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FULL PAPER

Olfactory ensheathing cells (OECs) degrade neurocan in injured spinal cord by secreting matrix metalloproteinase-2 in a rat contusion model

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Abstract

The mechanism by which olfactory ensheathing cells (OECs) exert their potential to promote functional recovery after transplantation into spinal cord injury (SCI) tissue is not fully understood, but the relevance of matrix metalloproteinases (MMPs) has been suggested. We evaluated the expression of MMPs in OECs *in vitro* and the MMP secretion by OECs transplanted in injured spinal cord *in vivo* using a rat SCI model. We also evaluated the degradation of neurocan, which is one of the axon-inhibitory chondroitin sulfate proteoglycans, using SCI model rats. The *in vitro* results showed that MMP-2 was the dominant MMP expressed by OECs. The *in vivo* results revealed that transplanted OECs secreted MMP-2 in injured spinal cord and that the expression of neurocan was significantly decreased by the transplantation of OECs. These results suggest that OECs transplanted into injured spinal cord degraded neurocan by secreting MMP-2.

Key Words: matrix metalloproteinase-2, neurocan, olfactory ensheathing cells, regenerative medicine, spinal cord injury

Introduction

Severe spinal cord injury (SCI) due to traffic accidents or intervertebral disc diseases is relatively common in dogs[9,11,15)]. Patients with severe SCI suffer from irreversible quadriplegia or paraplegia and incontinence. Although various clinical trials for SCI patients have been performed, a definitive treatment has not been established.

Cell transplantation therapy has been actively investigated, and promising results have been presented. Many types of cells were investigated in these studies, including embryonic

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stem cells, mesenchymal stem cells, neural stem and progenitor cells, and induced pluripotent stem cells[6,23].

Olfactory ensheathing cells (OECs) have been described as a promising candidate for cell transplantation therapy in SCI. OECs are specific glial cells in the olfactory system, and they have the notable ability to support axonal regeneration. A number of studies reported that functional recovery was achieved with OEC transplantation in SCI models[2,7,13,17,19,25,27,28]. However, most of these studies centered on the evaluation of functional recovery, and the mechanisms underlying the functional recovery achieved with OEC transplantation have not been investigated in detail. Although several mechanisms have been proposed, including axonal regeneration by secreting neurotrophic factor[4], remyelination of demyelinated axons[2,7,18,19], and the protection of axons that survived injury[27], no research has presented a definite answer to this question.

The irreversibility of SCI has been reported to be due to chondroitin sulfate proteoglycans (CSPGs); these prevent functional recovery from SCI by inhibiting axonal regeneration in the injured spinal cord[3,16,29]. CSPGs include several species, and an inhibitory effect on axonal regeneration has been reported for brevican, versican, neurocan, NG2, and phosphacan[8,12,20]. Of these five species, neurocan was recently suggested to be the major inhibitory CSPG in SCI[1]. The degradation of CSPGs, especially neurocan, promoted functional recovery in rodent SCI models[3,30].

The relevance of OECs and the degradation of CSPGs was suggested in 2006 by Pastrana *et al.*[26]. They demonstrated the mRNA expressions of matrix metalloproteinase-2 (MMP-2) and -9 in cultured OECs, and they showed that MMP-2 promoted the axonal regeneration of retinal neurons *in vitro*[26]. MMPs are a family of endopeptidases that contain Zn^{2+} in the catalytic domain; their main role in a living organism is the modulation of the extracellular matrix (ECM)[22]. Several MMPs, including MMP-2 and -3, have been reported to have the ability to degrade CSPGs, which are a type of ECM[21]. Gueye *et al.* presented a detailed report of MMP expression in OECs[10], but the expression of MMP-3—which has the ability to degrade all five species of CSPGs that inhibit axonal regeneration—has not been investigated in OECs, to our knowledge. Further, the relevance of MMP expression in OECs and the degradation of CSPGs, especially neurocan, in injured spinal cord has also not been examined in detail.

In this study, we evaluated the mRNA expression and protein secretion of MMP-2, -3 and -9 in cultured rat OECs, and we determined which MMPs are dominantly expressed by cultured OECs. We also evaluated the degradation of neurocan by the MMPs identified as the dominant MMPs in cultured OECs, using OEC transplantation into SCI model animals.

Materials and Methods

Animals: Thirty-six 8-week-old female Sprague-Dawley (SD) rats were used to culture OECs, six 10-week-old female SD rats were used for the induced SCI model, and one 13-week-old SD rat was used to provide intact spinal cord tissue for hematoxylin-eosin (HE) staining and immunofluorescence. All animals were housed in barrier cages in a temperature- and humidity-controlled room with *ad libitum* access to food and water. This study was approved by the Animal Care Committee of the Graduate School of Agricultural and Life Sciences, The University of Tokyo (approval number: P11-523).

Culture of OECs: A total of nine cultures was used for the following evaluations and transplantation: three of the nine cultures were used for reverse transcription-polymerase chain reaction (RT-PCR), three cultures were used for substrate zymography, three cultures were used for transplantation in SCI model rats, and a

small portion from five cultures was used for the evaluation of the purity of the cultured OECs. Four rats were sacrificed at a time; i.e., eight olfactory bulbs were used to establish one culture. The methods of OEC culture and purification were based on those used in earlier studies, with minor modifications[24,31].

Eight-week-old SD rats were anesthetized with an intraperitoneal (i.p.) injection of pentobarbital (50 mg/kg). After ensuring the loss of eyelid reflex, the animals were decapitated. Immediately after scarification, the olfactory bulbs were collected aseptically and placed in ice-cold Leibovitz L-15 medium (Invitrogen, Carlsbad, CA, USA). After the remnants of adherent meningeal membranes were stripped off, the outer layer of the olfactory bulb was dissected. The bulbs were chopped with a scalpel blade and digested in 0.5 ml of collagenase solution (155 U collagenase/mg, Wako, Osaka) for 20 min at 37°C in 5% CO_2. Then, 0.5 ml of 0.25% trypsin solution (Invitrogen) was added, and the mixture was incubated at 37°C for another 15 min.

After incubation, the samples were rinsed in 10 ml of DMEM containing 10% fetal bovine serum (FBS; Invitrogen), 1 mM glutamine (Nissui Pharmaceutical, Tokyo), and 2% gentamicin (Sigma, St. Louis, MO, USA) as a defined medium (DM (+)) and spun at 220 × g for 5 min. The pellets were resuspended in 1 ml of soybean trypsin inhibitor (0.25 mg/ml; Sigma) and bovine pancreas DNase (0.04 mg/ml; Sigma) solution containing bovine serum albumin (0.3 mg/ml; Sigma), and triturated 10 times using a 5-ml glass pipette and triturated once through 21- and 23-gauge needles with a 1-ml syringe, respectively.

The samples were then washed with 10 ml of DM (+) and spun at 220 × g for 5 min. The resulting cell pellets were resuspended in DM (+) containing 2 μM forskolin (Sigma) and 20 ng/ml neuregulin-1 (R & D Systems, Minneapolis, MN, USA) as a growth medium (GM (+)) and were plated on 90-mm-dia. dishes at 1×10^6 cells/dish in 10 ml of GM (+) for 24 hr. After the 24 hrs incubation, the supernatant containing floating cells was transferred to other dishes and incubated for 48 hr. After this incubation, the supernatant containing floating cells was transferred to poly-L-lysine-coated dishes and 5 ml of GM (+) was added to each dish. Cultures were maintained at 37°C in 5% CO_2, and 10 ml of culture medium was replaced with 5 ml of GM (+) at 6 days *in vitro* (DIV). After that, the culture medium was renewed every 2–3 days until the culture reached confluence.

After reaching confluence, the cells were used for RNA extraction, substrate zymography, and transplantation to SCI model rats. The purity of the cultured OECs was confirmed by immunocytochemistry using monoclonal mouse anti-$p75^{NTR}$ (1 : 100; Millipore, Temecula, CA, USA) and Cy3-conjugated goat anti-mouse (1 : 200; Jackson Immunoresearch Laboratories, West Grove, PA, USA). The results of immunocytochemistry ensured a purity of > 95% (data not shown).

RT-PCR: We used RT-PCR to analyze the mRNA expressions of MMPs in the OECs. For the experimental control, the mRNA of HT-1080, a human fibrosarcoma cell line, was also subjected to RT-PCR. Total RNA was extracted from OECs using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. During the procedure, genomic DNA was digested by DNase I (Qiagen) and cDNA was synthesized from 5 μg of total RNA using SuperScript™ III Reverse Transcriptase (Invitrogen) and 10 mM dNTP Mix (Invitrogen). The cDNA was diluted 10-fold and amplified with specific primers of *Mmp2*, *Mmp3*, *Mmp9* and glyceraldehyde-3-phosohate dehydrogenase (*Gapdh*).

We designed the forward and reverse primers for *Mmp2*, *Mmp3*, and *Mmp9* using Primer 3 software, and the primer sets for *Gapdh* given in a previous report were used[14] (Table 1). The following PCR cycling was used: started at 94°C for 2 min, repeating 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min. PCR

Table 1. Primer sequences for RT-PCR[a] to analyze the mRNA expressions of MMPs[b] in OECs[c]

Gene	Primer	Sequence (5'-3')
Mmp2	Forward	GCTCCACACTTCAGGTTCTCC
	Reverse	GCACACCTGTATCCCACTGC
Mmp3	Forward	GCTATGGCAGAGCAAAATGG
	Reverse	TGTCTGTAGCCCAGGAGTGTG
Mmp9	Forward	CTGGGCATTAGGGACAGAGG
	Reverse	TCCGGTTTCAGCATGTTTTG
Gapdh	Forward	TAAAGGGCATCCTGGGCTACACT
	Reverse	TTACTCCTTGGAGGCCATGTAGG

RT-PCR: reverse transcription-polymerase chain reaction; MMP: matrix metalloproteinase; OECs: olfactory ensheathing cells.

products were analyzed in 0.005% ethidium bromide-stained agarose gels, which were visualized using a Printgraph® device (Atto, Tokyo).

Substrate zymography: Substrate zymography was performed to detect the secretion of MMP-2, -3, and -9 from OECs. Primary OECs were passaged onto a poly-L-lysine-coated 60-mm-dia. dish at 2×10^6 cells/dish in 6 ml of GM (+). After 1 DIV, the OECs were rinsed twice in DM(+) without FBS (DM (−)) and then incubated in 6 ml of DM (−) containing 2 μM forskolin (Sigma) (GM (−)) for 72 hr. After incubation, the culture media were collected as OEC-conditioned media (OEC-CM) and centrifuged at 4°C, 1000 × g for 10 min to remove floating cells. Prior to performing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the OEC-CM were concentrated 10-fold using an Amicon® Ultra Centrifugal Filter Device (Millipore), and an equal volume of OEC-CM was dissolved in non-reducing 3× sample buffer.

Equal volumes of samples were separated on a 7.5% SDS gel containing 1 mg/ml gelatin (Sigma) for MMP-2 and -9, or 0.5 mg/ml casein (Sigma) for MMP-3. The gels were washed twice for 15 min and twice for 10 min on a shaker at room temperature with buffer containing 2.5% t-octylphenoxypolyethoxyethanol (Triton-X; Sigma), 100 mM Tris-HCl (pH 7.5), 5 μM $ZnCl_2$, and 5 mM $CaCl_2$. After that, the gels were incubated for 48 hrs at 37°C in re-activation buffer containing 100 mM Tris-HCl (pH 7.5), 5 μM $ZnCl_2$, and 5 mM $CaCl_2$. After incubation, the gels were stained with 0.1% Coomassie Brilliant Blue R-250 and destained in a solution of formic acid/methanol/water (1 : 30 : 70).

The presence of MMPs appeared as clear bands against a dark background, and we compared the co-migration of bands with MMPs from the conditioned media of rat meningeal fibroblasts (mFB-CM) and from HT-1080 cells as well as a stained molecular weight standard (Bio-Rad Laboratories, Hercules, CA, USA). Conditioned media of HT-1080 (HT-1080-CM) are widely used as a positive control for gelatin zymography and used as a technical control. Conditioned media from mFBs were used as a molecular weight control of rat MMP-2 and -9 because the HT-1080 line was established from human cells. The gels were scanned in a digital scanner, and the images of gels were converted to gray-scale using Adobe Photoshop 6.0.

Preparation of contusion model: Ten-week-old SD rats were anesthetized with an i.p. injection of pentobarbital (50 mg/kg), and a dorsal laminectomy was performed at the T10 vertebral level (Fig. 1A). The IH-0400 Impactor (Precision Systems and Instrumentation, Fairfax, VA, USA) was used to make a severe (250 kdyn; 1dyn =

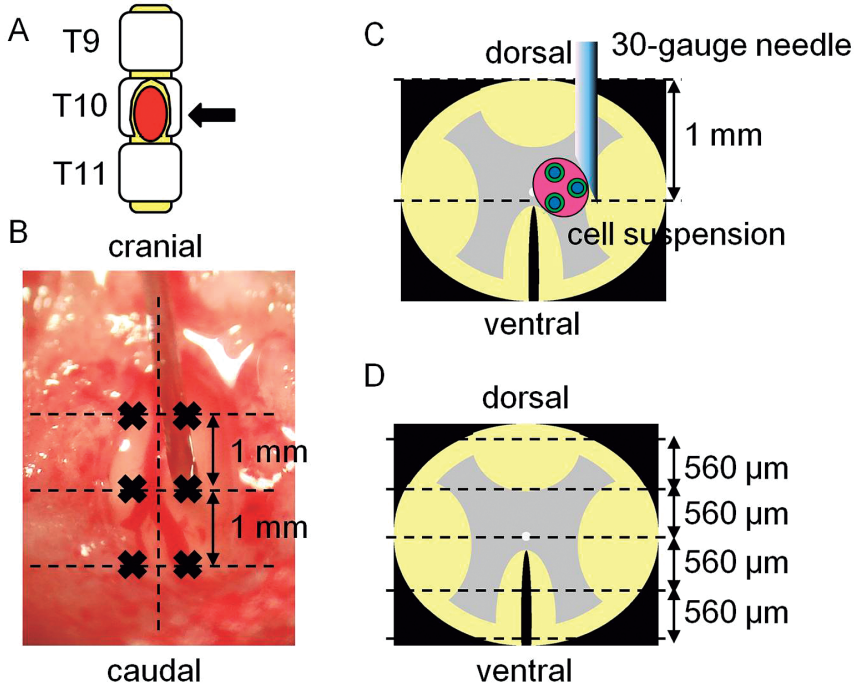

Fig. 1. Schema of the protocols. A: The site of SCI induction. Arrow and red circle: the site of impact. B, C: The sites of OEC transplantation in the injured spinal cord. X-marks: The sites of injection. D: Sectioning of spinal cord tissue. Dashed lines: Sampling point of sections.

10^{-5} N) contusion injury[1]. After impact, the overlying muscles were sutured and skin opening was closed. The animals received a subcutaneous administration of 20 mg/kg cefazolin sodium for the first 3 days post-injury (dpi), and their bladders were manually expressed twice a day until a bladder reflex was restored.

Transplantation of OECs: OEC transplantation was performed at 14–15 dpi. Prior to transplantation, OECs received fluorescent labeling as described[5]. OECs were labeled by 10 μg/ml Hoechst 33342 (Sigma) in DM(+) for 10 min and then resuspended in DM(+) at a density of 5×10^4 cells/μl. Three of six SCI rats were injected with OEC suspension (= the OEC group) and the other three were received a DM(+) injection as a negative control (= the media group). The animals were anesthetized with pentobarbital (50 mg/kg, i.p.), and the T10 level of the spinal cord was carefully exposed. After an incision in the dura matter, the animal was positioned on a stereotaxic instrument for rats (SR-6R; Narishige Scientific Instrument Laboratory, Tokyo), and the points of transplantation were decided using a microinjector (IMS-3; Narishige).

The cell suspension or DM(+) was injected into the injured spinal cord using a 10-μl microsyringe (Hamilton, Reno, NV, USA) and a 30-gauge needle (Becton, Dickinson and Co., Franklin Lakes, NJ, USA). Symmetrical injections about the vein running on the midline of the spinal cord were performed at the lesion epicenter and 1 mm cranial and caudal from the epicenter; i.e., a total of six injections were made (Fig. 1B). One μl of cell suspension was transplanted at each point at 1-mm depth from the surface of the spinal cord (Fig. 1C). To avoid reflux of the suspension from the injection site, 1 min was taken for each injection, and the needle was left in place for another 1 min after the injection was completed. After transplantation, muscles and skin were closed in the same manner as that used for the preparation of injury, and the animals were allowed to recover in warmed cages overnight. The administration of antibiotics and

manual urination were performed in the same manner as that used for the preparation of injury.

Tissue staining and immunofluorescence: The animals were sacrificed at 7–8 days after transplantation (21–22 dpi), and the spinal cord tissues were obtained. One 13-week-old female SD rat was also sacrificed to obtain intact spinal cord tissue. After deep anesthesia by an overdose of pentobarbital (i.p.), the rats were transcardially perfused with 4% paraformaldehyde-phosphate-buffered saline (PFA-PBS). After perfusion, the T8-T12 level of the spinal cord was dissected and immediately placed on ice-cold PBS. The meninges were then stripped off and the spinal roots were removed under stereomicroscopic observation. After trimming, the tissues were further fixed in 4% PFA-PBS at 4°C overnight, and then the fixed tissues were equilibrated in PBS solution containing 20% (w/v) sucrose at 4°C for more than 6 hr. Further equilibration was performed in PBS solution containing 30% (w/v) sucrose for 48 hr.

After equilibration, 10 mm of spinal cord centered on the lesion epicenter was cut with a scalpel blade. The tissues were embedded in Tissue-Tek® (OCT) compound (Sakura Finetek Japan, Tokyo) and frozen rapidly by liquid nitrogen. The frozen blocks were cut with a cryostat (Leica Microsystems, Tokyo) into longitudinal horizontal sections of 14 μm thickness, and three or four sections approx. 560-μm apart were thaw-mounted on a glass slide (MAS-GP; Matsunami Glass Industries, Osaka) (Fig. 1D). Several sections of the intact spinal cord and the injured spinal cord of the media group were stained by hematoxylin and eosin (HE) to evaluate the degree of injury by the power of 250 kDyne. For immunofluorescence, the slides were washed with Tris-buffered saline containing 0.1% polyoxyethylene sorbitan monolaurate (Tween® 20; Sigma) (TBS-T) three times for 5 min each time and then incubated in 5% normal goat serum (NGS) diluted in TBS-T for 1 hr at room temperature.

The slides were then incubated with primary antibodies diluted in TBS-T containing 5% NGS at 4°C overnight; 1 : 250 polyclonal rabbit anti-MMP-2 (Abcam, Cambridge, UK) and 1 : 300 monoclonal mouse anti-neurocan (Millipore). The slides were then washed with TBS-T three times for 5 min each time, and incubated with 1 : 200 secondary antibodies for 1 hr at room temperature: Cy3-conjugated goat anti-mouse (Jackson Immunoresearch Laboratories) and FITC-conjugated goat anti-rabbit (Invitrogen). After incubation, the slides were washed with TBS-T three times for 5 min each time and mounted in Mounting Medium for Fluorescent Microscopy (KPL, Gaithersburg, MD, USA).

Quantitative analysis of fluorescence-stained images: To evaluate the MMP-2 expression and neurocan degradation around the transplantation sites, we used laser scanning confocal microscopy. We evaluated the degradation of neurocan by analyzing the fluorescent signal intensity of neurocan. Fluorescence images of immunostained slides were photographed using the FluoView300 laser scanning confocal microscope (Olympus, Tokyo). A 100× objective lens was used for the observation of MMP-2, and a 10× objective lens was used for neurocan, with the camera gain and offset set to fixed values for all samples. The region of interest (ROI) was defined as a field centered on the most intensive accumulation of Hoechst 33342-positive nuclei to evaluate the MMP-2 secretion by transplanted OECs and neurocan degradation around transplanted OECs. The ROI in the media group was defined as the counterpart site.

The method used to analyze neurocan degradation from the digital images was as described[1)] with a minor modification. Images of neurocan stained with Cy3 were converted to gray-scale, and the mean intensity value (MIV) of each image was calculated from the histogram of the signal intensity using Image-J software. The results of the quantification are expressed as

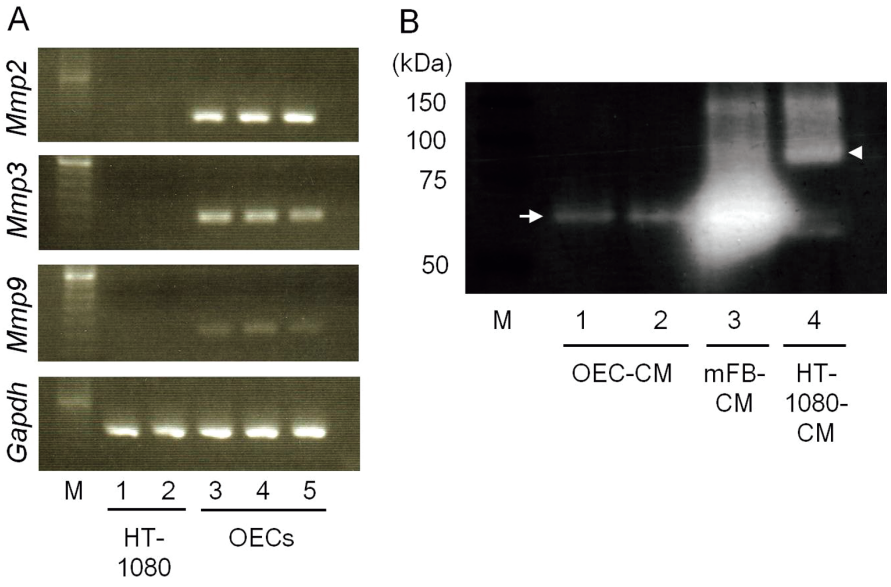

Fig. 2. OECs expressed the mRNA of *Mmp2*, *Mmp3* and *Mmp9* and secreted MMP-2 protein *in vitro*. A: The mRNA expressions of *Mmp2*, *Mmp3* and *Mmp9* in OECs *in vitro*. M: 50 base pair ladder. Lanes 1 and 2: HT-1080 (a human fibrosarcoma cell line). Lanes 3–5: OECs (three independent cultures). B: Gelatinolytic activity of MMP-2 and -9 in OEC conditioned media (OEC-CM). Arrow: MMP-2. Arrowhead: MMP-9. M: molecular weight standard. Lanes 1 and 2: OEC-CM. Lane 3: Rat meningeal fibroblast conditioned media (mFB-CM). Lane 4: HT-1080 conditioned media (HT-1080-CM). Lane 3 was used as a molecular weight control of rat MMP-2 and -9, and Lane 4 was used as an experimental control.

means ± SD.

Statistical analysis: We used Student's *t*-test to compare the MIVs of neurocan expression in the media and OEC groups, and *p*-values < 0.05 were considered significant.

Results

mRNA expression of MMPs in cultured OECs

The mRNA expressions of *Mmp2*, *Mmp3* and *Mmp9* were observed in cultured OECs (Fig. 2A). No significant difference was observed between three independent OEC cultures in each mRNA expression.

Secretion of MMPs from cultured OECs

The results of the gelatin zymography clarified that MMP-2 (68 kD) was contained in the OEC-CM, whereas MMP-9 (92 kD) was not detected (Fig. 2B). MMP-3 was not detected in the OEC-CM by casein zymography (data not shown).

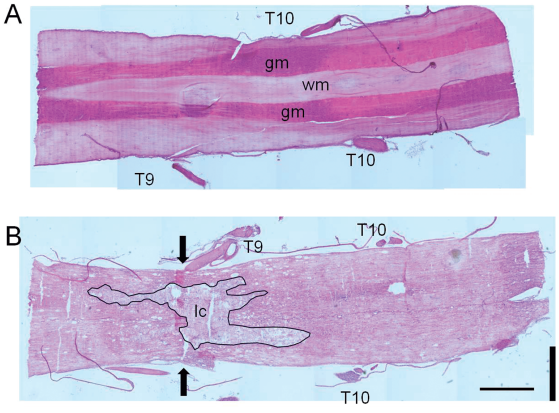

Fig. 3. Hematoxylin and eosin staining of the intact (A) and injured spinal cord in the media group (B). gm: gray matter. wm: white matter. T: thorasic cord. lc: lesion cavity. Arrows: lesion epicenter. Bar = 1 mm.

Evaluation of induced SCI by HE staining

In the intact rat spinal cord, gray matter, which was stained strongly by hematoxylin and eosin, and white matter, whose staining was faint, were clearly distinguishable (Fig. 3A). In

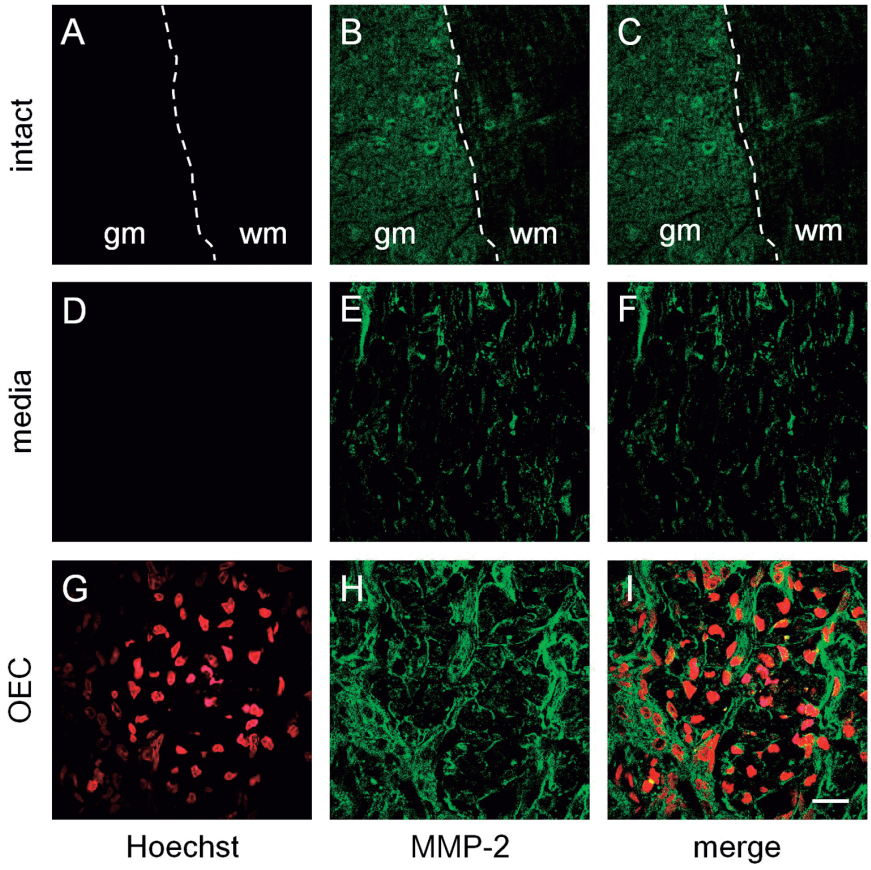

Fig. 4. Immunofluorescence of MMP-2 in the intact spinal cord (A–C) and in the media (D–F) and OEC (G–I) groups. gm: gray matter. wm: white matter. Red: Hoechst 33342. Green: MMP-2. Bar = 20 μm.

the induced SCI/injured spinal cord, the typical structure of gray matter and white matter was not observed. At the area of the lesion epicenter, a large cavity across the T9-T10 spinal level was formed, and eosinophilic cells appeared around the cavity (Fig. 3B). There were no tissue sections which contained normal structure of spinal cord among the induced SCI animals, suggesting that the injury made by the power of 250 kDyne spread at least 5 mm cranial and caudal from the point of impact.

Expression of MMP-2 by OECs transplanted in injured spinal cord

In intact spinal cord, the expression of MMP-2 was limited in the area of gray matter (Fig. 4A–C). In the media group, the structure consisting of gray matter and white matter was destroyed, and only a weak expression of MMP-2 was observed (Fig. 4D–F). In the OEC group, however, MMP-2 was intensively expressed in injured spinal cord, and the expression of MMP-2 was associated with Hoechst 33342-positive nuclei (Fig. 4G–I).

Fluorescent intensity of neurocan

In the intact spinal cords, neurocan expression was observed only in gray matter; faint or no expression was observed in white matter (Fig. 5A). In the injured spinal cords, neurocan was expressed wholly in the area of remaining tissue (Fig. 5B).

The evaluation of immunofluorescent intensity was carried out using only the media group (B) and the OEC group (C) because the intact spinal cords had a structure and staining pattern that were clearly different from those of the injured spinal cord. The immunoreactivity of neurocan in

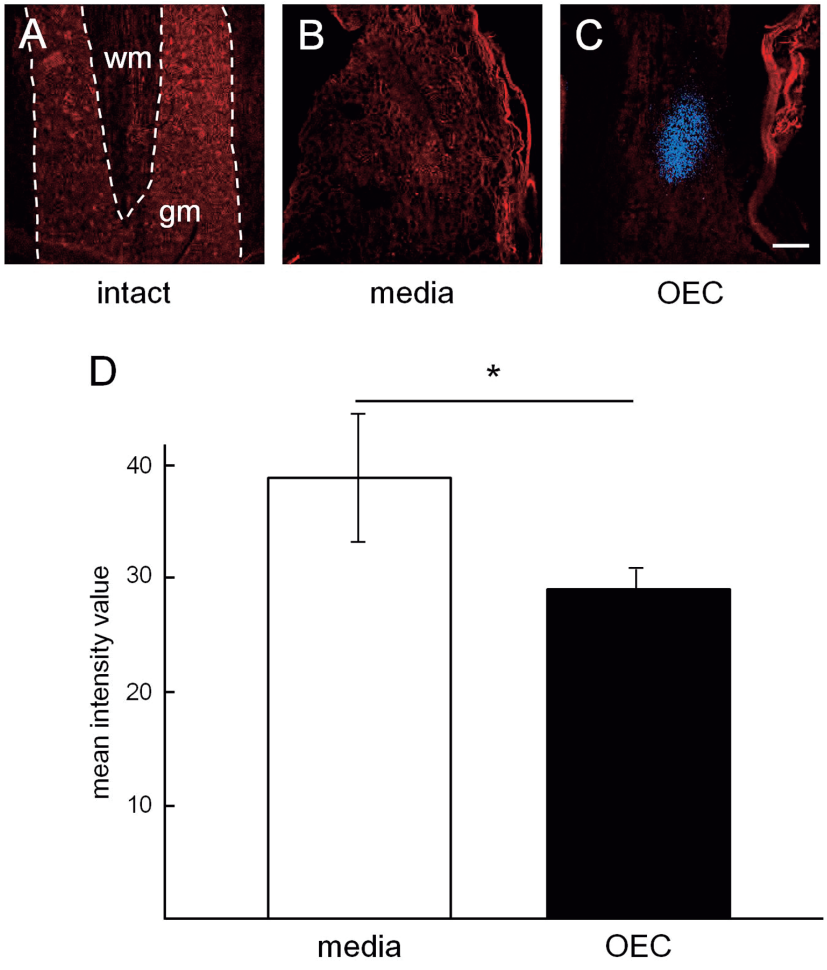

Fig. 5. Immunofluorescence of neurocan and the quantification of fluorescent signals of neurocan. A–C: Immunofluorescence of neurocan in the intact (A), media group (B), and OEC group(C). gm: gray matter. wm: white matter. Red: neurocan. Blue: Hoechst 33342. Bar = 200 μm. D: Quantification of fluorescent signals of neurocan in the media and OEC groups. Vertical bars: SD.

the OEC group was weaker than that in the media group (Fig. 5B, C). The quantification of immunoreactivity indicated a significant decrease of neurocan expression in the OEC group (MIVs: 38.48 ± 3.26 in the media group vs. 28.69 ± 1.10 in the OEC group, $p = 0.047$, Fig. 5D).

Discussion

The RT-PCR results showed that the cultured OECs expressed mRNA of *Mmp2*, *Mmp3* and *Mmp9*. To our knowledge, this is the first study reporting the mRNA expression of *Mmp3* in cultured OECs. Our substrate zymography results showed that the cultured OECs secreted MMP-2 into culture supernatants, whereas MMP-3 and -9 were not detected in the OEC-CM. These findings suggest that OECs expressed MMP-2 more abundantly than MMP-3 and -9 and that the mRNA expression of *Mmp3* and *Mmp9* in cultured OECs was not high enough to appear as protein expression. Although earlier studies reported MMP-9 secretion by OECs, their results also showed a weaker expression of MMP-9 protein compared to that of MMP-2[10,26].

As the results of substrate zymography suggested that MMP-2 was the major MMP secreted by OECs, we evaluated the relevance of MMP-2 secretion by OECs and the degradation

of neurocan in a rat injured spinal cord model. An intense immunofluorescent signal of MMP-2 in the OEC group associated with Hoechst 33342-positive nuclei was observed. We estimate that this finding suggests that the transplanted OECs secreted MMP-2 in the injured spinal cord. Although there was not a direct verification that the transplanted OECs secreted MMP-2 and there was a possibility that cells other than the transplanted OECs or parenchymal cells of spinal cord secreted MMP-2, our *in vitro* investigation showed the ability of OECs to secrete MMP-2, and an earlier study also suggested that OECs *in vivo* situation secrete MMP-2[10]. The above-cited study by Pastrana *et al.* showed that an immortalized cell line of OECs kept the ability to secrete MMP-2 in injured spinal cord[26]. We therefore conclude that the intense expression of MMP-2 associated with Hoechst 33342-positive nuclei indicates the secretion of MMP-2 from transplanted OECs.

In addition, the immunofluorescent signal of neurocan was significantly decreased in the present study's OEC group, and we suspect that this result indicates that neurocan was decreased by the transplantation of OECs. Neurocan has been reported to be expressed by reactive astrocytes in injured spinal cord[1]. However, it was difficult to distinguish which cells expressed neurocan in our immunofluorescence results and to verify that the transplanted OECs did not express neurocan. We estimated that the expression of neurocan would not decrease significantly if the transplanted OECs expressed neurocan at a significant level. Although there was still a possibility that the transplanted OECs expressed neurocan at an insignificant level, we considered that the significant decrease of neurocan by OEC transplantation had a larger sense than the possibility of OECs expressing neurocan at an insignificant level.

Taken together, our findings that OECs secreted MMP-2 in injured spinal cord and that neurocan was decreased by the transplantation of OECs led us to conclude that this decrease in neurocan may probably due to the degradation of neurocan by MMP-2 secreted from transplanted OECs.

Although this study has several limitations, including the lack of an investigation of the effect of OECs on other species of CSPGs and the lack of an evaluation of the effects of OECs on axonal regeneration or locomotor function, our results clarified a new property of OECs *in vitro* and in injured spinal cord. Further investigation is necessary to fully understand the properties of OECs and to establish OEC transplantation as a treatment for severe SCI.

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