

ORIGINAL ARTICLE

Chondroitinase ABC promotes corticospinal axon growth in organotypic cocultures

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Study design: Organotypic coculture model using brain cortex and spinal cord of neonatal rats was used to test the effect of chondroitinase ABC (ChABC) on corticospinal axon growth.

Objective: Chondroitin sulfate proteoglycan (CSPG) is neurite outgrowth inhibitory factor that combines with reactive astrocyte at the lesion site to form a dense scar that acts as a barrier to regenerating axons. ChABC is a bacteria enzyme that digests the glycosaminoglycan side chain of CSPG. We investigated the effect of ChABC on corticospinal axon growth quantitatively using the organotypic cocultures of brain cortex and spinal cord.

Setting: Department of Orthopaedic Surgery, Graduate School of Biomedical Sciences, Hiroshima University.

Method: We used organotypic cocultures with neonatal brain cortex and spinal cord as an *in vitro* assay system for assessing axon growth. After administering ChABC, we counted the number of axons passing through a reference line running parallel to the junction between the brain cortex and spinal cord 500 and 1000 μm from the junction. The immunoreactivity of CSPG was assessed.

Result: The average number of axons after ChABC administration was significantly greater than in the control group. Administration of ChABC decreased CSPG expression in this coculture system.

Conclusion: ChABC induces axonal regeneration by degrading CSPG after central nerve system injury. ChABC has great potential for future therapeutic use in spinal cord-injured patients.

Spinal Cord (2009) 47, 161–165; doi:10.1038/sc.2008.74; published online 10 June 2008

Keywords: regeneration; chondroitinase ABC; corticospinal tract; organotypic coculture; axon

Introduction

Chondroitinase ABC (ChABC) is a bacteria enzyme that digests the glycosaminoglycan (GAG) side chain of chondroitin sulfate proteoglycan (CSPG), a major component of the extracellular matrix. There are numerous reports on chemonucleolysis for the treatment of intervertebral disk herniation using ChABC in monkeys, dogs and many other animals.^{1–3} Preclinical treatments demonstrate no deleterious effect of ChABC, and a human clinical study is now underway. Therefore, ChABC is regarded as a safe agent for clinical use. One of the effects of ChABC was recently the focus of a report which stated that ChABC induces axonal regeneration and functional recovery by degrading chondroitin sulfate-GAG after central nerve system (CNS) injury.^{4–6} After CNS injury, failure of axonal regeneration is

thought to result in part from a nonpermissive milieu surrounding the lesion site.^{7,8} Several different families of inhibitory extracellular matrix molecules combine with reactive astrocytes at the lesion site to form a dense scar that acts as a barrier to regenerating axons.⁹ CSPG is upregulated by astrocytes and oligodendrocytes in the injury site after spinal cord injury (SCI) to limit axonal regeneration.¹⁰ It is reported that CSPG is among the most potent groups of repulsive scar-associated extracellular matrix molecules.¹¹ However, no study has addressed the details of the effect of ChABC on axonal regrowth quantitatively and these therapeutic effects are not well known.

Oishi *et al.*¹² suggested an organotypic coculture system using brain cortex and spinal cord from neonatal rats. The advantage of this coculture system is to assess corticospinal tract (CST) axon growth quantitatively and to facilitate the analysis of factors that regulate axonal growth.^{12,13} The purpose of the present study was to examine whether ChABC facilitates corticospinal axon growth quantitatively using the organotypic coculture system.

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Received 29 September 2007; revised 15 May 2008; accepted 16 May 2008; published online 10 June 2008

Materials and methods

Organotypic cocultures

Organotypic cocultures of brain cortex and spinal cord were prepared as reported previously.^{12,13} Brains and thoracic spinal cords were collected from Sprague–Dawley rats on postnatal day 3 (P3) or 7 (P7). The brains were sectioned using a Vibratome (Dosaka EM, Kyoto, Japan). The region of the sensorimotor cortex was dissected from the coronal sections, and thoracic spinal cord was bisected in the sagittal plane using a razor blade. The dissected cortex and spinal cord were placed on membranes (Millicell-CM; Millipore, Billerica, MA, USA), in 1 ml of serum-based medium (50% basal medium Eagle with Earle's Salts (Sigma, St Louis, MD, USA), 25% heat-inactivated horse serum (Gibco, Grand Island, NY, USA), 25% Earle's Balanced Salt Solution (Sigma), 1 mM L-glutamine and 0.5% D-glucose) in a six-well tissue culture plate. The cortex and the spinal cord were incubated for 1 day, then on the second day, the spinal cord pieces were aligned next to the white matter of the cortex. The cocultures were incubated in a humidified atmosphere with 5% CO₂ at 37 °C. The medium was replaced every 3 days. The cocultures were incubated for up to 14 days.

Two microliters of ChABC (1 U/ml, Seikagaku, Tokyo, Japan) was administered to the cocultures just after the cortex and the spinal cord had come into contact in the P7 ChABC group. In addition, ChABC was administered to the cocultures every 3 days during incubation. The cocultures without administration of ChABC were incubated as the control (P3 or P7 control group).

Tracing of axon growth

Axon projections from the cortex to the spinal cord were labeled by anterograde tracing with DiI. The cocultures were fixed for 5 days in 4% paraformaldehyde at 4 °C. Small crystals of DiI were placed on the center of the cortex, and the cocultures were incubated for another 14 days in 0.1M phosphate buffer in a humidified atmosphere with 5% CO₂ at 37 °C. The cocultures were mounted onto glass slides and covered with Vectashield (Vector, Burlingame, CA, USA) and glass coverslips. To analyze the axon growth, we counted the number of labeled axons passing through a reference line running parallel to the junction between the brain cortex and spinal cord 500, 1000, 1500 and 2000 μm from the junction. We evaluated the axonal growth using images that were taken at a magnification of ×200, at different focal planes. All fibers crossing the reference line were counted, and the counts were expressed as an average number of axons per culture. Results are expressed as mean ± standard errors (s.e.).

The statistical significance of differences in parameters was assessed by the Kruskal–Wallis test with a *post hoc* test using the Scheffe procedure. For all data collection, the researchers were blinded to the group identities.

Immunohistochemistry

The next stage was for the coculture tissues to be fixed onto the membranes and incubated overnight at 4 °C in 4% paraformaldehyde with mouse monoclonal antibodies against glial fibrillary acidic protein (GFAP) for astrocyte

(1:1000, Chemicon, Temecula, CA, USA), and rabbit polyclonal antibodies against CSPG (1:500, Chemicon). We assessed the degradation of CS-GAG with the antibody 2B6 (1:500, Seikagaku). This antibody recognized an epitope on CSPG core proteins but not intact CS-GAG. On the following day, the tissues were exposed for 1 hour to Alexa Fluor 488-conjugated goat anti-mouse antibodies (1:400, Molecular Probes, Eugene, OR, USA) and Alexa Fluor 568-conjugated goat anti-rabbit antibodies (1:400, Molecular Probes) at room temperature, and observed under a confocal laser microscope (Carl Zeiss, Oberkochen, Germany).

Results

Corticospinal axon growth in organotypic cocultures

In this study, 12 organotypic cocultures per group have been made successfully (Figure 1a). In the P3 control group, many axons labeled with DiI were detectable from the brain cortex to the spinal cord (Figures 1b and c). The average number of axons that extended 500 and 1000 μm past the junction was 17.3 ± 1.9 and 9.4 ± 1.0 in the P3 control group ($n = 12$ cultures per group), respectively. Compared with the P3 control group, fewer axons were seen in the P7 control group (Figures 1d and e). The average number of axons that extended 500 and 1000 μm past the junction was 0.6 ± 0.3 and 0.1 ± 0.1 ($n = 12$ cultures per group), respectively (Figure 2). The average number of axons in the P3 control group extending 500 and 1000 μm from the junction was significantly greater than that in the P7 control group ($P < 0.05$). In the P7 ChABC group, the axon growth was enhanced compared to that in the P7 control group (Figures 1f and g). The average number of axons that extended 500 and 1000 μm past the junction was 3.9 ± 0.4 and 0.8 ± 0.4 ($n = 12$ cultures per group), respectively ($P < 0.05$). The average number of axons in the P7 ChABC group extending 500 μm from the junction was significantly greater than that in the P7 control group. However, the average number of axons in the P7 ChABC group extending 500 and 1000 μm from the junction was significantly less than that in the P3 control group.

Immunohistochemistry

In the P3 control group, immunoreactivity of CSPG was observed around the junction of brain cortex and spinal cord (Figures 3a and b), and that increased in the P7 control group (Figures 3c and d). The GFAP expression was presented diffusely through the coculture in the P3 control group, and that increased in the P7 control group.

By administration of ChABC, immunoreactivity of CSPG was decreased (Figures 3e and f), whereas with 2B6 expression was increased (Figures 4a and b). 2B6 is the antibody used against CSPG when digested with ChABC and specifically recognizes the enzyme-generated epitope on chondroitin 4-sulfate GAG chains.

Discussion

Axon growth failure in the CNS of adult animals is thought to be attributable to several factors, including an inadequate

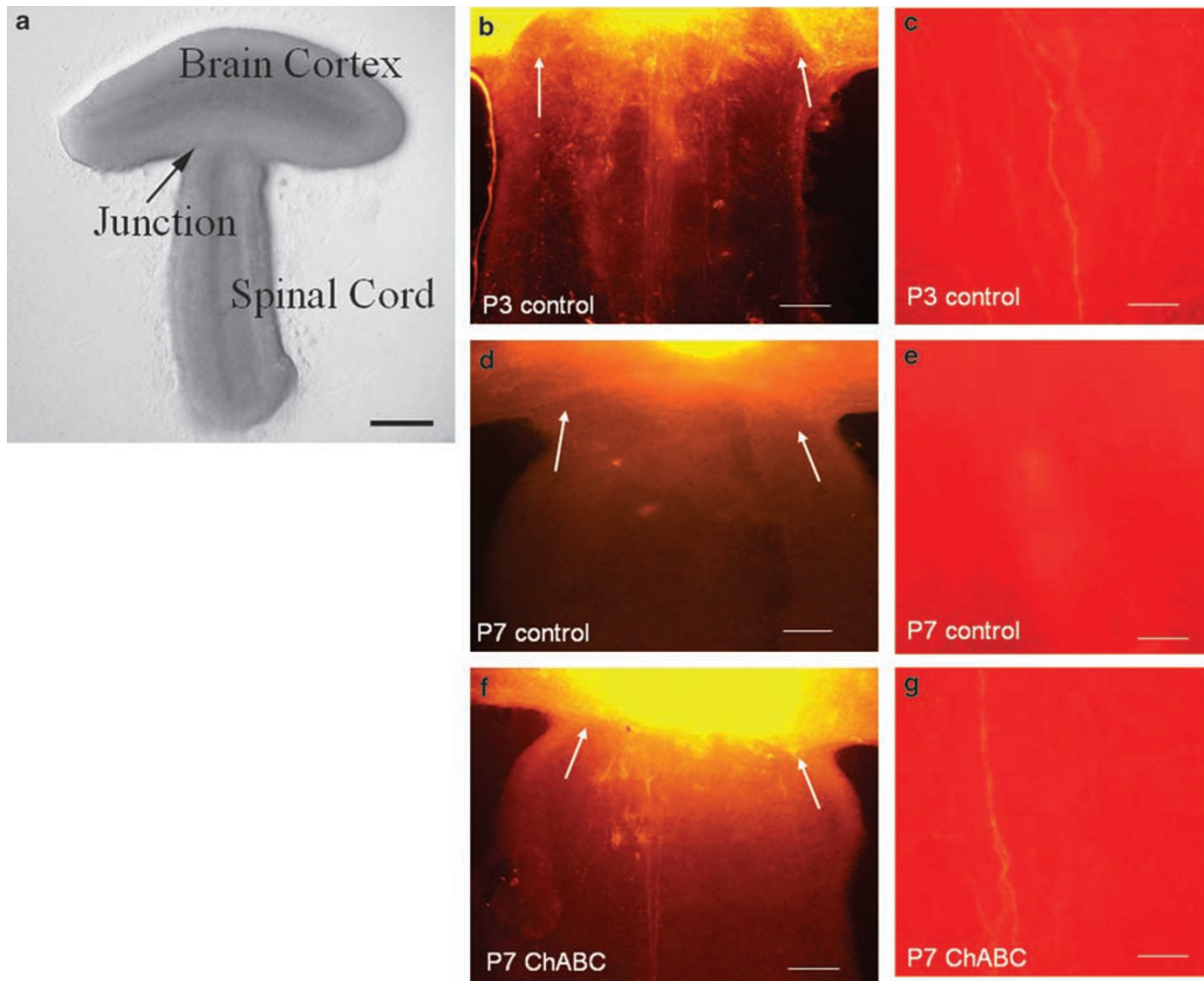


Figure 1 Axons projecting from the cortex to the spinal cord. A photomicrograph of a cortex/spinal cord organotypic coculture (a). Many axons extend in the P3 control (b, c). Minimal axonal growth is seen in the P7 control (d, e). Axon growth is promoted by the administration of chondroitinase ABC (ChABC) in P7 ChABC (f, g). Arrows indicate the junction between the brain cortex (upper side) and the spinal cord (lower side) (a) Bar = 1000 μm , (b, d, f) bar = 200 μm , (c, e, g) bar = 40 μm .

intrinsic growth response, the presence of inhibitory molecules and a lack of adequate neurotrophic support.^{8,14–16} Upregulation of CSPGs that takes place at various times after CNS injuries is a major factor that contributes to regeneration failure.^{10,17,18} CSPGs are extracellular matrix molecules and they are a family of molecules characterized by a protein core to which large sulfated GAG chains are attached. After injury, CSPG expression is rapidly upregulated by reactive astrocytes, forming an inhibitory gradient that is highest at the center of the lesion and diminishes gradually into the penumbra. It has been reported that ChABC, a bacterial enzyme that removes GAG chain from the protein core, enhances axonal regrowth and restores some downstream postsynaptic activity.^{4–6,19,20} However, the mechanisms by which these CSPG exert their inhibitory effects are still not entirely clear.

In this study, we have examined the organotypic coculture system using brain cortex and spinal cord from neonatal rats.^{12,13} The advantage of this coculture system is to assess CST axon growth quantitatively and to facilitate the analysis

of factors that regulate axonal growth. The data in a separate publication do indicate that neuronal cell bodies (identifiable by their characteristic appearance in Nissl-stained sections) were evident in both the cortical and spinal cord explants, residual lamination was seen in the cortex and longitudinally oriented cell columns could be seen in the spinal cord.¹² Moreover, in the paper, to identify the cortical neurons that projected to the spinal cord, DiI crystals were placed on the spinal cord and retrogradely labeled cortical neurons were identified. Retrogradely labeled neurons were seen in the cortex in some experiments, but most of these were located in the deep layers. The study has demonstrated that the failure of axon growth with development is due to development of a nonpermissive tissue substrate with organotypic coculture, and that the most prominent difference between the P3 and the P7 cocultures is the amount of gliosis. Glia scars not only present a physical barrier, but also produce repulsive molecules such as CSPG. In the current study, more CSPG expression in the P7 control group was detected than in the P3 control group, and fewer axons were

seen in the P7 control group compared to the P3 control group. The corticospinal axons growth depends on the age of the tissue, and it might correlate to the expression of CSPG.

The administration of ChABC causes CSPG expression to decrease and 2B6 expression to increase. These results indicate that CSPG is evidently degraded by the administration of ChABC. In addition, axon growth from the brain cortex to the spinal cord is promoted by the administration

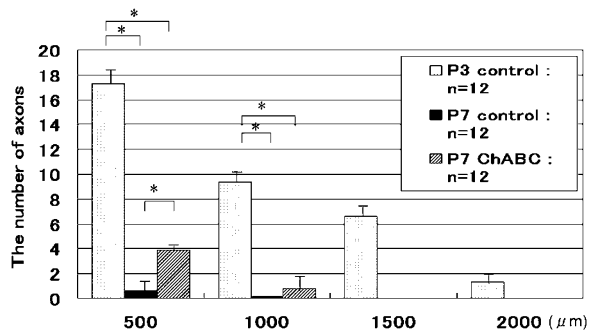


Figure 2 The quantitative assessment of axonal growth from the cortex to the spinal cord. The average number of axons in the P3 control group was significantly greater than that in the P7 control group at a distance of 500 and 1000 μm from the junction. The average number of axons in the P7 chondroitinase ABC (ChABC) group was significantly greater than that in the P7 control group at a distance of 500 μm from the junction. But the average number of axons in the P7 ChABC group extending 500 and 1000 μm from the junction was significantly less than that in the P3 control group and there was no significant difference between the average number of axons extending 1000 μm from the junction in the P7 ChABC group and that in the P7 control group (* $p < 0.05$, Scheffe).

of ChABC. This finding emphasizes that ChABC treatment may favor axonal regeneration by providing a more permissive substrate for axons through the degradation of CSPG. On the other hand, the effect of CSPG on axonal growth is unclear. Although ChABC promotes axon growth by digesting CSPG, the degree of axon growth is limited. In this study, the average number of axons in the P7 ChABC group extending 500 and 1000 μm from the junction was significantly less than that in the P3 control group and there was no significant difference between the average number of axons extending 1000 μm from the junction in the P7 ChABC group and that in the P7 control group. These findings suggest that other factors restrict axonal regeneration after SCI and it may be necessary to overcome these factors with a combination strategy. On the other hand, there is a possibility not only that spinal cord explants become inhibitory to axonal growth between P3 and P7 but that the regenerative capacity of cortical neurons may decrease between P3 and P7. The previous study showed that a moderate number of axons extended from P7 cortex into P3 spinal cord, and that the average number of axons in P7 cortex/P3 spinal cord cocultures was significantly greater than that in P7 cortex/P7 spinal cord cocultures but was not different from that in P3 cortex/P3 spinal cord cocultures.¹² These findings indicate that cortical neurons at P7 maintain their ability to regenerate axons in this coculture system. To clarify the mechanism of ChABC that reduce the inhibitory nature of CSPG, a further study using the mixed-age chimeric coculture will be performed. Nevertheless, there is a possibility that CSPG hampers axonal regeneration clinically after SCI. The ongoing development of pharmaceutical

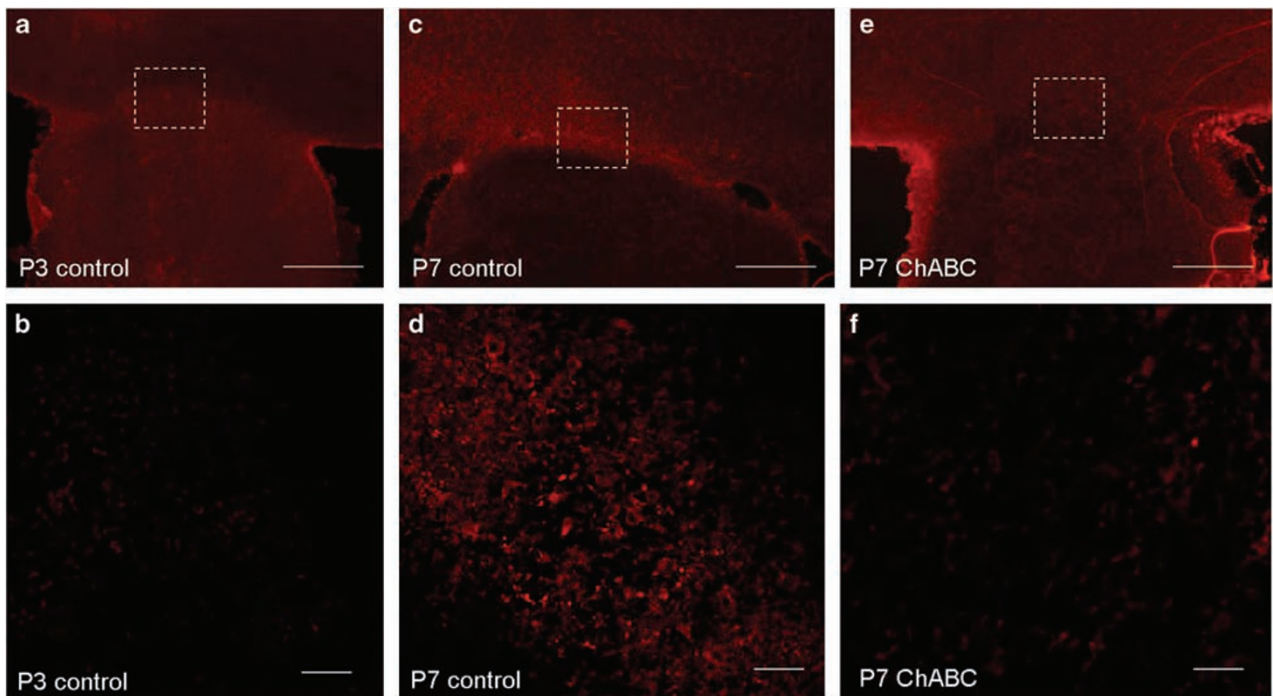


Figure 3 Immunological staining of chondroitin sulfate proteoglycan (CSPG). In the P3 control group, immunoreactivity of CSPG (red) was observed around the junction (a, b), and that increased in the P7 control group (c, d). The CSPG expression decreased in the P7 chondroitinase ABC (ChABC) group (e, f) compared with the P7 control group. (a, c, e) Bar = 200 μm, (b, d, f) bar = 20 μm.

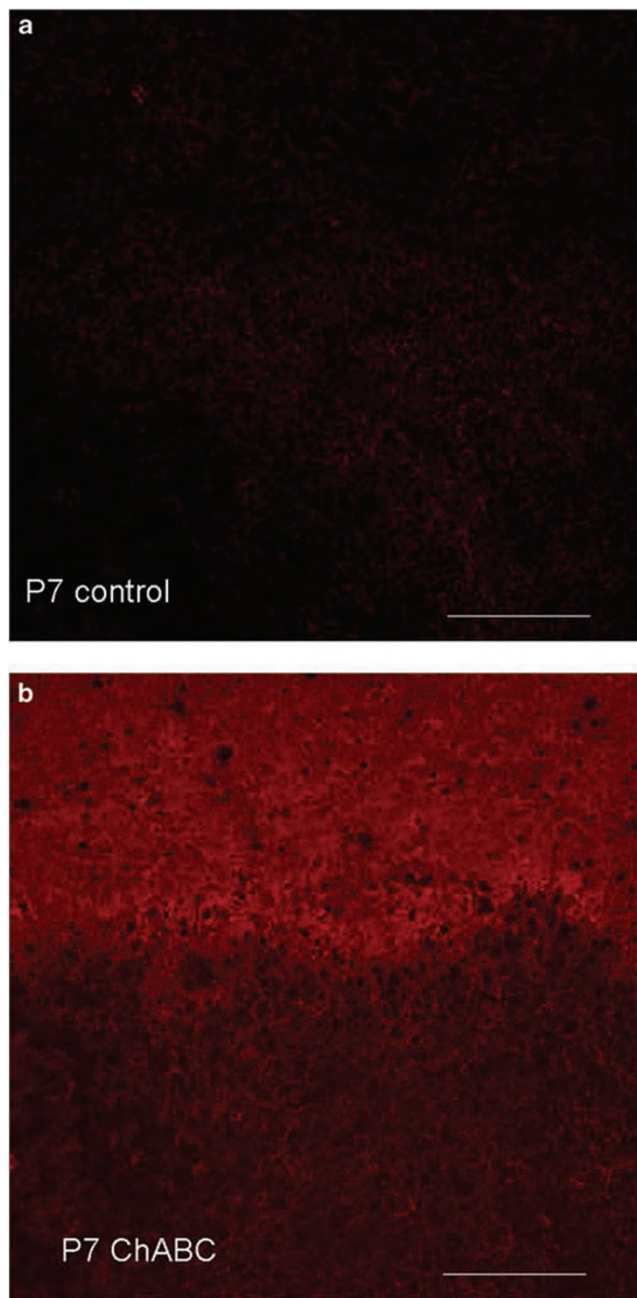


Figure 4 Immunological staining of 2B6 expression. The 2B6 (red) expression increased in the P7 chondroitinase ABC (ChABC) group (a) compared with the P7 control group (b). Bar = 20 μ m.

therapies such as ChABC has great potential for future therapeutic use in spinal cord-injured patients.

Acknowledgements

We thank Professor Norio Sakai (Department of Molecular and Pharmacological Neuroscience, Hiroshima University) for use of the instruments. We also thank Dr Yosuke Oishi for helpful comments.

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