

Isolation and Characterization of Chondroitin Sulfates from Sturgeon (*Acipenser sinensis*) and Their Effects on Growth of Fibroblasts

A-Rang IM, Youmie PARK, and Yeong Shik KIM*

Natural Products Research Institute, College of Pharmacy, Seoul National University; Seoul 151-741, Korea.

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Chondroitin sulfate (CS) is a glycosaminoglycan that composed of hexosamine (D-galactosamine) and hexuronic acid (D-glucuronic acid) unit arranged in an alternating unbranched sequence. CS is an essential component of the extracellular matrix (ECM) of connective tissue. It is mainly covalently attached to core proteins in the form of proteoglycans so that it exhibits specific interactions with proteins for cell growth, differentiation, division and migration. In this study, CSs were purified from the cartilage and backbone of sturgeon (*Acipenser sinensis*). To characterize their biochemical properties, we performed disaccharide compositional analysis after chondroitinase ABC digestion, high performance size exclusion chromatography (HPSEC) and ¹H-NMR spectroscopy. We also investigated the effects of CSs on fibroblast proliferation and adhesion to determine whether wound healing was accelerated *in vitro* and proliferation of different mitogen-activated protein kinases (MAPK) signaling pathways was facilitated. The CS purified from sturgeon cartilage was primarily composed of 4-sulfated CS (88.8%) and sturgeon backbone CS contains more than 60% 6-sulfated CS. The average molecular weights of CSs obtained from sturgeon cartilage and backbone were found to be 8 and 43 kDa, respectively. Our results showed that both CSs are able to increase cell adhesion, induce proliferation and migration on fibroblasts and may accelerate wound healing by inducing MAPK signaling pathways.

Key words chondroitin sulfate; sturgeon; characterization; fibroblast; proliferation

Glycosaminoglycans (GAGs) consist of a linear polysaccharide chain with a repeating disaccharide unit of hexosamine (D-glucosamine or D-galactosamine) and uronic acid (D-glucuronic acid or L-iduronic acid). Chondroitin sulfate (CS) is composed of hexosamine (D-galactosamine) and hexuronic acid (D-glucuronic acid) disaccharide unit, which is arranged in an alternating unbranched sequence. CS has been isolated from various natural sources including mammalian species (bovine, porcine, and chicken cartilage) and marine species (sharks, squids, and skates).^{1–4} CS is an essential component of the extracellular matrix (ECM) of connective tissue. It is mainly covalently attached to core proteins in the form of proteoglycans so that it exhibits specific interactions with proteins for cell growth, differentiation, division and migration.⁵ In addition, CS plays important roles in the elasticity, function of the articular cartilage, wound healing, the central nervous system, and chondrocytes.^{6–10}

Wound healing is a complex process that is a result of mechanical and chemical injuries. It has been divided into three main phases: inflammation, proliferation and tissue remodeling. Fibroblasts play an important role in wound healing and require a growth factor for their activation and proliferation.^{11–13} The action of CS in wounds affects the size, orientation and the degree of fibroblasts present in the wound. CS stimulates the proliferation of human fibroblasts derived from both normal skin and chronic wounds. CS helps to mediate fibroblast growth factor-2 (FGF-2)-induced cell proliferation by binding to FGF-2. It also helps to regulate cell adhesion and enhance cell spreading and migration.^{7,14,15}

Sturgeon (*Acipenser sinensis*) is a globally important wildlife resource that contributes as an important element of local food supply.¹⁶ Sturgeon's cartilage contains biologically active substances such as collagen, glucosamine, and CS.^{17–19} In this study, the purified CSs from sturgeon cartilage and backbone were characterized using strong anion-exchange (SAX) HPLC, high performance size-exclusion chro-

matography (HPSEC) and ¹H-NMR. We also determined the effect of the purified CS on fibroblast cell adhesion, proliferation and migration.

MATERIALS AND METHODS

Materials Sturgeon was purchased at a local store in Seoul, Korea. Control CS from bovine trachea was purchased from New Zealand Pharmaceuticals (Palmerston North, New Zealand) and its purity was assessed based on USP CS.²² Authentic CS from shark cartilage and two other CS samples used as MW standards were obtained from Seikagaku Co. (Tokyo, Japan). Hyaluronic acids (100, 130, 1690 kDa) were also acquired to use as MW standards. Chondroitinase ABC from *Proteus vulgaris*, sodium carbonate, trichloroacetic acid solution, cetylpyridinium chloride, sodium chloride and tris [hydroxymethyl] aminomethane (Trizma[®] base) were purchased from Sigma (St. Louis, MO, U.S.A.). The dialysis membrane (Spectra/Por[®] 1, MWCO 6–8 kDa) was purchased from Spectrum[®] Laboratories (Rancho Dominguez, CA, U.S.A.). Alcalase[®] (2.4 Anson units/g) from *Bacillus licheniformis* was purchased from Novozymes (Bagsvaerd, Denmark). Human dermal fibroblast cells were obtained from the Biospectrum Life Science Institute (Gyeonggi Province, Korea). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were obtained from Thermo Scientific HyClone (Waltham, MA, U.S.A.). Dulbecco's phosphate-buffered saline (D-PBS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Anti-p38, p-p38, c-Jun N-terminal kinases (JNK1), pJNK1, extracellular signal-regulated kinase 1 (ERK1), pERK1/2, β -actin, goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody and goat anti-rabbit (HRP)-conjugated secondary antibody were purchased from Santa Cruz (CA, U.S.A.). We used nitrocellulose membrane (PALL, Port

* To whom correspondence should be addressed. e-mail: kims@snu.ac.kr

Washington, NY, U.S.A.), WEST-SAVE UPTM luminal-based ECL reagent (LabFrontier, Seoul, Korea), dimethyl sulfoxide (Bioshop, Burlington, Canada), a wound healing assay kit (Culture-Insert, Ibidi, München, Germany) and a protein assay reagent (Bio-Rad, Vancouver, Canada).

Purification of CS from Sturgeon The bones and cartilage of sturgeon were cleaned, lyophilized and ground. CS was isolated using a method according to our previous study.²⁰ The dried powder was suspended in 50 mM sodium carbonate buffer (pH 7.0) containing alcalase solution (5%) and was then incubated for 12 h at 60 °C and 200 rpm in a shaking incubator. After boiling for 10 min to inactivate the protease, the sample solution was filtered and cooled at 4 °C. The trichloroacetic acid solution (6.1 M) was mixed with the filtrate to a final concentration of 5% (v/v), and the precipitated proteins were removed by spinning down at 887×*g* for 30 min at 4 °C. After ethanol (80%, v/v) precipitation, the solution was centrifuged at 887×*g* for 30 min at 4 °C. After the precipitate was dried, it was dissolved in water, mixed with cetylpyridinium chloride solution (final 1% w/v) and stored for 1 h at room temperature. It was then centrifuged again at 887×*g* for 30 min at 4 °C and the precipitate was recovered. The precipitate was dissolved in 2.5 M of sodium chloride, precipitated by ethanol (80%, v/v) and centrifuged at 887×*g* for 30 min at 4 °C. The final precipitate was dissolved in water, dialyzed (MWCO 6—8 kDa) for 2 d at 4 °C and freeze-dried.

Chemical Characterization of CS The chemical composition and purities of the CS were determined by the validated disaccharide analytical method after chondroitinase ABC digestion.²¹ To make the sample solution, 100 μl of control CS (10 mg/ml) and CS test samples (10 mg/ml) were mixed with 800 μl of Tris-acetate buffer (50 mM Trizma[®] base and 60 mM of sodium acetate, pH 8.0), respectively. The samples were depolymerized with 100 μl of chondroitinase ABC (1 mU/μl) overnight at 37 °C. After heating for 5 min and filtering on 0.2 μm syringe filters (PALL Life Sciences, Ann Arbor, MI, U.S.A.), reaction mixtures were injected (100 μg/μl) into a SAX-HPLC using a Hypersil SAX column (4.6×250 mm, 5 μm) from Thermo Hypersil-Keystone (Bellefonte, PA, U.S.A.). After the sample injection, the column was washed with water (pH 3.5) corresponding to one column volume (CV) for 4 min and a linear gradient of 0—1.0 M of NaCl (pH 3.5, 10 CV) was used. The absorbance was monitored at 232 nm and the flow rate was 1.0 ml/min. The chemical composition and purity of the samples were confirmed by the validated disaccharide analytical a method as previously described.²² Their purity was determined by comparing the total peak area of the disaccharides (ΔDi-0S, ΔDi-6S, ΔDi-4S, ΔDi-2,6diS) to control CS.

We used HPSEC equipped with a TSK G6000 PWXL column (7.5 mm×300 mm, Tokyo, Japan) and a TSK guard column (7.5 mm×75 mm, Tokyo, Japan) and ran MW-standard samples to determine the average MW of the CS test sample.²³ The mobile phase was 0.1 M of NaCl and the flow rate was 1.0 ml/min. The UV absorption was measured at 206 nm. Three kinds of hyaluronic acids (average MW 1690, 130, 100 kDa) and two CS samples (average MW 40, 15 kDa) were used as MW-standard samples. These samples were individually injected (500 μg/100 μl) into the high performance liquid chromatography (HPLC) in order to derive

a MW calibration curve, which was established by plotting the logarithm of the MW against each retention time of the MW standards. The average MW of the CS test sample was determined *via* the MW calibration curve.

¹H-NMR Spectroscopy The structural integrity of the CS test sample was observed using ¹H-NMR spectroscopy. One milligram of each sample was exchanged with 1 ml of D₂O (Sigma, St. Louis, MO, U.S.A.) and was then lyophilized. The dried samples were re-dissolved in 600 μl of D₂O and were analyzed using a 500 MHz BRUKER.

Cell Culture Fibroblasts were maintained at sub-confluence in 95% air and 5% CO₂ humidified atmosphere at 37 °C. The fibroblasts were grown in DMEM with 10% fetal bovine serum (FBS) containing 25 mM *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid (HEPES), 100 units/ml of penicillin and 100 μg/ml of streptomycin.

Cell Adhesion Assay and Proliferation Assay Fibroblasts were plated at a density of 5×10³ cells/well with DMEM containing 1% FBS (starvation medium) and various concentration of samples in a 96-well plate for adhesion assay. First, for proliferation assay, fibroblasts were plated at a density of 5×10³ cells/well with DMEM containing 1% FBS (starvation medium) in a 96-well plate and were incubated at 37 °C for 24 h. Samples of various concentrations were treated in a 96-well plate after additional incubation at 37 °C. Second, cell adhesion was determined after 3 h, 6 h, 12 h, 24 h and cell proliferation was determined after 72 h by reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) to formazan. After removing the medium, 100 μl of MTT (0.5 mg/ml in D-PBS) was added into each well and then incubated at 37 °C. Then, MTT was removed and dimethyl sulfoxide was added to dissolve the formazan crystals. The resulting absorbance was assayed at 540 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.).

***In Vitro* Wound Healing Assay** To determine the migration of fibroblasts, cells were seeded at a density of 1×10³ cells/well with DMEM containing 1% FBS (starvation medium) in a Culture-Insert (wound healing assay kit) and incubated at 37 °C for 24 h. We used sterile tweezers to remove the Culture-Insert from the dish filled with cell free starvation medium containing CS samples. After 6, 12 and 24 h of incubation we observed the samples by microscopy and took photographs using CKX41 microscopy (Olympus, Japan) at 100× magnification.

Protein Extraction and Western Blotting Fibroblasts (5×10⁴ cells/well) were seeded with DMEM containing 1% FBS (starvation medium) in a 6-well plate and incubated at 37 °C for 24 h. Cells were treated for an additional 0—6 h of incubation at 37 °C. Cells were then lysed in a Totex buffer (20 mM HEPES (pH 7.9), 350 mM NaCl, 20% glycerol, 1% NP-40, 1 mM MgCl₂, 0.5 mM ethylenediaminetetraacetic acid (EDTA) and 0.1 mM ethylene glycol bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA) (pH 8.0)). Samples were electrophoresed to separate 20 μg of protein on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel. The proteins were then transferred to a nitrocellulose membrane. After being blocked with 5% skim milk for 1 h, the membrane was incubated with the first antibody (anti-p38, p-p38, JNK1, pJNK, ERK1, pERK1/2, and actin, respectively). We then added the second antibody (goat anti-mouse (HRP)-con-

Table 1. Chemical Characterization of Authentic Chondroitin Sulfate and Sturgeon Chondroitin Sulfates

CS source	Purity (%)	Yield (%)	Disaccharide ratio (%) ^{a)}				Molecular weight (kDa)
			Δ Di-0S:	-6S:	-4S:	-2,6diS:	
Control CS	100	X	7.4	41.9	50.7	0	14
Shark cartilage	98.2	9.7	2.3	40.8	34.9	22	60.6
Sturgeon cartilage	91.1	10.5	4.0	7.2	88.8	0	8
Sturgeon backbone	100>	13.3	9.9	66.2	23.9	0	43.4

a) Δ Di-0S (Δ UA-[1 \rightarrow 3]-GalNAc); Δ Di-6S (CS C: Δ UA-[1 \rightarrow 3]-GalNAc-6S); Δ Di-4S (CS A: Δ UA-[1 \rightarrow 3]-GalNAc-4S); Δ Di-2,6diS (CS D: Δ UA-2S-[1 \rightarrow 3]-GalNAc-6S).

jugated and goat anti-rabbit (HRP)-conjugated secondary antibody. An enhanced chemiluminescence (ECL) detection kit was used to develop the membrane. The signal obtained from the membrane was photographed using an image analyzer (LAS 1000, Fuji, Japan). The target bands were quantified using UN-SCAN-ITTM software ver. 6.1 (Silk Scientific Corp., U.S.A.).

Statistics The data were summarized as means \pm standard deviation (S.D.) from three independent experiments. A one-way analysis of variance (ANOVA) followed by *t*-test was applied to assess the statistical significant of multiple comparisons. The level of statistically significant difference was set at $p < 0.05$.

RESULTS

Preparation of CS from Sturgeon Cartilage and Backbone Shark cartilage, which is a commercial CS source, was used as a raw material to compare the purity and the yield of CS purified from sturgeon cartilage and backbone. The CS samples were purified as white powders. The yield (%) and purity (%) were determined by SAX-HPLC analysis of CS disaccharides derived by enzymatic depolymerization as previously reported.²¹⁾ The purity of CS from sturgeon cartilage was 91.1%. However, the CS isolated from the sturgeon backbone exhibited more than 100% purity compared to control CS. The yield of CS from cartilage and backbone was 10.5% and 13.3%, respectively (Table 1).

Chemical Characterization of CS from Sturgeon Cartilage The samples purified from sturgeon cartilage and backbone were depolymerized to completion using chondroitinase ABC. CS disaccharides derived by enzymatic depolymerization were quantitatively analyzed using SAX-HPLC. The CS purified from sturgeon cartilage was composed of mono-sulfated disaccharides Δ Di-0S (4.0%, a), Δ Di-6S (7.2%, b) and Δ Di-4S (88.8%, c). The CS purified from sturgeon backbone consisted of mono-sulfated disaccharides Δ Di-0S (9.9%, a), Δ Di-6S (66.2%, b) and Δ Di-4S (23.8%, c) (Table 1). In order to determine the average molecular weight, the MW-standard samples and the CS samples were analyzed by HPSEC (Table 1). The average molecular weights of the CS obtained from shark cartilage, sturgeon cartilage and sturgeon backbone were found to be 60, 8, and 43 kDa, respectively. The CS obtained from the sturgeon cartilage has the lowest molecular weight among purified samples.

¹H-NMR spectroscopy was performed to confirm the disaccharide composition and the structural integrity of the CS samples. The results were compared to CS from shark cartilage (Fig. 1A). For sturgeon CS, a characteristic signal at

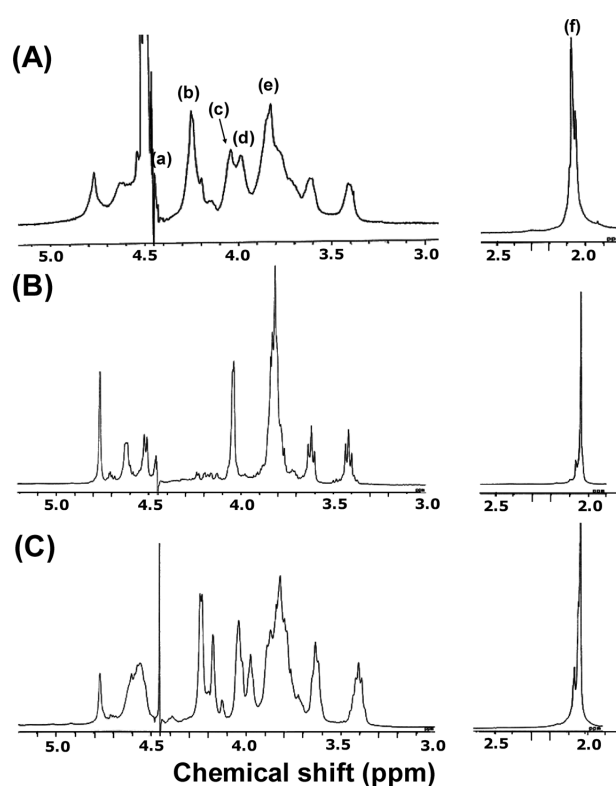


Fig. 1. ¹H-NMR Spectra of CS Samples (A) Shark Cartilage CS, (B) Sturgeon Cartilage CS and (C) Sturgeon Backbone CS

(a) H-4 of GalNAc, (b) H-1 of GalNAc, (c) H-6 of GalNAc6S, (d) H-4 of GalNAc6S, (e) H-2 of GalNAc and (f) *N*-acetyl signals of GalNAc4S and GalNAc6S.

4.4 ppm was assigned as H4 of the *O*-sulfate substituted on the C4-position of GalNAc.^{24,25)} The CS sample from shark cartilage had a characteristic signal at 4.0 ppm (Fig. 1A). The CS from sturgeon cartilage contained fewer sulfate groups on the C4-position of GalNAc, which showed a downfield signal at 4.4 ppm (Fig. 1B). This result is also consistent with the composition analysis of disaccharide by HPLC (Table 1). The signal intensity of the two overlapping peaks indicated methyl groups between 1.95 and 2.05 ppm and confirmed the above result.

Effect of CS on Fibroblast Adhesion and Proliferation

We determined the adhesion effect of CS on fibroblasts after 3, 6, 12, and 24 h of incubation with CS samples. After 3 h incubation, cell adhesion to wells treated with CS 100 μ g/ml purified from sturgeon cartilage was 113.8% compared to the control (Fig. 2A). The cell adhesion treated with CS from sturgeon backbone was 114.8% compared to the control (Fig. 2A). For each CS sample, cell adhesion to wells was significantly higher than that of control wells. After adhering to

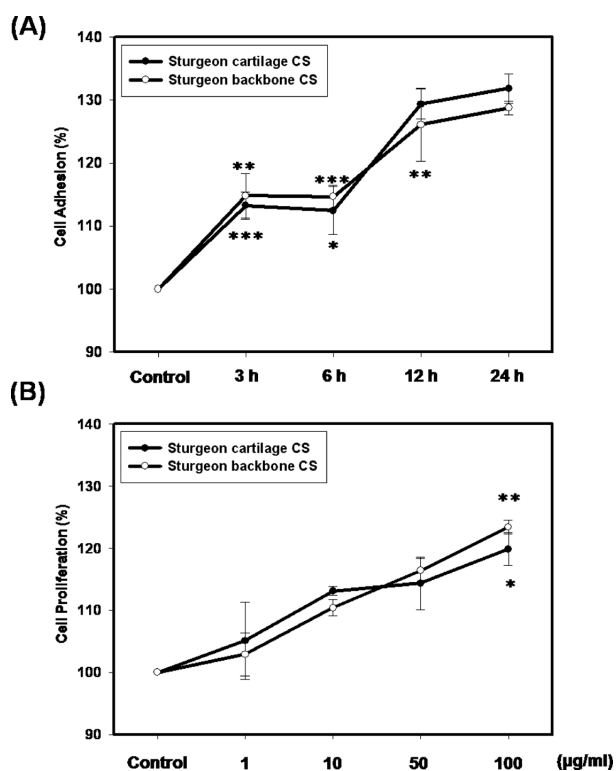


Fig. 2. The Effect of Sturgeon Cartilage and Backbone CS on (A) Cell Adhesion and (B) Cell Proliferation

The control indicates that cells are not treated with any samples. Data were obtained from at least three independent experiments and are expressed as mean \pm S.D. * $p < 0.01$, ** $p < 0.005$, and *** $p < 0.001$ versus untreated control cells.

wells within 24 h, cells started to proliferate. When incubation time was longer to 72 h, the ratio of cell proliferation compared to control increased in a dose-dependent manner and reached 119.9% on incubation with 100 $\mu\text{g/ml}$ of the purified CS from sturgeon cartilage. On the other hand, the proliferation increased to 123.4% when cells were incubated with 100 $\mu\text{g/ml}$ of purified CS from sturgeon backbone (Fig. 2B).

In Vitro Wound Healing Assay The effect of CSs from sturgeon cartilage and backbone on the migration of fibroblasts was tested in an *in vitro* model. The cell-free area was repopulated by fibroblasts through the combined action of migration and proliferation. Fibroblasts treated with CS started to migrate into the cell-free area at 6 h and the cell-free area was almost completely full at 24 h (Figs. 3G, J). In contrast, fibroblasts without CS treatment were less motile so there were fewer cells in the cell-free area after 24 h (Fig. 3D). In the wound area, fibroblasts treated with CS moved more quickly than control fibroblasts.

Effect of CS on pERK, pp38 and pJNK Kinases Mitogen-activated protein kinases (MAPKs) regulate physiological processes such as cell proliferation and differentiation. MAPKs form a large kinase complex with extracellular signal-regulated kinase (ERK), Jun kinase (JNK) and p38 MAPK.^{26,27} MAPKs play an important role in modulating many cellular events, including cell cycle progression, cell proliferation, differentiation, development and apoptosis.²⁸ Western blotting showed that both cartilage and bone CSs activate MAPK signaling pathways that are known to support cell proliferation. The levels of phosphorylated ERK and p38

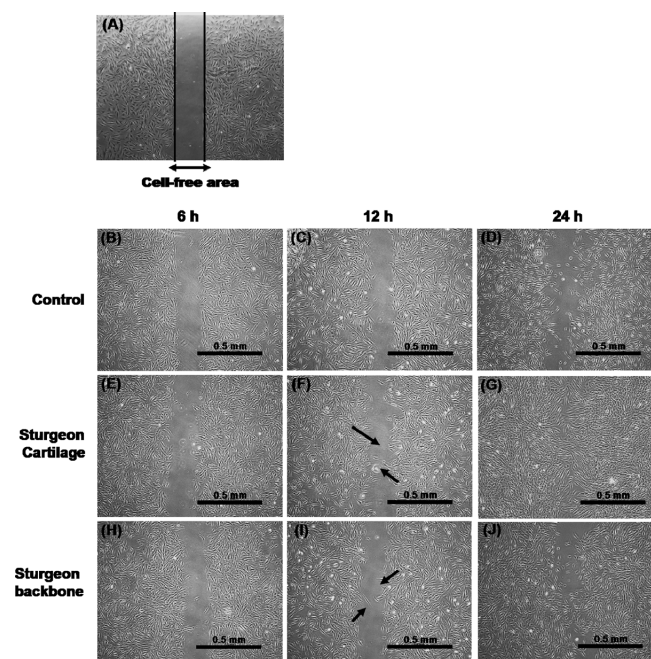


Fig. 3. The Effect of Sturgeon Cartilage and Backbone CS on Fibroblast Migration at Different Incubation Times (A) 0 h, (B) Control 6 h, (C) Control 12 h, (D) Control 24 h, (E) Sturgeon Cartilage 6 h, (F) Sturgeon Cartilage 12 h, (G) Sturgeon Cartilage 24 h, (H) Sturgeon Backbone 6 h, (I) Sturgeon Backbone 12 h and (J) Sturgeon Backbone 24 h

The control indicates that cells are not treated with any samples. These results were observed by a CKX41 microscopy at 100 \times magnification. The scale bar indicates 0.5 mm. Fibroblasts treated with CS started to migrate into the cell-free area at 6 h and the cell-free area was almost completely full at 24 h.

were increased, but the level of phosphorylated JNK was slightly weaker than ERK and p38. Especially the level of phosphorylated ERK was highly increased compared to control by treatment of backbone CS on incubation for 6 h (Fig. 4).

DISCUSSION

The quantification and characterization of CSs from sturgeon cartilage and backbone were performed based on disaccharide composition and average MW. Many spectrophotometric methods, including SAX-HPLC, have been used for the quality control of CS. The content of CS from sturgeon cartilage was 91.1% and sturgeon backbone CS content was 100%. The ratio of disaccharides showed that CS from sturgeon backbone has the typical disaccharide pattern of CS obtained from shark cartilage. However, CS from sturgeon cartilage has a different disaccharide pattern. Purified CS from sturgeon cartilage is rich in $\Delta\text{Di-4S}$ (88.8%). This finding is similar to purified CS from whale cartilage.²⁹ The CS-A is known to be rich [GlcA-GalNAc(4S)] and plays an important role in antioxidant activity³⁰ and prevention of atherosclerosis.³¹ Nowadays the main source of commercial CS is cartilage from marine animals. Commercial CS-C is prepared from shark cartilage, while commercial CS-A is obtained from whale or porcine cartilages. For centuries, whales have been caught for meat and as a source of raw materials. By the middle of the 20th century, however, whaling business had left many species seriously endangered, leading to the hot debate of whaling in the world. In this perspective, sturgeon can

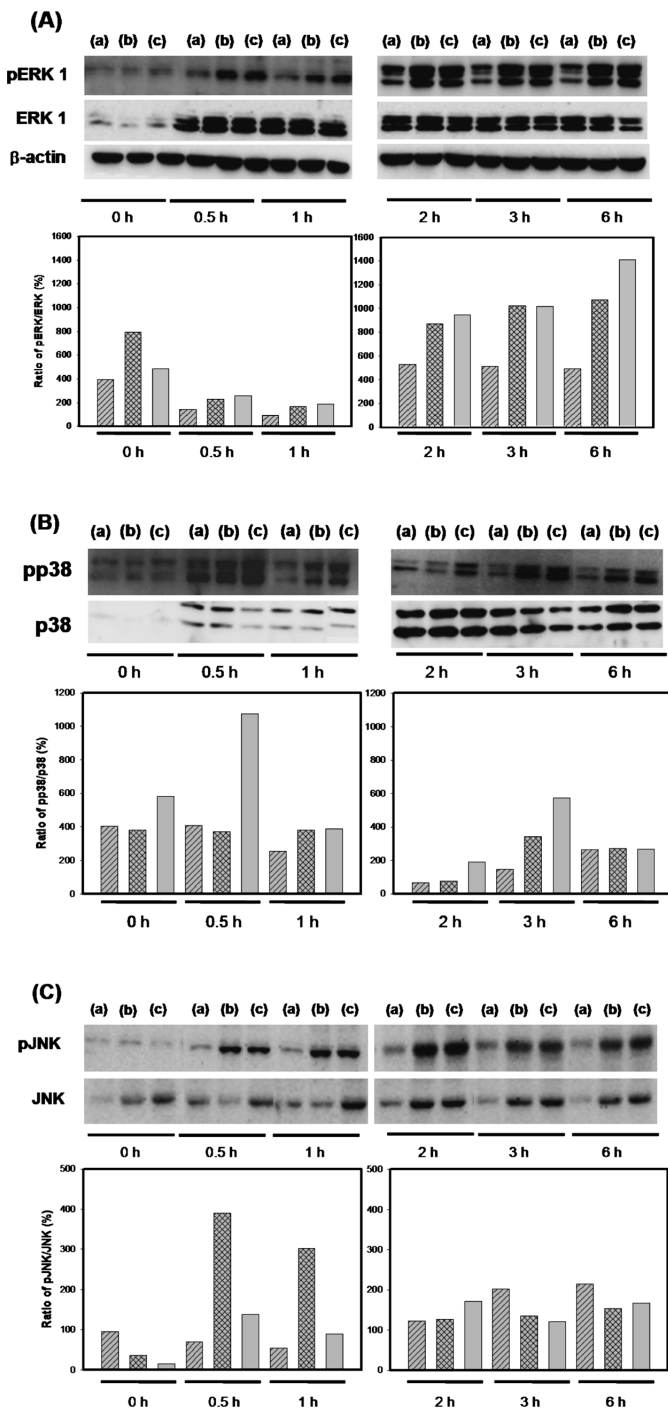


Fig. 4. The Effect of Sturgeon Cartilage and Backbone CS on Phosphorylation of ERK, p38 and JNK (A) pERK/ERK, (B) pp38/p38 and (C) pJNK/JNK in MAPK Signaling Pathway

(a) indicates control (the control indicates that cells are not treated with any samples), (b) indicates sturgeon cartilage CS at 10 $\mu\text{g}/\text{ml}$; (c) indicates sturgeon backbone CS at 10 $\mu\text{g}/\text{ml}$.

be an alternative for the new source of a raw material for CS and sturgeon farming is possible in some countries.³² Another uniqueness of CS from the sturgeon cartilage is its molecular weight of 8 kDa, because the average molecular weight of CS is generally more than 15 kDa. In our previous report, we determined the effect of low molecular weight CS on collagen-induced arthritis, which might be due to better absorption *via* the gastrointestinal tract and regulation in in-

flammatory responses.³³

In this study, we showed a stimulatory effect of CS on cell adhesion, proliferation and migration. As wound healing progresses, the injured skin matrix is replaced by a new, collagen-rich matrix synthesized by fibroblasts migrating into the wound. Fibroblasts migrated into the wound site from the surrounding tissue, become activated and begin to synthesize collagen. Therefore, fibroblasts adhesion and migration are essential to wound healing.³⁴ In this study, CS could stimulate fibroblasts to adhere, proliferate, and migrate into the wound space.

To explore the mechanism by which CS induces cell proliferation, we tested the proliferation-related signal transduction pathway (MAPK pathway). JNK and p38 kinases have been shown to play key roles in cellular stress, apoptosis and inflammation. The ERK1/2 pathway plays an important role in cell proliferation and differentiation.^{35,36} Our results show that CS induces MAPK pathways that may be casually linked to injury and proliferation of specific cell types. In previous reports, GAGs containing CS interact with the FGF family³⁷ and controlled the release of FGF.³⁸ CS interacts with dermal fibroblasts and has a potential role in wound healing, such as cell adhesion, proliferation and matrix deposition.^{39–41}

Based on our results, the purified CSs from sturgeon cartilage and backbone have the potential to enhance the proliferation of fibroblasts in wound healing. In addition, the purified CS in this study could be a valuable substitute for commercially available CS, which is mainly from shark cartilage.

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