they generally express high levels of mannose receptors, galactose receptors, and arginase-1 (Arg1) (Cherry et al., 2014). Arg1 converts arginine into ornithine and polyamines (Mantovani et al., 2013). Postinjury CNS repair is a process that involves the balanced action of M1/M2 macrophage populations (David and Kroner, 2011; Shechter and Schwartz, 2013). Following SCI, M1 macrophages produce high levels of nitric oxide (NO), inducible nitric oxide synthase (iNOS) and pro-inflammatory cytokines (such as IL-1β, tumor necrosis factor alpha and IL-6), which frequently cause tissue damage and axonal retrasction (Busch et al., 2009). In contrast, M2 macrophages secrete growth factors, such as glial cell-derived neurotrophic factor, brain-derived neurotrophic factor (BDNF), and oncomodulin, and are involved in tissue repair (Mantovani et al., 2013; Gensel and Zhang, 2015; Hu et al., 2015).

One treatment that uses peripheral nerve autografts to bridge a complete spinal cord lesion in combination with fibroblast growth factor (FGF)-1 leads to functional locomotor recovery in a complete spinal-transection rodent model

(Cheng et al., 1996). The Basso, Beattie and Bresnahan (BBB) score improved, and the detection of somatosensory-evoked potentials and motor-evoked potentials indicated that both sensory and motor information can cross the damaged site (Lee et al., 2002). The regeneration of axons through the graft and into the distal stump was demonstrated by retrograde labeling in the cortex and brain stem nuclei and by anterograde labeling in the distal stump (Cheng et al., 1996; Lee et al., 2004). Increased numbers of M2 macrophages were evident (Kuo et al., 2011), and the neurite inhibitory molecule chondroitin sulfate proteoglycan and glial reactive scar were also reduced in the treated rats (Lee et al., 2011). During the normal course of SCI, M1 macrophages sustain activities beyond the acute stage. M2 macrophages are present during the initial stage but are outnumbered by M1 macrophages at the injury site after the acute stage (Kigerl et al., 2009). Promoting an alternative macrophage phenotype may be a useful strategy for repairing a spinal cord injury. However, a method of consistently inducing a sustained, dominant M2 phenotype is presently unknown. Functional recovery in the aforementioned combination-treated rats suggests that the study of the origin and migration pathway of the M2 population may provide insight into how to harness the beneficial effects of M2 macrophages.

Following injury, the damaged tissue releases chemokines and attracts monocytes/microglia, which migrate to the lesioned area and become activated macrophages. As the migration of myeloid cells or inflammatory macrophages is matrix metalloproteinase (MMP) dependent (Gong et al., 2008; Letellier et al., 2010), in this study, we used a general MMP inhibitor to block the migration of macrophages to study the activities of macrophages for functional recovery observed in spinal cord-transected rats treated with fibroblast growth factor-1 and peripheral nerve grafts. MMP2 and MMP9 were reported to be regulated by factors that promote conversion of the M1 or M2 phenotype (Lolmede et al., 2009; Veeravalli et al., 2009; Huang et al., 2012). The levels of these two MMPs were examined in different treatment groups to determine whether there is an association with the M1 or M2 phenotype or with functional recovery.

Materials and Methods

Experimental animals

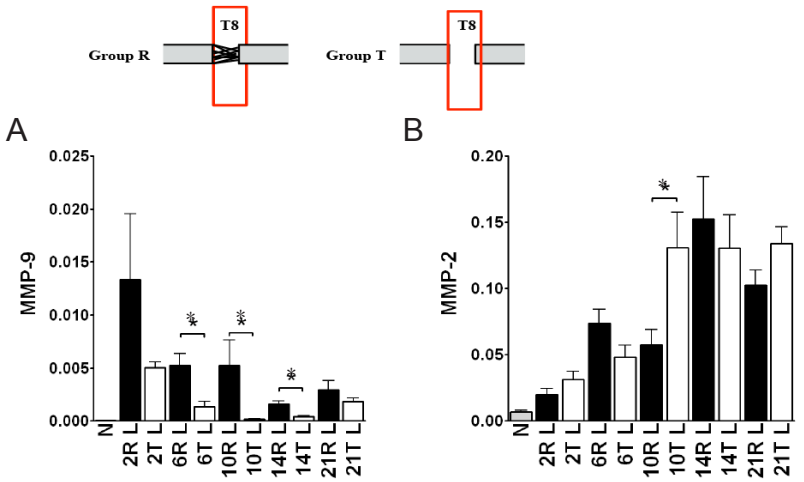
Young adult female Sprague-Dawley (SD) rats (250–280 g, ~8 weeks old) were used in this study. Surgical procedures, postoperative care, and monitoring were conducted as previously described (Kuo et al., 2007, 2011). The animal use protocol listed below was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Taipei Veterans General Hospital, China (IACUC approval number VGHTPE IACUC 2015-167). The rats were randomly separated to groups: N, T, R, RG, and U (see following). Group N is for rats those spinal cords were not transected. For group T spinal cords were completely transected at T_8, and 5 mm of spinal cord tissue was removed. In group R, spinal cord transected rats received a combined treatment with FGF-1 and a peripheral nerve graft that was developed by Cheng (Cheng et al., 1996). Briefly, autologous peripheral intercostal nerve segments combined with fibrin glue mixture (100 μL Hanks' balanced salt solution mixed with 10 μg fibrin glue and 1 μg FGF-1) (Beriplast P; Behringwerke Medical Inst., Marburg, Germany) was implanted to bridge a 5-mm gap in the transected spinal cord. In addition, to study the effects of MMP-2 and MMP-9 inhibition, GM6001 (an MMP inhibitor; MilliporeSigma) was mixed in the fibrin glue to a concentration of 200 μM in addition to FGF-1 (group RG). For quantitative real-time polymerase chain reaction (PCR) analysis, transection supplemented with glue (group U) was used as a control group for normalization. Manual emptying of the bladder was performed twice daily.

RNA isolation, reverse transcription, and quantitative real-time PCR analysis

On specified days following injury, rats were sacrificed with an overdose of anesthetic sodium pentobarbital intravenously (Health-Tech Pharmaceutical Co., Ltd., Taiwan, China). Then, 5 mm (length) of spinal cord surrounding the lesioned area was collected for RNA isolation, as shown in **Figure 1**. Total cellular RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. First-strand cDNA synthesis was primed with oligo dT and then reverse-transcribed using Superscript Reverse Transcriptase (Invitrogen) as previously described (Kuo et al., 2011). cDNA levels were quantified using MMP-2, MMP-9, Arg1, iNOS, and BDNF primer pairs (**Table 1**) with a QuantiTect SYBR Green PCR Kit (Qiagen) on a Light Cycler 480 (Roche Applied Science). Ribosomal protein L13A (RPL13A) was used as the reference gene in each set of reactions (Jesnowski et al., 2002; Tian et al., 2007). Melting curve analyses verified the formation of the single desired PCR product. Each sample was run in duplicate. The relative differences in expression between the groups were analyzed on the basis of cycle time values using the comparative threshold cycle (Ct) method (Livak and Schmittgen, 2001), in which the Ct value indicates the cycle in which there is the first demonstrable increase in SYBR Green fluorescence above the threshold. The target gene quantity was normalized to the reference gene using the formula: $2^{-(Ct(target)-Ct(reference))}$.

Immunofluorescence staining

At 10 days postinjury, rats received an overdose of sodium pentobarbital and were intravascularly perfused with 0.9% saline and 4% paraformaldehyde in phosphate buffered saline (PBS). The spinal cords were removed from the experimental animals, postfixed in 4% paraformaldehyde overnight, rinsed, cryoprotected in graded sucrose, embedded in an Tissue-Tek optimum cutting temperature compound (Sakura Fintek USA) at –20°C and longitudinally sectioned (20 μm thick) for staining. The primary antibodies included mouse anti-ED1 (1:250, Biorad, Hercules, CA, USA) and goat anti-Arg1 (1:100, Santa Cruz Biotechnology Inc, Dallas, TX, USA) at 4°C overnight, and nuclear staining was achieved *via* incubation with 1 μg/mL DAPI for 1 minute. The secondary antibodies were



The tissues from rats in group R (R) and group T (T) were collected at 2–21 days postoperation. In group T, rats underwent complete transection operation and T_8 spinal cord was removed. In group R, rats received treatment with fibroblast growth factor-1 and peripheral nerve grafts. The grey boxes in the small inset indicate the spinal cord, the lines indicated grafted nerves, and red boxes indicate area where tissues were collected. mRNA expression of MMP-2 and MMP-9 was analyzed using quantitative polymerase chain reaction. The Y axis is fold change relative to the gene expression of the non-transected group, the X axis indicates different treatment groups and days (*e.g.*, 2R means group R at 2 days postoperation). (A) Significant amount of MMP-9 was expressed at 2 days postoperation in group T and decreased afterwards. The expression of MMP-9 in group R was significantly higher than that in group T at 6 and 10 days postoperation. (B) MMP-2 was consistently increased following surgery in groups T and R until day 21 postoperation. Student's *t*-test was used for comparing T and R groups at the same day. No significant difference between the two groups was detected (mean ± SEM, $n = 4$, $*P < 0.05$).

Figure 1 Matrix metalloproteinase (MMP)-9 mRNA expression was upregulated in the graft area of the repaired spinal cord in rats.

Table 1 Sequences of quantitative polymerase chain reaction primer sets

Primers	Sequences (5' – 3')
MMP-2	F: TGT GTT CTT CGC AGG GAA TGA G
	R: AGG CTG GTC AGT GGC TTG G
MMP-9	F: TGT ATG GTC GTG GCT CTA AAC
	R: AAG GAT TGT CTA CTG GAG TCG
RPL13A	F: AGG TGG TGG TTG TAC GCT GTG
	R: GGT TGG TGT TCA TCC GCT TTC G
BDNF	F: ATT AGG TGG CTT CAT AGG AGA C
	R: ACT TTC TCC AGG ACT GTG AC
Arg1	F: TTG ATG TTG ATG GAC TGG AC
	R: TCT CTG GCT TAT GAT TAC CTT C
iNOS	F: AAG AGA CGC ACA GGC AGA G
	R: CAG GCA CAC GCA ATG ATG G

MMP: Matrix metalloproteinase; RPL13A: ribosomal protein L13A; BDNF: brain-derived neurotrophic factor; Arg1: arginase-1; iNOS: inducible nitric oxide synthase; F: forward; R: reverse.

donkey anti-mouse, and donkey anti-goat (1:250, Invitrogen, Carlsbad, CA, USA), incubated at 4°C for 1 hour. Photo images were obtained from stained slides of sections using an Axioscope fluorescence microscope and a CCD camera (Carl Zeiss AG, Oberkochen, Germany). The images were arranged in Adobe Photoshop (Adobe Systems Incorporated,San Jose, CA, USA). The immunoreactivities of Arg1 and ED1 were quantified in 3 sections from each rat using NIH ImageJ 1.44d software (software developed by National Institutes of Health, USA); three to four images were obtained through random sampling inside the graft area of each section. The threshold values of immunoreactivities were constant in all analyzed images.

Western blotting analysis

On days 10 and 14 post-injury, spinal cord segments (one-centimeter-long segments) were homogenized in lysis buffer containing 200 mM Tris-HCl (pH 6.8), 10% SDS, 5 mM ethylene glycol tetraacetic acid (EGTA), 5 mM EDTA, 10% glycerol, and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Lysates were centrifuged at 13,000 × *g* for 30 minutes. The supernatant fractions were collected for Western blotting analysis as previously described (Kuo et al., 2007). Proteins (20 μg/lane) were separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes. The membranes were blocked in PBS that contained 3% skim milk for 30 min. Each blot was incubated overnight at 4°C with a primary antibody against goat anti-β-actin (1:1000, Sigma), and goat anti-Arg1 (1:500, Santa Cruz Biotechnology Inc.). After washing in PBS, the membranes were incubated with donkey anti-goat IgG HRP (horseradish peroxidase)-conjugated secondary antibody (1:5000, Santa Cruz Biotechnology Inc) or goat anti-mouse IgG HRP-conjugated secondary antibody (1:5000, Santa Cruz Biotechnology Inc) for 2 hours at room temperature. Subsequent visualization was performed using SuperSignal Chemiluminescent Substrate (Pierce, Rockford, IL, USA).

In situ zymography

Rats received an overdose of sodium pentobarbital and were intravascularly perfused with 0.9% saline. The spinal cord samples were removed from the experimental animals, immediately embedded into OCT compound at –20°C and sectioned longitudinally on a cryostat (Leica Biosystems, Buffalo Grove, IL, USA). The sections were incubated in an in situ zymography reaction buffer consisting of 0.05 M Tris-HCl, 0.15 M NaCl, 5 mM $CaCl_2$, and 0.1 mM NaN_3 and 40 μg fluorescein-conjugated DQ gelatin (Molecular Probes, Eugene, Oregon, USA) at 37°C for 1 hour in the dark. The gelatin with a fluorescent tag remained caged until cleavage by MMP-2 or MMP-9. The reaction product was visualized using fluorescence microscopy (Carl Zeiss AG, Oberkochen, Germany).

Gelatin zymography

Rats received an overdose of sodium pentobarbital and were intravascularly perfused with 0.9% saline. One-centimeter spinal cord segments including grafts and caudal stumps of spinal cord were weighed and homogenized by sonication (1:10, w:v) in lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, and 0.1% sodium dodecyl sulfate (SDS). Soluble and insoluble extracts were separated by centrifugation, and the supernatant was stored at –20°C. Equal amounts of supernatant were separated using a 10% SDS gel with 0.1% gelatin (Invitrogen). After separation, the gel was renatured by washing in 2.5% Triton X-100 and incubating in substrate buffer (50 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, and 0.01% sodium azide) for 48 hours at 37°C to enable MMP-2 and MMP-9 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 indicated the locations of active protease bands. After exposure to sodium dodecyl sulfate during gel separation, the proenzymes that were present in the tissue extracts were activated without proteolytic cleavage.

Behavioral testing

The BBB (Basso, Bresnahan, and Beattie) open field score (Basso et al., 1995) was used to evaluate locomotion in terms of hindlimb functional improvement in rats with SCI. The BBB test was scored from 0 (no observable hindlimb movement) to 21 (normal hindlimb movement) points. In this study, behavioral analyses were conducted weekly for 8 weeks postoperation. The behavioral tests were recorded using a video camera and scored by two examiners who were blinded to each behavior evaluation group.

Statistical analysis

Data are expressed as mean ± SEM. Statistical comparisons were performed with GraphPad Prism software (version 7.0, GraphPad Software Inc., La Jolla, CA, USA) using a two-tailed Student's *t*-test for comparisons of two groups. For comparisons of three or more groups, one-way analysis of variance was performed, combined with Newman-Keuls multiple comparison tests for *post hoc* analysis. A P-value < 0.05 was used to indicate statistically significant differences between groups.

Results

MMP-2/9 was upregulated post-injury, and MMP-9 was further increased in the graft site

MMP-9 mRNA expression was acutely elevated in group T compared with uninjured rats 2 days following transection but quickly decreased after 6 days (**Figure 1A**). These data agreed with the results of a previous report (Noble et al., 2002) wherein upregulation was measured using zymography within 24 hours postinjury, before levels were downregulated at 72 hours postinjury. The levels of MMP-9 mRNA were further increased in the graft site in group R compared with those in group T (6, 10, and 14 days) (**Figure 1A**).

MMP-2 was previously reported to be upregulated 5 days after SCI in mice (Dang et al., 2008). Our data demonstrated that MMP-2 mRNA was elevated at 2 days postoperation and further upregulated until 21 days postoperation (final measurement) (**Figure 1B**). MMP-2 mRNA was not significantly elevated in group R compared with that in group T. These data suggest that MMP-2/9 were upregulated postinjury and that MMP-9 was further increased in the graft site in group R.

Local inhibition of MMP-2/9 activity was associated with the presence of Arg1$^+$ macrophages in the nerve grafts of repaired spinal cords in rats

To test whether MMP-2/9 inhibition altered the M2 macrophage response, we examined the M1 and M2 macrophage populations. Usually, a combination of markers can allow identification of a population of bona fide 'type II' macrophages. In our *in situ* study, Arg1 was chosen as the M2 macrophage marker, and iNOS was chosen as the M1 marker. Although the data obtained cannot be interpreted as changes in specific, homogenous populations, it is still possible to make some observations regarding changes in macrophage populations. The general MMP inhibitor GM6001 was mixed with fibrin glue and applied to the nerve grafts (group RG), and Arg1 and iNOS gene expression (used as M1 marker) levels were compared using qPCR. The fold change relative to the gene expression in transections with glue (group U) is demonstrated on day 10 postinjury. Arg1 expression was significantly reduced on day 10 with GM6001 application in group RG) compared to that in group R (**Figure 2A**, 10 R *vs.* 10 RG, $P < 0.001$), whereas the expression of iNOS was not significantly affected (**Figure 2A**). This finding suggests that the MMP inhibitor reduced the M2 macrophage population but did not affect the M1 macrophage population.

Immunohistological analysis demonstrated a significant reduction in M2 marker Arg1 immunoreactivity in the graft area when MMP was inhibited on day 10 postoperation (10 R *vs.* 10 RG, $P < 0.05$), whereas immunoreactivity for ED1 was not significantly affected (**Figure 2B**). ED1 is marker for both M1 and M2 macrophages. The lack of change in ED1 levels demonstrates that the total macrophage population was not significantly reduced and that the Arg1 reduction resulted from changes to a specific population of macrophages. Western blot analysis of the graft area showed a significant reduction in Arg1 levels at the nerve graft site in group RG compared to that in group R at 10 and 14 days postoperation (**Figure 2C**, $P < 0.05$ and $P < 0.001$, respectively)

Local inhibition of MMP-2/9 activity impedes BDNF expression and functional recovery

Following SCI, the expression of BDNF mRNA was reported to dramatically be increased within microglia, macrophages and astrocytes at the injury site (Dougherty et al., 2000). It has been previously reported that M2, but not M1, macrophages express BDNF in a myelopathy mouse model with severe compression of the spinal cord (Hirai et al., 2013). We previously reported that the M2 macrophages in the

group R expressed more BDNF in the grafted area at 14 days postoperation (Kuo et al., 2011). In the present experiment, we demonstrated that the mRNA expression of BDNF was decreased in the grafted area at 14 days postoperation in group RG relative to that in group R (**Figure 3**, 14 RG *vs.* 14 R, $P < 0.05$). Therefore, BDNF downregulation was associated with reduced expression of the M2 marker Arg1 when treated with the MMP inhibitor.

In situ zymography demonstrated that MMP-2/9 activity was detected at 10 and 14 days postoperation in group R but not in group RG (**Figure 4A**). Both the pro and active forms of the MMPs were inhibited (**Figure 4B**). Hindlimb locomotor function was tested using the BBB score on a weekly basis. At 8 weeks postoperation, the locomotor score of group RG was significantly lower than that of group R and was not significantly different from that of group T (**Figure 4C**, $P < 0.05$).

Discussion

Usually with combination of markers could one pinpoint a population that was bona fide 'type II' macrophage. In our *in situ* study Arg1 was chosen for the M2 macrophage and iNOS for M1. The data obtained cannot be interpreted as the activities for the specific populations, but rather, it approaches the reality to a certain degree that we found useful. In relevant places the M1 or M2 macrophage were used instead of $Arg1^+$ or $iNOS^+$ macrophage for a purpose of interpretation and the readers should be aware of the lack of multiple staining for these populations (Shechter et al., 2009, 2013). We previously demonstrated that a combination of peripheral grafts and FGF-1 treatment increased the M2 macrophage population and BDNF expression to promote functional recovery in spinal cord-transected rats (Kuo et al., 2011). In this study, we demonstrated that the expression of the M2 macrophage marker Arg1 was reduced by the MMP inhibitor in the grafted area. The expression of iNOS, an M1 macrophage marker, or ED1, a general marker for activated macrophages, was not significantly reduced, suggesting that the M2 population was preferentially reduced by the MMP inhibitor. At the same time, reduced BDNF expression and a loss of functional recovery were associated with the reduced Arg1 population.

MMP inhibition affects the $Arg1^+$ population significantly possibly because the M2 population secretes more MMP-9 and is more mobile than the M1 population

It is a generally accepted that MMP-9, not MMP-2, supports the migration and infiltration of macrophages into the injured spinal cord (Zhang et al., 2011). A discrepancy exists regarding the expression levels of MMP-9 among the M1 and M2 populations. It has been reported that IL-10-induced M2 macrophages have higher MMP-9 activity and mRNA levels than M1 macrophages *in vitro* (Chizzolini et al., 2000; Shimizu et al., 2004; Sica et al., 2006; Lolmede et al., 2009; Huang et al., 2012). In addition, as reported, M1 macrophages are relatively immobile, whereas M2 macrophages are more mobile (Lively and Schlichter, 2013). It is known that stem cell transplantation-induced M2 macrophage migration is accompanied by the upregulation of MMP-9 (Pastrana et al., 2006; Ding et al., 2009; Veeravalli et al., 2009). Human mesenchymal stem cell treatment significantly increased MMP-9 activity and M2 macrophage gene expression in kidneys with ischemia-reperfusion injury in mice (Wise et al., 2014). This evidence may explain the more active migration of the M2 population of macrophages. If the $Arg1^+$ population represents most of the M2 population, it follows that the more mobile population was affected when MMPs were inhibited in this study.

MMP inhibition may have differentially blocked the migration of a remote, endogenous M2 population after SCI

It has been recently discovered that the migration of M1 and M2 macrophages is distinctly regulated *via* different pathways in contusive SCI. Notably, M2 macrophages differentiate from monocytes, which are trafficked through the remote brain ventricular choroid plexus (CP) to reach the location of the injury (Shechter et al., 2013). In addition, the migration of the M1 and M2 populations can be regulated locally near the injury penumbra and is involved in the modulation of the ECM, which is an MMP target (Shechter et al., 2011; Vadivelu et al., 2015). Therefore, homing of M1 and M2 populations to the injury site is differentially regulated both chronologically and geographically. If the $Arg1^+$ population we observed in the nerve graft represents most of the incoming M2 population, our results indicate that some of the determinants of M2 entry into the injury site were locally distributed and may be regulated by MMPs. This result suggests that the $Arg1^+$ population, or at least some of the M2 population in our 'repair' model, may be a separate migratory cell population rather than the phenotypic conversion of early M1 cells.

MMP9 was associated with the M2 population and created a permissive environment for axonal regeneration

Recently, the M2-associated protein decoy receptor 3 has been observed to induce macrophages to secret more MMP-9 than IL-4-induced macrophages (Chang et al., 2008), to increase the population of M2 macrophages in the lesioned area and to promote functional recovery in contusive SCI (Chiu et al., 2016). In this study, we found that MMP-9 expression was increased and was associated primarily with macrophages in the graft area of the repaired spinal cord, where $Arg1^+$ macrophages were also increased compared to the transected spinal cord. In addition, we observed that the MMP inhibitor in the grafted area reduced $Arg1^+$ (M2 marker) gene expression, whereas iNOS (M1 marker) and ED1 (activated macrophages) expression was not significantly affected. This result suggests that MMP-9 may be associated with the M2 macrophage population in the grafted area, thereby creating a more permissive environment for functional recovery in our repaired model.

An increased M2 population was associated with many beneficial effects for recovery from SCI and should not be compromised

Several strategies, including cell therapy, molecular therapy,

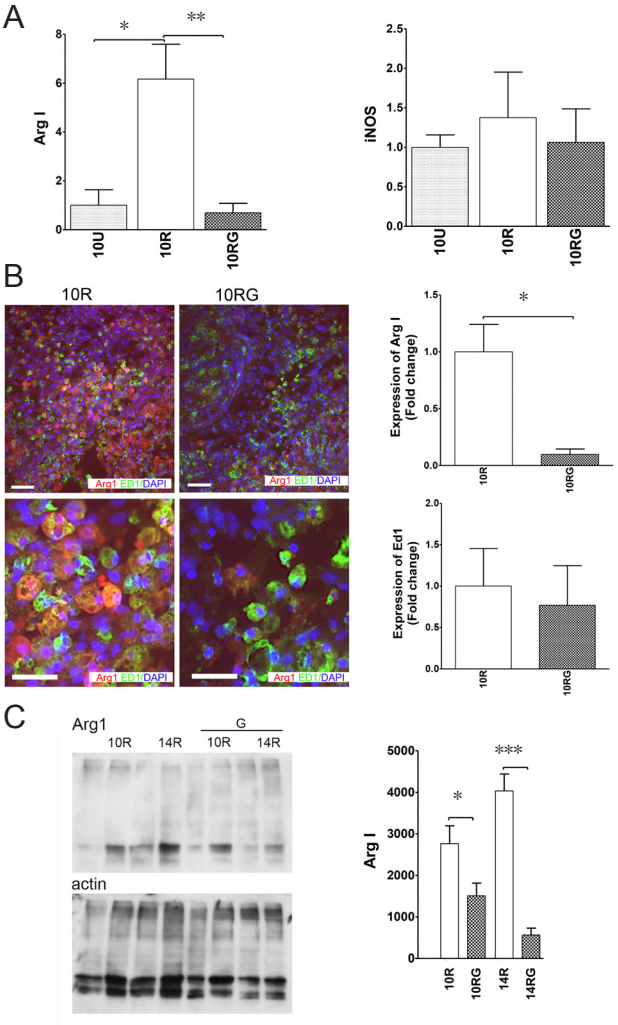


Figure 2 Blockage of matrix metalloproteinase (MMP) activity decreased the presence of M2 macrophages in grafted nerves at 10 days postoperation.

ED1 is a monoclonal antibody against the rat CD68 protein and detect activated macrophage. In group T, rats underwent complete transection operation and T_8 spinal cord was removed. In group R, spinal cord transected rats received treatment with fibroblast growth factor-1 and peripheral nerve grafts. In group RG, rats received the same treatment as group R with the addition of 200 μM GM6001 (an MMP inhibitor) to the fibrin mix. (A) Levels of gene expression were compared using quantitative polymerase chain reaction. The Y axis is fold change relative to the gene expression of group U (transection plus glue only). On day 10 postoperation, the rats in group R demonstrated a significant difference in Arg1 gene expression compared to the rats in the group U. This elevation was reduced when MMP activity was blocked (10 RG *vs.* 10R). Left, $n = 3, 4, 4$; mean ± SEM. Expression of inducible nitric oxide synthase (iNOS) was not significantly changed among groups U, R and RG (right) (mean ± SEM). $*P < 0.05$, $**P < 0.01$. (B) Left: Immunofluorescent images of cross sections of grafted nerves double stained with arginase-1 (Arg1) and ED1. The number of $Arg1^+$ macrophages was decreased in the GM6001-treated group (RG) compared with that in the R group (R), whereas the number of $ED1^+$ macrophages was not significantly different between groups R and RG. Right: Analysis of immunoreactivity in the graft area on day 10 postoperation ($n = 3$, mean ± SEM, $*P < 0.05$). (C) Left: Western blot analysis of Arg1 and ED1 protein levels in tissue collected from the grafted nerves. Y axis is the arbituary unit from the densitometry for analysing the blot. Right: quantitative analysis (Arg1, $n = 5$; ED1, $n = 4$). $*P < 0.05$, $***P < 0.001$.

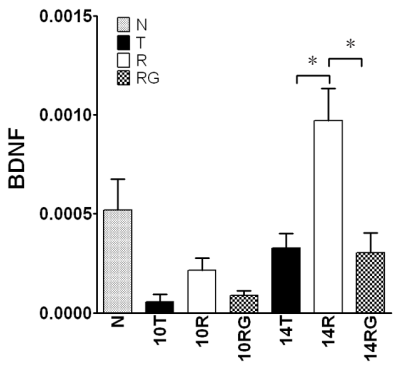


Figure 3 Brain-derived neurotrophy factor (BDNF) was decreased in the repaired spinal cords (R group) following treatment with matrix metalloproteinase (MMP) inhibitor GM6001.

BDNF gene expression level was compared using quantitative polymerase chain reaction. The Y axis is fold change relative to the BDNF gene expression of group T (transection plus glue only). In group T, rats underwent complete transection operation and T_8 spinal cord was removed. In group R, spinal cord transected rats received treatment with fibroblast growth factor-1 and peripheral nerve grafts. In group RG, rats received the same treatment as group R with the addition of 200 μM GM6001 (an MMP inhibitor) to the fibrin mix. BDNF was increased in the graft areas in group R compared with group T, but was reduced in group RG at 14 days postoperation. One-way analysis of variance combined with Newman-Keuls multiple comparison tests for *post hoc* analysis was used (mean ± SEM; $n = 4$; $*P < 0.05$).

and combinatorial treatment, have been developed in SCI animal models (Chang et al., 2006; Ahn et al., 2012; Silva et al., 2014). Increasing evidence demonstrates that combinatorial strategies employing multiple agents, such as pertinent neurotrophic factors, scar-resolving agents, or other axonal growth-promoting agents, are able to further improve locomotor functions after SCI (Didangelos et al., 2014). In our previous study, we demonstrated that local BDNF was increased on day 14 postoperation in the repaired spinal cord of rats (Kuo et al., 2011). In this study, we demonstrated that reduction of an $Arg1^+$ population by the MMP inhibitor was associated with BDNF reduction in grafted nerves and worse functional recovery. This result indicates that increased $Arg1^+$ cells, or at least some M2 macrophages, were associated with multiple beneficial effects. MMPs themselves have been considered a drug target for the promotion of CNS repair; however, if the mechanism of repair involves the elevation of M2 macrophage activity, treatment with an MMP inhibitor may compromise macrophage activity and should be used with caution.

Altogether, this study indicates that MMPs may contribute to the therapeutic features of M2 macrophages through the control of their motility in our repair model, as well as through providing a more permissive environment. The M2 population in group R may be a migratory population rather than the phenotypic conversion of early M1 macrophages. Application of the MMP inhibitor and the promotion of MMP activity require more consideration and investigation of the mechanisms of the coadministered treatments.

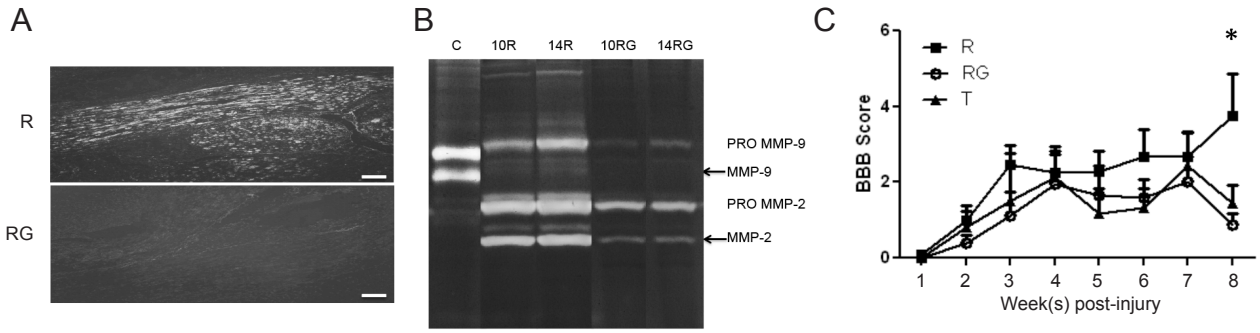


Figure 4 Local blockage of matrix metalloproteinase (MMP)-2/9 activity impeded functional recovery of spinal cord rats.
In group T, rats underwent complete transection operation and T_8 spinal cord was removed. In group R, spinal cord-transected rats received treatment with fibroblast growth factor-1 and peripheral nerve grafts. In group RG, rats received the same treatment as group R with the addition of 200 μM GM6001 (an MMP inhibitor) to the fibrin mix. (A) *In situ* zymography demonstrated that MMP-2/9 activity (white signal in photograph) in the graft area of the repaired spinal cord (R), was inhibited by GM6001 (RG) at 10 days postoperation. Scale bars: 1 mm. (B) MMP-2/9 activity in the graft area of the repaired spinal cord (R) at 10 and 14 days (10R, 14R) was reduced compared to group RG (10RG, 14RG), as demonstrated by zymography. (C) The locomotor recovery was evaluated over an 8-week period, using a 21-point scale (Basso, Beattie, Bresnahan (BBB) locomotor rating scale), in groups R, T, and RG. BBB scores were increased in group R (R *vs.* T, 8 weeks), whereas in group RG it was significantly lower than that in group R and was not different from that in group T (RG *vs.* T, 8 weeks). One-way analysis of variance combined with Newman-Keuls multiple comparison tests for *post hoc* analysis was used (mean ± SEM, $n = 4$, $*P < 0.05$).

About the ProCord clinical trial and our treatment

The ProCord clinical trial utilized the infusion of autologous macrophages for the treatment of completely transected spinal cord patients. Although the initial proof of safety was provided in 2005 for the phase I trial and several cases progress from AISA A to ASIA C (Knoller et al., 2005), the randomized controlled multicenter trial in phase II failed to show a significant difference in primary outcomes between the treated and control groups (Lammertse et al., 2012). Our group R treatment that resulted in vast $Arg1^+$ macrophage accumulation in the injury site could be similar to the peripheral nerve preincubated macrophage injection. However, the distribution and activities still differ. Further, the lack of details for the proprietary treatment of the clinical trials make it difficult for us to say whether the procedure would be different from our peripheral nerve grafts plus FGF-1 treatment. As one of the authors who published the result demonstrating behavioral improvement with activated macrophage treatment stated, the type of macrophages and the time and location of their activity would influence regeneration (Shechter and Schwartz, 2013). Our data suggest that macrophage activity for spinal regeneration remains an interesting topic.

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