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Chondroitin sulfate proteoglycans from salmon nasal cartilage inhibit angiogenesis

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ABSTRACT

Because cartilage lacks nerves, blood vessels, and lymphatic vessels, it is thought to contain factors that inhibit the growth and development of those tissues. Chondroitin sulfate proteoglycans (CSPGs) are a major extracellular component in cartilage. CSPGs contribute to joint flexibility and regulate extracellular signaling *via* their attached glycosaminoglycan, chondroitin sulfate (CS). CS and CSPG inhibit axonal regeneration; however, their role in blood vessel formation is largely unknown. To clarify the function of CSPG in blood vessel formation, we tested salmon nasal cartilage proteoglycan (PG), a member of the aggrecan family of CSPG, for endothelial capillary-like tube formation. Treatment with salmon PG inhibited endothelial cell adhesion and *in vitro* tube formation. The anti-angiogenic activity was derived from CS in the salmon PG but not the core protein. Salmon PG also reduced matrix metalloproteinase expression and inhibited angiogenesis in the chick chorioallantoic membrane. All of these data support an anti-angiogenic role for CSPG in cartilage.

1. Introduction

Chondroitin sulfate proteoglycans (CSPGs) are major components of cartilage, together with collagens and hyaluronan. Proteoglycan (PG) is a glycoprotein comprising a single core protein and attached glycosaminoglycan (GAG) polysaccharides. The GAG of CSPGs is mainly chondroitin sulfate (CS). Although CSPG is a major PG in cartilage, other PGs such as heparan sulfate PGs (HSPGs), dermatan sulfate PGs, and keratin sulfate PGs (KSPGs) are widely distributed in the cell surface and the extracellular matrix (ECM) of animal tissues [1].

CSPG and CS are major inhibitors of neuronal migration [2,3]. Cartilage lacks neurons, blood vessels, and lymphatic vessels, which led to the hypothesis that cartilage contains substances that inhibit the growth of those tissues. Therefore, CSPG and CS are expected to have an inhibitory effect on blood vessel formation. Indeed, loss of PG in osteoarthritic (OA) cartilage is associated with loss of resistance to vascular invasion [4]. Human intervertebral disc aggrecan was shown to inhibit endothelial cell migration [5]. Inhibition of endothelial cell adhesion and migration would inhibit tubulogenesis. Although the anti-migration activity of aggrecan was attributed to its CS moiety, CS alone showed both anti-angiogenic [6] and pro-angiogenic activities [7]. Moreover, treatment with chondroitinase, which is a specific GAG lyase, inhibits endothelial cell proliferation and angiogenesis, indicating that endogenous CS in endothelial cells is essential for angiogenesis [8]. Thus, it remains unclear whether CSPGs play an inhibitory role in blood vessel formation.

To clarify the role of CSPG in blood vessel formation, we aimed to identify effects of salmon nasal cartilage PG on angiogenesis. The conventional procedure for extraction of PG uses 4 M guanidine hydrochloride (GdnHCl) [9]. Recently, a simple, low-toxicity procedure for extraction of PG from salmon nasal cartilage was developed using acetic acid [10]. Our previous report showed that the major PG in this salmon PG fraction is from the aggrecan family of CSPGs [11]. Based on the cDNA sequence, globular domains G1, G2, and G3 are conserved between salmon and mammalian aggrecan. However, the core protein

Abbreviations: BME, basement membrane extract; BSA, bovine serum albumin; CAM, chorioallantoic membrane; CS, chondroitin sulfate; CSPG, chondroitin sulfate proteoglycan; ECM, extracellular matrix; FAK, focal adhesion kinase; FBS, fetal bovine serum; GAG, glycosaminoglycan; GalNAc, *N*-acetylgalactosamine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GdnHCl, guanidine hydrochloride; GlcUA, glucuronic acid; HSPG, heparan sulfate proteoglycan; KSPG, keratin sulfate proteoglycan; MMP, matrix metalloproteinase; OA, osteoarthritis; PBS, phosphate-buffered saline; PG, proteoglycan; UA, uronic acid

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of acetic acid-extracted salmon PG is partially fragmented compared to that of GdnHCl-extracted salmon PG [12]. Although the acetic acid-extracted PG is the fragmented CSPG fraction, it retains various biological activities and is the only commercially available PG for pharmacological use [13–17]. In this study, we show that salmon PG reduces the tube-like formation of vascular endothelial cells. The anti-angiogenic activity of salmon PG was derived from CS. In addition, salmon PG reduced matrix metalloproteinase (MMP) expression in endothelial cells. Our results support an anti-angiogenic function of CSPG and CS.

2. Materials and methods

2.1. Cell culture

The immortalized human umbilical vein endothelial cell line EA.hy926 was obtained from ATCC (CRL-2922, Manassas, VA, USA). The cells were maintained in Medium 199 (Life Technologies Japan, Tokyo, Japan) supplied with 10% fetal bovine serum (FBS) (GE Healthcare Japan, Tokyo, Japan) and penicillin/streptomycin (Life Technologies Japan). The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Purification of proteoglycan

Acetic acid-extracted salmon nasal cartilage PG was purchased from Ichimaru Pharcos Co., Ltd. (Gifu, Japan). The PG was repurified by ion-exchange chromatography as described previously with minor modifications [12]. Briefly, PG was dissolved in 7 M urea in 50 mM Tris-HCl buffer (pH 7.5) and applied to a column (2.5 cm×10 cm) filled with DEAE-sephacel (GE Healthcare Japan) at a flow rate of 0.5 mL/min. The column was washed with five column volumes of 7 M urea in 50 mM Tris–HCl buffer (pH 7.5) and eluted with five column volumes of 0.0–1.0 M NaCl in a linear gradient, and 5-mL fractions were collected. Uronic acid (UA)-rich fractions were pooled, dialyzed against pure water, and concentrated by Speedvac (Thermo Fisher Scientific, Waltham, MA, USA). The PG was sterilized with a 0.2-μm filter and stored at −80 °C until use. UA and protein contents in the purified PG were determined by the carbazole sulfuric acid method [18] and using Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA), respectively. In this study, we used UA as a unit of PG and CS. The composition of CS in salmon PG was approximately 14.4% ΔDi-0S [glucuronic acid (GlcUA)- *N*-acetylgalactosamine (GalNAc)], 27.0% ΔDi-4S [GlcUA-GalNAc(4S)], 57.8% ΔDi-6S [GlcUA-GalNAc(6S)], 0.8% ΔDi-S_D [GlcUA(2S)-GalNAc(6S)], and 0.0% ΔDi-S_E [GlcUA-GalNAc(4S, 6S)].

2.3. Cell viability test

EA.hy926 cells were seeded in a 96-well microplate at 16,000 cells/cm^2 and incubated in the culture medium at 37 °C for 24 h. After washing with phosphate-buffered saline (PBS), 0.1 mL of serum-free medium containing various concentrations of PG was added to the wells. After 24 or 48 h of incubation, 0.01 mL of WST-8 reagent (Cell count reagent SF, Nacalai Tesque Inc., Kyoto, Japan) was added to each well. The plate was incubated at 37 °C for 1 h. The absorbance at 450 nm of each well was measured using a Benchmark microplate reader (Bio-Rad).

2.4. Cell adhesion assay

A 96-well culture plate was incubated with 2 μg/well human plasma fibronectin (Life Technologies Japan) in PBS at 4 °C overnight. To block the uncoated area, the fibronectin-coated plate was incubated with 0.5% bovine serum albumin (BSA) (Sigma-Aldrich Japan, Tokyo, Japan) in PBS at 37 °C for 30 min and then washed with PBS three times. Thereafter, various amounts of PG in PBS (100 μL) were added to the well, and the plate was incubated at 37 °C for 30 min and then rinsed with PBS three times. Single-cell suspensions in serum-free media (20,000 cells/100 μL/well) were added to the well and incubated at 37 °C for 1 h. After incubation, the plate was vortexed on a plate shaker and washed with PBS three times to remove unattached cells. The numbers of attached cells were estimated by crystal violet assay. The crystal violet solution (0.2% in 25% methanol) was added to the well and incubated for 10 min. The plate was washed with water and then dried. Sodium dodecyl sulfate solution (1%) was added to the well to solubilize the stain. The number of cells was estimated by the absorbance at 570 nm of each well.

2.5. Enzymatic digestion of salmon PG

The PG (3 mg/mL) was incubated with 1 mg/mL Actinase E (Kaken Pharmaceutical Co. Ltd., Tokyo, Japan) for 16 h at 50 °C in 100 mM Tris–HCl (pH 8.0) and 10 mM $CaCl_2$. The reaction was terminated by boiling for 3 min. The PG (3 mg/mL) was incubated with 250 mU/mL protease-free chondroitinase ABC (C3667, Sigma-Aldrich Japan) for 16 h at 37 °C in 50 mM Tris–HCl (pH 8.0), 60 mM CH_3COONa, and 0.02% BSA. The reaction was terminated by boiling for 3 min. To remove digested CS oligosaccharides, the reaction was purified by ultrafiltration with Amicon Ultracel 30 kDa filters (Merck Millipore Ltd., Darmstadt, Germany). All reactions were sterilized with a Coaster Spin-X centrifuge filter unit (Corning Japan K.K., Tokyo, Japan). The efficiency of enzymatic digestion was evaluated using a combination of agarose gel electrophoresis and Stains-All staining (Sigma-Aldrich Japan).

2.6. In vitro *tube formation*

The *in vitro* angiogenesis assay was performed as described by Arnaoutova and Kleinman with minor modifications [19]. Briefly, 50 μL of Basement Membrane Extract (BME, Trevigen Inc., Gaithersburg, MD, USA) was added to a 96-well plate on ice. The plate was incubated for 30 min at 37 °C for gelling. EA.hy926 cells (5000 cells) in 100 μL of serum-free media with or without PG were added to the plate and cultured on the BME gel for 16–20 h. In some experiments, the enzyme-digested PG (mentioned above), CS4S (400655, Seikagaku Corp., Tokyo, Japan), or CS6S (400675, Seikagaku Corp.) was used instead of PG. The composition of CS4S was 1.7% ΔDi-0S, 74.1% ΔDi-4S, 23.0% ΔDi-6S, 1.0% ΔDi-S_D, and 0.2% ΔDi-S_E and that of CS6S was 1.9% ΔDi-0S, 12.7% ΔDi-4S, 75.0% ΔDi-6S, 7.6% ΔDi-S_D, and 2.8% ΔDi-S_E. Photographs were taken using a digital camera (E-330, Olympus Corp., Tokyo, Japan) attached to an Olympus CKX41 microscope. Tube formation was analyzed with ImageJ [20] using the Angiogenesis Analyzer Plug-in [21].

2.7. Real-time quantitative PCR and gelatin zymography

Confluent cells were incubated in media supplemented with 0.5% FBS and 0.5% BSA with or without PG for 24 h. Culture supernatants were used for gelatin zymography to analyze MMP expression. Gelatin zymography was performed as described previously [22]. To analyze the mRNA level, total RNA was isolated from the cells using an RNeasy mini kit (QIAGEN Japan, Tokyo, Japan) according to manufacturer's procedure. To generate cDNA, reverse transcription was performed with 1 μg of RNA using a High-Capacity cDNA Reverse Transcription Kit (Life Technologies Japan). Quantitative real-time PCR was performed using a StepOnePlus Real-time PCR system (Life Technologies Japan). The reaction mixtures contained each cDNA, FastStart Universal Probe Master [ROX] (Roche Diagnostics GmbH, Mannheim, Germany), and TaqMan Probes (Life Technologies Japan) for each gene (Table 1). The reaction was performed for 40 cycles of 95 °C for 10 s and 60 °C for 30 s followed by 95 °C for 10 min.