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The Spine Journal 15 (2015) 1060–1069

THE SPINE JOURNAL

Basic Science

Intact glycosaminoglycans from intervertebral disc-derived notochordal cell-conditioned media inhibit neurite growth while maintaining neuronal cell viability

Devina Purmessur, PhD[a], Marisa C. Cornejo, BA[a], Samuel K. Cho, MD[a], Peter J. Roughley, PhD[b], Robert J. Linhardt, PhD[c], Andrew C. Hecht, MD[a], James C. Iatridis, PhD[a,*]

[a]*Leni & Peter W. May Department of Orthopedics, Icahn School of Medicine at Mount Sinai, One Gustave L.levy place, box 1188 New York, NY 10029, USA*
[b]*Genetics Unit, Shriners Hospital for Children, 1529 Cedar Avenue Montreal, Quebec H3G 1A6, Canada*
[c]*Biocatalysis & Metabolic Engineering Department, Rensselaer Polytechnic Institute Biotech 4005, 110 8th St., Troy, NY, 12180, USA*

Received 19 June 2014; revised 8 December 2014; accepted 1 February 2015

Abstract

BACKGROUND CONTEXT: Painful human intervertebral discs (IVDs) exhibit nerve growth deep into the IVD. Current treatments for discogenic back pain do not address the underlying mechanisms propagating pain and are often highly invasive or only offer temporary symptom relief. The notochord produces factors during development that pattern the spine and inhibit the growth of dorsal root ganglion (DRG) axons into the IVD. We hypothesize that notochordal cell (NC)-conditioned medium (NCCM) includes soluble factors capable of inhibiting neurite growth and may represent a future therapeutic target.

PURPOSE: To test if NCCM can inhibit neurite growth and determine if NC-derived glycosaminoglycans (GAGs) are necessary candidates for this inhibition.

STUDY DESIGN: Human neuroblastoma (SH-SY5Y) cells and rat DRG cells were treated with NCCM in two-dimensional culture in vitro, and digestion and mechanistic studies determined if specific GAGs were responsible for inhibitory effects.

METHODS: Notochordal cell-conditioned medium was generated from porcine nucleus pulposus tissue that was cultured in Dulbecco's modified eagle's medium for 4 days. A dose study was performed using SH-SY5Y cells that were seeded in basal medium for 24 hours and neurite outgrowth and cell viability were assessed after treatment with basal media or NCCM (10% and 100%) for 48 hours. Glycosaminoglycans from NCCM were characterized using multiple digestions and liquid chromatography mass spectroscopy (LC-MS). Neurite growth was assessed on both SH-SY5Y and DRG cells after treatment with NCCM with and without GAG digestion.

RESULTS: Notochordal cell-conditioned medium significantly inhibited the neurite outgrowth from SH-SY5Y cells compared with basal controls without dose or cytotoxic effects; % of neurite expressing cells were 39.0±2.9%, 27.3±3.6%, and 30.2±2.7% and mean neurite length was 60.3±3.5, 50.8±2.4, 53.2±3.7 μm for basal, 10% NCCM, and 100% NCCM, respectively. Digestions and LC-MS determined that chondroitin-6-sulfate was the major GAG chain in NCCM. Neurite growth from SH-SY5Y and DRG cells was not inhibited when cells were treated with NCCM with digested chondroitin sulfate (CS).

FDA device/drug status: Not applicable.

Author disclosures: ***DP:*** Grant: AO Foundation (No. S-13-50P, E, Paid directly to institution); Board of Directors: Unpaid position on Orthopedic Research Society Board of Directors. ***MCC:*** Nothing to disclose. ***SKC:*** Grant: OREF (D, Paid directly to institution); Consulting: Stryker (B). ***PJR:*** Nothing to disclose. ***RJL:*** Stock Ownership: Solarzyme, Momenta (F); Consulting: Kabi, Daniels LLC, Reliable Pharm, Mylan (D); Grants: NIH (G, Paid directly to institution). ***ACH:*** Royalties: Zimmer (D); Consulting: Zimmer (D); Speaking/Teaching Arrangements: Stryker (B), Medtronic (B), Depuy (B). ***JCI:*** Grant: NIH (No. R01 AR064157, H, Paid directly to institution); Grants: NIH (No. R01 AR057397, H, Paid directly to institution).

The disclosure key can be found on the Table of Contents and at www.TheSpineJournalOnline.com.

* Corresponding author. Leni & Peter W. May Department of Orthopedics, Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Place, Box 1188, New York, NY 10029, USA. Tel.: 212-241-1517.

E-mail address: james.iatridis@mssm.edu (J.C. Iatridis)

http://dx.doi.org/10.1016/j.spinee.2015.02.003

CONCLUSIONS: Soluble factors derived from NCCM were capable of inhibiting neurite outgrowth in multiple neural cell types without any negative effects on cell viability. Cleavage of GAGs via digestion was necessary to reverse the neurite inhibition capacity of NCCM. We conclude that intact GAGs such as CS secreted from NCs are potential candidates that could be useful to reduce neurite growth in painful IVDs.

Keywords: Notochordal cells; Intervertebral disc; Neuronal cells; Glycosaminoglycans; Chondroitin sulfate; Neurite outgrowth

Introduction

Low back pain is the leading global cause of disability causing substantial socioeconomic burden, and intervertebral disc (IVD) disease is commonly implicated in its pathogenesis [1,2]. Although the etiology of discogenic back pain is not completely understood, back pain patients have demonstrated increased nerve growth into diseased IVDs [3]. Analgesics provide only short-term pain relief and current biological strategies to treat painful IVDs focus largely on repair and regeneration of the IVD rather than targeting the source of pain itself. There is a need to develop therapies that focus on the mechanisms associated with the induction and propagation of discogenic back pain, and addressing neurovascular invasion is a natural target [4].

The healthy immature IVD is largely avascular and aneural and rich in proteoglycans. The gelatinous nucleus pulposus (NP) is surrounded circumferentially by the fibrous annulus fibrosus and contained cranially and caudally by the cartilage end plates, providing the IVD with the ability to withstand high mechanical forces and maintain motion [5]. Aging and degeneration of IVD results in increased matrix degradation, proinflammatory cytokine expression, decreased water content, and inferior mechanical properties [6]. These degenerative changes, including fissures, may provide a permissive microenvironment for neurovascular growth and sensitization of nerve fibers in the IVD [7]. Small unmyelinated nociceptive neurons expressing the neuropeptide Substance P and axonal elongation marker GAP43 have been demonstrated to grow into the painful human IVD [3,8]. These nerves also express the high affinity receptor for nerve growth factor (NGF), tyrosine kinase A, and accompany microvascular blood vessels that express NGF [9]. The likely sources of neoinnervation and neovascularization are defects in the annulus fibrosus or vertebral end plates [9]. Neurovascularization has been identified in posterior radial and transdiscal tears of human cadaveric IVDs [10], and in such tears, a decrease in the stress profile along the defect and also focal depletion of proteoglycans were observed, providing a path for nerves and blood vessels to grow into the IVD [7]. Defects in the vertebral end plate are also associated with neoinnervation, where nerve growth has shown to be the greatest in fibrovascular end plate marrow defects compared with annular tears or other end plate pathologies [11].

The healthy IVD produces factors with the ability to inhibit growth of nerves and blood vessels into the IVD, yet expression of such factors decreases with age and their absence may be associated with promoting neurovascular growth in diseased states [7,12–14]. Disc cells express vascular endothelial growth factor, NGF, and brain-derived neurotrophic factor, and expression of these nerve promoting factors increases with the severity of degeneration [15–18]. These growth factors are also upregulated by the proinflammatory cytokines TNFα and IL-1β in vitro, and as such, they may enhance the increase in expression observed with disease progression. The disc also produces neuropeptides (ie, Substance P) involved in pain perception that can upregulate proinflammatory cytokines themselves and increase with the severity of degeneration [19,20]. Human neuronal cells cocultured with human degenerate NP cells show increased neurite growth [21], whereas coculture with human NP cells isolated from the healthy nondegenerate IVDs had inhibitory effects on neurite growth. Human aggrecan from the healthy IVD can inhibit both neurite growth and endothelial migration in vitro, and taken together, these studies suggest that soluble factors from the healthy IVD could be harnessed to repel neurovascular growth in painful IVD [12,13].

Recapitulation of the processes that occur during the developmental patterning of the healthy immature notochordal cell (NC)-rich IVD may help inform therapeutic strategies to treat painful IVD degeneration [4]. The notochord patterns the IVD during development through secretion of diverse developmental ligands that give rise to the aneural and avascular structure. These include soluble neurovascular repulsive factors, such as semaphorin 3A and noggin [22,23]. Chondroitin sulfate (CS) proteoglycans expressed by the notochordal sheath are also associated with neuronal patterning and inhibition during the development of spine [24]. Understanding the processes that occur during development may inform treatments for discogenic back pain, and we believe that such natural processes should be emulated to enhance therapeutic strategies. A number of species such as pig and rabbit retain NCs from development through to adulthood and do not experience the disc disease common in humans [25,26]. As a consequence, NCs have received much attention with regard to the possible mechanisms associated with their loss and also their therapeutic potential [4,27–30]. It has previously been shown that

NC-conditioned media (NCCM) promotes a healthy NP phenotype in human degenerate NP cells, human mesenchymal stem cells, and mouse-induced pluripotent stem cells [31–35] via stimulation of substantial glycosaminoglycan (GAG) production and upregulation of phenotypic markers. The long-term objectives of this work are to use factors secreted by NCs to treat painful IVDs by preventing or limiting neurovascularization in the diseased state. We previously demonstrated that notochordal-rich NP tissue contains soluble factors that have the capacity to inhibit axonal growth, namely semaphorin 3A and CS, and showed increased proteoglycan accumulation after mechanical stimulation of NCs [29]. However, it is not known if NCCM is capable of inhibiting axonal growth and what soluble factors in NCCM are responsible for such neurite inhibition.

The aim of this study was to examine the dose effects of NCCM on neurite growth and establish the soluble factors necessary for these observed effects. We investigated human neuroblastoma (SH-SY5Y) cells because they are a well-characterized readily available human neural cell source previously used for these types of studies [12,21]. For mechanistic studies, we validate the effects also on rat dorsal root ganglion (DRG) cells because of their physiologic relevance, since DRGs are responsible for innervating the IVD in situ and human DRGs are not readily available. Notochordal cell-conditioned medium was derived from porcine NP tissue that comprises ~80% NCs [33]. Glycosaminoglycans in NCCM were characterized using a series of enzymatic digestions and NCCM digested with chondroitinase ABC (Chon-ABC) was then used to treat neural cells to probe the mechanism of GAGs as a potential neural inhibitory factor in NCCM.

Materials and methods

SH-SY5Y and DRG cell culturing

All materials and reagents were obtained from Sigma-Aldrich (St. Louis, MI, USA) unless otherwise stated. Human neuroblastoma SH-SY5Y cells (Passage 19) (Sigma-Aldrich, # 94030304) were maintained in Eagle minimum essential medium (EMEM) (ATCC, # 30-2003, Manassas, VA, USA) and F-12K medium (ATCC, # 30-2004) in 1:1 ratio with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C 5% CO_2 and expanded to 70% confluence before treatment. Primary rat DRGs (Innoprot, Biscay, Spain) were maintained at 37°C 5% CO_2 and cultured according to the manufacturer's instructions. Dorsal root ganglion cells were cultured in neuronal medium (Cat #1521; supplemented with neural growth serum and 1% penicillin/streptomycin, Innoprot, Biscay Spain) to produce neuronal supplemented media.

Generation of NCCM

Porcine spines (6 to 8 weeks of age) were obtained within 24 hours of death (Animal Facility Research 87, Inc., Boylston, MA, USA; animals are routinely slaughtered for human consumption [permit from USDA] and waste tissue taken for research) and notochordal-rich NP tissue aseptically dissected from a total of six spines (n=6) (Fig. 1). Nucleus pulposus tissue from four discs of the same spine were cultured in 50 mL falcon tubes with 30 mL of basal media, which consisted of high glucose DMEM, ×1 insulin transferrin and selenium, salt solution (5 M NaCl/0.4 M KCl), and 50 μg/mL ascorbic acid, and incubated (1% O_2, 5% CO_2, and 37°C) for 4 days to generate NCCM. Medium was then filtered through 3,000 MW Amicon Ultra-15 (Millipore, Bedford, MA, USA) to remove small metabolites and waste products associated with altered pH and the concentrate resuspended in an equal volume of either fresh control SH-SY5Y basal media (EMEM: F12K without FBS) or rat DRG basal media (neuronal medium+growth serum).

Dose response study of NCCM on SH-SY5Y cells

SH-SY5Y cells were treated with NCCM at 100% and 10% or control basal media. Cells were seeded at a density of 50,000 cells/well in a six-well poly-D-lysine coated plate in 2 mL of EMEM: F12K with 10% FBS and 1% penicillin/streptomycin for 24 hours 37°C 5% CO_2. After incubation, wells were washed twice with 2 mL phosphate buffered saline and then 2 mL of NCCM (n=6) at 100% concentration, NCCM at 10% in basal media, or a basal media control (SH-SY5Y basal media: EMEM: F12K without FBS) was added. All media conditions contained 40 ng/mL platelet-derived growth factor to induce neurite growth. SH-SY5Y cells were incubated at 37°C 5% CO_2 for a further 48 hours.

Digestion of NCCM to investigate the content of GAGs in NCCM

A series of digestions were performed to investigate which GAGs are present in NCCM. Notochordal cell-conditioned medium (n=4) was predigested with either chondroitinase B (Chon-B) (Sigma # C8058), Chon-ABC (Sigma # C3667), keratanase (Seikagaku AM, Inc., Japan # 100810-1), or heparinase II (Sigma # H6512) to digest dermatan sulfate (DS), CS and DS, keratan sulfate (KS), or heparan sulfate (HS), respectively. Glycosaminoglycan content of control and digested NCCM was assessed by the dimethyl methylene blue (DMB) assay [36]. The DMB assay measured the amount of intact GAG in NCCM treated with enzymes. Dimethyl methylene blue analyses were performed on both NCCM samples and NCCM with selected enzymatic digestions. Chondroitin sulfate (Sigma # C4384), DS (Sigma # C3788), HS (Sigma # H7640), and KS (provided by Dr. Robert Linhardt) standard curves were used as appropriate. Digested NCCM was then normalized to the undigested control to give percentage degradation for each enzyme treatment.

To investigate the CS composition of NCCM, GAGs were isolated, purified, and fractionated as described

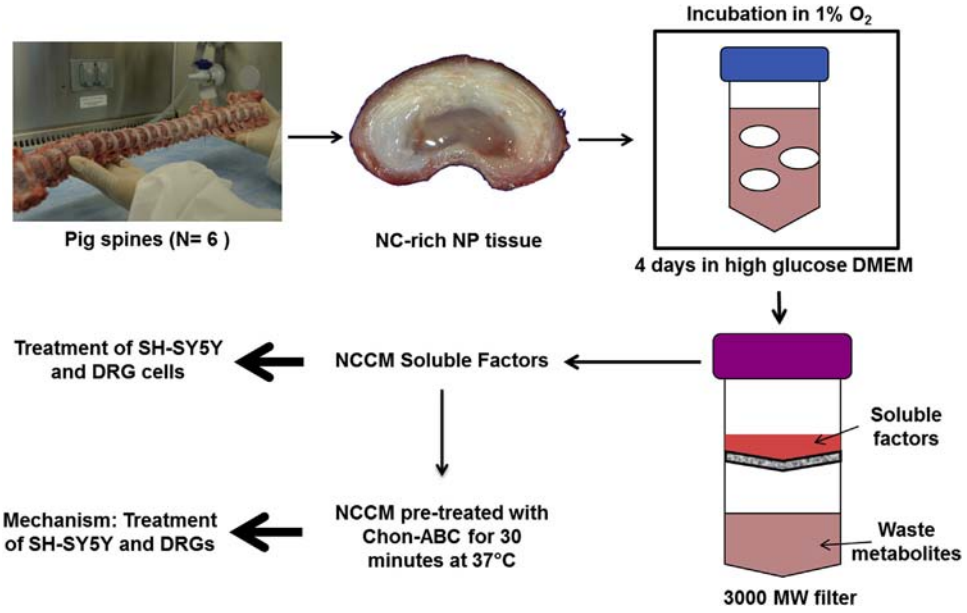

Fig. 1. Study design: Generation of NCCM and treatment of SH-SY5Y and DRG cells. NCCM was generated from NC-rich NP tissue from porcine pig spines (n=6) preconditioned in high glucose DMEM (Dulbecco's modified eagle's medium) with 10% insulin transferrin and selenium, 50 μg/mL ascorbic acid, 1% penicillin/streptomycin, and 0.5 Fungizone for 4 days at 1% O_2 5% CO_2, 37°C. The media was then filtered with Amicon-Ultra 15 filters and the filtrate (soluble factors) resuspended in an equal volume of basal serum-free media. NCCM was either used directly or predigested with Chon-ABC before addition to SH-SY5Y or DRG cells. NC, notochordal cell; NP, nucleus pulposus; DRG, dorsal root ganglion; NCCM, notochordal cell-conditioned media; Chon-ABC, chondroitinase ABC.

previously [37,38]. Liquid chromatography mass spectroscopy (LC-MS) analyses were performed on an Agilent 1200 LC/MCD instrument (Agilent Technologies, Inc., Wilmington, DE, USA) and quantitation analysis of disaccharides performed using calibration curves by separation of increasing amounts of unsaturated disaccharide standards. Linearity was assessed based on the amount of disaccharide and peak intensity in extract ion chromatography.

Treatment of SH-SY5Y cells and DRGs with NCCM predigested with chondroitinase ABC

Chondroitinase-ABC was the only enzyme to significantly digest GAGs in NCCM (see Results); therefore, we examined the effects of NCCM digested with Chon-ABC on SH-SY5Y and DRG cells. Notochordal cell-conditioned medium (100%) (n=4) was predigested with Chon-ABC at 0.05 U/mL at 37°C for 30 minutes to generate the NCCM+Chon-ABC media group. SH-SY5Y cells were then seeded as described previously for NCCM dose study treatment, with the addition of the NCCM+Chon-ABC group. SH-SY5Y cells were incubated at 37°C 5% CO_2 for 48 hours. For rat DRG cells, cells were seeded onto laminin-coated poly-D-lysine 24-well plates at a cell density of 8,333 cells per well in 1 mL of DRG basal media, and DRG cells were incubated at 37°C 5% CO_2 for 4 hours to allow adherence. After incubation, DRG cells were treated with 1 mL of DRG basal media, 100% NCCM (n=4) in DRG basal media, and 100% NCCM predigested with Chon-ABC together with recombinant rat beta-NGF at 100 ng/mL and incubated for 48 hours, as for SH-SY5Y.

Cell viability

Cell viability of SH-SY5Y and rat DRG cells was assessed using the live/dead assay (L3224; Invitrogen, Grand Island, NY, USA). Briefly, cells were incubated for 15 minutes in 10 mL of high glucose DMEM containing 10 μL of 2 mM ethidium homodimer-1 stock and 20 μL of 4 mM calcein AM stock at 37°C protected from light. After incubation, cell viability was assessed and images captured at ×10 magnification using a fluorescent inverted Zeiss microscope (Carl Zeiss Microscopy, Thornwood NY, USA) (emission/excitation; calcein=494/517 nm and ethidium homodimer-1=528/617 nm).

Image analysis

To assess neurite growth from SH-SY5Y and DRG cells after 48 hours incubation, 10 phase contrast images at ×10 magnification were captured using a Zeiss inverted microscope and Axiovision software for each treatment group, and the percentage of neurite expressing cells, mean neurite length, and total numbers of neurites were calculated using ImageJ software for both SH-SY5Y and DRG cells [12,21]. For the analysis, a neurite was defined as a cell process greater than 20 μm, for branching neurites, only the longest branch was traced and only cells with the entire cell body and neurite in the field of view were included (Fig. 2).

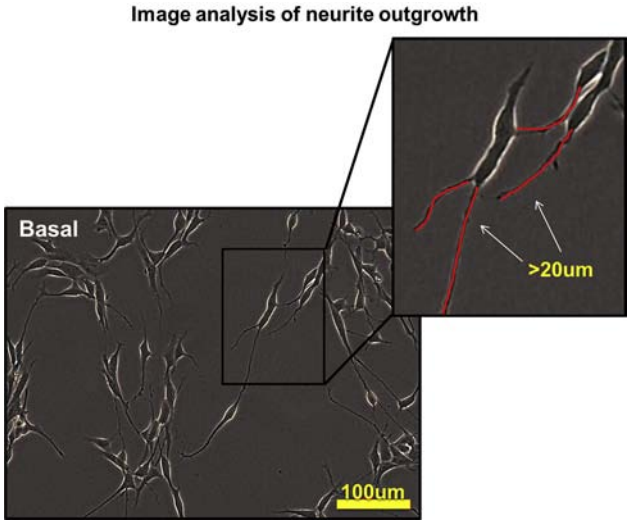

Fig. 2. Image analysis of neurite growth: A representative phase contrast image of SH-SY5Y cells at ×10 magnification captured using a Zeiss inverted microscope and Axiovision software. A neurite was defined as a cell process greater than 20 μm, as shown in red. For branching neurites, only the longest branch was traced and only cells with the entire cell body and neurite in the field of view were included. The percentage of neurite expressing cells, mean neurite length, total number of neurites, and cell number were calculated for each image using ImageJ software.

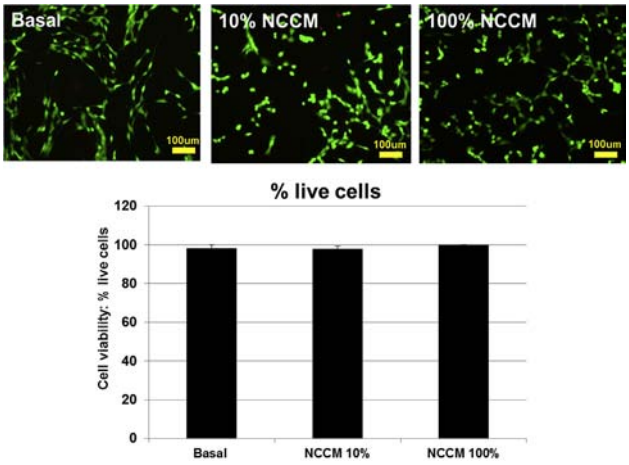

Fig. 3. (Top) Live/dead staining of SH-SY5Y cells with calcein and ethidium: SH-SY5Y cells demonstrated high viability in all groups tested: basal and 10% and 100% NCCM as indicated by most cells stained green with calcein and fewer dead cells stained red with ethidium. (Bottom) No significant differences were observed in cell viability between basal and NCCM groups. Images captured at ×10 magnification. NCCM, notochordal cell-conditioned media.

Statistics

For all neurite growth and DMB assay data, a one-way analysis of variance with post hoc Tukey test was performed comparing basal with NCCM 10% and NCCM 100% for SH-SY5Y cells. For GAG characterization/digestion studies, the undigested NCCM group (control) was compared with different enzyme concentration groups for each respective enzyme assessed (Chon-ABC, Chon-B, keratanase, and heparanase). For mechanistic studies, comparisons were made between basal, NCCM, and NCCM+Chon-ABC groups for SH-SY5Y and DRG cells. GraphPad Prism 3 (Graphpad software, La Jolla, CA, USA) was used with $p<.05$ significant.

Results

Effects of NCCM dose on SH-SY5Y cells

Notochordal cell-conditioned medium at doses of 10% and 100% did not demonstrate any detrimental effects on cell viability for SH-SY5Y cells, with no significant differences compared with basal controls (green calcein stained cells=live; red ethidium stained cells=dead) (Fig. 3). Notable differences in cell morphology were observed for SH-SY5Y cells treated with NCCM at both 10% and 100% (Fig. 4A-C), with NCCM-treated cells exhibiting a more spread out, less elongated appearance with fewer neurites than the basal condition. Notochordal cell-conditioned medium significantly reduced the percentage of neurite expressing cells and mean neurite length at both 10% and 100% compared with the basal control group with no differences between doses (Fig. 4D-F) ($p<.05$). No significant effects were observed on the total number of neurites.

Digestion of NCCM to investigate the abundance of intact GAGs in NCCM

Chondroitinase-ABC was the only enzyme to significantly decrease the amount of GAG in NCCM at all doses assessed compared with untreated controls suggesting that CS/DS formed the predominant GAGs in NCCM (Fig. 5) ($p<.05$). To determine whether CS or DS forms the predominant GAG, NCCM was digested with Chon-B that specifically digests DS. At all doses assessed, we observed no significant changes in GAG content compared with undigested NCCM, suggesting that CS forms the majority of GAG in NCCM. To ensure that Chon-B was active, as the suggested incubation conditions differed from that of Chon-ABC, we ran a DS positive control. Dermatan sulphate was digested with the same concentrations of Chon-B as for Chon-ABC and decreases in GAG were observed at all doses, in particular for 0.1 U/mL. Keratanase and heparanase at all doses did not degrade GAG in NCCM compared with NCCM controls, further confirming that CS was the dominant GAG in NCCM.

To determine the CS disaccharide composition of NCCM, GAGs were isolated, purified, and subject to digestion with chondroitinase ACII and Chon-ABC, followed by analysis using LC-MS with appropriate standards for identification and quantification. Chondroitin-4-sulfate (C-4-S), chondroitin-6-sulfate (C-6-S), and chondroitin-0-sulfate disaccharides were identified in NCCM with a significantly greater percentage of C-6-S compared with either C-4-S or chondroitin-0-sulfate alone (Fig. 5) ($p<.05$), indicating that C-6-S is the most abundant disaccharide in NCCM.

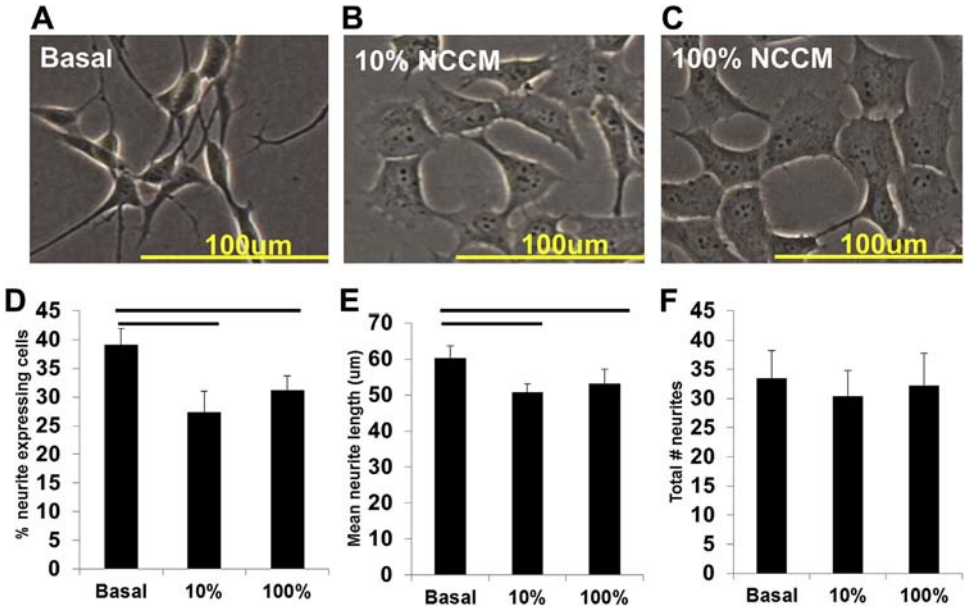

Fig. 4. NCCM inhibits neurite growth of human SH-SY5Y cells with no dose effect: (A) SH-SY5Y cells in basal media treated with 40 ng/mL platelet-derived growth factor for 48 hours demonstrated an elongated morphology with long neurites; (B and C) however, addition of 10% and 100% NCCM caused cells to exhibit a more spread-out appearance with fewer neurites. Images were captured at ×10 magnification. (D) Quantitative analysis of neurite growth demonstrated significantly reduced percentage of neurite expressing cells with NCCM at 10% and 100% compared with basal. (E) We also observed significant decreases in mean neurite length with 10% and 100% NCCM compared with basal controls ($p<.05$), (F) but no differences were observed for total number of neurites. NCCM, notochordal cell-conditioned media.

Treatment of SH-SY5Y and DRG cells with NCCM predigested with chondroitinase ABC

SH-SY5Y cells treated with NCCM+Chon-ABC resembled that of basal controls with an elongated appearance and significantly more neurites (Fig. 6A–C) ($p<.05$). Notochordal cell-conditioned medium+Chon-ABC was able to restore the percentage of neurite expressing cells back to basal levels and significantly increased mean neurite length and total number of neurites compared with NCCM treatment alone (Fig. 6D–F) ($p<.05$). To investigate

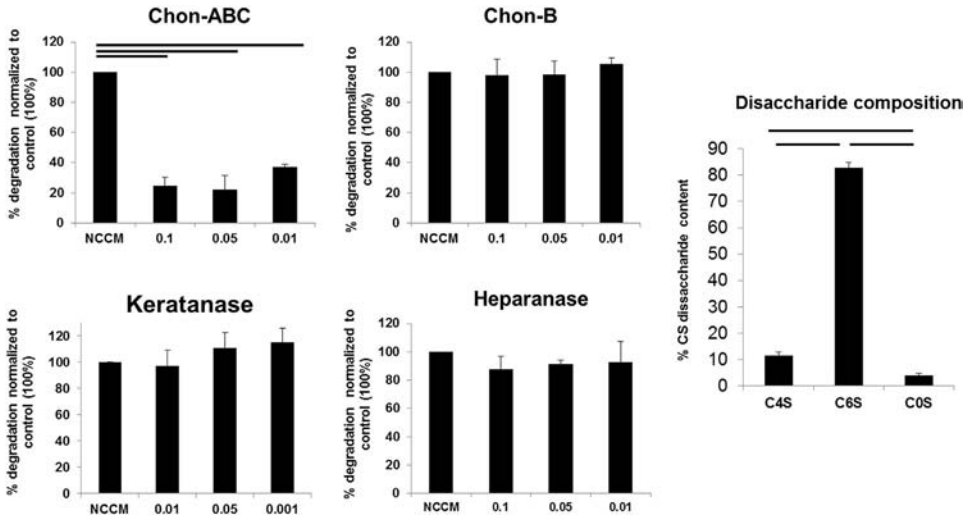

Fig. 5. CS is the most abundant glycosaminoglycan (GAG) in NCCM and C-6-S the most abundant disaccharide. GAG in NCCM was characterized using multiple digestions using (Left) Chon-ABC specific for CS and dermatan sulfate (DS), Chon-B specific for DS only, keratanase for keratan sulphate, and heparanase for heparan sulphate. The dimethyl methylene blue analysis indicated that Chon-ABC was the only enzyme at all doses tested to significantly decrease the amount of GAG in NCCM compared with untreated controls suggesting that CS is the most abundant GAG in NCCM ($p<.05$). (Right) Further characterization using liquid chromatography mass spectroscopy identified C-6-S as the most abundant disaccharide in NCCM ($p<.05$). NCCM, notochordal cell-conditioned media; Chon-ABC, chondroitinase ABC; Chon-B, chondroitinase B; CS, chondroitin sulfate; C-6-S, chondroitin-6-sulfate; C-4-S, chondroitin-4-sulfate; C-0-S, chondroitin-0-sulfate.

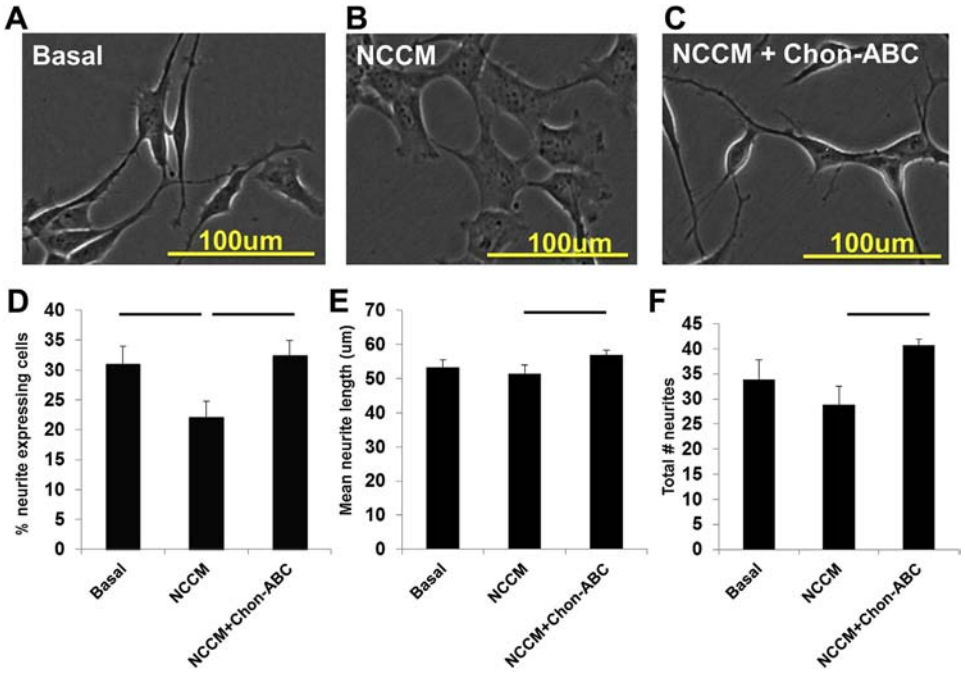

Fig. 6. Intact glycosaminoglycans in NCCM such as chondroitin sulfate are necessary to inhibit neurite growth of human SH-SY5Y cells: NCCM was pretreated with 0.05 U/mL of Chon-ABC for 30 minutes at 37°C and then added to SH-SY5Y cells seeded as described previously. (C) SH-SY5Y cells treated with NCCM digested with Chon-ABC (NCCM+Chon-ABC) demonstrated a similar cellular morphology as the (A) basal control group and this was confirmed after quantitative analysis of neurite growth, where (D) NCCM+Chon-ABC group restored the percentage of neurite expressing cells to basal levels ($p<.05$). (C and F) NCCM pretreated with Chon-ABC significantly increased mean neurite length and total number of neurites compared with NCCM alone (B and E) ($p<.05$). NCCM, notochordal cell-conditioned media; Chon-ABC, chondroitinase ABC.

whether NCCM and Chon-ABC digested NCCM had similar effects on more physiologically relevant neuronal cell type, rat DRG cells were treated as described for SH-SY5Y cells. No qualitative changes in cell morphology were observed with any of the treatments, although NCCM significantly decreased the percentage of neurite expressing cells, mean neurite length, and total number of neurites (Fig. 7A–F) ($p<.05$). Notochordal cell-conditioned medium did not demonstrate any cytotoxic effects on DRG cell viability compared with basal conditions (Supplemental Figure). No differences were observed between basal and NCCM+Chon-ABC groups for all parameters assessed, indicating that digestion of GAGs of which CS is the predominant form in NCCM restored rat DRG cells to basal conditions.

Discussion

This study evaluated the hypotheses that soluble factors secreted from NCs can be used to inhibit neurite growth and that intact GAGs such as CS are likely important factors in this inhibitory process. Notochordal cell-conditioned medium generated from immature porcine NP tissue that is rich in NCs inhibited the neurite growth in both human SH-SY5Y neuroblastoma and rat DRG cells. Glycosaminoglycan from NCCM was characterized using digestion studies that targeted CS, DS, KS, and HS and also used LC-MS to determine CS disaccharide composition. Chondroitin sulfate consisting largely of C-6-S disaccharides was determined to be the predominant GAG in NCCM and we suggest that CS in its intact form was a candidate factor for inducing the observed neurite inhibition. There was no dose effect of NCCM on this neural inhibiting characteristic and no loss of cell viability at high or low concentrations compared with controls. SH-SY5Y cells treated with NCCM demonstrated an altered cell morphology that may be associated with a change in cell phenotype. Results, therefore, suggest that intact GAGs produced from NCs could be used for therapeutic effect, targeting neoinnervation into painful IVDs, and the lack of any observed cytotoxicity further suggests that components of NCCM are likely to be safe for surrounding neuronal structures.

Previous studies demonstrated that NP cells from the healthy human IVD have the ability to inhibit neurite growth from SH-SY5Y cells when in coculture, suggesting that these healthy NP cells are able to produce soluble factors that have the potential to limit neoinnervation [21]. Rat NP and NC cells cocultured in different ratios showed significant inhibition of neurite growth from rat DRGs [39], further corroborating these results. The results from our present study support the literature, demonstrating that soluble factors from healthy NP cells or immature NCs can inhibit neurite growth and suggests that intact GAGs such as CS are important neural inhibitory factors that can be produced by NCs and healthy NP cells. During early patterning of the spine, the notochordal sheath expresses

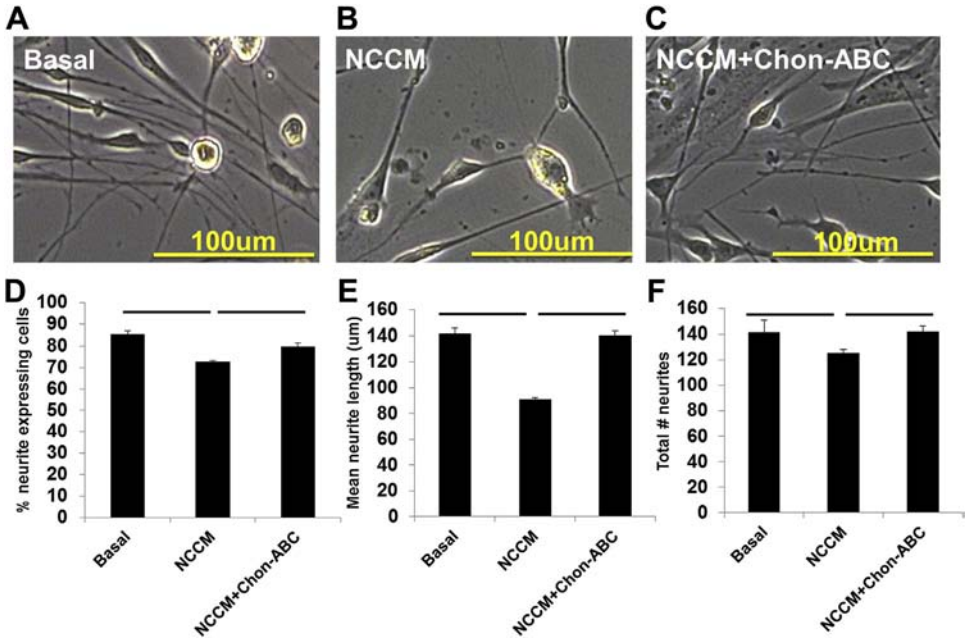

Fig. 7. Intact glycosaminoglycans in NCCM such as chondroitin sulfate are necessary to inhibit neurite growth of rat dorsal root ganglia (DRGs): NCCM was pretreated with 0.05 U/mL of Chon-ABC for 30 minutes at 37°C and added to rat DRGs. Rat DRGs demonstrated no differences in cell morphology between groups; however, differences in the number of neurites was observed with (B) NCCM, showing the fewest compared with (A) basal and (C) NCCM+Chon-ABC groups. NCCM+Chon-ABC restored neurite growth to basal levels compared with NCCM alone for parameters of (D) percentage of neurite expressing cells, (E) mean neurite length, and (F) total number of neurites ($p<.05$). NCCM, notochordal cell-conditioned media; Chon-ABC, chondroitinase ABC.

CS. This is one of the mechanisms by which the notochord repels axonal elongation from DRGs along the midline [24]. Johnson et al. [12] demonstrated that aggrecan derived from the mature human IVD can inhibit neurite growth; however, when Chon-ABC was used to digest the CS side-chains on the aggrecan core protein, its inhibitory effects were reduced. Studies have suggested that a loss of proteoglycan during disc disease, together with a decrease in pressurization, may provide a permissive environment for nerve growth [7]. Therefore changes in CS/proteoglycan content may be a key mechanism associated with neurovascular growth and pain during disease. Neoangiogenesis has been suggested to be a precursor for neoinnervation in the painful degenerate IVD [9], providing the nerve fibers with an oxygen supply as the IVD is hypoxic and neurons will undergo apoptosis/cell death in these conditions [40]. As such NCCM was generated under hypoxic conditions to maintain NC cell phenotype and generation of soluble factors; however, neuronal cells were treated with NCCM in normoxic conditions to maintain neuronal cell viability.

During growth, aging, and disease, the IVD undergoes dramatic changes in matrix composition [41]. Specifically, the composition and length of GAG chains change with aging as does the length of the aggrecan core protein [41]. There is a decrease in the length and number of CS side-chains in favor of KS, and KS has been shown to have a reduced effect on neural inhibition compared with CS [12]. Early research demonstrated that the composition of CS subtypes in the IVD was primarily C-4-S and C-6-S [42]. Chondroitin-6-sulfate was abundant in the developing notochordal-rich NP and declined with aging, whereas the converse was true for C-4-S. However, more recent studies have suggested a role for native 7-D-4 CS sulfation motifs that contain a mix of C-4-S and C-6-S oligosaccharides in the IVD anlagen and they have been described as a ''marker of tissue development'' that decline with aging [43]. In our study, we demonstrated that the major CS disaccharide in NCCM is C-6-S, suggesting that this disaccharide could likely be responsible for the observed inhibitory effects. Additional experiments such as rescue studies validating the role of C-6-S would be valuable but currently C-6-S is not commercially available. Both C-6-S and C-4-S disaccharides have been shown to inhibit neurite growth from embryonic neurons in cell culture; however, their role with respect to adult neurons, such as those that innervate the adult IVD, is less clear [44,45]. These studies suggest that free intact GAGs such as CS side-chains could be effective at inhibiting neurite growth, and this is supported by work demonstrating that recombinant aggrecan depleted of CS side-chains was unable to inhibit neurite extension [46]. At present, it is uncertain whether a specific CS sulfation pattern is required or any CS structure will suffice, or if other GAGs of low abundance are also important in repelling neurite extension. Answers to these key questions will be important for determining how to use intact CS in a therapeutic manner.

This study used multiple cell sources from different species; porcine-derived NCCM was used as the pig is rich in

NCs and readily available in contrast to the human. The SH-SY5Y neuroblastoma cell line represents the most relevant human neural cell available. Dorsal root ganglion cells were the most anatomically relevant cell population innervating the IVD and were readily available to be isolated from rats unlike for humans. Although this can complicate data interpretation, it does not detract from the ultimate conclusions. Autologous human-derived NCCM is not a feasible treatment option in humans, which lose their large vacuolated NCs during adolescence. Therefore, minimally invasive strategies using these NC-derived concepts rely on isolation and injection of specifically identified factors in NCCM. Our study points toward CS as a candidate factor for our observed neuronal inhibiting effects. Additional ligands present in NCCM, such as semaphorin 3A and noggin, are also suggested as a mechanism for neuronal inhibition and avascular patterning of the IVD during development [22–24]. The presence of multiple anabolic and neural-inhibiting factors in NCCM may have contributed to the lack of NCCM dose effects, giving more justification for the use of a single factor that may have the potential to repel invading nerves into the painful IVD. The lack of dose effects may also suggest that neural inhibition was saturated at 10%. Furthermore, it is possible that other factors can play a neural inhibiting role at lower GAG concentrations. It is also reasonable to stimulate the resident NP cells to produce a continuous source of CS rather than inject CS directly as a single dose. In general, we believe that maintaining, or restoring, the structure of the IVD as a barrier high in GAGs such as CS is a key element to repelling neural growth and pain.

In conclusion, our study demonstrated that intact GAGs are the principal neural inhibitory factors in NCCM; we hypothesize that restoration of the CS matrix barrier rich in C-6-S is essential to preventing neural growth and discogenic pain, but note that additional low abundant GAGs that are difficult to detect may also play roles in neurite inhibition. We also demonstrated that human SH-SY5Y and rat DRGs have similar neurite growth behaviors in vitro, suggesting both are reasonable cell choices for such screening studies but recommend the use of DRGs as a neuronal cell model of choice because of their greater physiologic relevance. Soluble factors from NCs were not cytotoxic, suggesting these factors have the potential to be injected into the IVD with minimal detrimental effects on surrounding neuronal structures. Whether NCs produce an immature type of CS that is more inhibitory compared with mature NP cells remains unknown and represents a future area of exploration.

Acknowledgments

The authors gratefully acknowledge the technical assistance of Dr Svenja Illien-Junger and Ms Ilana Stock for NP tissue isolation and cell culture and Dr Lingyun Li for technical assistance with the disaccharide compositional analysis. The authors also acknowledge the support of Dr Hongyan Zhou with DRG cell culture. This study was supported by the NIH (R01 AR064157), Orthopedic Education Research Foundation, & S-13-50P of the AO Foundation.

Supplementary data

Supplementary data related to this chapter can be found at http://dx.doi.org/10.1016/j.spinee.2015.02.003.

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