Inhibitory Effects of Neurocan and Phosphacan on Neurite Outgrowth from Retinal Ganglion Cells in Culture

Masaru Inatani,[1] Megumi Honjo,[1] Yasumasa Otori,[2] Atsuhiko Oohira,[3] Noriaki Kido,[1] Yasuo Tano,[2] Yoshihito Honda,[1] and Hidenobu Tanihara[4]

Purpose. Neurocan and phosphacan are nervous tissue–specific chondroitin sulfate proteoglycans (CSPGs) that are highly expressed in postnatal rat retina. To elucidate potential roles of neurocan and phosphacan on neurite outgrowth from retinal ganglion cells (RGCs), in vitro experiments were conducted with purified RGCs.

Methods. Neurocan and phosphacan were purified from postnatal rat brain by DEAE-column chromatography and subsequent gel chromatography. RGCs were obtained from postnatal rat retinas by a two-step immunopanning procedure using an anti-Thy 1,1 antibody and an anti-macrophage antibody. Neurite outgrowth from RGCs was examined on poly-L-lysine (PLL)-conditioned plates, and PLL-conditioned plates treated with neurocan or phosphacan.

Results. Compared with PLL-conditioned plates, neurocan and phosphacan inhibited neurite outgrowth from RGCs at 48 and 72 hours after seeding. When chondroitin sulfate side chains linked to the core proteins were digested by chondroitinase ABC, the inhibitory effect remained, indicating that the core proteins are related to the effect. Furthermore, the digestion of chondroitin sulfate side chains linked to phosphacan core protein significantly promoted the inhibitory effect of phosphacan on neurite outgrowth from RGCs.

Conclusions. Neurocan and phosphacan, which are highly expressed in postnatal rat retina, inhibit neurite outgrowth from postnatal rat RGCs, indicating that these proteoglycans may be inhibitory factors against neurite outgrowth from RGCs during retinal development. (*Invest Ophthalmol Vis Sci.* 2001;42: 1930–1938)

It is known that multiple types of proteoglycans are expressed in developing and pathologic retinas.[1] Among these, some chondroitin sulfate proteoglycans (CSPGs) have been identified on developing retinal neurites.[2–6] In addition, it has been reported that, in embryonal rat retinal tissue, disturbance of chondroitin sulfate causes ectopic differentiation and aberrant orientation of neurites in retinal ganglion cells (RGCs).[7] Moreover, elongation of RGC axons is regulated by CSPGs derived from bovine nasal cartilage and chick limb,[8] whereas rat superior colliculus–derived CSPGs promote survival of RGCs and elongation of their axons in vitro.[9] These findings demonstrate that CSPGs elicit both inhibitory and stimulatory responses in neurite outgrowth from RGCs, suggesting that CSPGs play complicated and crucial roles in the retinal neural network.

Neurocan and phosphacan are nervous tissue–specific proteoglycans and are two major constituents of CSPGs in postnatal rat central nervous system (CNS).[10] Neurocan belongs to the aggrecan family, which constitutes hyaluronan-binding proteoglycans.[11] The 130- and 150-kDa neurocan core proteins are created from the 220-kDa full-length neurocan core protein by proteolytic processes during CNS development.[10,11] At the same time, the 300-kDa core protein of phosphacan (also known as 6B4 proteoglycan[12] or DSD-1-proteoglycan[13]) is an alternatively spliced product of the receptor-type protein tyrosine phosphatase (RPTP ζ/β).[14] Neurocan[15,16] and phosphacan[17,18] show spatiotemporal expression patterns during the development of the CNS and exhibit upregulated expression after CNS injury.[19–24] There are also many reports that neurocan and/or phosphacan regulate neurite outgrowth from neural cells in vitro.[13,24–29] Furthermore, the CSPGs have been reported to alter neural cellular behaviors through binding to extracellular matrices,[30–33] cell adhesion molecules,[26,27,34] and growth factors.[32,35,36] Thus, these experimental results suggest that neurocan and phosphacan play a major role in neural network formation.

We previously demonstrated that neurocan,[5] the C-terminal proteolytic variant of neurocan core protein,[5] and phosphacan[6] are highly expressed in nerve fiber–rich layers, such as the nerve fiber layer (NFL), inner plexiform layer (IPL), and outer plexiform layer (OPL) in postnatal rat retina, indicating their involvement with neural network formation during retinal development. However, so far, knowledge about the biological significance of the CSPGs in the retinal neural network is limited. Recently, Li et al.[37] showed that recombinant neurocan core protein inhibits neurite outgrowth from chick embryonal retinal cells. We previously showed that the major form of phosphacan in postnatal rat retina has no chondroitin sulfate side chains, termed nonproteoglycan-type,[6] whereas neurocan has chondroitin sulfate side chains.[5] At present, the effects of chondroitin sulfate side-chain–bound neurocan and phosphacan or phosphacan core protein, the major form in the retina, on RGCs remain unknown. Thus, to elucidate potential roles of neurocan and phosphacan on neurite outgrowth from RGCs, we conducted in vitro experiments using purified RGCs.

Materials and Methods

Preparation of the PBS-Soluble Protein Fraction

All animals were given water and food ad libitum, and all studies were conducted in accordance with the ARVO Statement for the Use of

From the [1]Department of Ophthalmology and Visual Sciences, Kyoto University Graduate School of Medicine, Japan; the [2]Department of Ophthalmology and Visual Science, Osaka University Medical School, Japan; the [3]Department of Perinatology and Neuroglycoscience, Institute for Developmental Research, Aichi, Japan; and the [4]Department of Ophthalmology, Kumamoto University School of Medicine, Japan.

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Corresponding author: Hidenobu Tanihara, Department of Ophthalmology, Kumamoto University School of Medicine, 1-1-1 Honjo, Kumamoto 860-8556, Japan. tanihara@pearl.ocn.ne.jp

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Animals in Ophthalmic and Vision Research and the guidelines for animal experimentation of our institute. Preparation of the phosphate-buffered saline (PBS)–soluble protein fraction was performed according to a previous report,[18] with slight modification. In brief, 10-day-old Sprague-Dawley rats were anesthetized with diethyl ether and killed by decapitation. One hundred grams of whole brain tissue from 100 pups was homogenized in 200 ml ice-cold PBS containing 20 mM EDTA, 10 mM *N*-ethylmaleimide (NEM), and 2 mM phenylmethylsulfonyl fluoride (PMSF) as protease inhibitors. The homogenate was centrifuged at 27,000*g* for 40 minutes at 4°C, and the supernatant (SUP-I) was stored. The pellet was homogenized in 100 ml ice-cold PBS containing protease inhibitors, and the homogenate was again subjected to centrifugation. The resultant supernatant (SUP-II) was added to SUP-I. The supernatant containing the PBS-soluble protein fractions was then lyophilized with a vacuum pump.

Purification of Neurocan and Phosphacan

Purification of the two proteoglycans was performed according to a previous report,[38] with slight modification. The lyophilized materials were suspended in 100 ml of 2 M urea and 50 mM Tris-HCl (pH 7.5), containing 0.15 M NaCl and protease inhibitors, and then dialyzed against the same buffer at 4°C. After insoluble materials were removed by centrifugation at 15,000*g* for 30 minutes at 4°C, the supernatants were applied to a DEAE-Sephacel column (60 ml; Amersham Pharmacia Biotech, Uppsala, Sweden). The columns were then washed with 180 ml of the same urea buffer. Elution was performed at 4°C in 600 ml of the buffer with a linear gradient of the NaCl concentration from 0.15 to 0.7 M at a flow rate of 20 ml/h. Fractions of 5 ml were collected and measured for concentrations of hexuronate.

The amount of hexuronate was determined by the method of Bitter and Muir.[39] DEAE column chromatography showed the peak in the material extracted from brains. Because the peak was enriched with chondroitin sulfate,[38] the component was concentrated to 4 ml on a membrane (Diaflo YM-10; Millipore, Bedford, MA). The proteoglycan-rich solution was chromatographed at 4°C on a Sepharose column (CL-4B; Amersham Pharmacia Biotech) in 1 M guanidine HCl containing 50 mM Tris-HCl (pH 7.5) at a flow rate of 10 ml/h. Then, fractions of 3 ml were collected and measured for concentration of hexuronate. The eluted fractions were separated into three fractions, designated Ia, IIa, and IIIa, in the order of elution (Fig. 1A). Proteoglycans were precipitated from fractions Ia and IIIa by adding 3 volumes of 95% ethanol containing 1.3% potassium acetate at 4°C, and then, each precipitated material was suspended in 2 ml of 1 M guanidine HCl buffer. The concentrated fractions Ia and IIIa were chromatographed at 4°C on a Sepharose column (CL-6B; Amersham Pharmacia Biotech) in 1 M guanidine HCl and 50 mM Tris-HCl (pH 7.5) at a flow rate of 7 ml/h.

After measuring the concentration of hexuronate in fractions of 2 ml, the eluted fractions were again separated into two fractions, designated I and IIb from fraction Ia (Fig. 1B) and IIc and III from fraction IIIa (Fig. 1C). The fractions IIb and IIc were added to the fraction IIa, designated as II (Fig. 1D). Proteins were precipitated from each fraction (I, II, and III) with ethanol. Each fraction was further purified by ultracentrifugation in a cesium chloride (CsCl) density gradient at an initial density of 1.4 g/ml in the guanidine HCl buffer at 10°C, using a rotor (RPS-65T; Hitachi, Ltd., Tokyo, Japan) at 40,000 rpm for 40 hours. After the concentration of hexuronate from the aliquot (each 650 μl)

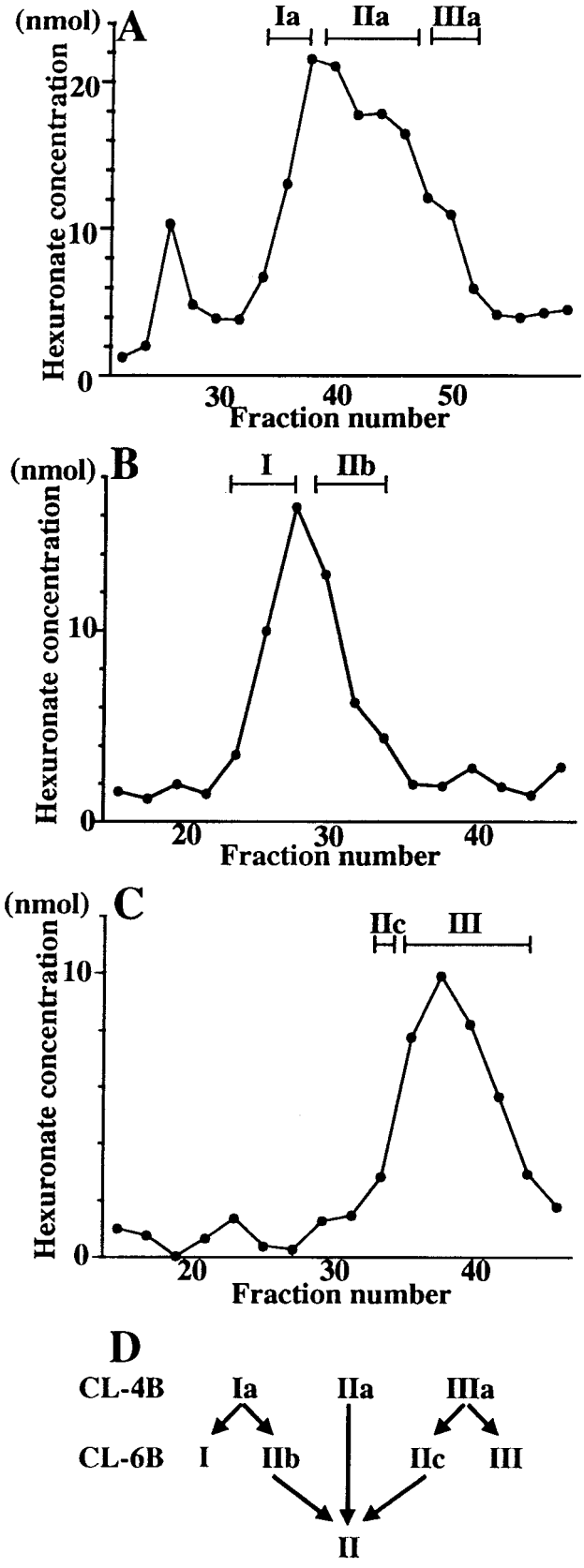

Figure 1. Hexuronate concentrations of the fractions after column chromatography. The proteoglycan-rich solution separated by DEAE column chromatography was applied to a CL-4B column (1.6 × 100 cm). (**A**) The samples of fraction numbers 33 to 37, 38 to 46, and 47 to 51 were designated as fractions Ia, IIa and IIIa, respectively. The samples derived from fractions Ia and IIIa were further chromatographed on a CL-6B column (1.4 × 75 cm). The eluted fractions were again separated into two fractions, designated fraction I (fraction numbers 23–27) and fraction IIb (fraction numbers 28–33) from fraction Ia (**B**), and fraction IIc (fraction numbers 33–34) and fraction III (fraction numbers 35–43) from fraction IIIa (**C**). Fractions IIa, IIb, and IIc were combined, and the combined sample was designated fraction II (**D**).

was measured, the aliquots with high hexuronate concentration were combined.

Detection of Purified Proteoglycans

To remove CsCl in the proteoglycan solutions, the aliquots (10 nanomoles hexuronate) of solutions derived from the fractions I, II, and III were diluted with three volumes of distilled water, and proteoglycans were then precipitated with ethanol. The precipitated materials were dissolved in 50 μl distilled water and again precipitated with ethanol. The materials were suspended in 21.5 μl distilled water. To this suspension, 2.5 μl of 1 M Tris-HCl buffer (pH 7.5), including 0.3 M sodium acetate, and 1 μl (10 mU) of protease-free chondroitinase ABC (EC 4.2.2.4; Seikagaku Co., Tokyo, Japan) solution were added. Chondroitinase ABC was diluted with 20 mM Tris-HCl (pH 7.5) buffer containing 0.1% bovine serum albumin (BSA). The mixture (total, 25 μl) was incubated at 37°C for 30 minutes and precipitated with ethanol.

The precipitated material was separated by SDS-PAGE on a 3% stacking gel and a 6% separating gel, as described previously.[5] The electrophoresed gel was stained with Coomassie blue. Moreover, to confirm whether neurocan and phosphacan were completely separated from each other, we performed immunoblot analyses using an anti-neurocan monoclonal antibody, mAb 1G2,[16] and an anti-phosphacan antibody (mAb) 6B4,[18] as described previously. In brief, the electrophoresed sample was transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was incubated in the blocking solution for 1 hour at room temperature, incubated in the first antibody (mAb 1G2 or mAb 6B4) for 2 hours, and subsequently incubated in the biotinylated second antibody for 30 minutes at room temperature. After incubation with an ABC kit (Vectastain Elite; Vector Laboratories, Burlingame, CA), immunoreactive materials on the membrane were detected using 4-chloro-1-naphthol.

Culture of RGCs

As described previously,[40,41] RGCs from retinas of 6- to 8-day-old rats were purified by a two-step immunopanning procedure. Briefly, the retinal tissue was dissociated into single cells in Eagle's minimum essential medium (EMEM) containing 15 U/ml papain and 70 U/ml collagenase. The dissociated cells were incubated in a polypropylene tube coated with an anti-rat macrophage monoclonal IgG (Chemicon International, Inc., Temecula, CA) to exclude macrophages, and then incubated in a tube coated with an anti-rat Thy 1.1 monoclonal IgG (Chemicon International, Inc.). The tube was gently washed with PBS five times, and adherent RGCs were collected by centrifugation at 700*g* for 5 minutes.

Before beginning the examinations of the neurite outgrowth of RGCs, a preliminary study was conducted to determine purity of RGCs after the two-step immunopanning procedure. In brief, RGCs were labeled in a retrograde manner by injecting 1 mg/ml 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiI) into the superior colliculi of anesthetized 4- to 5-day-old rats. As described previously,[41] after this immunopanning method, approximately 85% of the collected cells were labeled by DiI. Next, in further examinations of the neurite outgrowth of RGCs, RGCs were used from rats without DiI injection. Purified RGCs were seeded at low density of approximately 5000 cells/cm^2 on 96-well plastic tissue culture plates. Before seeding, the plates were coated with 50 μg/ml poly-L-lysine (PLL plates; Life Technologies, Rockville, MD) overnight at 4°C. After the plates were washed three times with PBS, the plates were coated with other materials, such as the purified proteoglycans, 10 μg/ml ornithine (Sigma, St. Louis, MO), 10 μg/ml fibronectin (Sigma), or 10 μg/ml laminin (Life Technologies) overnight at 4°C.

Moreover, to compare another proteoglycan with the purified proteoglycans, rat chondrosarcoma proteoglycan (ICN Biomedicals, Aurora, OH) was used as a coating material. To remove CsCl from our purified proteoglycan fractions, the fractions were diluted with three volumes of distilled water, and proteoglycans were precipitated from the diluted fractions with ethanol, as described earlier. Proteoglycans thus precipitated were washed with 75% ethanol containing 1% potassium acetate. Finally, the precipitated materials were suspended in PBS in the same manner as the other coating materials. To use the proteoglycan core proteins bearing no chondroitin sulfate as coating materials, we digested chondroitin sulfate with chondroitinase ABC, as described earlier. After the treatment with chondroitinase ABC, the proteoglycan materials were precipitated with 75% ethanol containing 1% potassium acetate, dried up, and suspended in PBS, in a manner similar to the other coating materials. The plates coated with materials were washed three times with PBS before the cells were seeded. The cells were then cultured (Neurobasal culturing medium; Life Technologies) with 1 mM glutamine, 10 μg/ml gentamicin, B27 supplement (Life Technologies), 40 ng/ml human brain-derived neurotrophic factor (Diaclone Research, Besaçn, France), 40 ng/ml rat ciliary neurotrophic factor (Diaclone Research), and 5 μM forskolin (Sigma). Cultures were maintained at 37°C in a 5% CO_2 incubator.

Assay of the Lengths of Neurites from RGCs

To measure the lengths of neurites from living RGCs only, 1 μM calcein-acetoxymethyl ester (calcein-AM; Molecular Probes, Eugene, OR) was added to the culture wells.[41] After incubation for 60 minutes at 37°C, the cell bodies and neurites of living cells were stained by calcein-AM, which becomes fluorescent when activated by an intracellular esterase. The lengths of neurites from calcein-AM–stained cells were examined. In each well, five fields were photographed through the microscope at a ×200 scale. The first field was selected in the center of the well. The other fields were selected in the four directions next to the first field. In each substratum condition, three wells were used (15 fields in each condition). All calcein-AM–stained cells in each photograph were selected to measure neurite length. The clusters of cells were excluded from the measurement of neurite length. A total of approximately 400 cells in 15 fields were examined for each condition. The longest neurite from each cell was selected for measurement. Neurite length was defined as the distance between the cell body and the farthest tip of the neurite.

Results

Purification of Neurocan and Phosphacan from Rat Brain

After purification, we obtained proteoglycans equivalent to 400 nanomoles (fraction I), 2800 nanomoles (fraction II), and 300 nanomoles (fraction III) of hexuronate. These fractions (containing proteoglycans) were analyzed by SDS-PAGE, with or without chondroitinase ABC treatment. When fractions I and II were electrophoresed, Coomassie blue staining demonstrated that most proteoglycans in the fractions remained at the top of the 6% polyacrylamide separating gel because of their high molecular weights, due to glycosaminoglycan side chains (Fig. 2A, lanes 1 and 3). In fraction III, a broad smearing band was observed at more than 200 kDa (lane 5). Subsequent chondroitinase ABC treatment produced one band of 300 kDa (fraction I; lane 2), four bands of 300, 220, 150, and 130 kDa (fraction II; lane 4), and two bands of 150 and 130 kDa (fraction III; lane 6). As described before,[16,18,38] the highest molecular mass (300 kDa) was equal to that of the reported phosphacan core protein, and the other three corresponded to those of the reported core proteins of the full-length neurocan (220 kDa) and its C-terminal half (150 kDa) and N-terminal half (130 kDa) proteolytic variants.

To confirm whether neurocan and phosphacan were completely separated in fractions I and III, we performed immunoblot analyses using an anti-neurocan monoclonal antibody (mAb 1G2) and an anti-phosphacan mAb, 6B4. After the immunoblot analysis using the anti-phosphacan antibody (mAb 6B4) in fractions I (Fig. 2B, lane 2) and II (lane 4) treated with

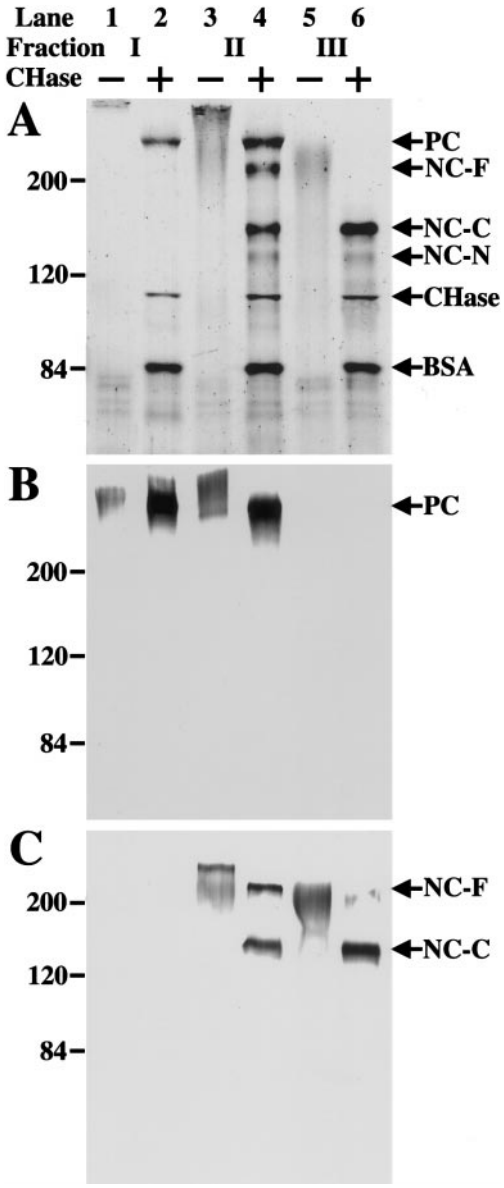

FIGURE 2. SDS-PAGE and subsequent immunoblot analyses of proteoglycan fractions I, II, and III. When the intact proteoglycans from fractions I and II were electrophoresed, most of the proteoglycans remained at the top of the 6% polyacrylamide separating gel (**A**, *lanes 1* and *3*). In fraction III, a broad smearing band was observed above 200 kDa (*lane 5*). Digestion of proteoglycans with chondroitinase ABC (CHase) produced one band from the fraction I (*lane 2*), four bands from the fraction II (*lane 4*), and two bands from fraction III (*lane 6*). The molecular masses of the protein bands were 300 kDa (phosphacan core protein; PC), 220 kDa (the full-length core protein of neurocan; NC-F), 150 kDa (the C-terminal half proteolytic core protein of neurocan; NC-C), and 130 kDa (the N-terminal half of the proteolytic core protein of neurocan; NC-N). BSA was added to the chondroitinase ABC preparation as a stabilizer. After the immunoblot analysis with (**B**) an anti-phosphacan antibody (mAb 6B4), in fractions I (*lane 2*) and II (*lane 4*) treated with chondroitinase ABC, an intense immunopositive band was observed at 300 kDa whereas, in fraction III treated with chondroitinase ABC (*lane 6*), no immunopositive bands were detected. Furthermore, after immunoblot analysis with (**C**) the anti-neurocan antibody (mAb 1G2), which recognizes both the 220-kDa full-length core protein of neurocan and a 150-kDa proteolytic C-terminal half product, in fraction II treated with chondroitinase ABC (*lane 4*), two intense immunopositive bands (220 and 150 kDa) were detected. In fraction III treated with chondroitinase ABC (*lane 6*), the intense 150-kDa band and faint 220-kDa band were detected. In fraction I treated with chondroitinase ABC (*lane 2*), no immunopositive bands were detected.

chondroitinase ABC, an intense immunopositive band was observed at 300 kDa, whereas in fraction III treated with chondroitinase ABC (lane 6), no immunopositive bands were detected. The same procedure was performed using the anti-neurocan antibody (mAb 1G2). As described previously,[16] mAb 1G2 recognizes both the 220-kDa full-length core protein of neurocan and a 150-kDa proteolytic C-terminal half product. In fraction II treated with chondroitinase ABC (Fig. 2C, lane 4), two intense immunopositive bands (220 and 150 kDa) were detected, indicating the full-length core protein of neurocan and the proteolytic C-terminal half product. In fraction III treated with chondroitinase ABC (lane 6), the intense 150-kDa band and faint 220-kDa band were detected. In contrast, in fraction I treated with chondroitinase ABC (lane 2), no immunopositive bands were detected. Thus, the purification procedures and subsequent electrophoretic analyses of the purified products clearly show that we obtained purified neurocan (fraction III) and phosphacan (fraction I).

Neurite Outgrowth from RGCs on PLL Plates Coated with Neurocan and Phosphacan

At first, we conducted preliminary studies to confirm the neurite-extending effects of laminin in our experimental design. As described previously,[41] RGCs in serum-free medium (including neurotrophic factors and forskolin) extended their neurites on 50 μg/ml PLL plates subsequently coated with 10 μg/ml laminin (Fig. 3A). At 48 hours after seeding, the average length ($\pm$SE) of the extended neurites was 41.5 $\pm$ 3.1 μm. When PLL plates were subsequently coated with 10 μg/ml fibronectin, instead of laminin, the average length of the extended neurites was 28.6 $\pm$ 1.6 μm. Similar experiments, using PLL plates coated with 10 μg/ml ornithine, instead of laminin, resulted in 25.7 $\pm$ 1.8 μm of the neurite length. When RGCs were cultured on PLL plates without any further coating (Fig. 3B), the average length of the extended neurites was 25.6 $\pm$ 1.6 μm, which was significantly shorter than that on PLL plates coated with laminin ($P < 0.001$, Mann-Whitney test). These results demonstrated the promoting effects of laminin on neurite outgrowth in comparison with PLL, ornithine, and fibronectin, which is agreement with previous reports.[41,42]

When PLL plates were subsequently coated with purified neurocan (fraction III; Fig. 3D) or purified phosphacan (fraction I; Fig. 3E), inhibitory effects on neurite outgrowth were observed after 48 hours of culture. Moreover, when PLL plates were coated with neurocan or phosphacan after treatments with chondroitinase ABC (Figs. 3F, 3G), inhibitory effects were also observed. An interesting observation was that the RGCs on phosphacan after the treatment with chondroitinase ABC had shorter neurites than those on intact phosphacan. In comparison with another CSPG, PLL plates were coated with rat chondrosarcoma proteoglycan. Inhibitory effects on neurite outgrowth were not observed on PLL plates coated with rat chondrosarcoma proteoglycan (Fig. 3C).

When PLL plates were coated with the CSPGs, we estimated 1 μg core protein of neurocan as 5 nanomoles hexuronate and 1 μg protein of phosphacan as 11 nanomoles hexuronate in the same procedure as described previously.[25] In measurement of neurite outgrowth, approximately 400 calcein AM–positive cells (living cells) in each condition were examined as described earlier. The numbers of calcein AM–positive cells in all fields of the microscope did not differ significantly. When compared with PLL, neurocan and phosphacan inhibited neurite outgrowth from RGCs after 48 hours of culture in a dose-dependent manner (Fig. 4). Measurements of neurite outgrowth demonstrated the average lengths ($\pm$SE) of the neurites to be 16.3 $\pm$ 1.0 μm on PLL plates coated with 10 μg/ml neurocan and 13.1 $\pm$ 1.0 μm on PLL plates coated with 10 μg/ml phosphacan. In contrast, the average length of the

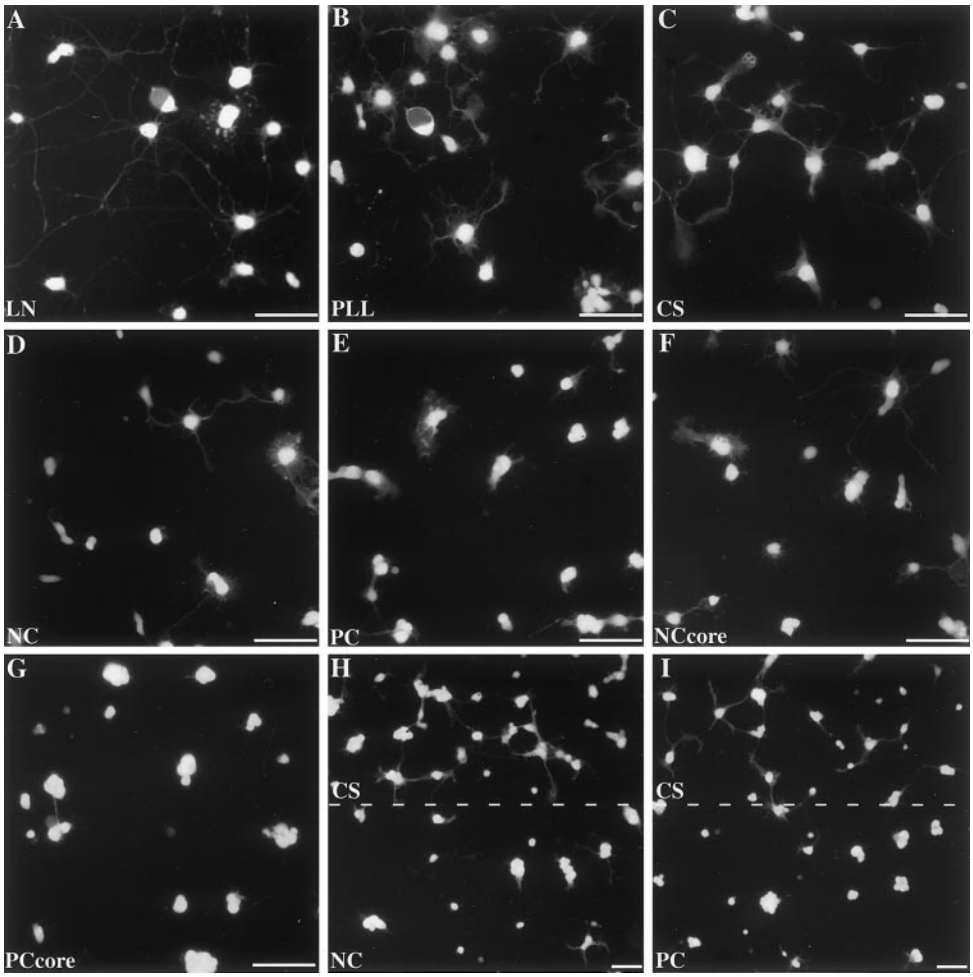

FIGURE 3. Inhibition of neurite outgrowth by neurocan and phosphacan. To observe the neurites of living cells, calcein-AM was used. Many RGCs extended their long neurites on 50-μg/ml PLL plates coated with 10 μg/ml laminin at 48 hours after seeding (**A**). Even on plates coated only with PLL, many RGCs extended their neurites (**B**). Additionally, when PLL plates were coated with rat chondrosarcoma proteoglycan, many RGCs bearing long neurites were also observed (**C**). On PLL plates coated with the 10 μg/ml neurocan (**D**) or phosphacan (**E**), strong inhibitory effects on neurite outgrowth were observed. When PLL plates were coated with neurocan (**F**) or phosphacan (**G**) after treatment with chondroitinase ABC, the inhibitory effects also remained. Of interest, the RGCs on phosphacan treated with chondroitinase ABC had shorter neurites than those on intact phosphacan. RGCs extended long neurites on the region coated only with rat chondrosarcoma proteoglycan, whereas neurite outgrowth was inhibited on the region coated with neurocan (**H**) or phosphacan (**I**). LN, laminin; PLL, poly-L-lysine; CS, rat chondrosarcoma proteoglycan; NC, neurocan; PC, phosphacan; NCcore, neurocan digested with chondroitinase ABC; PCcore, phosphacan digested with chondroitinase ABC. Scale bar, 50 μm.

neurites on PLL plates coated with 10 μg/ml rat chondrosarcoma proteoglycan was 27.2 $\pm$ 1.5 μm, indicating that the proteoglycan showed no inhibitory effects.

To identify whether the inhibitory effects of neurocan and phosphacan are derived from the core proteins or the chondroitin sulfate side chains, we coated PLL plates with the proteoglycans digested by chondroitinase ABC. The inhibitory effect was also observed on plates coated with neurocan and phosphacan after treatment with chondroitinase ABC again in a dose-dependent manner. Unexpectedly, on PLL plates coated with 10 μg/ml phosphacan, neurite length was 13.1 $\pm$ 1.0 μm after 48 hours of culture, whereas on PLL plates coated with 10 μg/ml phosphacan treated with chondroitinase ABC (phosphacan core protein), it was 4.0 $\pm$ 0.5 μm, indicating that digestion of the chondroitin sulfate side chains significantly promoted the inhibitory effect of phosphacan on neurite outgrowth from RGCs ($P < 0.0001$, Mann-Whitney test). However, the digestion of the chondroitin sulfate side chains did not show any significant changes in the inhibitory effects of neurocan.

In addition, to exclude the possibility that CsCl contained in the purified neurocan and phosphacan fractions exert an inhibitory effect on neuritogenesis, we compared the neurite outgrowth inhibitory effects of neurocan and phosphacan with that of rat chondrosarcoma proteoglycan on the same PLL plate. After PLL plates were coated with rat chondrosarcoma proteoglycan, the half regions of the plates were coated with neurocan or phosphacan. RGCs extended long neurites on the region coated only with rat chondrosarcoma proteoglycan, as described earlier. Neurite outgrowth was inhibited, however, on the region with neurocan (Fig. 3H) or phosphacan (Fig. 3I). Thus, the data showed that neurocan and phosphacan in the purified fractions truly have inhibitory effects on neurite outgrowth.

We further cultured RGCs for 72 hours on each experimental substrate (Fig. 5). After 72 hours of culture, neurocan and phosphacan inhibited neurite outgrowth. Moreover, after treatment with chondroitinase ABC, the inhibitory effects of phosphacan, not neurocan, were significantly promoted ($P < 0.0001$, Mann-Whitney test).

Inhibitory Effects of Neurocan and Phosphacan on Neurite Outgrowth from RGCs on PLL Plates Coated with Laminin

We examined whether the CSPGs inhibit neurite outgrowth from RGCs on PLL plates coated with laminin, which is a substrate that intensely promotes neurite outgrowth.[41,42] After cultivating RGCs for 24 hours on PLL plates coated with 10 μg/ml laminin, we added 1- or 10-μg/ml (final concentration) neurocan or phosphacan to the medium and continued cultivation for another 24 hours. We then measured neurite lengths in the same manner as described earlier (Fig. 6). When RGCs were cultured on PLL plates coated with laminin, the average of neurite lengths was 41.5 $\pm$ 3.1 μm at 48 hours after seeding, as described earlier. In the cases in which 10 μg/ml (final concentration) rat chondrosarcoma proteoglycan was added to the medium at 24 hours after seeding and then cultured for an additional 24 hours, the average neurite length was 30.7 $\pm$ 3.2 μm, which indicates that rat chondrosarcoma proteoglycan

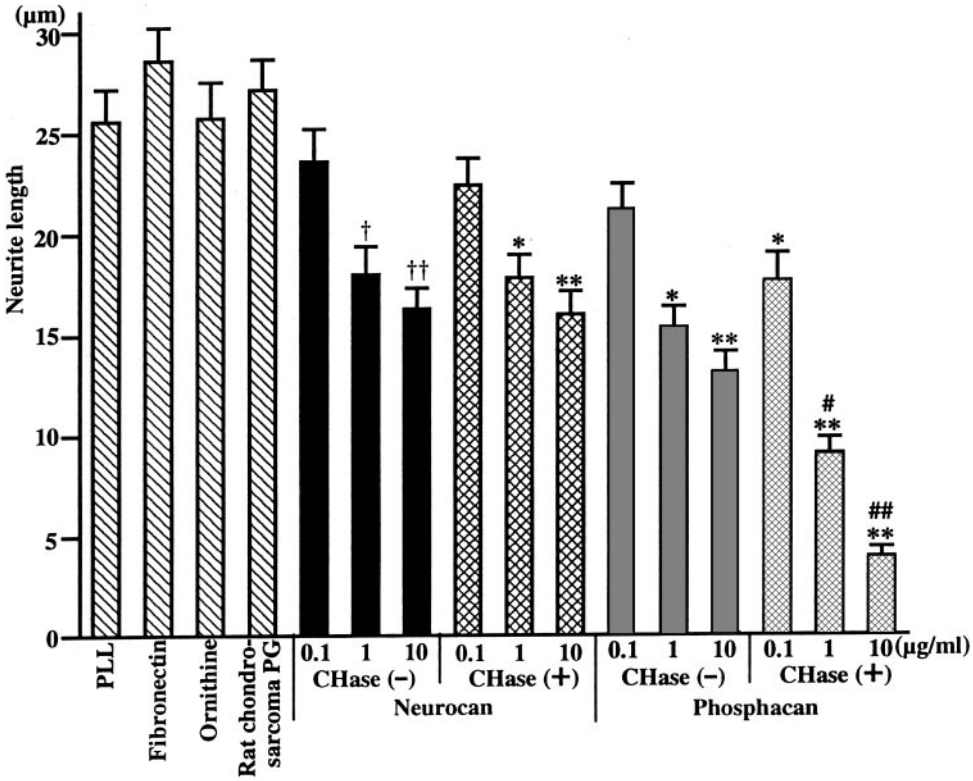

FIGURE 4. Neurite lengths from RGCs at 48 hours after seeding. Compared with PLL plates, neurite outgrowth from RGCs was significantly inhibited in a dose-dependent manner on PLL plates coated with neurocan or phosphacan (†$P < 0.05$, ††$P < 0.01$, *$P <$ 0.001, **$P < 0.0001$ compared with PLL, Mann-Whitney test). Moreover, phosphacan core protein had a greater inhibitory effect on neurite outgrowth than did phosphacan-bearing chondroitin sulfate side chains (#$P < 0.01$, ##$P < 0.0001$ compared with 10 μg/ml phosphacan without chondroitinase ABC, Mann-Whitney test). CHase, chondroitinase ABC. Error bar, SE.

attenuated the promoting effect of laminin on neurite outgrowth. Instead of the proteoglycan, in the cases in which 10 μg/ml neurocan was used, neurite length decreased to 19.4 ± 2.0 μm, whereas after treatment with chondroitinase ABC, the length was 19.4 ± 3.2 μm in the presence of 10 μg/ml neurocan. In contrast, when we added 10 μg/ml phosphacan to the medium, the neurite length decreased to 12.0 ± 1.2 μm, and when we added 10 μg/ml phosphacan core protein, which was created by chondroitinase ABC treatment, to the medium, the neurite length was 8.5 ± 1.2 μm, indicating that digestion of the chondroitin sulfate side chains significantly promoted the inhibitory effect ($P < 0.01$, Mann-Whitney test).

DISCUSSION

The studies described herein show that two nervous tissue–specific CSPGs, neurocan and phosphacan, inhibited neurite outgrowth from postnatal rat RGCs in vitro. Numerous kinds of proteoglycans are expressed in mammalian retinas.[1] In particular, CSPGs are expressed primarily in two regions in the retina: the interphotoreceptor matrix (IPM)[43–47] and the nerve fiber–rich layers,[2–6] such as the NFL, IPL, and OPL. It has been thought that the hyaluronan-binding property of proteoglycans in the IPM is involved in the organization of the IPM and in retinal attachment.[48] However, most proteoglycans in the nerve fiber–rich layers are expressed only transiently during developmental stages.[3–6] Previously, we showed that neurocan[5] and phosphacan[6] are highly expressed in the NFL and IPL between postnatal day (P)7 and P14, which corresponds to the stage when the growth of axons and dendrites from RGCs is complete.[49–51] Thus, it is likely that the CSPGs may be related to stopping the neurite outgrowth from RGCs during retinal development.

In our assay of neurite length from RGCs on plates coated with the CSPGs, we defined PLL plates as the control. It is widely known that laminin is a substrate that strongly promotes neurite outgrowth in vitro.[42] Our studies showed that

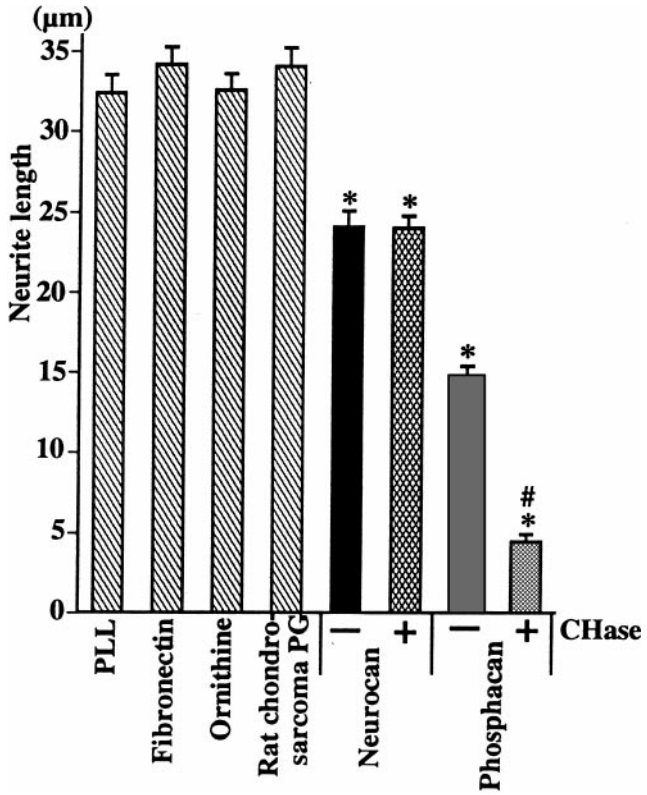

FIGURE 5. Length of neurites from RGCs at 72 hours after seeding. Compared with plates coated with PLL alone, on PLL plates coated with the CSPGs (each concentration, 10 μg/ml), neurite outgrowth was significantly inhibited at 72 hours after seeding (*$P < 0.0001$ compared with PLL, Mann-Whitney test). After treatment with chondroitinase ABC, the inhibitory effects of phosphacan, not neurocan, were significantly promoted (#$P <$ 0.0001 compared with phosphacan before treatment with chondroitinase ABC, Mann-Whitney test). CHase, chondroitinase ABC. Error bar, SE.

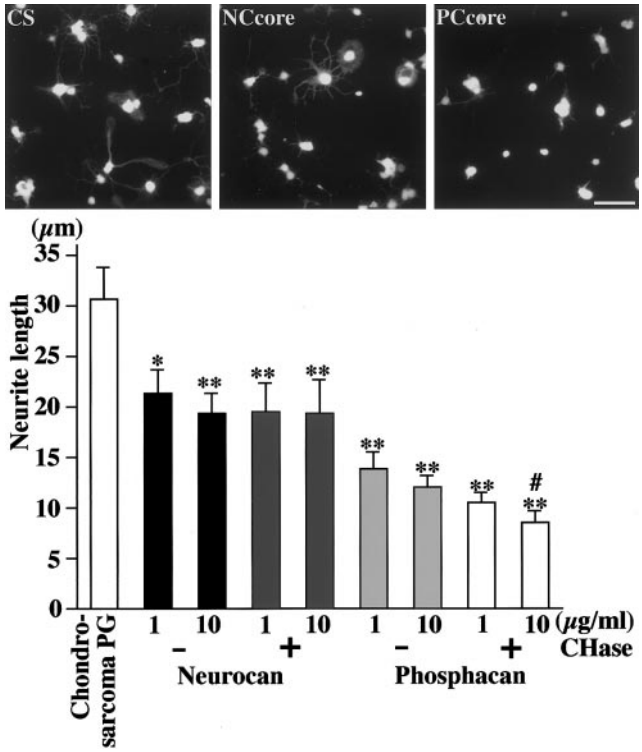

FIGURE 6. *Top*: Inhibitory effects of neurocan and phosphacan on neurite outgrowth from RGCs on PLL plates coated with laminin. When 10 μg/ml (final concentration) rat chondrosarcoma proteoglycan (CS), instead of neurocan and phosphacan, was added to the medium, RGCs extended long neurites. *Bottom*: When 10 μg/ml neurocan or phosphacan after the treatments with chondroitinase ABC (NCcore and PCcore), neurite outgrowth was inhibited. Neurocan and phosphacan significantly inhibited neurite outgrowth from RGCs, even on laminin ($^{*}P < 0.01$, $^{**}P < 0.0001$ compared with rat chondrosarcoma proteoglycan, Mann-Whitney test). In addition, digestion of the chondroitin sulfate side chains linked to phosphacan core protein significantly promoted the inhibitory effect ($\#P < 0.01$ compared with 10 μg/ml phosphacan bearing chondroitin sulfate side chains, Mann-Whitney test). CHase, chondroitinase ABC. Error bar, SE.

neurites from RGCs on PLL plates coated with laminin were much longer than those on laminin-free PLL plates. Compared with PLL plates, striking inhibitory effects on neurite outgrowth were observed on PLL plates coated with the CSPGs. In addition, we examined neurite outgrowth using other coating substrates, such as ornithine, fibronectin, and another CSPG, rat chondrosarcoma proteoglycan. The neurites from RGCs on PLL plates coated with such substrates were much longer than those on PLL plates coated with neurocan or phosphacan.

Furthermore, we added the CSPGs to the culture of RGCs that were extending their neurites on PLL plates coated with laminin in serum-free medium containing neurotrophic factors and forskolin. Even in an environment that was sufficient for the cells to extend neurites, neurite outgrowth was inhibited by the CSPGs. In our results, the CSPGs clearly had an inhibitory effect on neurite outgrowth from RGCs obtained from postnatal rat retinas. Accordingly, neurocan and phosphacan, coexpressed in the nerve fiber–rich layers, may be important inhibitory factors against further neurite outgrowth from RGCs during postnatal stages.

There is some evidence that neurocan and phosphacan inhibit neurite outgrowth of brain neural cells[24–27,29] and dorsal root ganglion cells[13] in vitro, and some studies suggest that core proteins of the CSPGs cause this inhibitory effect.[13,24–26] It is now thought that the inhibitory effect is associated with the core proteins, rather than with chondroitin sulfate side chains. Furthermore, in our studies, the inhibitory effect of phosphacan core protein was stronger than that of phosphacan bearing chondroitin sulfate side chains, but chondroitin sulfate side chains linked to the core protein may attenuate the inhibitory effect. We previously reported that the major form of phosphacan in postnatal rat retina, termed nonproteoglycan phosphacan, has no chondroitin sulfate side chains.[6] This nonproteoglycan phosphacan, characteristic of retinal tissue during retinal development, may be expressed to inhibit further neurite outgrowth from RGCs more effectively than phosphacan bearing chondroitin sulfate.

Neurocan and phosphacan bind with high affinity to cell-adhesion molecules, such as N-CAM,[26,27] Ng-CAM/L1[26,27] and TAG-1/axonin-1,[34] and it is thought that the interaction with these cell-adhesion molecules is involved in neural cell migration and axon pathfinding.[11] Moreover, RGCs express cell-adhesion molecules during development.[52–55] The recent finding by Li et al.[37] that the recombinant core protein of neurocan inhibits neurite outgrowth from chick embryonal retinal cells in vitro supports our results. Their data suggest that the core protein binds to its GalNAcPTase receptor and that this interaction inhibits both N-cadherin– and β1-integrin–mediated adhesion of retinal cells, resulting in an inhibitory effect on neurite outgrowth.[37] These prior findings, taken together with our results from the present study, may show that the inhibitory effects of neurocan and phosphacan on neurite outgrowth from RGCs are due to the interaction with cell adhesion molecules on the neurites.

In conclusion, we have shown that both neurocan and phosphacan, highly expressed in postnatal rat retina, inhibit neurite outgrowth from RGCs, and our data suggest that these proteoglycans play a role in regulating neurite outgrowth from RGCs during retinal development.

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