Roles of Chondroitin Sulfate and Dermatan Sulfate in the Formation of a Lesion Scar and Axonal Regeneration after Traumatic Injury of the Mouse Brain

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Abstract

Dermatan sulfate (DS) is synthesized from chondroitin sulfate (CS) by epimerization of glucuronic acid of CS to yield iduronic acid. In the present study, the role of CS and DS was examined in mice that received transection of nigrostriatal dopaminergic pathway followed by injection of glycosaminoglycan degrading enzymes into the lesion site. Two weeks after injury, fibrotic and glial scars were formed around the lesion, and transected axons did not regenerate beyond the fibrotic scar. Injection of chondroitinase ABC (ChABC), which degrades both CS and DS, completely suppressed the fibrotic scar formation, reduced the glial scar, and promoted the regeneration of dopaminergic axons. Injection of the DS-degrading enzyme chondroitinase B (ChB) also yielded similar results. By contrast, injection of chondroitinase AC (ChAC), a CS-degrading enzyme, did not suppress the fibrotic and glial scar formation, but reduced CS immunoreactivity and promoted the axonal regeneration. Addition of transforming growth factor- β 1 (TGF- β 1) to a co-culture of meningeal fibroblasts and cerebral astrocytes induces a fibrotic scar-like cell cluster. The effect of TGF- β 1 on cluster formation was suppressed by treatment with ChABC or ChB, but not by ChAC. TGF- β 1-induced cell cluster repelled neurites of neonatal cerebellar neurons, but addition of ChABC or ChAC suppressed the inhibitory property of clusters on neurite outgrowth. The present study is the first to demonstrate that DS and CS play different functions after brain injury: DS is involved in the lesion scar formation, and CS inhibits axonal regeneration.

Key words: axonal regeneration; extracellular matrix; glial response to injury; *in vitro* study; traumatic brain injury

Introduction

CHONDROITIN SULFATE (CS) is a glycosaminoglycan (GAG) that consists of disaccharide units containing glucuronic acid and N-acetyl galactosamine. CS is attached covalently via O-xylose linkage to serine residues of the core protein to form CS proteoglycans. CS proteoglycans are known to inhibit the neurite outgrowth of central neurons *in vitro* and markedly increase after traumatic central nervous system (CNS) injury. Therefore, CS proteoglycans have been generally considered as major impediments for axonal regeneration in the injured CNS.^{1–5} The inhibitory property of CS proteoglycans may reside in the CS side chains, because the continuous infusion of chondroitinase ABC (ChABC), a CS-degrading enzyme, into the CNS lesion site, effectively promotes regeneration of severed axons in the nigrostriatal dopaminergic⁶ and corticospinal descending⁷ axonal pathways.

On the other hand, a fibrotic scar containing type IV collagen (Col IV) deposits is also proposed as an obstacle of axonal regen-

eration after CNS injury.⁸ Elimination of the fibrotic scar has been shown to allow axonal regeneration in a variety of animal models, including inhibition of Col IV biosynthesis,⁹ newborn mice,¹⁰ and transplantation of olfactory ensheathing cells.¹¹ Injection of ChABC into the lesion site also suppresses the fibrotic scar formation to promote axonal regeneration,¹² which suggests that CS not only inhibits axonal regeneration, but also promotes the fibrotic scar formation. CS has, however, a structural heterogeneity depending on postsynthetic modification, such as sulfation and epimerization. When C5 of glucuronic acid in CS is epimerized to yield iduronic acid, this type of CS is called dermatan sulfate (DS). DS is abundantly present in the dermis as its name indicates and has a variety of roles in development, homeostasis, and disease of peripheral tissues.¹³

Recently, DS was reported to be involved in the fibrosis and scar formation in the repair process of dermal injury.¹⁴ We postulated that CS and DS share diverse functional roles in the damaged CNS; CS inhibits axonal regeneration and DS plays some role in the

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fibrotic scar formation. To verify this hypothesis, effects of enzymatic degradation of CS and DS were examined in the mouse brain that received surgical transection of nigrostriatal dopaminergic pathway. Just after the lesion, the following chondroitinases were separately injected to the lesion site: ChABC, which degrades both DS and CS; chondroitinase AC (ChAC), which degrades CS; and chondroitinase B (ChB), which degrades DS.^{15,16} Further, we have recently established an *in vitro* model of lesion scar formation in which addition of transforming growth factor- β 1 (TGF- β 1) to the co-culture of meningeal fibroblasts and cerebral astrocytes induces the formation of fibrotic scar-like cell clusters.¹⁷ Using this model, effects of chondroitinases on the cell cluster formation and neurite outgrowth were also examined.

Methods

In vivo experiments

Transection of nigrostriatal dopaminergic pathway. Male ICR mice at 2 months old purchased from Japan CLEA (Tokyo, Japan) were used in the present study. The experimental protocols were approved by the Animal Use and Care Committee of the Tokyo Metropolitan Institute of Medical Science, and all efforts were made to minimize the number of animals used and their suffering. The right side of the nigrostriatal dopaminergic pathway was transected as reported previously.¹⁰ Briefly, mice were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight) and fixed on a stereotaxic instrument (Narishige, Tokyo, Japan), with the incisor bar set 3 mm below the intra-aural line. A middle skin incision was made on the pre-shaved scalp, periosteum was cleared from the cranium, and a small oval hole was made with a dental drill where the knife with a width of 2.0 mm made of a razor blade was inserted. The knife was attached to the vertical bar of the stereotaxic frame so that the blade could be directed mediolaterally, and the tip of knife was inserted into the right side of the brain at 0.5 mm lateral to the midline, at 2.0 mm posterior to the bregma, and at a depth of 6.0 mm from the surface of the brain. A total of 53 mice were subjected to the unilateral transection of the ascending dopaminergic pathway. Other five intact mice were used as controls.

Chondroitinase injection. Immediately after the transection, 5 μ L of ChABC, ChAC, or ChB solution (Seikagaku Corporation, Tokyo, Japan) dissolved in physiological saline was slowly injected into the lesion site using a glass micropipette connected to a 50 μ L Hamilton syringe that was attached to the vertical bar of the stereotaxic frame. After the injection, the tip of the micropipette was allowed to remain in place for a further 3 min before withdrawing. Mice with unilateral transection were injected with 1 U/mL ($n=3$), 5 U/mL ($n=8$), and 50 U/mL ($n=4$) of ChABC; or 1 U/mL ($n=3$), 5 U/mL ($n=7$), and 50 U/mL ($n=4$) of ChAC; 1 U/mL ($n=3$) and 5 U/mL ($n=8$) of ChB. Ten mice were only transected.

In addition, for 2 weeks until sacrifice, three mice received continuous infusion of ChB into the lesion site through a cannula attached via polyethylene tube to the subcutaneously implanted Alzet osmotic pump (model 2002; Alzet Corp, Cupertino, CA) as described previously.¹⁸

Tissue preparation. The operated mice were killed at 2 weeks after injury. Under deep anesthesia with intraperitoneal injection of sodium pentobarbital (75 mg/kg body weight), the brain was fixed by cardiac perfusion with saline followed by ice-cold 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.4. The brains were dissected free, immersed in the fixative overnight, and transferred to 20% sucrose in 20 mM phosphate buffered saline (PBS), pH 7.4, until they sank. The brains were frozen in crushed

dry ice, and 40 μ m-thick horizontal consecutive sections were cut on a sliding cryotome. The free-floating sections were stored in cryoprotectant solution (0.4 M phosphate buffer, pH 7.4, containing 30% sucrose, 30% ethylene glycol, and 1% polyvinyl pyrrolidone) at -20°C to process for immunohistochemistry.

Immunohistochemical staining. Free-floating sections were initially rinsed in 20 mM PBS and incubated in a mixture of 3% hydrogen peroxide and 0.1% Triton X-100 for 15 min at room temperature. After rinsing in 20 mM PBS, the sections were incubated overnight at 4°C with one of the following primary antibodies: (1) rabbit polyclonal antibody against Col IV (LSL, Tokyo, Japan, 1:5000); (2) rabbit polyclonal antibody against glial fibrillary acidic protein (GFAP) (Dako, Carpinteria, CA, 1:50); (3) mouse monoclonal antibody against CS-D unit (MO-225, Seikagaku Corporation, 1:100); (4) Antibody against DS (LKN1, 1:50); (5) rabbit polyclonal antibody against tyrosine hydroxylase (TH,²⁰ 1:2000).

All primary antibodies were diluted with 20 mM PBS containing 0.5% skim milk. Following incubation of rabbit polyclonal antibodies against Col IV, GFAP, and TH, sections were rinsed and sequentially incubated with biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA, 1:100) at 37°C for 30 min. For mouse monoclonal anti-CS staining, the primary antibody was mixed with secondary biotinylated anti-mouse IgM (Invitrogen, Carlsbad, CA, 1:100) overnight and with 0.1% normal mouse serum before incubation on tissue sections according to Hierck and coworkers²¹ to prevent nonspecific binding to mouse IgM present in the lesion site. To detect DS, a VSV-tagged phage display-derived single chain antibody (LKN1, 1:50) was applied, followed by incubation with rabbit anti-VSV antibody (Medical and Biological Laboratories [MBL], Nagoya, Japan, 1:100) and biotinylated anti-rabbit IgG (Vector Laboratories, 1:100) at 37°C for 30 min each. All the sections were finally incubated with avidin-biotin peroxidase complex (Vectastain ABC kit, Vector Laboratories) at 37°C for 30 min. All sections were rinsed in 20 mM PBS for 15 min between incubations. The immunoreaction was visualized in 50 mM Tris buffer (pH 7.4) containing 0.01% diaminobenzidine tetrahydrochloride (DAB) and 0.01% hydrogen peroxide at 37°C for 4 min.

Sections were finally mounted on MAS-coated glass slides (Matsunami Glass, Osaka, Japan), air-dried on a hot plate at 40°C , and cover-slipped after dehydration through ethanol and xylene. Digital images of horizontal sections at the level of the nigrostriatal dopaminergic pathway were taken on AxioVision CCD system (Carl Zeiss, Oberkochen, Germany), and the TIF files were processed with Photoshop software (Adobe, San Jose, CA). To verify the specificity of GAG immunostaining, tissue sections were incubated with each chondroitinase at a concentration of 0.1 U/mL for 2 h at 37°C before immunostaining.

Triple-color immunofluorescent staining. For triple immunofluorescent staining of TH/Col IV/CS, sections were initially incubated with anti-TH (1:2000) for 4 h at 37°C . Sections were sequentially incubated with biotinylated secondary antibody (1:100) and streptavidin-Alexa Fluor[®] 488 conjugate (1:100) at 37°C for 1 h each. Then, sections were incubated with the mixture of anti-Col IV (1:500) and anti-CS-mouse IgM complex (as previously described in the section on immunohistochemical staining, 1:100) overnight at 4°C . Sections were then incubated with the mixture of Alexa Fluor 594-conjugated anti-rabbit IgG (Molecular Probes, Eugene, OR, 1:200) and streptavidin-Alexa 647 (Invitrogen, 1:100) for 1 h at 37°C . Mounted sections were air-dried on a hot plate at 40°C and cover-slipped with Vectashield Hard Set Mounting Medium (Vector Laboratories). Sections were observed and digital images were recorded by confocal laser scanning microscopy (FV-1000, Olympus Tokyo, Japan). TIF files were processed with Adobe Photoshop software (Adobe Systems). The red

color in all micrographs was converted to magenta, and both brightness and background adjusted for ease of viewing.

Quantitative analysis. Measurement of areas of the Col IV, GFAP, CS, and DS immunoreactivity was performed by an image analyzing system (MetaMorph, Molecular Devices, Sunnyvale, CA) as described previously.¹⁸ In immunohistochemistry, using ABC kit, sections selected from each experimental group were simultaneously immunostained, and time and temperature of the immunohistochemical procedure were performed in the same conditions. Digital micrographs of sections (magnification: 10×2.5) including the lesion site were obtained using the Axio-Vision CCD system (Carl Zeiss). The micrographs of GFAP were set at $100\ \mu\text{m}$ caudal to the lesion site (Fig. 1C). The micrographs were analyzed by MetaMorph as follows: First, pixel sizes of the digital image were calibrated to micrometer. The area of visual field was $168,750\ \mu\text{m}^2$. Next, the specific immunostained area in each digital image was thresholded with integer intensity values (IIV) between 0 and 255 (255 represents the maximum intensity). For each antibody staining, threshold regions were set to include specific staining intensity from 150 to 255 IIV. Total area of thresholded regions was measured in each image. Average of the stained area was calculated from five samples, and data were statistically analyzed by using SPSS software (v.11.0) between the injured group and chondroitinase-treated groups using a Student *t* test. Statistical significance was represented as $p < 0.01$ and $p < 0.05$.

Quantitative evaluation of the regeneration of the transected nigrostriatal dopaminergic axons was performed as described previously¹⁰ by using an automatic counting method (macroprogram for KS400, ver. 3.0 supplied by Zeiss, Germany). Micrographs of sections immunostained with anti-TH antibody were obtained using an AxioCam microscope (Zeiss) equipped with color CCD camera at a final magnification of 20×2.5 . A rectangular area ($42,188\ \mu\text{m}^2$) was set up at $100\ \mu\text{m}$ distance distal to the central part of lesion site (Fig. 1B). The images were then automatically converted to binary images by thresholding. Since TH-immunoreactive

axons are stained brown by oxidation of DAB, the images were first converted to grey scale images, and the brightness of the pixels in the image was inversely correlated to the staining intensity. Then the images were represented by an array of pixels with IIV between 0 and 255 (255 represents the maximum brightness). Labeled objects with brightness of 30 IIV more than the background and in the size range exceeding 20×20 pixels were recorded as positive. Almost all of immunopositive structures proved to be axonal varicosities. Statistical analyses were performed as mentioned above. The validity of the automatic counting method was confirmed by comparison with counts from human observers.

In vitro experiments

Cultures of meningeal fibroblasts and cerebral astrocytes. Rats of the Sprague-Dawley strain (Japan CLEA) were used in all experiments. The meninges were stripped from the cortices of 1- or 2-day-old neonates that were decapitated under isoflurane anesthesia. Dissected meninges were chopped into small pieces, plated on culture flasks ($25\ \text{cm}^2$, Nunc Nalgene Inc., Waltham, MA) coated with poly-L-lysine (PLL; Sigma-Aldrich, St Louis, MO), and cultured for about 1 week in Dulbecco modified Eagle medium (DMEM; Gibco®, Invitrogen, Carlsbad, CA) containing 10% fetal calf serum (FCS; PAA Labo, Pasching, Austria). When meningeal cells were propagated to confluent culture from the sliced small tissue, the cells were dissociated with 0.25% trypsin, resuspended with culture medium, and cultured in flasks for a further 2–12 weeks. Before passage of the cultured cells, culture flasks were shaken to detach more weakly adherent microglia/macrophages.

For cultures of astrocytes, cerebral cortices were dissected from the neonatal rats, and the meninges and blood vessels were carefully removed. The dissected cortices were digested with papain (Worthington Bio. Co. Lakewood, NJ), and dissociated cells were plated on PLL-coated culture flasks ($75\ \text{cm}^2$, Nunc Nalgene Inc.) and cultured for 1 week using DMEM including 10% FCS. When confluency was reached at the end of each passage of the culture,

FIG. 1. A schematic drawing of the mouse brain that shows the regions for quantitative analysis. (A) Horizontal view of mouse brain showing the lesion site placed in the nigrostriatal dopaminergic pathway (black lines). (B) A rectangular area ($225\times 188\ \mu\text{m}$) $100\ \mu\text{m}$ rostral to the lesion indicates the region for quantitative analysis of tyrosine hydroxylase (TH)-immunoreactive axons. (C) Rectangular areas ($450\times 375\ \mu\text{m}$) including the lesion site (blue) and $100\ \mu\text{m}$ caudal to the lesion site (red) represent the regions for quantification of type IV collagen, chondroitin sulfate, and dermatan sulfate immunoreactivities and glial fibrillary acidic protein-immunoreactivity, respectively. Color image is available online at www.liebertonline.com/neu

the culture flasks were shaken for 10 min to remove any loosely attached microglia/macrophages, oligodendrocyte progenitor cells, and neurons. After removal of the medium containing these detached cells, the cells were trypsinized, resuspended with culture medium, and cultured in flasks for a further 2–12 weeks.

Co-culture of fibroblasts and astrocytes. Independent cell suspensions of meningeal fibroblasts or astrocytes (5×10^4 cells / 50 μ L) were plated separately in the opposite corner of the chamber slide well (Nunc Nalgene Inc.) pre-coated with PLL. One day later, medium was added to cover the surface of the well and cultivated for 7–14 days until two types of cells contact each other. Half volume of culture medium was changed every 4–5 days. For all experiments, DMEM including 10% FCS was used. TGF- β 1 (R&D Systems, Minneapolis, MN) was added to the co-culture at a concentration of 8 or 10 ng/mL and then cultivated for 2 days. Three hours before application of TGF- β 1, each chondroitinase was added to the co-culture at a concentration of 0.025–0.2 U/mL. To confirm the specificity of enzymes on degradation of GAGs, the co-culture was incubated with each chondroitinase (0.1 U/mL), fixed 3 h later, and immunostained for GAGs.

Effects of chondroitinases on neurite extension of cerebellar neurons were examined to clarify which GAGs contribute a neurite outgrowth-inhibiting property of cell clusters. TGF- β 1 (10 ng/mL) was added to the co-culture in which the cell clusters were induced, and chondroitinases were added after 7 days. Three hours later, dissociated cerebellar neurons (3×10^4 cells/100 μ L) were plated to the co-cultures. Cerebellar neurons were prepared from neonatal rat cerebella that were dissected and digested with papain.²² The mixed cultures were cultivated for 2 days, fixed with 4% PFA, and immunostained with each specific antibody under equal conditions. All the co-culture experiments were performed four times, and representative results are shown in the Figures.

Immunohistochemistry of cultured cells. The cultured cells were fixed with 4% PFA in 0.1M phosphate buffer for 20 min at room temperature. The fixed cells were treated with cold methanol for 5 min to permeabilize the cell membrane. For avidin-biotin system, an endogenous avidin-biotin blocking kit (Zymed Laboratories Inc., South San Francisco, CA) was used to avoid non-specific staining.

For detection of specific antigens, the following primary antibodies were used: mouse anti-GFAP (Progen Biotechnique, Heidelberg, Germany; 1:50); chicken anti-fibronectin (FN) (Abcam Cambridge, UK; 1:500); mouse monoclonal anti-CS-D (Seikagaku Corporation, 1:100); anti-DS (LKN1, 1:50); mouse monoclonal anti-neuronal class III β -tubulin (Tuj1, Covance Madison, WI, 1:500). To detect the anti-DS antibody (1:50), an anti-VSV antibody (1:100) was used as a secondary antibody as described in the *in vivo* experiments. For triple labeling, the cells were incubated with primary antibodies against Tuj1, CS, and DS, followed by anti-VSV and biotinylated anti-mouse IgM (Jackson ImmunoResearch Lab, West Grove, PA, 1:200), and then with Alexa647-conjugated avidin, goat Alexa488-conjugated anti-mouse IgG, and goat Alexa594-conjugated anti-rabbit IgG (Molecular Probe; 1:200).

For four-color staining, the cells were incubated with primary antibodies against GFAP, CS, DS, and FN, followed by anti-VSV and biotinylated anti-chicken IgY (Vector; 1:200), and then with Alexa647-conjugated avidin (Molecular Probe; 1:200), goat Alexa405-conjugated anti-mouse IgG (Molecular Probe; 1:200), goat Alexa488-conjugated rabbit IgG (Molecular Probe), and donkey DyLight594-conjugated anti-mouse IgM (Jackson ImmunoResearch Lab, antibodies; 1:200).

Stained cells were mounted in an anti-fade reagent (ProLong Gold, Molecular Probe). All the cultured cells were observed and images were taken using a confocal laser microscopy (Zeiss, LSM780).

Quantitative analysis of cluster size. To measure cluster size, micrographs of the cluster area in each culture well were obtained at 10×5 magnification by AxioVert 135 microscopy (Zeiss) and CCD camera (Hamamatsu Orca-ER). Using MetaMorph, pixels of obtained digital images were calibrated to micrometer, and border lines of the clusters were drawn by region tool, to exclude ambiguous cluster area. The sizes were measured on five separate experiments, and the data were statistically analyzed by using SPSS software (v.11.0).

To measure relative expressions of DS and CS, digital images of stained clusters were obtained using Z stack tool ($1 \mu\text{m} \times 20 \sim 30$ sections) by confocal laser microscopy (Zeiss, LSM780) at equal conditions including each laser strength, image size, and digital gains, etc. Using MetaMorph, pixels of the stacked images were calibrated to micrometer, and resulted images were thresholded using a threshold tool. The total fluorescent intensities and area of the thresholded regions of clusters were measured.

For quantitative analysis of neurite extension, digital images of Tuj1-stained neurites were calibrated and drawn by traced line of region tool, and measured by region measurement tool of MetaMorph. The average of four separate experiments were measured and statistically analyzed by using SPSS software (v.11.0).

Statistical analyses were conducted using the Student *t* test or one-way analysis of variance (ANOVA). *Post hoc* comparisons were performed using the Bonferroni/Dunn test. *p* values < 0.05 were considered significant.

Results

Animals

Among 53 mice that were subjected to the unilateral transection of the ascending dopaminergic pathway, 15 mice were excluded from the analysis because of dislocation of the transection or a large cavity formation in the lesion site. A total of 43 mice were, therefore, used for the present analyses: 5 intact, 7 transected, and 31 chondroitinase-treated brains. In successful cases, the transection was placed in the ascending dopaminergic pathway at the site of entry to the striatum.¹⁰

Scar formation, GAG expression, and axonal regeneration in injured brain. In the mouse brain at 2 weeks after injury, the fibrotic scar containing dense Col IV deposits was formed in the lesion center (Fig. 2Ai). GFAP-immunoreactive reactive astrocytes formed the glial scar and surrounded the fibrotic scar (Fig. 2Bi). In the intact brain, DS immunoreactivity was found only in the meninges and blood vessels of the brain surface and rarely detected in the brain parenchyma. In the injured brain, DS immunoreactivity was densely accumulated in the fibrotic scar (Fig. 2Ci). CS immunoreactivity was faintly distributed in the intact brain, while in the injured brain, intense CS immunoreactivity was found in not only the fibrotic scar but also the perilesional area (Fig. 2Di). After pre-incubation of tissue sections with chondroitinases, DS and CS immunoreactivity specifically disappeared; incubation of sections with ChABC eliminated DS and CS immunoreactivity, ChAC incubation eliminated CS but not DS, and ChB incubation eliminated DS but not CS. The nigrostriatal dopaminergic pathway was recognized by TH immunostaining in horizontal sections of the mouse brain.¹⁰ Two weeks after the transection, a mass of TH immunoreactive fibers accumulated at the proximal part of the fibrotic scar, and TH fibers were barely detected beyond the lesion (Fig. 2Ei).

Effect of chondroitinase injection on scar formation, GAG expression, and axonal regeneration. The role of CS in the injured brain was examined by CS degradation with ChAC treatment.

FIG. 2. Type IV collagen (Col IV), glial fibrillary acidic protein (GFAP), dermatan sulfate (DS), chondroitin sulfate (CS), and tyrosine hydroxylase (TH) immunoreactivity in mouse brains injured and injected with 5 U/mL of chondroitinase AC (ChAC), chondroitinase B (ChB), or chondroitinase ABC (ChABC). **(A)** The fibrotic scar (FS) containing Col IV deposition was formed at the injured (Ai) and ChAC-treated (Aii) lesion site. Treatment with ChB (Aiii) or ChABC (Aiv) completely suppressed FS formation. **(B)** The glial scar containing GFAP-positive reactive astrocytes was formed around the FS in injured (Bi) and ChAC-treated (Bii) brains. Expanded astrocytes with intense GFAP immunoreactivity were frequently seen (insets). The glial scar formation was remarkably suppressed in ChB- (Biii) or ChABC- (Biv) treated brains. Most of astrocytes were small and weakly immunoreactive for GFAP (insets). **(C)** DS immunoreactivity was localized in the FS in injured (Ci) and ChAC-treated (Cii) brains and was remarkably suppressed in ChB- (Ciii) or ChABC- (Civ) treated brains. **(D)** Intense CS immunoreactivity was localized in both the fibrotic and glial scars in injured brains (Di). Less intense CS immunoreactivity was found in ChAC-treated brains (Dii). Little CS immunoreactivity was observed in ChB- (Diii) and ChABC- (Div) treated brains. **(E)** Regenerating TH-immunoreactive axons extended into and beyond the fibrotic scar in ChAC-treated brains (arrows in Eii), but not in injured brains (Ei). Regenerating TH axons extended beyond the lesion site in ChB- (Eiii) and ChABC- (Eiv) treated brains. Asterisks indicate lesion sites that lack the FS. Scale bar = 100 μ m in A also apply to B and C; = 200 μ m in D; = 50 μ m in E; = 10 μ m in inset. Color image is available online at www.liebertonline.com/neu

The lesion scar formation in the ChAC-treated brain resembled the injured brain. The fibrotic and glial scars were similar to those observed in the injured brain (Fig. 2Aii, Bii). DS immunoreactivity was densely accumulated in the fibrotic scar of ChAC-treated brain as in the injured brain (Fig. 2Cii). In contrast, CS immunoreactivity was also observed in and around the fibrotic scar, but the intensity of reaction was apparently reduced (Fig. 2Dii) in brains injected with

5 U/mL and 50 U/mL ChAC ($n=5$ and 3, respectively). In these brains, a considerable number of TH fibers accumulated within the fibrotic scar, and regenerated TH fibers were detected beyond the lesion (Fig. 2Eii). The brains injected with 1 U/mL ChAC ($n=2$) were not changed compared with the injured brains.

The role of DS in the injured brain was examined by DS degradation with ChB treatment. The fibrotic scar formation was

completely suppressed in all of the brains injected with 5 U/mL of ChB ($n=5$) (Fig. 2Aiii). In these brains, only a little deposition of Col IV immunoreactivity was observed in the lesion site and on the wall of blood vessels. Compared with mice that were only transected, the lesion sites in the ChB-treated animals were notably narrower (Fig. 2Aiii). Injection of a lower concentration of ChB (1 U/mL) ($n=3$) or continuous infusion of ChB into the lesion site ($n=3$) proved to be insufficient to suppress the fibrotic scar formation. In scar-suppressed brains of ChB-treated mice, astrocytes completely occupied the entire lesion site (Fig. 2Biii). In these brains, the size of astrocytes was smaller, and GFAP immunoreactivity was less intense than in injured and ChAC-treated brains. DS immunoreactivity almost disappeared with elimination of the fibrotic scar in ChB-treated lesion site (Fig. 2Ciii). In ChB-injected brains, CS immunoreactivity was remarkably reduced in the lesion site (Fig. 2Dii). In these brains, many fine varicose TH-immunoreactive fibers traversed the lesion site in which the fibrotic scar was eliminated (Fig. 2Eiii).

Effects of both CS and DS degradation in the injured brain were examined by ChABC treatment. The result of ChABC injection at concentrations of 5 U/mL ($n=5$) and 50 U/mL ($n=3$) on scar for-

mation, GAG expression, and axonal regeneration was very similar to that of ChB injection. The fibrotic scar was not formed (Fig. 2Aiv) and the glial scar was reduced (Fig. 2Biv). Both DS and CS immunoreactivity was remarkably decreased (Fig. 2Civ, Div), and regeneration of dopaminergic axons was enhanced (Fig. 1Eiv). Injection of the lower dose of ChABC (1 U/mL) ($n=2$) did not completely suppress the fibrotic scar formation.

Quantitative analysis. Quantitative evaluation of the effect of chondroitinases on the extent of the deposition of Col IV (Fig. 3A), astrocytes (Fig. 3B) and GAGs (Fig. 3C, D) and regeneration of nigrostriatal dopaminergic axons (Fig. 3E) was performed by using the automatic counting systems. At 2 weeks after injury, a similar extent of Col IV deposition was observed in the injured group (control) and ChAC-treated group, while ChB and ChABC treatment significantly reduced the area of Col IV deposition, compared with the injured group (control: $24.4 \pm 2.1 \times 10^3 \mu\text{m}^2$; ChAC: $21.4 \pm 3.9 \times 10^3 \mu\text{m}^2$; ChB: $3.6 \pm 0.8 \times 10^3 \mu\text{m}^2$; ChABC: $1.5 \pm 0.4 \times 10^3 \mu\text{m}^2$). The extent of GFAP immunoreactivity was also reduced in ChB- and ChABC-treated groups compared with the injured group

FIG. 3. Quantitative analysis of the areas of type IV collagen (Col IV) deposition (A), glial fibrillary acidic protein (GFAP) immunoreactivity (B), chondroitin sulfate (CS) immunoreactivity (C), and dermatan sulfate (DS) immunoreactivity (D), and numbers of regenerated tyrosine hydroxylase (TH)-immunoreactive axons (E) in brains of intact, with lesion and injected with 5 U/mL chondroitinases. Number of mice in each group used for statistical analysis was five. Statistical difference between injection groups and injured group is represented as ** ($p < 0.01$) and * ($p < 0.05$). ChAC, chondroitinase AC; ChB, chondroitinase B; ChABC, chondroitinase ABC.

(control: $4.9 \pm 0.6 \times 10^3 \mu\text{m}^2$; ChAC: $4.6 \pm 0.2 \times 10^3 \mu\text{m}^2$; ChB: $2.2 \pm 0.4 \times 10^3 \mu\text{m}^2$; ChABC: $1.6 \pm 0.4 \times 10^3 \mu\text{m}^2$). The extent of DS immunoreactivity was not altered by ChAC treatment, but significantly reduced in ChB- and ChABC-treated groups (control: $22.6 \pm 3.6 \times 10^3 \mu\text{m}^2$; ChAC: $20.7 \pm 3.2 \times 10^3 \mu\text{m}^2$; ChB: $3.1 \pm 2.2 \times 10^3 \mu\text{m}^2$; ChABC: $2.0 \pm 0.7 \times 10^3 \mu\text{m}^2$). The extent of CS immunoreactivity was reduced in the ChAC-treated group and further decreased in ChB- and ChABC-treated groups (control: $22 \pm 3.2 \times 10^3 \mu\text{m}^2$; ChAC: $7.1 \pm 0.8 \times 10^3 \mu\text{m}^2$; ChB: $0.4 \pm 0.1 \times 10^3 \mu\text{m}^2$; ChABC: $0.7 \pm 0.2 \times 10^3 \mu\text{m}^2$). The number of TH-immunoreactive fibers was remarkably decreased by transection of the dopaminergic pathway. Treatment of ChAC, ChB, and ChABC significantly augmented the number of regenerated TH-containing fibers, compared

with the injured group (intact: 1571 ± 268 ; control: 149 ± 23 ; ChAC: 848 ± 111 ; ChB: 973 ± 74 ; ChABC: 1267 ± 282).

Relationship among the fibrotic scar, CS, and regenerating axons. In triple immunofluorescence, the spatial relationships of the fibrotic scar, CS, and TH axons were clearly visible. In the injured brain, TH axons stopped at the fibrotic scar, which was intensely CS immunoreactive (Fig. 4A). The glial scar also expressed intense CS immunoreactivity. In ChAC-treated brains, CS immunoreactivity was less prominent than in injured brains (Fig. 4Bii). TH-immunoreactive fibers were detected not only in the fibrotic scar but also beyond the lesion (Fig. 4Biii). In mice injected with ChB or ChABC, CS immunoreactivity was remarkably reduced

FIG. 4. Triple immunofluorescent staining of type IV collagen (Col IV), chondroitin sulfate (CS), and tyrosine hydroxylase (TH) in sections of injured brains (A), and of brains treated with 5 U/mL of chondroitin AC (ChAC) (B), chondroitin B (ChB) (C), and chondroitin ABC (ChABC) (D). The bottom of figures is caudal. (A) In injured brains, the fibrotic scar (FS) containing Col IV deposition was formed (Ai), and CS-immunoreactivity increased around the lesion site (Aii). TH-immunoreactive axons stop at the FS (Aiii). In ChAC-treated brains, the FS was also formed (Bi), but an increase in CS-immunoreactivity was suppressed (Bii). TH-immunoreactive axons traverse the FS and extend in the glial scar (Biii). (C, D) In ChB- and ChABC-treated brains, Col IV (Ci, Di) and CS (Cii, Dii) immunoreactivity was remarkably decreased, and TH-immunoreactive axons robustly regenerated beyond the lesion site (Ciii, Diii). Scale bar = 200 μm . Color image is available online at www.liebertonline.com/neu

(Fig. 4Cii, Dii), and TH fibers traversed the lesion site in which the fibrotic scar formation was completely suppressed (Fig. 4Civ, Div).

Effects of chondroitinases on cluster formation in the co-culture. The formation of fibrotic scar is promoted by TGF- β 1. Two days after TGF- β 1 (8 ng/mL) was added to the co-culture of meningeal fibroblasts and cerebral astrocytes, fibroblasts actively proliferated and aggregated to form fibrotic scar-like clusters (Fig. 5Ai), which expressed a high amount of CS and intensely repelled neuritis.¹⁷ In the present study, how GAGs are involved in the scar-forming activity of TGF- β 1 was examined. Incubation of the coculture with 0.1 U/mL of ChB or ChABC significantly suppressed the cluster formation (Fig. 5Aiii, Aiv), while preincubation with 0.1 U/mL of ChAC was without effect (Fig. 5Aii). In the absence of TGF- β 1, treatment of co-culture with chondroitinases did not yield any effect (Fig. 5B). Dose response of each chondroitinase was examined at a concentration ranging from 0.025 U/mL to 0.2 U/mL. Cluster formation induced by TGF- β 1 was suppressed by 0.025 U–0.2 U/mL of ChB and 0.1 U–0.2 U/mL of ChABC, but not by 0.025 U–0.2 U/mL of ChAC. Quantitatively, the areas of cell clusters formed were not changed by ChAC treatment, but significantly reduced after addition of ChB or ChABC (Fig. 8A).

Expression of GAGs in the co-culture. Specificity of chondroitinases on degradation of GAGs was demonstrated in the co-culture. When meningeal fibroblasts and cerebral astrocytes were co-cultivated, they formed separate flat colonies. At the boundary of colonies, both the cell types contacted each other and often overlapped within a distance of 100 μ m from the boundary. DS and CS immunoreactivity was weakly observed in the boundary area of co-culture (Fig. 6). Both DS and CS immunoreactivity was more abundant on fibroblasts than astrocytes (Fig. 6Aii, Aiii). DS immunoreactivity decreased by treatment with ChB or ChABC (Fig. 6Cii, Dii), and CS immunoreactivity decreased by treatment with ChAC or

ChABC (Fig. 6Biii, Diii). The appearance of astrocytes and fibroblasts were not affected by chondroitinase treatment.

Cell clusters induced by TGF- β 1 were intensely immunoreactive for both DS and CS (Fig. 7Aii, Aiii). When chondroitinases were added to the co-culture containing cell clusters, DS immunoreactivity on the cluster was significantly reduced by treatment with ChB or ChABC (Figs. 7Cii, Dii, 7B), and CS immunoreactivity was significantly decreased by treatment with ChAC or ChABC (Figs. 7Biii, Diii, 8C).

Effects of chondroitinases on neurite outgrowth on the cluster. When neonatal cerebellar neurons were grown on the co-culture in the absence of TGF- β 1, neurons expressed an intense preference for astrocytes over fibroblasts as reported previously.¹⁷ Treatment of this co-culture with three kinds of chondroitinases did not affect the neurite outgrowth. Further, neurite outgrowth on PLL-coated dishes was not changed by treatment with any of three kinds of chondroitinases.

Seven days after treatment with TGF- β 1, only a few neurons were attached to the cell cluster, and they had a few short neurites (Fig. 7Ai). To explore the neurite growth-inhibiting property of the cell cluster, chondroitinases were added to the cluster formed by TGF- β 1. After treatment of these co-cultures with ChAC or ChABC, cerebellar neurons attached well and extended their neurites on cell clusters (Fig. 7Bi, Di), while ChB treatment did not promote the attachment and neurite extension of cerebellar neurons on the cluster (Fig. 7Ci). Neurite outgrowth on the cluster was significantly less than on PLL-coated dishes. Quantitatively, addition of ChAC or ChABC but not ChB to the co-culture significantly promoted neurite outgrowth of cerebellar neurons (Fig. 8D).

Discussion

Roles of GAGs on the lesion scar formation

We have previously demonstrated that a single injection of ChABC into the injured mouse brain completely suppresses the

FIG. 5. Effects of chondroitinases on cluster formation by transforming growth factor- β 1 (TGF- β 1) in the co-culture of meningeal fibroblasts and cerebral astrocytes. Two days after addition of TGF- β 1 to the co-culture, cell clusters were formed (Ai), whereas pretreatment with chondroitinase B (ChB) (Aiii) and chondroitinase ABC (ChABC) (Aiv) suppressed the effect of TGF- β 1. Chondroitinase AC (ChAC) treatment did not change the effect of TGF- β 1 (Aii). Cell clusters were not formed in the absence of TGF- β 1 (Bi-iv). Scale bar = 100 μ m.

FIG. 6. Effects of chondroitinases on dermatan sulfate (DS) and chondroitin sulfate (CS) immunoreactivity in the co-culture of meningeal fibroblasts and cerebral astrocytes. In control, DS (Aii) and CS (Aiii) immunoreactivity (asterisks) was mainly expressed in fibroblasts (Ai-iv). After incubation with chondroitinase AC (ChAC) (Biii) or chondroitinase ABC (ChABC) (Diii), CS immunoreactivity remarkably decreased, while DS immunoreactivity was eliminated after treatment with chondroitinase B (ChB) (Cii) or ChABC (Dii). Scale bar = 100 μ m. TGF- β 1, transforming growth factor- β 1; GFAP, glial fibrillary acidic protein; FN, fibronectin. Color image is available online at www.liebertonline.com/neu

formation of a fibrotic scar in the lesion site.¹² In these brains, axons labeled with the lipophilic dye DiI placed in the substantia nigra extended beyond the lesion site, indicating ChABC injection actually promoted axonal regeneration.¹² In the present study, administration of the DS-degrading enzyme ChB, in addition to ChABC, also suppressed the fibrotic scar formation (Fig. 2). Because injection of the CS-degrading enzyme ChAC did not eliminate the fibrotic scar, it is proposed that the fibrotic scar formation requires DS but not CS.

We have recently reported that the addition of TGF- β 1 to the co-culture of meningeal fibroblasts and cerebral astrocytes induces the

formation of cell clusters that resemble the fibrotic scar in the structure, molecular expression, and inhibitory property of neurite outgrowth.²⁴ The present *in vitro* experiment demonstrates that addition of ChABC or ChB inhibited the effect of TGF- β 1 on cluster formation in the coculture (Fig. 5). As ChAC treatment was ineffective in this respect, it is likely that DS, but not CS, is a downstream molecule that mediates the effect of TGF- β 1.

In the injured brain, intense DS immunoreactivity is localized in the fibrotic scar (Fig. 2C) that expresses TGF- β receptors.²⁴ DS immunoreactivity is also localized in the TGF- β 1-induced cluster

FIG. 7. Effects of chondroitinases on attachment and neurite outgrowth of cerebellar neurons on the cluster formed by transforming growth factor- $\beta 1$ (TGF- $\beta 1$). In control cultures, cell clusters repelled neurons and suppressed neurite outgrowth (Ai-iv), whereas neurons were well attached to the cluster and extended their neurites after chondroitinase AC (ChAC) (Bi-iv) or chondroitinase ABC (ChABC) (Di-iv) treatment. Treatment with chondroitinase B (ChB) had no effect (Ci-iv). After incubation with ChB (Cii) or ChABC (Dii), dermatan sulfate (DS) immunoreactivity was eliminated, while chondroitin sulfate (CS) immunoreactivity remarkably decreased after treatment with ChAC (Biii) or ChABC (Diii). Scale bar = 100 μ m. Tuj1, class III β -tubulin. Color image is available online at www.liebertonline.com/neu

of cultured meningeal fibroblasts (Fig. 7). TGF- $\beta 1$ enhances the expression of DS-containing proteoglycans, biglycan, and decorin in various types of mesenchymal cells.^{25–27} Therefore, it is likely that upregulated TGF- $\beta 1$ after CNS injury enhances the expression of DS proteoglycans in meningeal fibroblasts, which, in turn, promotes the formation of fibrotic scar.

The fibrotic scar formation, a kind of fibrosis, includes the process of migration, proliferation, and extracellular matrix molecule (ECM) production of meningeal fibroblasts.²⁸ Degradation of

DS by ChB inhibits proliferation of human dermal fibroblasts, suggesting the promotion of fibroblast proliferation by DS.²⁹ In mice with genetically deleted DS epimerase-1, an enzyme that converts CS to DS, collagen fibril formation is altered in the skin.³⁰ After CNS injury, several kinds of proteoglycans containing DS, such as decorin and biglycan,³¹ versican,³² and CD44,^{33,34} are upregulated around the lesion site. DS and the DS proteoglycans CD44, decorin, and biglycan are known to bind to collagens.^{35–37} The binding of these proteoglycans to the ECM and growth factors

FIG. 8. Quantitative analysis of effects of chondroitinase pre-treatment of the co-culture on the cluster formation by transforming growth factor- β 1 (TGF- β 1) (A) and effects of chondroitinases treatment on dermatan sulfate (DS) immunoreactivity (B), chondroitin sulfate (CS) immunoreactivity (C) and neurite outgrowth of cerebellar neurons on the TGF- β 1-induced cell clusters (D). (A) Chondroitinase B (ChB) or chondroitinase ABC (ChABC) pre-treatment significantly suppressed the effect of TGF- β 1 on cluster formation. Addition of ChB or ChABC significantly decreased DS immunoreactivity (B), while addition of chondroitinase AC (ChAC) or ChABC decreased CS immunoreactivity (C) and promoted neurite outgrowth of cerebellar neurons (D). Neurite outgrowth on the cluster was significantly less than on poly-L-lysine (PLL)-coated dishes. Statistical differences compared with the non-treated group (None) are represented as ** ($p < 0.01$) and * ($p < 0.05$).

including fibroblast growth factor-2 and platelet derived growth factor BB increases during the fibrosis of fascia.¹⁴ Actually, cutaneous wound healing is severely impaired in decorin-deficient mice.³⁸ Further, DS of CD44 proteoglycan is essential for fibroblast migration into fibrin clots in an *in vitro* migration assay of tissue injury model.³⁹ CD44 is expressed after myocardial infarction and involved in infarct healing by regulating the inflammatory and fibrotic response.⁴⁰ Thus, these findings support the contention that DS and DS proteoglycans are a key facilitator of the fibrosis,¹³ including the fibrotic scar formation.

Glial scar formation was also suppressed by administration of ChABC or ChB, but not by ChAC (Figs. 2 and 3). In rats and mice treated with DPY, an inhibitor of Col IV synthesis, the fibrotic scar is not formed, but the glial scar formation was reported to be unchanged.^{9,10,41} On the other hand, elimination of the fibrotic scar by inhibition of TGF- β function has been reported to suppress the glial scar formation. This includes administration of decorin, an endogenous inhibitor of TGF- β function,⁴² anti-TGF- β 2-treatment,²³ anti-TGF- β 1 and anti-TGF- β 2 treatment,⁴³ genetic deletion of Smad3, an intracellular signal transducer of TGF- β ,⁴⁴ and administration of type I TGF- β receptor inhibitor.¹⁸ Whether suppression of the glial scar formation by enzymatic removal of DS is attributed to elimination of the fibrotic scar or inhibition in TGF- β signaling remains to be elucidated.

Role of GAGs on axonal regeneration in the CNS

Injection of ChABC or ChB completely suppressed the formation of a fibrotic scar and promoted regeneration of transected dopaminergic axons (Figs. 2E and 3E), which supports previous findings that elimination of the fibrotic scar enhances axonal

regeneration.^{9–12} The promoting effect of ChABC and ChB on axonal regeneration may be because of both the suppression of glial scar and elimination of the fibrotic scar, which contain high levels of CS. Reduction of the fibrotic scar by inhibition of TGF- β signaling has been reported to decrease expression of CS proteoglycans in the glial scar.^{18,42,44} In fact, TGF- β 1 is known to upregulate expression of CS proteoglycans in cultured astrocytes.^{45,46} Because enzymatic removal of DS inhibits the formation of TGF- β 1-induced cell clusters (Fig. 5), which express a high amount of CS, DS may be involved in TGF- β function on CS upregulation.

The interesting finding of the present study is that in ChAC-treated mice, regeneration of dopaminergic axons was promoted in spite of the presence of the fibrotic scar (Figs. 2–4). Transected axons rarely enter the fibrotic scar in the damaged CNS,^{10,47} while in ChAC-treated animals, regenerating axons extended into and beyond the fibrotic scar, which express a decreased amount of CS immunoreactivity. This implicates CS as a major molecular inhibitor for axonal regeneration in both fibrotic and glial scars as proposed previously.^{1–5} This result accords well with the findings obtained by the present *in vitro* experiments (Fig. 7). We have previously demonstrated that neurite outgrowth of cerebellar neurons is severely inhibited on cell clusters induced by TGF- β 1, but the mechanism was not yet known.¹⁷ Addition of ChABC or ChAC to TGF- β 1-induced cell clusters reduced not only the CS immunoreactivity but also the inhibitory property of the cluster (Fig. 7). Therefore, the present *in vivo* and *in vitro* findings indicate that CS is responsible for inhibitory property of the fibrotic scar/TGF- β 1-induced cluster.

On the other hand, the effect of DS on axonal regeneration seems to be not inhibitory. In ChAC-treated mice, ascending dopaminergic axons regenerated through the fibrotic scar, which is

intensely immunoreactive for DS (Figs. 2 and 4). Also in the ChAC-treated cell cluster, the inhibitory property was reduced in spite of the presence of DS immunoreactivity (Fig. 7). In fact, most of the *in vitro* studies have demonstrated the stimulatory effect of DS on neurite outgrowth,^{48–51} although only a few studies reported the inhibitory effect of DS.⁵² Oversulfated DS binds to various heparin-binding growth factors including pleiotrophin, midkine, and fibroblast growth factor-2, 7, 10, and 18,^{53,54} and the neurite outgrowth-promoting activity of DS is dependent on these growth factors.⁵⁵ As a model of CNS injury, neurite growth of chick dorsal root ganglionic neurons was inhibited in injured adult spinal cord tissue sections, but was substantially greater on sections treated with ChABC, whereas degradation of DS by ChB does not promote neurite growth.⁵⁶

In the present study, addition of ChB to TGF- β 1-induced cell clusters reduced DS immunoreactivity, but did not suppress the inhibitory property of the cluster (Fig. 6). Therefore, it seems unlikely that DS serves as an impediment for axonal regeneration in injured CNS.

Acknowledgments

We thank Dr. G. Raisman for reviewing the manuscript. This study was supported by grants from Grant-in-Aid for Scientific Research (C) from Ministry of Education, Culture, Sports, Science and Technology in Japan Contract (Mext) (23500422) and from The National Natural Science Foundation of China (NSFC-81171248).

Author Disclosure Statement

No competing financial interests exist.

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