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Phosphacan and neurocan are repulsive substrata for adhesion and neurite extension of adult rat dorsal root ganglion neurons in vitro

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Abstract

Phosphacan (PC) and neurocan (NC) are major chondroitin sulfate proteoglycans (CS-PGs) in nervous tissue and are involved in the modulation of cell adhesion and neurite outgrowth during neural development and regeneration. In the present study, we examined the effects of PC and NC on the attachment and neurite extension of adult rat dorsal root ganglion (DRG) neurons in vitro. Treatment with PC and NC on poly-L-lysine (PL) significantly impaired both neuronal attachment and neurite extension in a concentration-dependent manner (10 μg/ml $>$ 1 μg/ml $>>$ 0.1 μg/ml), and they were partially suppressed by chondroitinase ABC (ChABC) digestion. The CS-PGs applied to culture medium (1 μg/ml) also displayed inhibitory effects on neurite extension, which were not altered by ChABC treatment. These results show that PC and NC are repulsive substrata for adhesion and neurite regeneration of adult DRG neurons in vitro and suggest that both chondroitin sulfate moieties and core proteins are responsible for the inhibitory actions of the CS-PGs. We also conducted immunohistochemical analyses with the monoclonal antibodies to core proteins of PC (mAb 6B4) and NC (mAb 1G2), which revealed that only a few neurons in the DRG section were stained with these antibodies. In contrast, most DRG neurons at different stages (12 h, 1 day, 2 days, and 4 days) in culture were immunoreactive to mAb 6B4 and mAb 1G2. Taking these findings together, it is plausible that both CS-PGs expressed in the cultured neurons may play a role in the modulation of attachment, survival, and neurite regeneration.

Keywords: Phosphacan; Neurocan; Dorsal root ganglion; Neuron; Cell culture; Attachment; Neurite extension; Adult rat; Immunohistochemistry

Introduction

Proteoglycans (PGs) are constituents of the extracellular matrix (ECM) and composed of a core protein on which sulfated glycosaminoglycans (GAGs) are covalently attached (Lander, 1999). In recent years many studies have been conducted on PGs in association with the development, plasticity, and regeneration of the nervous system (Bovolenta and Fernaud-Espinosa, 2000). According to the molecular nature of the GAG chains, PGs are classified as heparan sulfate PGs (HS-PGs), chondroitin sulfate PGs (CS-PGs), dermatan sulfate PGs (DS-PGs), and keratan sulfate PGs (KS-PGs). In addition to the GAG chains, the structure of a large number of PG core proteins has been determined based on their cDNA sequences. Such molecular and structural diversity of PGs may account for a wide variety of effects of PGs on developing and regenerating neurons in vivo and in vitro.

Phosphacan (PC) and neurocan (NC) are major CS-PGs in the nervous system (Margolis et al., 1996; Bovolenta and Fernaud-Espinosa, 2000). Phosphacan core protein is an alternative splicing variant of the receptor-type protein tyrosine phosphatase (RPTPζ/β), whereas neurocan is a multidomain hyaluronan-binding CS-PG. Although PC and NC are structurally unrelated, they share several biological properties. First, both CS-PGs are expressed by cultured astrocytes or in the glial scar resulting from brain injury and may contribute to the

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failure of axonal regeneration (Snyder et al., 1996; Fawcett and Asher, 1999; McKeon et al., 1999; Asher et al., 2000). Second, both CS-PGs can interact with several cell adhesion molecules (i.e., neural cell adhesion molecule (NCAM), L1, and TAG-1/axonin-1) (Friedlander et al., 1994, Milev et al., 1994, 1996) and ECM proteins (i.e., tenascin-C, tenascin-R, and heparin-binding proteins) (Grumet et al., 1994; Milev et al., 1997, 1998). Third, both CS-PGs, when used as coating substrata, display significant inhibitory actions on attachment and/or neurite outgrowth of cultured CNS neurons [i.e., embryonic chick brain neurons (Friedlander et al., 1994; Milev et al., 1994) and postnatal (6–8 days) rat retinal ganglion cells (Inatani et al., 2001)]. These findings suggest that both CS-PGs may play a pivotal role in the modulation of cell adhesion and neurite outgrowth during neural development and in scar formation following brain injury. On the other hand, these CS-PGs have shown cell-type-specific neurite outgrowth promoting activity [i.e., PC on embryonic rat cortical neurons (Maeda and Noda, 1996), DSD-1-PG (mouse homolog of PC) on embryonic rat hippocampal neurons (Garwood et al., 1999), and NC on olfactory neuroepithelial cells (Clarris et al., 2000)]. The precise mechanisms accounting for the opposing effects of PC and NC on neurite outgrowth that are dependent on neuronal lineage remain unknown, but these CS-PGs are unlikely to act as a simple barrier to axonal regeneration (Margolis and Margolis, 1997). Compared with the large number of studies with PC and NC in CNS as described above, little is known about the localization of these CS-PGs in the peripheral nervous system (PNS) or their functional roles in peripheral nerve regeneration. Li et al. (1998) observed up-regulation of PC and RPTPζ/β mRNA in segments of the rat sciatic nerves distal to crush injury and suggested its biological relevance to nerve regeneration. Introduction of peripheral neuron cultures into adult animals is considered to be useful for the study of nerve regeneration. Dorsal root ganglion (DRG) neurons are constituents of the sensory system, and a method for their primary culture from mature animals has been established (Goldenberg and De Boni, 1983; Sango et al., 1991). We observed that ECM proteins such as type I collagen, type IV collagen, laminin (LM), and fibronectin (FN) enhanced attachment and/or neurite extension of adult mouse DRG neurons in vitro and that neuronal attachment to these substrata was impaired by experimental diabetes and aging (Sango et al., 1993, 1995, 1999). In a similar manner, we examined the effects of PC and NC on the attachment and neurite regeneration of adult rat DRG neurons in vitro. We also observed immunohistochemical localization of these CS-PGs in DRG both in vivo and in vitro.

Materials and methods

Tissue culture of adult rat DRG neurons

Three-month-old female Sprague–Dawley rats were sacrificed by ether exposure. Primary culture of adult DRG neurons was performed as previously described (Sango et al., 1991). Briefly, 20–25 ganglia (from the thoracolumbar level) were dissected from the spinal column of each animal and were dissociated with collagenase (Worthington Biochem., Freehold, NJ, USA) and trypsin (Sigma, St. Louis, MO, USA). These ganglia were subjected to density gradient centrifugation (5 min, 200 g) with 30% Percoll (Pharmacia Biotech, Uppsala, Sweden) to eliminate the myelin sheath (Goldenberg and De Boni, 1983). This procedure resulted in a yield of more than 5×10^4 neurons together with a smaller number of non-neuronal cells such as Schwann cells, satellite cells, and fibroblasts.

Assays for cell attachment and neurite extension

For cell adhesion experiments, wells of 12-well tissue culture plates (Nalge Nunc International, Naperville, IL, USA) were coated with the following substrata: (1) Poly-L-lysine (10 μg/ml) (PL, Sigma) (control), (2) PL and phosphacan (10 μg/ml, 1 μg/ml, or 0.1 μg/ml) (PC), (3) PL and neurocan (10 μg/ml, 1 μg/ml, or 0.1 μg/ml) (NC), (4) PL and laminin (10 μg/ml; Becton–Dickinson, Bedford, MA, USA) (LM), (5) PL and fibronectin (Becton–Dickinson, 10 μg/ml) (FN), (6) PL and [PC (5 μg/ml) and LM (5 μg/ml)] (PC + LM), (7) PL and [PC (5 μg/ml) and FN (5 μg/ml)] (PC + FN), (8) PL and [NC (5 μg/ml) and LM (5 μg/ml)] (NC + LM), and (9) PL and [NC (5 μg/ml) and FN (5 μg/ml)] (NC + FN).

PC and NC were purified from postnatal rat brain as described previously (Oohira et al., 1991; Inatani et al., 2001). PL diluted in distilled water (100 μl in volume) was spotted on the central area of each well for 30 min at room temperature. After removal of PL and following air-drying for 1 h, the PL-coated wells were immediately used for experiments or further treated with each of the other substrata [(2)–(9)] (100 μl in volume) for 1 h at room temperature. DRG neurons dissociated from each rat were suspended in a serum-free medium [Ham's F12 with B27 supplement (Invitrogen, Groningen, Netherlands)] and were seeded on the substratum-coated wells. Areas of 5×5 mm^2 were delineated by lines on the bottom of the wells prior to cells seeding, and the density of neurons was adjusted to approximately 500 cells within a specified area (2×10^3/cm^2) in each well. Thirty minutes after incubation of the cells in a humidified atmosphere containing 5% CO_2 at 37°C, the number of DRG neurons within the designated area of each well was counted under a phase-contrast light microscope. DRG neurons were easily distinguished from nonneuronal cells by their larger cell size with round phase-bright cell bodies (Petersen, 1999). These cells were kept in serum-free medium and allowed to adhere to the substratum for 3 h and then washed three times with the medium to exclude non-attached cells. The number of viable neurons remaining within the area of each well was counted under the microscope. The ratio (%) of neurons attached to the substratum in each well was expressed as B/A (A = number

of neurons measured at 30 min after seeding and B = number of neurons measured after washing with the serum-free medium at 3 h of culture) (Sango et al., 1993, 1995, 1999).

For neurite extension experiments, wells of 8-well chamber slides (Nalge Nunc) were coated with substrata (1)–(9) as described above. The dissociated DRG neurons were suspended in a serum-containing medium (F12 supplemented with 10% fetal calf serum; Mitsubishi Kasei Corp., Tokyo, Japan) and were seeded on the substratum-coated wells. The neuronal density was adjusted to approximately 200 cells per well. After remaining in the serum-containing medium for 12 h, cells were cultured in the serum-free medium for 36 h. We then counted the number of neurite-bearing cells, defined as neurons with neurites with lengths that were longer than the cell body diameter. The ratios (%) of neurite-bearing cells were expressed relative to the total number of neurons in each well (Sango et al., 1993). We measured the length of the 5 longest neurites in each well under the microscope with the help of a measuring eyepiece. The neurite length (in micrometers) was expressed as the average value calculated from at least 20 neurites from 4 different cultures on each substratum/substrata; the maximum and minimum length values in each substratum/substrata group were excluded from the data.

Digestion of phosphacan and neurocan with chondroitinase ABC

One microgram of PC or NC in 100 μl of 0.1 M Tris–HCl, pH 8, and 30 mM sodium acetate was treated for 2 h at 37°C with 1 mU of protease-free chondroitinase ABC (Seikagaku Corporation, Tokyo, Japan). The samples were diluted to 1 μg/ml with PBS and added to PL-coated wells (Maeda and Noda, 1996).

Phosphacan and neurocan in culture medium

DRG neurons dissociated from each rat were suspended in serum-containing medium, and seeded on PL- or LM-coated wells of the chamber slides. Cells were allowed to adhere to the substrata for 12 h and were then incubated in the following media for 36 h:(1) Serum-free medium, (2) Serum-free medium containing 1 μg/ml of PC or NC, and (3) Serum-free medium containing 1 μg/ml of chondroitinase ABC-treated PC or NC. Neurite extension from the neurons after 2 days in culture with each medium was evaluated as described above.

Immunohistochemical localization of CS-PGs and cell adhesion molecules in DRG in vivo and in vitro

The rats were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg) and then briefly perfused through the left cardiac ventricle with heparinized physiologic saline followed by ice-cold fixative. Two different fixatives were used, depending on the antigen to be examined by immunohistochemistry: 4% paraformaldehyde for detection of PC and NC and acid–alcohol (95% ethanol and 5% acetic acid) for L1 and NCAM. DRG dissected from the rats were processed for paraffin embedding and sectioned into 5-μm-thick slices. Sections were deparaffinized with 100% xylene and hydrated through a graded alcohol series (Katoh-Semba et al., 1995).

Adult rat DRG neurons were dissociated as described above, seeded on PL-coated wells of chamber slides, and kept in the serum-free medium. After 12 h, 1 day, 2 days, and 4 days in culture, cells were fixed with 4% paraformaldehyde or acid–alcohol at room temperature for 30 min.

These sections and fixed cells were incubated overnight at 4°C with the following antibodies [diluted with 20 mM phosphate-buffered saline (PBS) containing 0.5% skimmed milk]: (1) Mouse monoclonal antibody 6B4 (mAb 6B4), which recognizes the core protein of phosphacan (1:50; Maeda et al., 1992); (2) Mouse monoclonal antibody 1G2 (mAb 1G2), which recognizes the core protein of neurocan (1:5; Oohira et al., 1994); (3) Rabbit antiserum against rat L1 (1:5000; from Dr. Asou, Tokyo Metropolitan Institute for Gerontology, Japan) (Miura et al., 1991); and (4) Mouse monoclonal antibody against rat NCAM (1:100; from Dr. Ono, Shimane Medical University, Izumo, Japan) (Ono et al., 1992).

After rinsing in PBS for 15 min, samples were incubated at 37°C for 1 h with biotinylated secondary antibodies (i.e., antimouse IgM for 6B4, antimouse IgG for 1G2 and NCAM, and antirabbit IgG for L1), followed by reaction at room temperature for 30 min with avidin and biotinylated horseradish peroxidase complex (ABC Elite kit; Vector Laboratories Inc., Burlingame, CA, USA). After rinsing in PBS for 15 min, the immunoreaction was visualized under a light microscope by 0.01% diaminobenzidine tetrahydrochloride (DAB; Wako Co., Tokyo, Japan) and 0.01% hydrogen peroxide in 50 mM Tris buffer (pH 7.4) at 37°C for 15 min (Toba et al., 2002).

Preabsorption tests of mAb 6B4 and 1G2

Staining specificity of mAb 6B4 and 1G2 was confirmed by preabsorption tests in which sections and fixed cells were incubated with mAb 6B4 or mAb 1G2 that had been mixed with different concentrations of antigen. Positive reactions with mAb 6B4 (1:50) and mAb 1G2 (1:5) were completely abolished by addition of 25 nM of PC and NC, respectively.

Statistical analysis

For a statistical comparison of the ratios of cell attachment, the ratios of neurite-bearing cells and neurite length, post hoc tests were performed using Bonferroni/Dunn post hoc analyses. P values of <0.05 were considered significant.

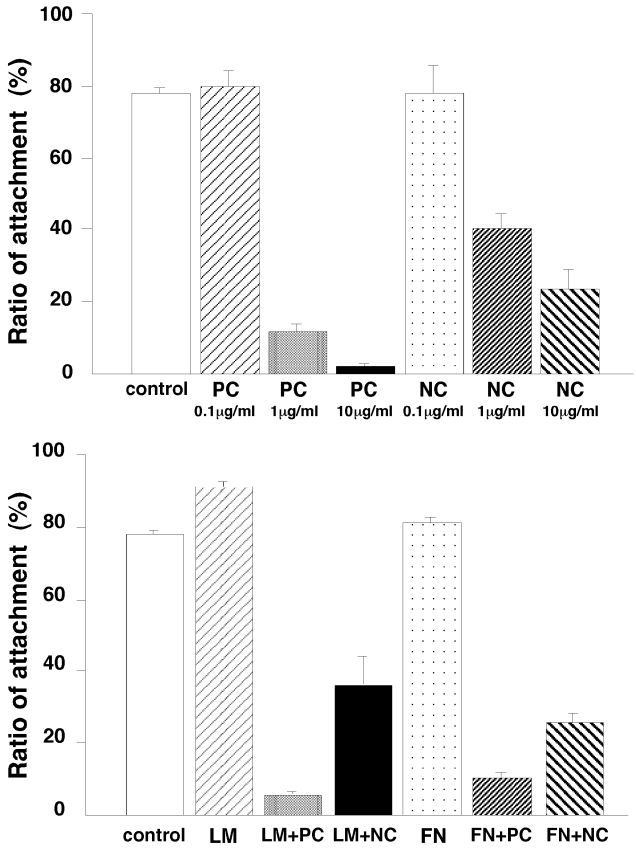


Fig. 1. PC and NC were repulsive substrata for the attachment of adult rat DRG neurons in culture. (Upper panel) The inhibitory effects of PC and NC on DRG neuronal attachment to PL were dose dependent (10 μg/ml >1 μg/ml >>0.1 μg/ml). (Lower panel) PC and NC abolished the enhancing effects of LM and FN on DRG neuronal attachment. Each substratum/substrata (LM, FN, and the mixture of PC or NC with LM or FN) was applied to culture dishes precoated with PL. The concentration of each substratum was 10 μg/ml. The ratio of attachment (%) is expressed as B/A (A = number of neurons measured at 30 min after seeding; B = number of neurons measured after washing with the serum-free medium). Values in the both panels represent the mean + SEM of three to four experiments with four animals. Significant differences ($P < 0.001$) are as follows: (upper panel) control *vs* PC (1 μg/ml), PC (10 μg/ml), NC (1 μg/ml), or NC (10 μg/ml), (lower panel) control *vs* LM, LM + PC, LM + NC, FN + PC or FN + NC, LM *vs* LM + PC or LM + NC, FN *vs* FN + PC or FN + NC.

Results

PC and NC inhibited attachment and neurite extension of DRG neurons in a dose-dependent manner

The average ratios of attachment to PL (indicated as "control" in Fig. 1), PC (0.1 μg/ml), PC (1 μg/ml), PC (10 μg/ml), NC (0.1 μg/ml), NC (1 μg/ml), and NC (10 μg/ml) were 77.9, 79.5, 12.1, 2.1, 77.8, 40.4, and 23.9%, respectively (Fig. 1, upper panel). PC and NC at concentrations of 1 and 10 μg/ml significantly ($P < 0.0001$) inhibited neuronal attachment in a dose-dependent manner, whereas neither substratum at 0.1 μg/ml had any significant effect on the attachment. Treatment with LM and FN (10 μg/ml) on PL increased the ratio of attachment from 77.9 to 91.3% and 81.3%, respectively, but the addition of PC or NC to LM or FN (5 μg/ml each at the final concentration) detrimentally diminished the number of adhesive neurons (Fig. 1, lower panel). These findings are in agreement with previous studies from other laboratories (Condic et al., 1999; Talts et al., 2000; Li et al., 2000), showing an anti-adhesive role for CS-PGs.

In the neurite extension assay, DRG neurons were allowed to adhere to the substrata for 12 h in the serum-containing medium and subsequently maintained in the serum-free medium. Treatment with 10 μg/ml of PC and NC significantly ($P < 0.001$) reduced the average ratio of neurite-bearing cells from 49 to 5.8% and 19.5%, and the neurite length from 290 to 68.8 μm and 120 μm, respectively (Fig. 2). PC and NC inhibited neurite extension in a concentration-dependent manner (10 μg/ml > 1 μg/ml >> 0.1 μg/ml), which was similar to the results of the cell attachment assay. In sharp contrast to PC and NC, treatment

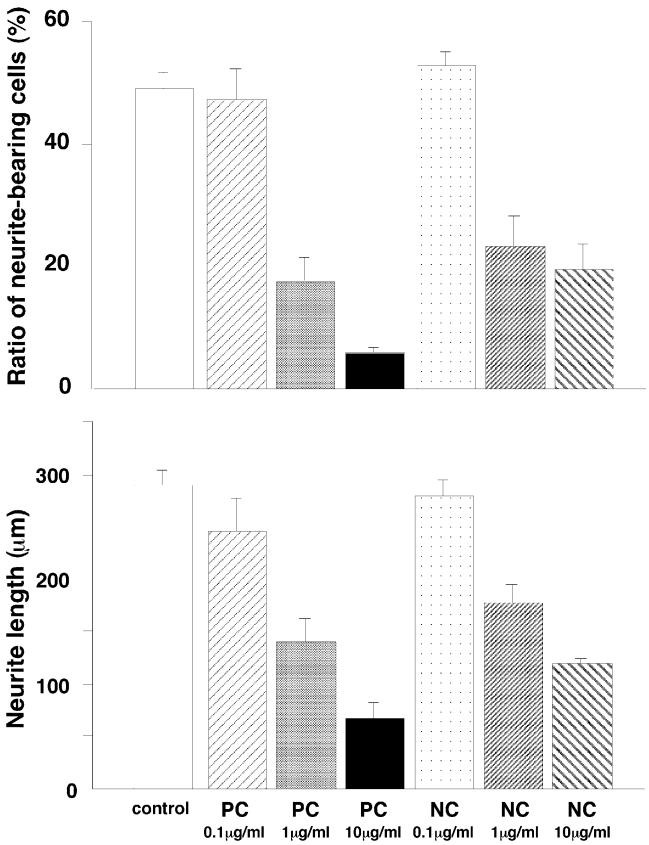


Fig. 2. Inhibitory effects of PC and NC on neurite extension of DRG neurons were dose dependent (10 μg/ml > 1 μg/ml >> 0.1 μg/ml). The ratio of neurite-bearing cells (%) (upper panel) is expressed as B/A (A = total number of viable neurons on each substratum at 48 h in culture; B = number of neurons with neurites longer than the cell body diameter at 48 h in culture). Neurite length (μm) (lower panel) was expressed as the average length of the five longest neurites measured in each culture. Values represent the mean + SEM of 3–13 experiments with five animals. Significant differences ($P < 0.001$) are noted between control and PC (1 μg/ml), PC (10 μg/ml), NC (1 μg/ml), or NC (10 μg/ml) in both panels.

Table 1
PC and NC abolished the enhancing effects of LM and FN on neurite extension of DRG neurons at 48 h in culture

Substratum/substrata	Neurite-bearing cells (%)	Neurite length (μm)
Control	49.0 ± 2.5	290.0 ± 14.3
LM (10 μg/ml)	84.8 ± 4.5	618.0 ± 30.0
LM + PC (5 μg/ml each)	17.7 ± 2.1	256.0 ± 34.6
LM + NC (5 μg/ml each)	59.0 ± 5.0	643.1 ± 25.6
FN (10 μg/ml)	67.4 ± 2.6	475.7 ± 24.4
FN + PC (5 μg/ml each)	2.8 ± 1.2	75.0 ± 16.5
FN + NC (5 μg/ml each)	35.0 ± 4.8	328.6 ± 23.7

Note. Each substratum/substrata (LM, FN, and the mixture of PC or NC with LM or FN) was applied to culture dishes precoated with PL (10 μg/ml). The ratio of neurite-bearing cells and neurite length are expressed as shown in the text and Fig. 2. Values represent the mean ± SEM of 3–13 experiments with five animals. Significant differences ($P < 0.001$) are as follows: (neurite-bearing cells) control *vs* LM, LM + PC, FN or FN + PC; LM *vs* LM + PC or LM + NC; FN *vs* FN + PC or FN + NC; (neurite length) control *vs* LM, LM + NC, FN or FN + PC; LM *vs* LM + PC; and FN *vs* FN + PC or FN + NC.

with 10 μg/ml of LM and FN significantly ($P < 0.001$) increased the ratio of neurite-bearing cells from 49 to 84.8% and 67.4% and the neurite length from 290 μm to 618 μm and 476 μm, respectively. However, the enhancing effects of LM and FN on neurite extension were totally abolished by PC and significantly diminished by NC (Table 1). These findings confirm the results of previous studies using embryonic and neonatal DRG neurons (Braunewell et al., 1995; Katoh-Semba et al., 1995; Snow et al., 1996; Garwood et al., 1999) and suggest that PC and NC are repulsive substrata for neurite extension of DRG neurons. Our findings also imply that PC is a more potent inhibitor of adult rat DRG neurons than NC.

Digestion of CS-PGs with chondroitinase ABC (ChABC) partially suppressed their inhibitory effects on attachment and neurite extension of DRG neurons in culture

To clarify whether the inhibitory effects of PC and NC are derived from their core proteins or CS chains, both CS-PGs were digested by ChABC prior to their use as substrata. Treatment with ChABC significantly ($P < 0.05$) increased the ratio of cell attachment from 8.0 to 31.3% on PC and from 44.8 to 76.4% on NC (Table 2). After treatment, PC still inhibited neuronal attachment while the repulsive effect of NC was totally abolished [the attachment ratio on ChABC-treated NC was almost equal to that on PL (77.9%)]. ChABC-treatment also increased the ratio of neurite-bearing cells and/or neurite length on PC and NC but was not able to restore the values to those on the control (Table 2). These findings suggest that digestion of CS chains significantly, but not completely, suppressed the inhibitory actions of both CS-PGs in vitro.

PC and NC applied to the culture medium inhibited neurite extension of DRG neurons in a manner independent of CS chains

Next, we examined the effects of PC and NC in the soluble form on the neurite extension of DRG neurons in culture. Neurons were allowed to adhere to PL or LM in the serum-containing medium for 12 h. They were then kept in serum-free medium with or without 1 μg/ml (final concentration) of each CS-PG for another 36 h in culture. Treatment with PC and NC significantly ($P < 0.05$) reduced the ratio of neurite-bearing cells on PL-coated wells from 53.3 to 17.3% and 33.3%, respectively (Table 3). More than 70% of neurons on LM-coated wells showed extended neurites after 2 days in culture in the presence or absence of the CS-PGs, although treatment with PC and NC decreased the ratio of neurite-bearing cells from 84.2 to 71.7% and 75.5%, respectively. In contrast to the study in which PC and NC were used as coating substrata (Table 2), the digestion of CS chains of PC or NC by ChABC failed to improve neurite extension on either PL or LM.

Immunohistochemical localization of PC and NC in DRG in vivo and in vitro

In DRG sections (Fig. 3), cells with large round shapes were identified as neurons (Lieberman, 1976; Petersen, 1999), whereas the small flattened cells surrounding the neurons (indicated by arrowheads in Fig. 3B) were identified as satellite cells (Lieberman, 1976). This was confirmed by immunohistochemistry with the antibody to glial fibrillary acidic protein (GFAP; DAKO, Carpinteria, CA, USA), in which satellite cells, but not neurons, were immunoreactive for GFAP (not shown) (Stephenson and Byers, 1995;

Table 2
Digestion of PC and NC by chondroitinase ABC (ChABC) partially suppressed their inhibitory effects on attachment and neurite extension of DRG neurons at 48 h in culture

Substratum	Cell attachment (%)	Neurite-bearing cells (%)	Neurite length (μm)
Control	77.9 ± 1.1	51.0 ± 2.0	295.7 ± 24.4
PC	8.0 ± 1.4	9.0 ± 2.1	127.1 ± 7.5
PC-ChABC	31.3 ± 6.9	22.5 ± 4.2	186.2 ± 13.7
NC	44.8 ± 7.3	23.5 ± 4.8	178.6 ± 16.1
NC-ChABC	76.4 ± 4.6	40.0 ± 3.8	185.7 ± 9.2

Note. Prior to coating, 1 μg of PC or NC was treated with 1 mU of protease-free ChABC at 37°C for 2 h. One microgram per milliliter of each CS-PG with or without ChABC treatment was added to the PL-coated dishes. The ratio of cell attachment, ratio of neurite-bearing cells, and neurite length are expressed as shown in the text and Figs. 1 and 2. Values represent the means ± SEM of 3–13 experiments with five animals. Significant differences ($P < 0.05$) are as follows: (cell attachment) control *vs* PC, PC-ChABC or NC; PC *vs* PC-ChABC; NC *vs* NC-ChABC; (neurite-bearing cells) control *vs* PC, PC-ChABC or NC; PC *vs* PC-ChABC; NC *vs* NC-ChABC; (neurite length) control *vs* PC, PC-ChABC, NC or NC-ChABC; and PC *vs* PC-ChABC.

Table 3
PC and NC added to the culture medium inhibited neurite extension of DRG neurons on PL but not on LM at 48 h in culture

Substratum	Additive in culture medium	Neurite-bearing cells (%)
PL	Control	53.3 ± 1.0
	PC	17.3 ± 2.5
	PC-ChABC	17.9 ± 5.1
	NC	33.3 ± 2.8
	NC-ChABC	37.8 ± 2.2
LM	Control	84.2 ± 2.5
	PC	71.7 ± 2.7
	PC-ChABC	71.0 ± 3.1
	NC	75.5 ± 4.6
	NC-ChABC	74.2 ± 3.8

Note. DRG neurons were suspended in serum-containing medium and were plated on culture dishes coated with 10 μg/ml of PL or LM. Twelve hours after plating, the neurons were cultured in the following five different media for 36 h: (1) serum-free medium (SFM) in the absence of PC or NC (control), (2) and (3) SFM in the presence of 1 μg/ml of PC or NC, and (4) and (5) SFM in the presence of 1 μg/ml of PC or NC treated with ChABC. Digestion of PC and NC with ChABC did not change their inhibitory effects on neurite extension of DRG neurons on either substratum. Values represent the means ± SEM of 3–16 experiments with six animals. Significant differences ($P < 0.05$) are as follows: (PL) control *vs* PC, PC-ChABC, NC, or NC-ChABC; and (LM) control *vs* PC or PC-ChABC.

Saito et al., 2002). Intense immunoreactivity for L1 and NCAM was observed in DRG neurons, satellite cells, and Schwann cells in the sections (Fig. 3C and D). Compared with the abundant and ubiquitous expression of L1 and NCAM, immunoreactivity for PC and NC in DRG was more subdued and restricted. Only a few DRG neurons (4–5 per section) were immunoreactive for 6B4, and all of them were small neurons with diameters shorter than 30 μm (indicated by arrows in Fig. 3A). Immunoreactivity for 1G2 in DRG was restricted to a few small neurons (5–10 per section, indicated by arrows in Fig. 3B) and satellite cells (indicated by arrowheads in Fig. 3B). No immunoreactivity for either CS-PG was detected in spinal nerve sections (Fig. 3A and B). In contrast to the weak immunoreactivity for 6B4 and 1G2 in vivo, almost all DRG neurons in culture were immunoreactive to all antigens (Fig. 4). The whole cell soma was immunoreactive for 6B4 and 1G2 (Fig. 4A and B), whereas the immunoreactivities for L1 and NCAM were localized on the cell surface of DRG neurons and regenerating neurites (Fig. 4C and D). Immunoreactivity to all antigens in the neurons was observed from a very early stage (12 h) to the later stages (1 day, 2 days, and 4 days) in culture (not shown). These positive reactions were completely eliminated when sections and fixed cells were incubated with preabsorbed mAb 6B4 and mAb 1G2 antibodies, indicating their specificity for PC and NC, respectively (not shown).

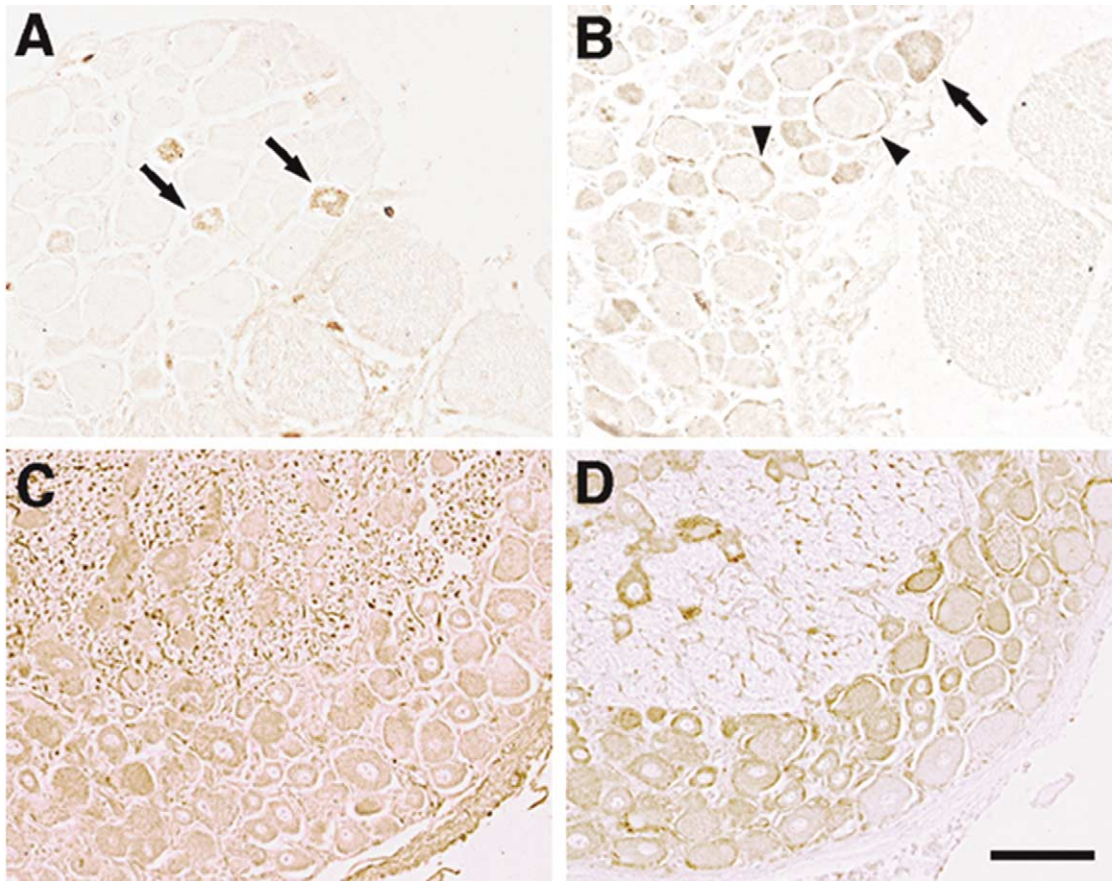
Discussion

While many studies have shown repulsive actions of CS-PGs on attachment and/or neurite outgrowth of DRG neurons from *immature* (embryonic and neonatal) animals in vitro (Braunewell et al., 1995; Katoh-Semba et al., 1995; Snow et al., 1996; Condic et al., 1999; Garwood et al., 1999), much less attention has been paid to the effects of CS-PGs on adult DRG neurons. It is recognized that some biological properties of DRG neurons change with maturation and aging. For example, nerve growth factor (NGF) is essential for the survival of immature DRG neurons but not for the survival of *mature* (older than 1 month after birth) neurons in vitro (Lindsay, 1988). Fundamental functions of neural cell membranes, such as fluidity and elasticity, have also been found to decrease with age (Horie et al., 1986, 1990). Furthermore, mRNA and protein expressions of neurofilaments in rat DRG neurons were up-regulated (Schlaepfer and Bruce, 1990), whereas fibronectin receptor density of mouse DRG neurons was down-regulated during postnatal development (Kawasaki et al., 1986). These changes may contribute to the decrease in the capability of neurons to attach to extracellular substrata and/or regenerate neurites in vitro (Sango et al., 1993; Mukhopadhyay et al., 1994; Ng et al., 1999). Taking such age-related changes into consideration, it appears that the culture system of adult DRG neurons mimics peripheral nerve regeneration better than that of immature neurons. In a recent study by Ferguson and Muir (2000), neurite outgrowth from adult mouse DRG neurons cultured on sciatic nerve sections was increased by degradation of CS-PGs in the sections with ChABC or metalloproteinase. Also, inhibitory effects of CS-PGs on mature neurons were suggested in several articles (Asher et al., 2000; Lemons et al., 2001; Grimpe and Silver, 2002). These reports led us to believe that a systemic examination of adult DRG neuron responses to CS-PGs is advantageous to better understand the roles of CS-PGs in peripheral nerve regeneration.

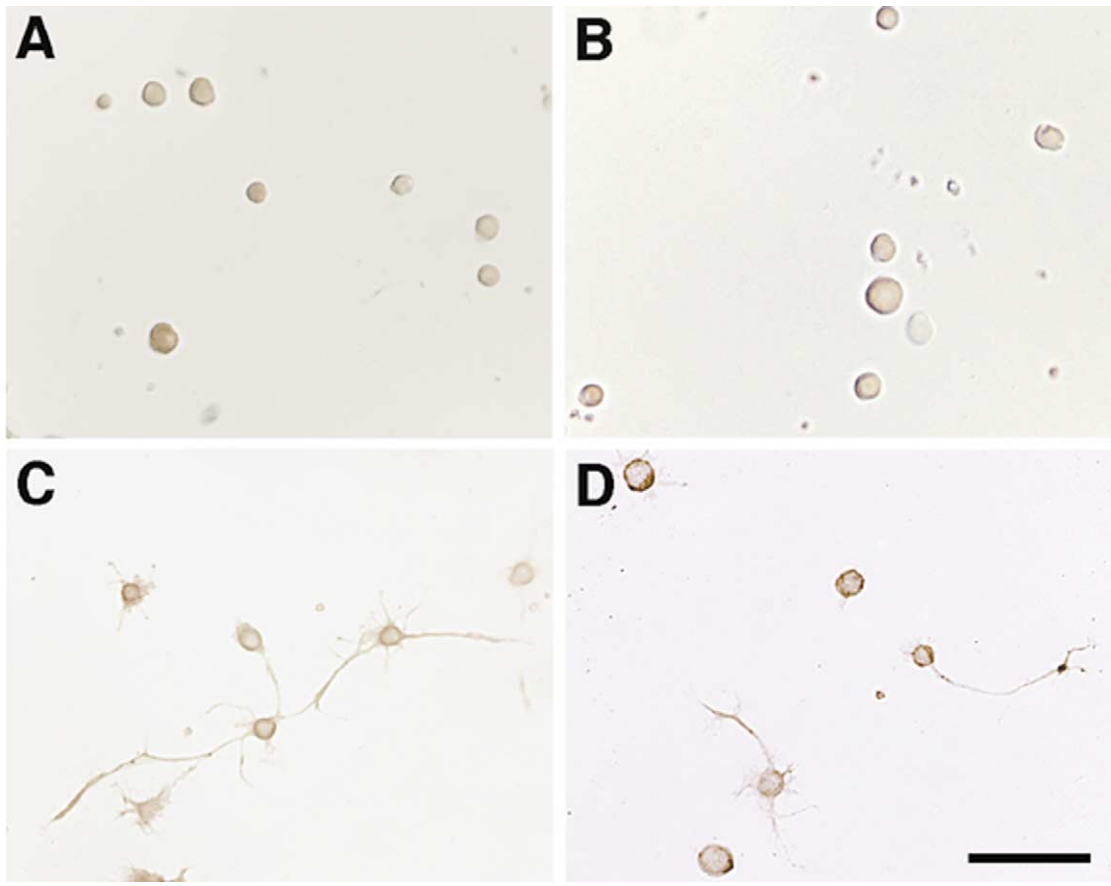
The results in the present study show that two major CS-PGs in the nervous tissue, PC and NC, inhibited attach-

Fig. 3. Immunohistochemical localization of chondroitin sulfate proteoglycans (PC and NC) and cell adhesion molecules (L1 and NCAM) in sections of adult rat DRG. Proteoglycans immunoreactivity is subdued and restricted to only a few small neurons (indicated by arrows in A). Neurocan immunoreactivity is restricted to a few small neurons (indicated by arrows in B) and satellite cells (indicated by arrowheads in B). No immunoreactivity for either CS-PG was detected in spinal nerve sections (right side in A and B). In contrast, intense immunoreactivities of L1 (C) and NCAM (D) are localized in neurons, satellite cells, and Schwann cells. (Bar = 50 μm)

Fig. 4. Immunocytochemical localization of core proteins of chondroitin sulfate proteoglycans (PC and NC) and cell adhesion molecules (L1 and NCAM) in adult rat DRG neurons after 12 h (A and B) and 2 days (C and D) in culture. The whole-cell soma of the neuron was immunoreactive for PC (A) and NC (B). Immunoreactivities for L1 (C) and NCAM (D) were localized on the surface of cell bodies and growing neurites. (Bar = 100 μm)



3



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ment and neurite extension of adult rat DRG neurons in a concentration-dependent manner (Figs. 1 and 2). These findings agree with those obtained in previous studies using immature DRG neurons (Braunewell et al., 1995; Katoh-Semba et al., 1995; Snow et al., 1996; Condic et al., 1999; Garwood et al., 1999). Adult DRG neurons, in the same way as other primary cultured neurons, are "anchorage dependent" cells and require attachment to the surface of dishes for survival and neurite extension in vitro (Horie and Kim, 1984). Poly-L-lysine enhances cell attachment by means of electrostatic interaction between anionic sites on plasma membranes and cationic sites of polylysine molecules (Yavin and Yavin, 1974). On the other hand, CS and other glycosamonoglycans are negatively charged molecules and one of the major constituents of anionic sites on the surface of cells (Nilsson et al., 1983). When PC or NC is applied to PL on culture dishes, negative charges on CS chains of PC or NC may interact with positive charges on PL, thereby abolishing the electrostatic interaction between neurons and PL. Since digestion of the CS-PGs with ChABC significantly improved neuronal attachment, the inhibitory effects of CS-PGs on cell adhesion might be attributed in part to the negative charges on CS chains. If this were the case, the CS-PGs applied to PL would abolish attachment of any kind of cells in vitro. However, Maeda and Noda (1996) reported that PC spotted on PL disturbed adhesion of rat cortical and thalamic neurons but not purified astrocytes in culture. Their findings suggest that the repulsive nature of CS-PGs is specific to neuronal cells and does not depend on negative charges on CS chains. ChABC treatment improved both neuronal attachment and neurite extension (Table 2), suggesting that chondroitin sulfate moieties contribute to the repulsive nature of CS-PGs. These findings are consistent with those of Zuo et al. (1998b), who showed that neurite outgrowth from embryonic chick DRG neurons on adult rat spinal cord tissue sections in vitro was enhanced by pretreating sections with ChABC. They also observed that axonal regeneration after sciatic nerve transection was significantly enhanced by injection of ChABC into the injury site (Zuo et al., 2002). On the other hand, PC treated with ChABC was still a potent inhibitor for attachment and neurite extension of DRG neurons in this study. Similarly, NC treated with ChABC displayed inhibitory actions on neurite extension. These results are in agreement with the recent studies by Bandtlow and Zimmermann (2000) and Inatani et al. (2001), showing that NC inhibited neurite outgrowth from embryonic neurons even after the removal of CS chains. Consequently, it appears that not only CS chains but also core proteins participate in the repulsive actions of CS-PGs. Furthermore, both PC and NC in soluble form significantly inhibited neurite extension from DRG neurons on PL-coated wells, and ChABC digestion of the CS-PGs had no effect on these inhibitory actions (Table 3). In this experiment, neurons were seeded in serum-containing medium and allowed to adhere to PL for 12 h prior to exposure to CS-PGs. Even in such an environment sufficient for neurons to survive and extend neurites, neurite extension was impaired by the CS-PGs with or without ChABC treatment. Taking these findings together, the core proteins of CS-PGs may play a major role in the inhibition of neurite regeneration from DRG neurons. In contrast, chondroitin sulfate moieties of the molecules may affect neurite extension through inhibition of neuronal attachment.

ECM proteins such as LM and FN have also been shown to enhance the neuronal attachment in vitro (Carbonetto et al., 1983, 1987; Faivre-Bauman et al., 1984; Horie and Kim, 1984), and this enhancement results from specific binding of cell-surface receptors (e.g., integrins) to the ligands [e.g., Arg-Gly-Asp (RGD)] on ECM proteins (Clark and Brugge, 1995). In this study, we applied a mixture (1:1) of LM or FN and PC or NC (5 μg/ml each) to dishes coated with PL. Phosphacan and NC inhibited the enhancing actions of LM and FN on neuronal attachment and neurite extension (Fig. 1 and Table 1). This result is consistent with that in a previous study on neonatal rat DRG neurons (Katoh-Semba et al., 1995) and suggests that the affinity of PC or NC for PL may be greater than that of LM or FN. In contrast to substratum-bound CS-PGs, soluble CS-PGs only slightly inhibited LM-induced neurite extension. It has been reported that there are specific sites with active neurite elongation on the laminin molecule (Malinda and Kleinman, 1996), which are different from the sites responsible for attachment. Once neurons are attached to LM, they extend neurites in response to these active sites. Thus, the results in this study suggest that PC and NC may affect the sites for attachment rather than the sites for neurite elongation on LM. In agreement with our study, Snow et al. (1996) observed that substratum-bound CSPG, but not soluble CS-PG, inhibited neurite outgrowth from embryonic chick DRG neurons cultured on LM.

The precise mechanisms by which PC and NC act on DRG neurons to inhibit neurite extension in culture remain to be determined. Since PC and NC bind with high affinity to cell adhesion molecules such as L1 and NCAM (Friedlander et al., 1994; Milev et al., 1994, 1996), it is conceivable that CS-PGs interact with these molecules on DRG neurons to disturb sprouting and/or elongation of neurites. Our immunocytochemical analysis supported this possibility: the surface area of cultured DRG neurons was immunoreactive for L1 and NCAM (Fig. 4). We hope to obtain evidence that PC and NC inhibit L1/NCAM-induced neuronal attachment and neurite extension of DRG neurons.

While immunoreactivity specific for mAb 6B4 was detected in neurons of embryonic rat DRG in vivo (Katoh-Semba et al., 1998), localization of CS-PGs in DRG and peripheral nerves of mature animals has not yet been fully examined. Our immunohistochemical studies with mAb 6B4 and mAb 1G2 revealed subdued and restricted expressions of PC in neurons and NC in both neurons and satellite cells in DRG in vivo (Fig. 3). Only a few small neurons (less than 10 in each section) were stained with mAb 6B4 or

1G2, and it is difficult to speculate any functional role of CS-PGs in the sensory system. In contrast, most DRG neurons in vitro were stained with these antibodies (Fig. 4). It is noteworthy that immunoreactivities of both CS-PGs in the neurons were observed from a very early stage in culture (i.e., 12 h after seeding). Enzymatic and mechanical treatments for the dissociation of DRG cells, together with disruption of the interactions between neurons and satellite cells are detrimental to the survival of neurons, and some neurons die during in vivo/in vitro replacement (Sango et al., 1991; Kasper et al., 1999). Therefore, it seems possible that both PC and NC expressed in cultured DRG neurons play a role in the repair of neurons from injury caused by the dissociation procedure and/or protection of neurons from cell death at the initial phase in culture. Moreover, endogenous CS-PGs may modulate attachment and neurite extension of DRG neurons. In our preliminary experiment, DRG neurons seeded on PL or LM were kept in serum-free medium in the presence or absence of 10 mU/ml of ChABC. Treatment with ChABC failed to increase the ratios of neurite-bearing cells on either substratum after 48 h in culture (Sango et al., personal observation). This finding implies that the CS chains expressed on the neurons are not inhibitory molecules for neurite extension. In a way similar to that used in this ChABC experiment, we are about to determine if the application of mAb 6B4 or mAb 1G2 to the culture enhances neurite outgrowth. There is room for further investigations to elucidate the functional roles of endogenous PC and NC in peripheral nerve regeneration. We plan to examine mRNA and protein expressions of CS-PGs in adult DRG and sciatic nerves after injury, using the axotomy/crush injury models in vivo (Li et al., 1998) and explant culture models in vitro (Kasper et al., 1999; Sango et al., 2002).

The repulsive effects of PC and NC on attachment and neurite regeneration of DRG neurons do not necessarily mean that these CS-PGs are simple barrier molecules for peripheral nerve regeneration. It is also possible that CS-PGs play a role in the avoidance of excess fiber regeneration and the proper guidance of target reinnervation after axonal injury. Zuo et al. (1998a) suggested that up-regulation of inhibitory CS-PGs at the site of crush injury in adult rat sciatic nerves might contribute to preclusion of “dysfunctional” neurite growth and/or direction of growth cones to appropriate pathways. The idea of dysfunctional neurite growth was further supported by our recent studies with streptozotocin-induced diabetic mice: Neurite regeneration from transected nerve terminals of sensory ganglia (DRG and nodose ganglia) in vitro was enhanced in diabetic mice (Saito et al., 1999; Sango et al., 2002). Since diabetes is one of the major causes of peripheral neuropathies, those studies imply that excess fiber regeneration in diabetes may impair proper target reinnervation and subsequent functional repair.

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