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Isolation and Characterization of Proteoglycan Derived From Human Placenta and its Biological Activities

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Chondroitin sulfates proteoglycans were isolated from human placenta. For the identification of enzymatic digestion products of isolated proteoglycan, strong anion exchange-high performance liquid chromatography (SAX-HPLC) was performed. By the action of chondroitin ABC and chondroitin B lyase, three unsaturated disaccharides 2-acetamide-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose (ΔDi-*O*S), 2-acetamide-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose (ΔDi-6S) and 2-acetamide-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose (ΔDi-4S) were produced from the human placenta proteoglycan. The anticoagulant activity of chondroitin sulfate proteoglycan was evaluated by activated partial thromboplastin time (aPTT) assay and thrombin time (TT) assay. The clotting times of aPTT and TT were increased from 72 to 144 sec and 19 to 27 sec, respectively. The immuno-modulating activity of chondroitin sulfate proteoglycan was examined by cell proliferation assay and these results suggest that it may play a role in suppression of the function of immune-related cells.

Key words: Human placenta, Chondroitin sulfates, Proteoglycan, Anion-exchange HPLC, Cell proliferation assay, Anticoagulant

INTRODUCTION

The placenta is the organ that facilitate the metabolic interchange between fetus and mother. The glycosaminoglycans (GAG) are the major constituents of placenta connective tissue. The glycosaminoglycan composition of human placenta has been reported (Calatroni *et al.*, 1969) and variations in GAG patterns have been observed to occur with maturation and with the development of pathological conditions (Lee, T.Y. *et al.*, 1973). The analysis of the proteoglycan extracted from placenta confirmed the presence of chondroitin sulfate which consists of various proportions of both 4-sulfated and 6-sulfated disaccharide repeats. Chondrotin is increasingly gaining attention as important regulators of many biological processes, such as cell migration, and recognition, extracellular matrix deposition, and morpho-genesis (Rogerson *et al.*, 1995; Silbert *et al.*, 1995). Anticoagulant activity of dermatan sulfate proteoglycan in the term human placenta has been reported. (Delorme *et al.*, 1998). Therefore, to examine the anticoagulant activity of isolated proteoglycan, aPTT assay and TT assay were performed. It is yet unexplained that the pregnant mother tolerates the growth of the fetal organism in her own body. It prompted us to perform the cell proliferation assay of isolated proteoglycan. This paper reports the presence of chondroitin sulfate from the human placenta and its anticoagulant activity as well as suppression activity of the function of immunerelated cells.

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MATERIALS AND METHODS

Materials

Human placentas were obtained at Baek Jae Hospital of Obstetrics and Gynecology, Nonsan, Chung Nam. Strong anion exchange HPLC (SAX-HPLC) was performed

using Shimadzu LC-10 Avp system equipped with a 5-μm Spherisorb 0.46 × 25 cm column (Phenomenox, Torells, CA). Activated partial thromboplastin time (aPTT) reagent, thrombin time (TT) reagent, chondroitin ABC and chondroitin B lyase were purchased from Sigma (St. Louis, MO, USA). Diethylaminoethyl cellulose (DE52) was purchased from Whatman (Fairfield, NJ 07004, USA). Standard unsaturated disaccharides 2-acetamide-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose (ΔDi-OS), 2-acetamide-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose (ΔDi-4S) and 2-acetamide-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose (ΔDi-6S) were from Seikagaku (Tokyo, Japan). UV spectrometer (JASCO model V550) equipped with a thermostated cell was used. RPMI was purchased from GIBCO BRL (Grand Island, NY. USA). Coagulator (Amelung KC1A) was purchased from Sigma.

Methods

Preparation of proteoglycan

The proteoglycan was prepared as described previously. (Isemura *et al.*, 1987). Human placentas were cut into fine pieces with scissors and rinsed several times with 0.15 M NaCl, then extracted with 4M urea in 50 mM Tris-HCl buffer (pH 7.5) containing 2 mM EDTA, 10 mM 6-amino-*n*-carpronic acid, 1 mM phenylmethylsulfonyl-fluoride, 1 mM benzamidine HCl (buffer A) containing 0.1 M NaCl. Extraction was performed three times and the combined extracts were applied onto a column of DEAE-cellulose (5 × 12 cm) pre-equilibrated with the same buffer. After the column was washed with 0.2 M NaCl, in buffer A, a stepwise elution was performed with 500 ml each of 0.5 M NaCl and 2 M NaCl in buffer A. The effluents were collected in 5 ml fractions and monitored by the absorbance at 280 nm and uronic acid assay.

Enzymatic digestion of the sample with chondroitin ABC and chondroitin B lyase

Sample (0.1 mg) in 490 ml of 0.02 M sodium phosphate buffer (pH 8.0) were depolymerized by treating with 10 ml of 100 mU chondroitin ABC lyase at 37°C in the cuvette. Sample (0.1 mg) in 490 ml of 0.02 M sodium phosphate buffer (pH 7.5) were depolymerized by treating with 10 ml of 1 U chondroitin B lyase at 25°C in the cuvette. The reactions were monitored by UV absorbance at 232 nm. The increase in UV absorbance was continually recorded as a function of time.

HPLC analysis of oligosaccharides

The composition of oligosaccharide produced from enzyme treatment was analyzed by SAX-HPLC (Linhardt *et al.*, 1988). The column (Spherisorb, 0.46 × 25 cm) was equilibrated with 3% of 2.5 M NaCl (Eluant 2, pH 3.5) in distilled water (Eluant 1, pH 3.5). After injection of the 20 μl of digestion mixture, the column was eluted isocratically at a flow rate of 1 ml/min with 3% of Eluant 2 in Eluant 1 for the first 5 min. For the next 50 min, the column was eluted with a linear gradient of Eluant 2 from 3% to 30%, monitoring A_{232nm} at 0.01AUFS (absorbance unit at full scale). Each peak was identified by co-injection with disaccharide standard.(Lee, K. B. *et al.*, 1998)

Effects on blood coagulation

APTT assay: APTT test was performed on a Amelung KC 1A micro coagulation analyzer (St. Louis, MO, USA). $CaCl_2$ solution (20 mM) and aPTT reagent were pre-incubated at 37°C. Sample (50 μl) and human plasma (100 μl) were mixed well and 50 μl of above mixture was taken into the bottom of cuvette and incubated for 2 min. APTT reagent (50 μl) was added and incubated for 3 min then 50 μl of 20 mM $CaCl_2$ was added and the clotting time was measured. (Lee, H. J. *et al.*1999; Song, J. W. *et al.*, 1998)

.TT assay: TT reagent and fibrinogen reference reagent were preincubated at 37°C. Sample 20 μl and TT reagent 30 μl were mixed well and incubated for 2 min. Fibrinogen reference 100 μl was added and the clotting time was measured.

Cell proliferation assay

The specific pathogenfree female Balb/c mice (6-8 weeks old) were used. The spleen cells isolated aseptically were resuspended in RPMI 1640 complete media containing 7% fetal bovine serum, 2 mM L-glutamine, 100 units/ml of penicillin, 100 mg/ml of streptomycin, 15 mM HEPES, and 50 μM 2-mercaptoethanol. The splenocytes (2×10^5 cells/well) were transferred to each well of 96-well culture plate and cultured in a humidified 5% CO_2 incubator at 37°C for 72 hr in the presence of chondroitin sulfates and either 10 μg/ml of lipopolysaccahride (LPS) or 10 μg/ml of concanavalin A (ConA). The proliferation of splenocytes was measured spectrometrically by using Cell Counting Kit (Dojindo, Japan) under the manufacturers instruction.

RESULTS AND DISCUSSION

Preparation of human placenta proteoglycan

The proteoglycan components of the human placenta were isolated by urea extraction and separated by DEAE-cellulose ion-exchange chromatography (Fig. 1). The efficiency of proteoglycans extraction was followed by carbazole assay and the yield at each step is presented in Table I. This assay demonstrated that the starting material

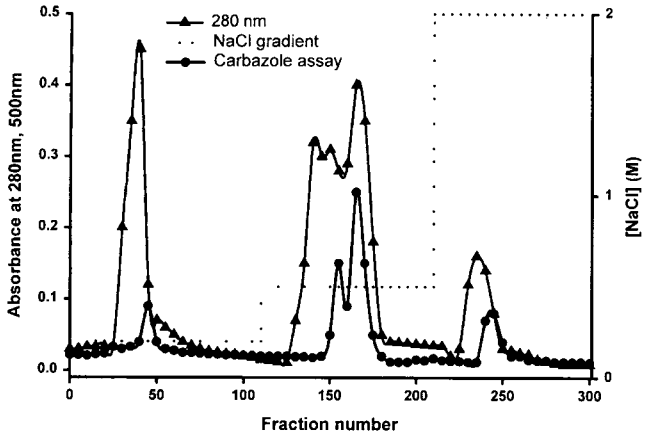

Fig. 1. DEAE-cellulose ion-exchange chromatography of proteoglycan derived from human placenta. After the column was washed with 0.2 M NaCl in extraction buffer, stepwise elution was performed with 500 ml each of 0.5 M NaCl and 2 M NaCl in extraction buffer. The effluents were collected in 5 ml fractions and monitored by the absorbance at 280 nm(▲)and uronic acid assay(□). The NaCl concentration on the elution buffer applied to the column is indicated by the dashed line.

Table I. Purification of proteoglycan from human placenta

Purification step	Uronic acid/wet tissue μg/g
Starting material	280
4M Urea extraction	197
DEAE-cellulose ion-exchange chromatography, 0.2 M NaCl fraction	19
DEAE-cellulose ion-exchange chromatography, 0.5 M NaCl fraction	102
DEAE-cellulose ion-exchange chromatography, 2 M NaCl fraction	35

contained uronic acid at a concentration 280 μg/g wet tissue. About 9.6%, 51.5% and 17.8% of proteoglycans in terms of uronic acid were found to be eluted from the DEAE column with 0.2 M NaCl, 0.5 M NaCl and 2 M NaCl, respectively (Fig. 1).

Enzymatic degradation of human placenta proteoglycan

The depolymerization of proteoglycan using various lyases has been useful method for the structural analysis of glycosaminoglycans(GAGs). To identify the oligosaccharide composition of prepared proteoglycan, the sample was treated with chondroitin ABC lyase and chondroitin B lyase to produce unsaturated disaccharides (Fig. 2). The depolymerization of sample by chondroitin ABC lyase was followed by measuring the change in absorbance at 232 nm (Fig. 3). The conjugated system of double bonds created by this elimination produced a chromophore at 232 nm in the resulting disaccharides (Fig. 2). After treatment with chondroitin B lyase, we

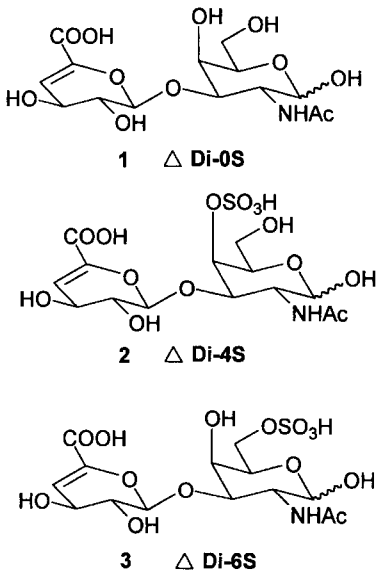

Fig. 2. Structures of unsaturated disaccharides formed from the human placenta proteoglycan by the action of chondroitin ABC lyase

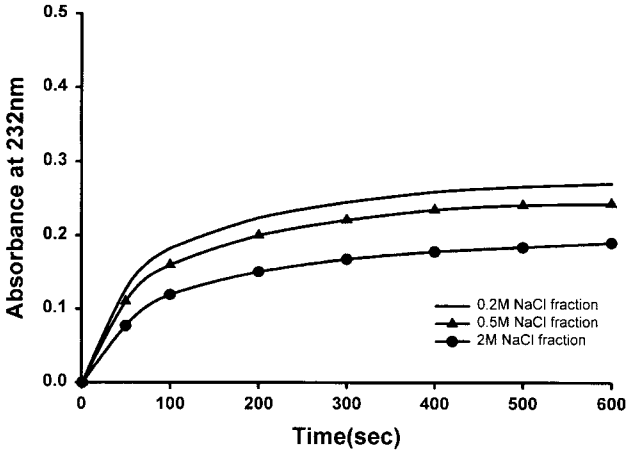

Fig. 3. Degradation of human placenta proteoglycan by chondroitin ABC lyase.
Enzyme digestion was performed as described under "Material and Methods". The reactions were monitored by UV absorbance at 232 nm. The increase in UV absorbance is continually recorded as a function of time.

failed to measure any increase in UV absorbance. Above results suggest that prepared proteoglycans contain chondrotin 4-, 6- and *O* sulfates, not dermatan sulfate.

Characterization of unsaturated disaccharides

Analyses of unsaturated disaccharides produced by chondroitinase ABC and chondroitinase B lyase digestion are performed by using SAX-HPLC (Fig. 4). Three disaccharides were observed in the human placenta chondroitin sulfate. The structures of unsaturated oligosaccharides were first assigned by comparing each elution

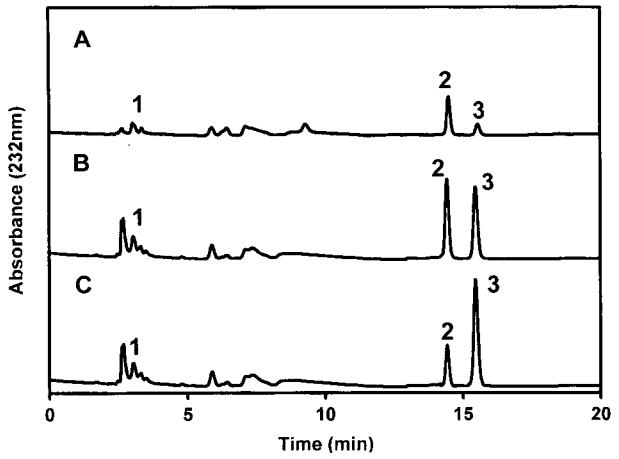

Fig. 4. The SAX-HPLC analysis of human placenta pro-teoglycan on treatment with chondroitin ABC lyase. A, 0.2 M NaCl fraction was treated with chondroitin ABC lyase. B, 0.5 M NaCl fraction was treated with chondroitin ABC lyase. C, 2 M NaCl fraction was treated with chondroitin ABC lyase. The peak 1 was co-eluted with DDi-OS and the peak 2 was co-eluted ΔDi-4S. The peak 3 was co-eluted with ΔDi-6S.

Table II. Relative proportion of disaccharide obtained from human placenta proteoglycan by the action of chondroitin ABC lyase.

Elution from DEAE column	Relative proportion of disaccharides		
	ΔDi-OS	ΔDi-4S	ΔDi-4S
0.2 M NaCl	17.7%	60.1%	21.2%
0.5 M NaCl	11.3%	46.7%	42.0%
2.0 M NaCl	11.9%	25.2%	62.9%

Table III. Effects of proteoglycans on blood coagulation parameters.

Sample	Time (sec)	
	aPTT	TT
Control	72±2	19±1
0.2M NaCl fraction	89±3	21±0
0.5M NaCl fraction	144±4	27±1
2M NaCl fraction	92±3	20±1

Coagulation tests (aPTT and TT) were performed as described under "Material and Methods". Values represented the mean ±SD (n=5).

time with those of authentic standards and the major peaks were confirmed by the co-injection of oligosaccharide standards. The separation of disaccharides is mostly dependent on the number of sulfate charges. The peak 1 in Fig. 4 was co-eluted with ΔDi-OS and the peak 2 was co-eluted ΔDi-4S. The peak 3 was co-eluted with ΔDi-6S. The relative proportion of ΔDi-OS : ΔDi-4S : ΔDi-6S was as shown in Table II.

Effects on blood coagulation

The anticoagulant activity of chondroitin sulfate proteo-

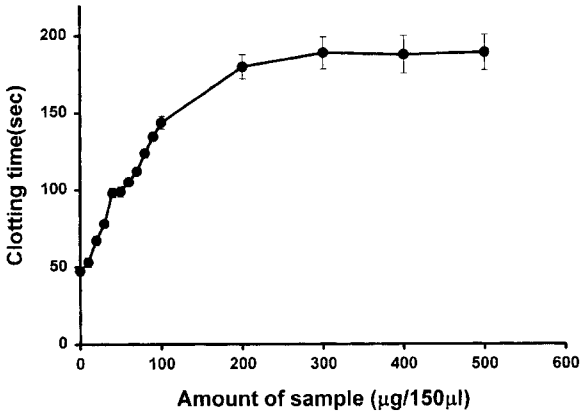

Fig. 5. Effect of 0.5M NaCl fraction on blood coagulation. The clotting time was measured by aPTT assay. Results were expressed as mean±SD (n=3).

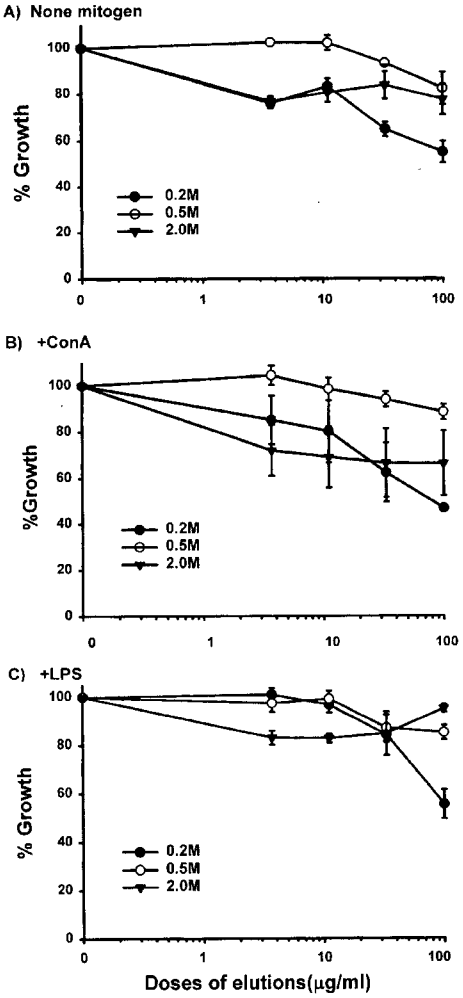

Fig. 6. Effect of chondroitin sulfates on the proliferation of murine splenocytes. Splenic cells obtained from Balb/c mice were co-incubated with the indicated doses of 0.2 M, 0.5 M and 2.0 M NaCl-eluted fractions of chodroitin sulfates admixed with either of Con A or LPS (10 μg/ml) for 24 h. After incubation, the proliferation of splenic cells was measured using Cell Counting Kit (Dojindo, Japan).

glycan was examined by aPTT and TT assay (Table III). With 0.5 M NaCl eluted fraction, we observed prolongation in clotting time (aPTT) in a dose-dependent manner and approximately three times prolongation was obtained at 200 μg/150 μl (Fig. 5).

Regulatory effect on the proliferation of splenic cells

To investigate the activity of placenta chondroitin sulfates regulating the function of immune-related cells, we examined their effects on responsiveness of splenocytes to mitogens such as ConA and LPS. As depicted in Fig. 6-A, chondroitin sulfate fractions eluted with 0.2 M and 2.0 M, but not 0.5 M, NaCl suppressed the proliferation of splenocytes in a dose-dependent manner. When the cultures were co-incubated with T-cell mitogen, Con A, both 0.2 M- and 2.0 M-eluted chondroitin sulfate fractions also markedly suppressed the proliferation of splenocytes (Fig. 6-B), indicating that the chondroitin sulfate fractions play a role in down-regulation of T-cell activation. In contrast, in an experiment that B-cell mitogen, LPS, was added to the cultures, these chondroitin sulfates (0.2 M- and 2.0 M-eluted fractions) showed just a slight effect on LPS-stimulated splenocytes (Fig. 6-C). Interestingly, 0.5 M-eluted chondroitin sulfate fraction had no effect on the proliferation of both ConA- and LPS-stimulated lymphocytes (Fig. 6-B and 6-C). Taken together, it suggests that human placenta contains chondroitin sulfates which are effective for regulation of immune-related cells, and those proteoglycans from human placenta with 0.2 M as well as 2.0 M NaCl are responsible for the immuno-regulatory effect, especially down-regulation of ConA-stimulated T-lymphocytes.

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