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ORIGINAL ARTICLE

CONSTITUENT PROTEOGLYCANS OF THE HUMAN YELLOW LIGAMENT EXTRACELLULAR MATRIX

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Abstract Three types of proteoglycans (PG-I, II and III) present in the human yellow ligament extracellular matrix were separated using a combination of CsCl isopycnic density gradient centrifugation, DEAE-Sephacel ion-exchange chromatography, gel-filtration on Sepharose CL-4B and CL-2B, and Octyl-Sepharose CL-4B chromatography. Proteoglycan-I was a high-molecular-weight proteoglycan, which was excluded by Sepharose CL-4B, and its molecular weight, estimated using sodium dedecyl sulfate-polyacrylamide gel electrophoresis, was over 350,000. The glycosaminoglycan chain of PG-I was composed of chondroitin 6-sulfate, and PG-I appeared to be an aggrecan-type proteoglycan. Proteoglycan-II and III were low-molecular-weight proteoglycans with molecular weights of 190,000 and 105,000, respectively. The core protein sizes of PG-II and III were similar, 44,000, but anti-human decorin antibody reacted with PG-III, not with PG-II. The glycosaminoglycan chain of PG-II contained dermatan sulfate and chondroitin 6-sulfate, whereas that of PG-III contained the former only. In the light of these results, PG-II and III were suggested to be biglycan- and decorin-type proteoglycans, respectively. The major proteoglycan in the human yellow ligament extracellular matrix was PG-III.

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Key words: human yellow ligament; proteoglycan; extracellular matrix; decorin; biglycan.

原 著

ヒト黄色靭帯の細胞外マトリックスを構成するプロテオグリカン

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抄録 ヒト黄色靭帯の細胞外マトリックスを構成するプロテオグリカン(PG-I, II, III)をCsCl等密度勾配超遠心の後，DEAE-Sepharose, Sepharose CL-4BおよびCL-2B, Octyl-Sepharose CL-4Bの各クロマトで分離した．PG-Iは分子量350,000以上の高分子プロテオグリカンで，aggrecanタイプのプロテオグリカンと推定された．そのグリコサミノグリカン糖鎖はコンドロイチン6-硫酸であった．PG-IIおよびPG-IIIは分子量がそれぞれ190,000および105,000の低分子プロテオグリカンで，コア蛋白の分子量は44,000であった．抗ヒトデコリン抗体にPG-IIIは反応し，PG-IIは反応しなかった．PG-IIのグリコサミノグリカン糖鎖はデルマタン硫酸とコンドロイチン6-硫酸で，PG-IIIのグリコサミノグリカン糖鎖はデルマタン硫酸であった．PG-IIおよびPG-IIIはそれぞれバイグリカンおよびデコリンタイプのプロテオグリカンであった．ヒト黄色靭帯の細胞外マトリックスの主たるプロテオグリカンはPG-IIIであった．

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キーワード: ヒト黄色靭帯; 細胞外マトリックス; プロテオグリカン; デコリン; バイグリカン.

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Introduction

Proteoglycan is the major component of the extracellular matrix[1]. High-molecular-weight proteoglycans, such as aggrecan which aggregates with hyaluronic acid[2], and versican isolated from chick embryo limb buds[3], have been found in other species and low-molecular-weight proteoglycans, such as decorin[4], biglycan[4] and fibromodulin[5] have been reported. Furthermore, it has become clear that type IX[6] and XII[7] collagens contain glycosaminoglycans.

The human yellow ligaments are a series of short ligaments that pass between the vertebra along the length of the spine. These ligaments are rich in elastic tissues, but endochondral ossification may occur with aging[8]. As proteoglycans bind to calcium ions, they are considered to play an important role in the process of ossification[9]. However, the role of the proteoglycans in human yellow ligaments is not well understood.

Both high- and low-molecular-weight proteoglycans have been reported to be present in the extracellular matrix of the human yellow ligaments[10]. The high-molecular-weight proteoglycan content increases with aging and their glycosaminoglycan chains contain chondroitin sulfate. Low-molecular-weight proteoglycans are the major components of the human yellow ligament extracellular matrix. Their glycosaminoglycan chains are composed of dermatan sulfate in young subjects, but change to chondroitin sulfate with aging[10]. However, detailed nature of these proteoglycans is still unclear. In this paper, the proteoglycans of the human yellow ligament were isolated and their biochemical properties were characterized.

Materials and Methods

Materials

Chondroitin 6-sulfate (Ch6S, from shark cartilage), dermatan sulfate (DS, from pig skin), heparin (Hep, from bovine lung), chondroitinase ABC (from *Proteus vulgaris*) and chondroitinase AC-II (from *Arthrobacter aurescens*) were purchased from Seikagaku Kogyo Co., Tokyo, Japan. Hyaluronic acid (HA, from human umbilical cord) and endo-β-xylosidase were prepared according to the methods reported by Nakamura et al.[11] and Takagaki et al.[12] Actinase E was purchased from Kaken Kagaku Co., Tokyo, Japan, and the immunoblotting ABC-POD (R) Kit, CsCl and 2-aminopyridine were from Wako Pure Chemical Co., Osaka, Japan. DEAE-Sephacel, Sepharose CL-2B and CL-4B, Octyl-Sepharose CL-4B and the electrophoresis calibration kit were from Pharmacia LKB Biotechnology AB, Uppsala, Sweden. Cellulose acetate membranes and 5-20% acrylamide gradient slab gels were from Jookoo Co., Tokyo and Atto Co., Tokyo, Japan, respectively, and rabbit polyclonal anti-human decorin antibody (PG-40) was from Life Technology Co., Tokyo, Japan. All the other reagents used were obtained from commercial sources.

Extraction and separation of proteoglycans from human yellow ligaments

For these experiments, normal human yellow ligaments (20 g) were taken from 31 patients, all of whom were in their forties, at the time of operation for intervertebral disk herniation or lumbar spinal canal stenosis.

The extraction of proteoglycans from human yellow ligaments was carried out in 5 volumes of 4 M guanidine-HCl/50 mM sodium acetate buffer (pH 5.8) containing protease inhibitors at 4 ℃ for 24 h, as described by Sajdera and Hascall[13]. The extracts were collected and designated the

crude proteoglycan fraction.

Cesium chloride to produce a density of 1.45 g/ml was added to the crude proteoglycan fraction, this solution was subjected to isopycnic density-gradient centrifugation at 228,000 x g at 10℃ for 70 h and 500-μl fractions were collected. Each fraction was assayed for uronic acid by the carbazole reaction[14], and for protein by the protein-dye binding method[15]. The fractions containing uronic acid were collected and designated proteoglycan fractions, which were dialyzed against 7 M urea/50 mM Tris-HCl buffer (pH 7.0) at 4 ℃ for 48 h, after which, the undialyzable fractions were applied to a DEAE-Sephacel column (1.0 x 15.0 cm) equilibrated with 7 M urea/50 mM Tris-HCl buffer (pH 7.0), which was eluted with a linear concentration-gradient of NaCl (0-1.0 M) at a flow rate of 10 ml/h. The uronic acid-containing (proteoglycan) fractions were pooled and concentrated using a Centricon 10 (Amicon Co., Beverly, MA, U.S.A.). Under dissociative conditions (4 M guanidine-HCl/50 mM sodium acetate buffer, pH 5.8), these fractions were subjected to gel-filtration chromatography on a Sepharose CL-4B column (1.2 x 153 cm) at a flow rate of 10 ml/h and 3-ml fractions were collected. Each uronic acid-containing peak fraction was re-chromatographed on a Sepharose CL-4B (1.2 x 153 cm) or Sepharose CL-2B (1.2 x 153 cm) column.

For further purification, chromatography on an Octyl-Sepharose CL-4B column (1.0 x 15 cm) was carried out under the conditions described by Choi et al.[16] Briefly, the proteoglycan fractions were applied to the column, which was equilibrated with 2 M guanidine-HCl/0.1 M sodium acetate buffer (pH 6.3), and eluted with a linear concentration-gradient of 2-6 M guanidine-HCl/0.1 M sodium acetate buffer (pH 6.3) at a flow rate of 30 ml/h and 3-ml fractions were collected.

Preparation of proteoglycan core proteins by chondroitinase ABC digestion

Proteoglycan core proteins were obtained by chondroitinase ABC digestion, as described by Oike et al.[17], and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Separation and characterization of glycosaminoglycan chains

The proteoglycan glycosaminoglycan chains were separated by digestion with Actinase E and endo-β-xylosidase, as described by Takagaki et al.[18], and identified by carrying out two-dimensional electrophoresis on cellulose acetate membranes (10 x 10 cm) using the method of Yokoyama et al.[19]

The dermatan sulfate content of the glycosaminoglycan chains was estimated from the difference between the amount of unsaturated disaccharides obtained by digesting glycosaminoglycan chains with chondroitinase ABC[20] and AC-II[21]. The unsaturated disaccharides produced by each chondroitinase digestion were coupled with 2-aminopyridine at their reducing termini by the method of Kon et al.[22] The resulting pyridylaminated unsaturated disaccharides were analyzed by high-performance liquid chromatography (HPLC), as described by Kodama et al.[23]

Analysis of proteoglycans and their core proteins by SDS-PAGE

The proteoglycans and their core proteins were subjected to SDS-PAGE on 5-20% acrylamide gradient slab gels at 4 ℃ at 200 V for 4 h, as described by Rosenberg et al.[24] The gels were stained successively with Coomassie brilliant blue R-250 followed by Alcian blue, as described by Fisher et al.[25]

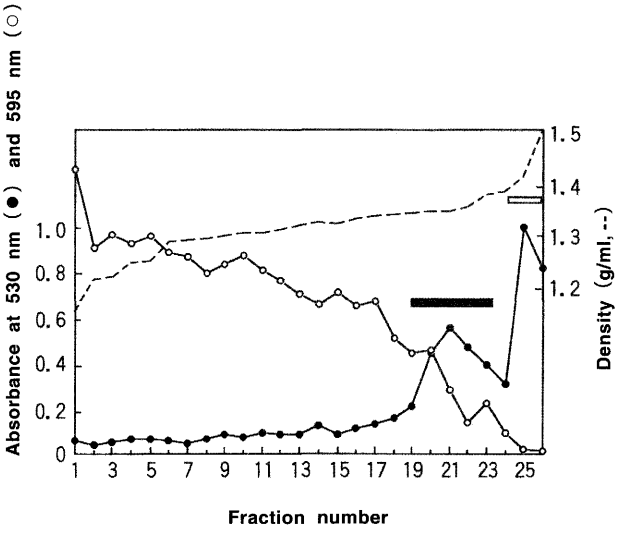

Figure 1 CsCl Isopycnic density gradient centrifugation. The crude proteoglycan fraction extracted from human yellow ligament was subjected to CsCl isopycnic density gradient centrifugation. Each fraction was monitored for uronic acid (530 nm), and protein (595 nm). Closed bar, density of 1.35-1.38 g/ml; open bar, density of 1.40-1.50 g/ml.

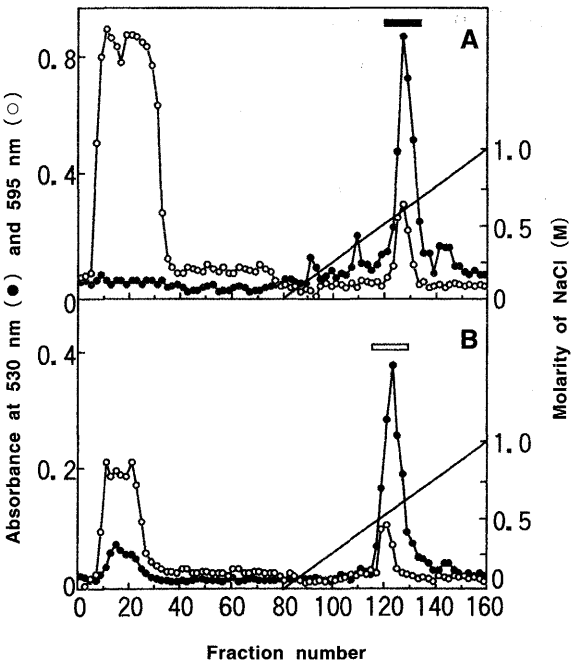

Figure 2 DEAE-Sephacel ion-exchange chromatograms. Two proteoglycan fractions obtained by CsCl isopycnic density gradient centrifugation was applied to a DEAE-Sephacel column, which was eluted with a linear concentration-gradient of NaCl (0-1.0 M). The fractions eluted at a NaCl concentration of 0.65 M (closed bar and open bar) were collected, concentrated and subjected to Sepharose CL-4B gel-filtration chromatography. Each fraction was monitored for uronic acid (530 nm) and protein (595 nm). A, low-density proteoglycan fraction; B, high-density proteoglycan fraction.

Immunoassay

The Western blotting immunoassay for proteoglycans using anti-human decorin antibody was carried out, as described by Towbin et al.[26]

Results

CsCl Isopycnic density gradient centrifugation of the crude proteoglycan fraction

The crude proteoglycan fraction extracted with 4 M uanidine-HCl/50 mM sodium acetate buffer (pH 5.8) was subjected to CsCl isopycnic gradient centrifugation and 500-μl fractions were collected. Two uronic acid-containing fractions were collected and designated the low-density (closed bar in Figure 1, density of 1.35-1.38 g/ml) and high-density (open bar in Figure 1, density of 1.40-1.50 g/ml) proteoglycan fractions. Total amount of uronic acid they contained were 5.3 and 1.8 mg, respectively.

Ion-exchange and gel-filtration chromatography of proteoglycan fractions

Two proteoglycan fractions obtained by CsCl isopycnic density gradient centrifugation were applied to a DEAE-Sephacel column and the fractions eluted at a NaCl concentration of

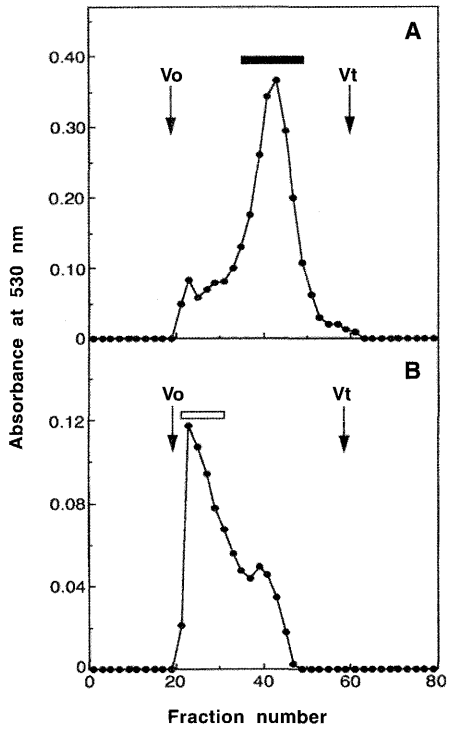

Figure 3 Sepharose CL-4B gel-filtration chromatograms of the proteoglycan fractions.
Under dissociative conditions (4 M guanidine-HCl/50 mM sodium acetate buffer, pH 5.8), the proteoglycan fractions from DEAE-Sephacel chromatography were applied to a Sepharose CL-4B column. The low-density proteoglycan fraction was eluted at Kav = 0.61, collected and designated the low-molecular-weight proteoglycan fraction (closed bar in A). The high-density proteoglycan fraction was excluded (open bar in B). Vo, Void volume; Vt, total volume.

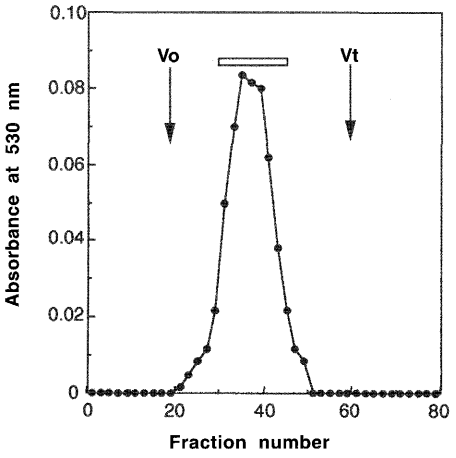

Figure 4 Sepharose CL-2B gel-filtration chromatogram of the high-density proteoglycan fraction.
The proteoglycan fractions excluded by the Sepharose CL-4B column were re-chromatographed on a Sepharose CL-2B column. The proteoglycan fractions eluted at Kav=0.41 were collected and designated high-molecular-weight proteoglycan fractions.

0.65 M (Figure 2) were collected, concentrated and subjected to Sepharose CL-4B gel-filtration chromatography. The low-density proteoglycan fraction was eluted at Kav = 0.61 (Figure 3-A). The uronic acid-containing fractions were re-chromatographed on a Sepharose CL-4B column and collected. They were designated low-molecular-weight proteoglycan fractions, and the total amount of uronic acid they contained was 1.9 mg.

As the high-density proteoglycan fraction was excluded by the Sepharose CL-4B column (Figure 3-B), gel-filtration chromatography of this fraction on a Sepharose CL-2B column was performed. The fractions that eluted at Kav = 0.41 were collected and designated high-molecular-weight proteoglycan fractions (Figure 4). The total amount of uronic acid they contained was 0.5 mg.

Analysis of proteoglycans by SDS-PAGE on 5 to 20% acrylamide gradient slab gels

The proteoglycan fractions separated by the column chromatographic procedures described above were subjected to SDS-PAGE

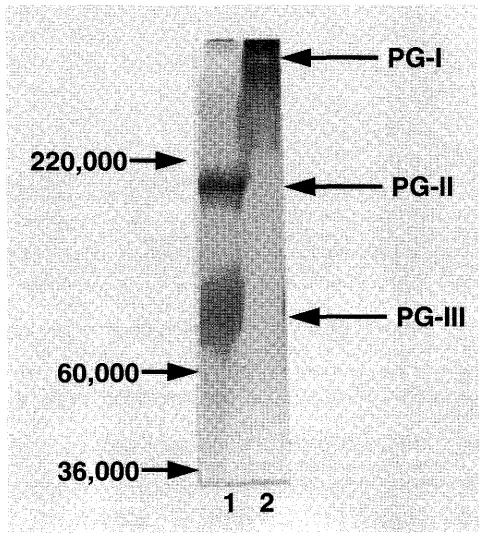

Figure 5 Analysis of proteoglycans on 5 to 20% acrylamide gradient slab gels electrophoresis.
The proteoglycans eluted from the Sepharose CL columns (indicated by bars in Figs. 3 and 4) were subjected to SDS-PAGE. The high-molecular-weight proteoglycan was designated PG-I. The low-molecular-weight proteoglycan fractions contained two types of proteoglycan (designated PG-II and III). With reference to a standard calibration curve, the molecular weight of each proteoglycan was determined. Lane 1, low-molecular-weight proteoglycan fractions; lane 2, high-molecular-weight proteoglycan fractions.

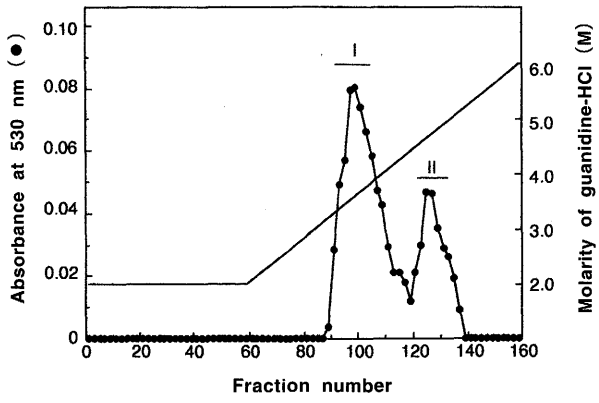

Figure 6 Separation of the low-molecular-weight proteoglycans by Octyl-Sepharose CL-4B.
Chromatography on an Octyl-Sepharose CL-4B column was performed to separate PG-II and PG-III. The column was eluted with a linear concentration-gradient of 2-6 M guanidine-HCl/0.1 M sodium acetate buffer (pH 6.3). The fractions eluted at guanidine-HCl concentrations of 3.59 M (peak I) and 4.67 M (peak II) were collected and subjected to SDS-PAGE and immunoblotting.

on 5 to 20% acrylamide gradient slab gels, with reference to a standard calibration curve, and their molecular weights were estimated (Figure 5). The molecular weight of the high-molecular-weight proteoglycan, designated PG-I, was over 350,000. The low-molecular-weight proteoglycan fractions contained two types of proteoglycan (designated PG-II and PG-III) with molecular weights of about 190,000 and 105,000, respectively.

Separation of the low-molecular-weight proteoglycans by Octyl-Sepharose CL-4B

Chromatography on an Octyl-Sepharose CL-4B column was performed to separate PG-II and III (Figure 6). The column was eluted with a linear-concentration gradient of 2-6 M guanidine-HCl/0.1 M sodium acetate buffer (pH 6.3), and the uronic acid-containing fractions were eluted at guanidine-HCl concentrations of 3.59 M (peak I) and 4.67 M (peak II). The total amounts of uronic acid in peak I and II were 1.2 and 0.5 mg, respectively. Analysis by SDS-PAGE, showed that peaks I and II corresponded to PG-III and II, respectively (Figure 7-A).

The PG-II and III core proteins prepared by the chondroitinase ABC digestion were subjected to SDS-PAGE, which showed that their molecular weights were similar (about 44,000, data not shown). The Western blotting for PG-II and III using anti-human decorin antibody was carried out (Figure 7-B). Anti-human decorin antibody reacted with

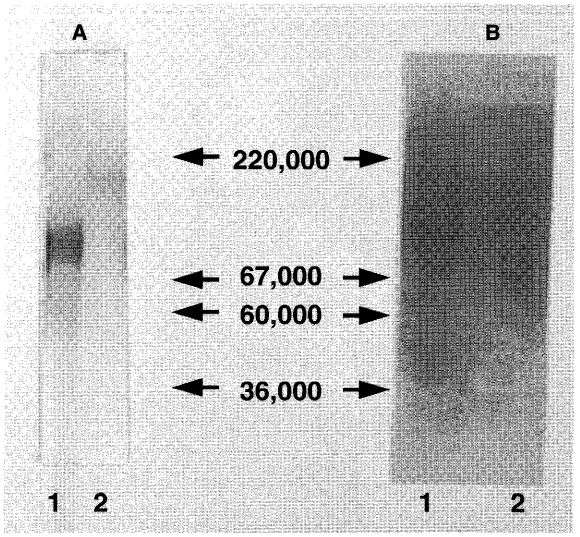

Figure 7 Analysis of PG-II and PG-III by SDS-PAGE and immunoblotting.
The fractions corresponding to the peaks shown in Figure 6 were subjected to SDS-PAGE (A) and the Western blotting using anti-human decorin antibody(B). Lane 1, PG-III (peak I shown in Figure 6); lane 2, PG-II (peak II shown in Figure 6).

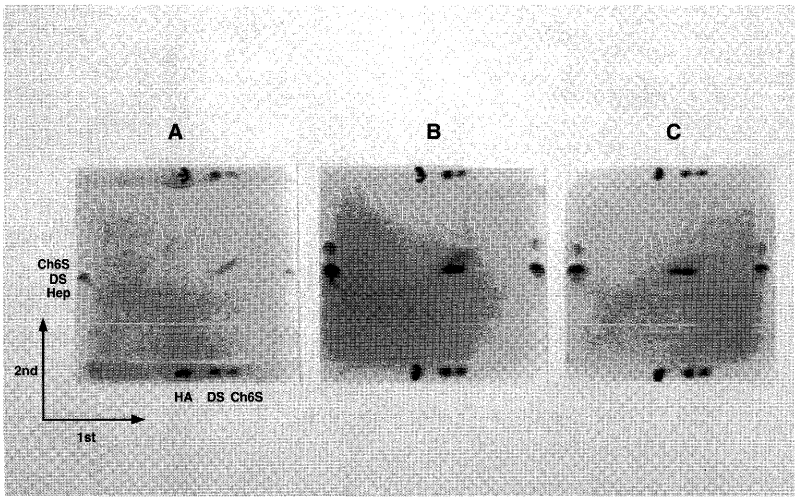

Figure 8 Two-dimensional electrophoresis on cellulose acetate membranes of the glycosaminoglycan chains from PG-I, II and III.
The glycosaminoglycan chains from PG-I, II and III obtained by Actinase E digestion were subjected to electrophoresis on cellulose acetate membranes. HA, hyarulonic acid; DS, dermatan sulfate; Ch6S, chondroitin 6-sulfate; Hep, heparin. A, glycosaminoglycans from PG-I; B, PG-II; C, PG-III.

PG-III, but not with PG-II, indicating that PG-II and III possess different core proteins.

Identification and characterization of glycosaminoglycan chains

In order to identify the glycosaminoglycan chains of PG-I, II and III, glycosaminoglycan chains were prepared by digesting each proteoglycan with Actinase E and endo-β-xylosidase followed by two-dimensional electrophoresis on cellulose acetate membranes (Figure 8). The glycosaminoglycan chains of PG-I were composed of chondroitin 6-sulfate, those of PG-II were composed of chondroitin 6-sulfate

and dermatan sulfate, whereas those of PG-III were composed of dermatan sulfate.

High-performance liquid chromatography of the pyridylaminated unsaturated disaccharides showed that the glycosaminoglycan chains of PG-I contained no dermatan sulfate, whereas 31.1 and 42.0% of glycosaminoglycan chains of PG-II and PG-III, respectively, consisted of dermatan sulfate (data not shown).

Discussion

The human yellow ligament extracellular matrix contained three types of proteoglycan (PG-I, II and III) (Figure 5), which were separated and characterized in this study. PG-I was fractionated in the density region of over 1.40 g/ml by CsCl isopycnic density gradient centrifugation (Figure 1) and its molecular weight, estimated by SDS-PAGE, was in excess of 350,000. Its glycosaminoglycan chains contained chondroitin 6-sulfate, which was identified by two-dimensional electrophoresis on a cellulose acetate membrane (Figure 8-A). The PG-I content of human yellow ligament was reported to increase[16] and endochondral ossification has been found to occur with aging[8]. In the light of these results, PG-I was suggested to be an aggrecan-type proteoglycan.

Both PG-II and III were fractionated in the density region of 1.35-1.38 g/ml by CsCl isopycnic density gradient centrifugation (Figure 1). When subjected to Sepharose CL-4B gel-filtration chromatography, they were eluted at Kav = 0.61 and yielded a single uronic acid peak (Figure 3). They were separated by the method of Choi et al.[16] using Octyl-Sepharose CL-4B chromatography (Figure 6), which separated proteoglycans with core proteins with different hydrophobic properties, and PG-II and III were eluted at similar concentrations of guanidine-HCl as biglycan and decorin, respectively. Analysis by SDS-PAGE showed that the respective molecular weights of PG-II and III were 190,000 and 105,000 (Figure 5). The molecular weights of their core proteins were similar, 44,000. However, the Western blotting for PG-II and III using anti-human decorin indicated that they possess different core proteins (Figure 7-B). These characteristics of PG-II and III resemble those of biglycan and decorin, respectively, isolated from other species[7, 20]. These results suggested that PG-II and III were biglycan- and decorin-type proteoglycans, respectively.

The glycosaminoglycan chains of PG-II contained 31.1% dermatan sulfate, and two-dimensional electrophoresis on cellulose acetate membrane showed that they contained both chondroitin 6-sulfate and dermatan sulfate (Figure 8-B). Okada et al. reported that the amount of low-molecular-weight proteoglycans with glycosaminoglycan chains composed of chondroitin 6-sulfate in human yellow ligaments increased with aging[10]. The result of our study, suggested that chondroitin 6-sulfate was from PG-II. It remains to be elucidated whether chondroitin 6-sulfate and dermatan sulfate bind to one core protein or they bind to different core proteins of PG-II.

The major proteoglycan component of the human yellow ligament extracellular matrix was PG-III, which was suggested to be decorin. The glycosaminoglycan chains of PG-III were composed of dermatan sulfate (Figure 8-C). The decorin content of the extracellular matrix of human articular cartilage has been reported to decrease with age[27] However, whether a similar change in the amount of PG-III in the extracellular matrix of human yellow ligament occurs with aging is still unclear. Decorin has been

reported to bind to collagens, thus controlling fibrillogenesis[28] and inhibiting tissue calcification[29]. Therefore, PG-III may possess these functions.

Differential distribution of decorin and biglycan in adult bovine tissues has been reported. Adult bovine tendon and skin contained only decorin[30], whereas bone and cartilage contained both decorin and biglycan [24], bovine nasal cartilage contained biglycan only[30]. These distinct distributions suggest that decorin and biglycan have different functions in the extracellular matrix.

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