Purification and characterization of human platelet proteoglycan

Minoru OKAYAMA,*§ Kayoko OGURI,* Yoshihisa FUJIWARA,† Hayao NAKANISHI,*
Hideto YONEKURA,* Toshihiko KONDO‡ and Nobuo UI‡

*Clinical Research Institute and †Blood Disease Centre, National Nagoya Hospital, 4-1-1 Sannomaru, Nakaku, Nagoya 460, Japan, and ‡Department of Physical Biochemistry, Institute of Endocrinology, Gunma University, Maebashi 371, Japan

Freshly prepared platelets were shown to contain glycosaminoglycans equivalent to 530 μg of hexuronate/ 10^{11} platelets. When the platelets were extracted with 4 M-guanidinium chloride containing proteinase inhibitors, and the extract was dialysed extensively against 7 M-urea solution, almost all of proteoglycan was recovered in the urea-soluble fraction. The proteoglycan was purified from the urea-soluble fraction with a yield of 47% by DEAE-Sephacel chromatography, CsCl-density-gradient centrifugation, Bio-Gel A-15m gel filtration and then rechromatography on DEAE-Sephacel. The purified proteoglycan contained 30% glucuronic acid, 32% *N*-acetylgalactosamine, 14% sulphate and 15% protein. Serine, glutamic acid, glycine, aspartic acid and leucine accounted for 64% of the total amino acids. The M_r of the proteoglycan was assessed to be approx. 136000 by sedimentation-equilibrium methods. The galactosaminoglycan released by alkaline-borohydride treatment of the proteoglycan was converted stoichiometrically into 4-sulphated unsaturated disaccharide by digestion with chondroitinase AC-II, indicating that the galactosaminoglycan was fully sulphated chondroitin 4-sulphate. The apparent M_r of the chondroitin sulphate was assessed to be 28000 by gel filtration on Bio-Gel A-0.5m (K_D 0.18). On two-dimensional electrophoresis on a cellulose acetate membrane, the chondroitin sulphate gave a single compact spot co-migrating with a reference chondroitin sulphate, indicating that the chondroitin sulphate chains were homogeneous in both length and charge density. On the basis of these results, the proteoglycan in human platelets was concluded to be a macromolecule of M_r 136000 containing four chondroitin 4-sulphate chains each with the apparent M_r of 28000.

INTRODUCTION

The presence of glycosaminoglycan in platelets has been well established biochemically and histochemically (Kharchenko, 1982), and this glycosaminoglycan has been demonstrated to be chondroitin 4-sulphate (Olsson & Gardell, 1967; Ward *et al.*, 1976; Ward & Packham, 1979). Electron-microscopic observations have indicated that the glycosaminoglycan is mainly located in α -granules with a little on the platelet surface (Behnke, 1968; Spicer *et al.*, 1969; MacPherson, 1972). Ward & Packham (1979) confirmed this with rabbit platelets prelabelled with [^{35}S]sulphate *in vivo*: 15% of the total chondroitin 4-sulphate could be released from the platelet surface by ADP treatment, and the remaining 85% from the α -granules by thrombin treatment. Although the biological role of platelet proteoglycan has not been elucidated, platelet factor 4, which is present in α -granules and has neutralizing activity for the anticoagulant effect of heparin (Niewiarowski & Thomas, 1969), is released by thrombin in the form of a high- M_r proteoglycan-platelet factor 4 complex (Barber *et al.*, 1972; Huang *et al.*, 1982).

For isolation of the platelet proteoglycan in an intact form, it is necessary to obtain the platelet fraction free from other blood constituents and to avoid proteolysis during the purification procedure, since other blood constituents such as leucocytes (Olsson, 1968; Taniguchi *et al.*, 1974) and plasma (Calatroni *et al.*, 1969; Juvani *et al.*, 1975; Hata *et al.*, 1978) contain a considerable amount of chondroitin sulphate, and moreover platelets

themselves (Legrand *et al.*, 1973) and plasma have been demonstrated to have high proteolytic activity. In the present paper we describe the purification and characterization of platelet proteoglycan obtained in an intact form from freshly prepared human platelets.

MATERIALS AND METHODS

Materials

A 400 ml sample of fresh concentrated platelet-rich human plasma (20 units from 20 random donors, O-type) was obtained from Aichi Prefecture Red Cross Blood Centre. It was obtained freshly, when not required for an intended recipient. The cell population of the sample is shown in Table 1.

Pronase P was obtained from Kaken Kagaku Co. (Tokyo, Japan). Chondroitinase ABC (chondroitin ABC lyase, EC 4.2.2.4), chondroitinase AC-II (chondroitin AC-II lyase, EC 4.2.2.5), chondro-4-sulphatase (EC 3.1.6.9) and chondro-6-sulphatase (EC 3.1.6.10), and standard materials for cellulose acetate membrane electrophoresis, chondroitin sulphate A, chondroitin sulphate B, chondroitin sulphate C, heparan sulphate and hyaluronic acid, and for paper chromatography, 2-acetamido-2-deoxy-3-*O*-(β -D-glucopyranosyluronic acid)-4-*O*-sulpho-D-galactose ($\Delta\text{Di-4S}$), 2-acetamido-2-deoxy-3-*O*-(β -D-glucopyranosyluronic acid)-6-*O*-sulpho-D-galactose and 2-acetamido-2-deoxy-3-*O*-(β -D-glucopyranosyluronic acid)-D-galactose ($\Delta\text{Di-}$

§ To whom correspondence should be addressed.

Table 1. Cell population in 400 ml of fresh concentrated platelet-rich human plasma

	$10^{-8} \times$ Cell number	Proportion (%)
Platelets	4480	89.4
Erythrocytes	520	10.4
Leucocytes	9.2	0.2
Granulocytes	1.1	
Monocytes	1.0	
Lymphocytes	7.1	

OS), were purchased from Seikagaku Kogyo Co. (Tokyo, Japan). Heparins of known M_r (22000, 13000, 9400, 7300 and 5700) were gifts from Dr. Suguru Suzuki of Eizai Co. (Tsukuba, Japan). The proteinase inhibitors used were *N*-ethylmaleimide from Nakarai Chemical Co. (Kyoto, Japan), phenylmethanesulphonyl fluoride from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and pepstatin A from the Protein Research Foundation (Osaka, Japan). Cellulose acetate membranes, Separax (10 cm $\times$ 10 cm), were from Jookoo Sangyo Co. (Tokyo, Japan). Bio-Gel A-10.5m and A-15m were obtained from Nippon Bio-Rad Laboratories K.K. (Tokyo, Japan) and DEAE-Sephacel was from Pharmacia Japan (Tokyo, Japan). Hanks balanced salt solution was obtained from GIBCO (Grand Island, NY, U.S.A.). All reagents used were analytical grade.

Extraction of proteoglycan

Platelets in platelet-rich plasma (400 ml) were collected by centrifugation with 6000 *g* for 10 min at 4 °C and washed three times with 100 ml of 1 vol. of citrate/phosphate/dextrose solution and 5 vol. of Hanks balanced salt solution. They were then suspended in 100 ml of 4 M-guanidinium chloride/50 mM-sodium acetate buffer, pH 6.0, containing 10 mM-EDTA, 10 mM-*N*-ethylmaleimide, 1 mM-phenylmethanesulphonyl fluoride and 0.036 mM-pepstatin A as proteinase inhibitors (Oike *et al.*, 1980). The suspension was stirred at 4 °C for 20 h. The extract (120 ml) was equilibrated with 7 M-urea/20 mM-NaCl/20 mM-Tris/HCl buffer, pH 7.3 (urea solution), by dialysis against five 1-litre volumes of the urea solution. The precipitate was collected by centrifugation at 10000 *g* for 20 min at 4 °C and washed with the urea solution once more. The washed precipitate was suspended in 50 ml of the urea solution (urea-insoluble fraction). The combined supernatant was adjusted to 200 ml (urea-soluble fraction).

Analytical methods

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. Hexuronate was determined by the carbazole method of Bitter & Muir (1962), with glucuronolactone as a standard. When samples contained a substantial quantity of proteins and/or glycoproteins, the samples were treated for 12 h at room temperature with 0.3 M-NaOH/1 M-NaBH₄ to release glycosaminoglycans and then digested extensively with Pronase as described previously (Kobayashi *et al.*, 1983). The digest was adjusted to 7% (w/v) trichloroacetic acid by addition of 50% trichloroacetic acid. The mixture was kept for 1 h on ice to precipitate peptides, and the precipitate was removed by

centrifugation at 8000 *g* for 20 min. The supernatant was mixed with 3 vol. of 95% ethanol containing 1.3% (w/v) potassium acetate/0.23 mM-EDTA (referred to below as ethanol precipitation treatment) and then the mixture was kept on ice for 2 h to precipitate glycosaminoglycans. The precipitated glycosaminoglycans were then collected by centrifugation and assayed for hexuronate content.

Enzymic assay of chondroitin sulphate isomers

The molecular species of chondroitin sulphate was determined by the method described by Saito *et al.* (1968): the borate-catalysed Morgan-Elson reaction (Reissig *et al.*, 1955), with Δ Di-OS as a standard, after digestion of the samples with appropriate combinations of chondroitinase AC-II, chondroitinase ABC, chondro-4-sulphatase and chondro-6-sulphatase.

Analytical ultracentrifugation

Sedimentation-equilibrium studies were performed by the meniscus-depletion method (Yphantis, 1964) with a Beckman model E analytical ultracentrifuge at a rotor

Fig. 1. Gel filtration on Bio-Gel A-0.5m of Pronase digests of the urea-soluble and urea-insoluble fractions

Each fraction (10 ml each) was desalted by ethanol precipitation treatment, treated with alkaline borohydride and digested with Pronase P. The digests were dissolved in 3 ml of 1 M-NaCl/50 mM-Tris/HCl buffer, pH 7.3, and applied to a column (1.5 cm $\times$ 70 cm) of Bio-Gel A-0.5m. The materials were eluted with the same buffer at a flow rate of 4 ml/h, and 1.6 ml fractions were collected. The absorbance at 280 nm (●) and hexuronate content, determined by the carbazole reaction (○), of each fraction were measured. (a) Urea-soluble fraction; (b) urea-insoluble fraction. The arrows show the void volume and the total volume of the column.

Fig. 2. DEAE-Sephacel column chromatography of the urea-soluble fraction (Fig. 1)

The urea-soluble fraction (185 ml) was applied to a column (2.2 cm $\times$ 29 cm) of DEAE-Sephacel previously equilibrated with 20 mM-NaCl/7 M-urea/20 mM-Tris/HCl buffer, pH 7.2. Material was eluted stepwise with 600 ml each of 20 mM-, 0.2 M- and 1 M-NaCl, and then 0.5 M-NaOH. The flow rate was 37 ml/h and 10 ml fractions were collected. The absorbance at 280 nm (●) and hexuronate content (○) of the fractions eluted with 1 M-NaCl were determined. Arrows show the starting position of each solvent. The fractions indicated by thin bars were pooled, and their protein and hexuronate contents were measured as described in the Materials and methods section. The 1 M-NaCl fractions shown by a thick bar were pooled as the proteoglycan fraction for further study.

temperature of 18.1 °C and speed of 16000 rev./min. Proteoglycan was used at concentrations of 0.2, 0.5 and 1.0 mg/ml in 1 M-NaCl/20 mM-sodium phosphate buffer, pH 7.0. The M_r was calculated by using a value of 0.54 cm³/g (Hopwood & Robinson, 1975) for the partial specific volume of the proteoglycan sample.

Amino acid and hexosamine analyses

Purified proteoglycan was hydrolysed in sealed evacuated tubes with 6 M-HCl at 110 °C for 16 h for amino acid analysis and with 3 M-HCl at 100 °C for 16 h for hexosamine analysis. Analyses were carried out in an automatic amino acid analyser (Hitachi model 835) by the method of Spackman *et al.* (1958).

Cellulose acetate membrane electrophoresis

Two-dimensional electrophoresis of glycosaminoglycan was carried out on cellulose acetate membrane by the method of Hata & Nagai (1973).

RESULTS

Extraction of proteoglycan from platelets

When platelets (4.48×10^{11} cells) were extracted with 4 M-guanidinium chloride/50 mM-acetate buffer, pH 6.0, containing proteinase inhibitors, the platelets were almost completely dissolved in the solution. Extensive dialysis of this clear solution against 7 M-urea solution, pH 7.3, resulted in appearance of a significant amount of aggregates, which were removed by centrifugation. The amounts of protein recovered in the urea-soluble and urea-insoluble fractions were determined to be approx. 950 mg and 60 mg respectively. To determine accurately the hexuronate contents in these fractions, a small

portion of each sample was treated with alkaline borohydride, and then digested extensively with Pronase. The digests were chromatographed on Bio-Gel A-0.5m. As shown in Fig. 1(b), no hexuronate was detected in the urea-insoluble fraction. Material containing hexuronate in the sample derived from the urea-soluble fraction was eluted close to the void volume of the column (Fig. 1a), and its hexuronate content was calculated to be 2.37 mg/ 4.48×10^{11} platelets.

Purification of proteoglycan

The proteoglycan was purified from the urea-soluble fraction by DEAE-Sephacel chromatography, CsCl-density-gradient centrifugation, Bio-Gel A-15m gel filtration and then rechromatography on DEAE-Sephacel. All the steps were carried out at 4 °C except for dialysis at 0 °C, unless otherwise stated. Details of these steps were as follows. The urea-soluble fraction (equivalent to 4.14×10^{11} platelets) was applied to a column of DEAE-Sephacel previously equilibrated with the same buffer. Materials were eluted stepwise with 20 mM-, 0.2 M- and 1 M-NaCl/7 M-urea/20 mM-Tris/HCl buffer, pH 7.3, and then with 0.5 M-NaOH. Fractions eluted were assayed for hexuronate content and protein as indicated in Fig. 2 legend. Approx. 80% of the proteoglycan was eluted with 1 M-NaCl solution, and more than 99% of the total protein was eliminated from that fraction (Fig. 2). The 1 M-NaCl fraction contained 1.77 mg of hexuronate and 3.11 mg of protein. Although a significant amount of hexuronate (0.33 mg) was washed out from the column with 0.5 M-NaOH, this fraction was not investigated further in the present study. The 1 M-NaCl fraction was dialysed against water. The non-diffusible materials were freeze-dried and dissolved in 4 M-guanidinium chloride solution, pH 6.0, containing

Fig. 3. CsCl-density-gradient centrifugation on DEAE-Sephacel of the 1 M-NaCl fraction (Fig. 2)

The 1 M-NaCl fraction was dialysed against water at 0 °C and freeze-dried and the residue was dissolved in 17 ml of 4 M-guanidinium chloride/50 mM-acetate buffer, pH 6.0, containing the proteinase inhibitors. Solid CsCl (0.5 g/g of solution) was added to give a density of 1.4 g/ml, and the solution was divided into two portions. A density gradient was formed by centrifugation at 170000 *g* for 72 h at 15 °C. The gradient was collected in eight fractions and their hexuronate (○) and protein (●) contents were measured. The densities of fractions are shown in the upper part of the Figure (◆). Bars show fractions pooled for further purification.

proteinase inhibitors. The solution was adjusted to an initial density of 1.4 g/ml by adding solid CsCl, centrifuged at 170000 *g* for 72 h at 15 °C and then collected in eight fractions. Approx. 80% of the proteoglycan was recovered in the bottom four fractions, with densities higher than 1.39 g/ml, and the remainder was distributed with major contaminating proteins (equivalent to approx. 93% of the total protein in the 1 M-NaCl fraction) in the upper four fractions, with densities lower than that (Fig. 3). The proteoglycan recovered in the upper fraction is described below under 'Minor components'. The bottom fraction had an absorption maximum at 260 nm and was considered to be contaminated with nucleic acid. The proteoglycan in this fraction was therefore further purified by gel filtration in Bio-Gel A-15m. Material having an absorption maximum at 260 nm was eluted at the void volume of the column and was clearly separated from the proteoglycan (Fig. 4). Approx. 82% of hexuronate in the sample was eluted from the column at partition coefficient K_D 0.22 as a sharp peak, coinciding with a peak of absorption at 280 nm, indicating that the proteoglycan was reasonably purified. The remaining 18% of hexuron-

Fig. 4. Gel filtration on Bio-Gel A-15m of the bottom fraction from the CsCl density gradient (Fig. 3)

The bottom fraction was dialysed against water at 0 °C and freeze-dried. The residue was dissolved in 2 ml of 1 M-NaCl/50 mM-Tris/HCl buffer, pH 7.3, and applied to a column (1.6 cm × 90 cm) of Bio-Gel A-15m. Material was eluted with the same buffer at a flow rate of 6.5 ml/h, and 3 ml fractions were collected. The absorbance at 280 nm (●) and hexuronate content determined by the carbazole reaction (○) of each fraction were measured. The arrows show the void volume and the total volume of the column. Bars show fractions pooled for further study.

Scheme 1. Fractionation of proteoglycans present in human platelets

ate was eluted in the more-retarded fractions and was processed as described under 'Minor components'. The yields of material containing hexuronate in the respective fractions are summarized in Scheme 1. The proteoglycan in the main fraction on the gel was finally purified by ion-exchange chromatography on DEAE-Sephacel. On elution of the column with a linear gradient of 20 mM–1 M-NaCl, the proteoglycan was eluted as a single sharp peak at 0.5 M-NaCl concentration (Fig. 5). This

Fig. 5. DEAE-Sephacel column chromatography of the proteoglycan fraction from Bio-Gel A-15m (Fig. 4)

The main fraction from Bio-Gel A-15m was dialysed against water at 0 °C and freeze-dried. The residue was dissolved in 10 ml of 20 mM-NaCl/20 mM-Tris/HCl buffer, pH 7.3, and applied to a column (1 cm × 10 cm) of DEAE-Sephacel previously equilibrated with the same buffer. The column was washed extensively with the equilibrating buffer, and material was eluted with a linear gradient (—) formed with 100 ml of the equilibrating buffer in the mixing chamber and 100 ml of 1 M-NaCl/20 mM-Tris/HCl buffer, pH 7.3, in the reservoir, at a flow rate of 6 ml/h. Fractions (2 ml) were collected, and their absorbance at 280 nm (●) and hexuronate content (○) were measured.

Fig. 6. Concentration-dependence of the apparent M_r of the purified proteoglycan

The purified proteoglycan was dissolved in 1 M-NaCl/20 mM-phosphate buffer, pH 7.0. Centrifugation was performed at 16 000 rev./min at 18.1 °C.

elution profile indicates that the proteoglycan was almost completely purified. The purified proteoglycan had an absorption maximum at 275 nm in 0.5 M-NaCl/50 mM-Tris/HCl buffer, pH 7.3.

Characterization of proteoglycan

The M_r of the purified proteoglycan was measured by sedimentation-equilibrium centrifugation in 1 M-NaCl/20 mM-phosphate buffer, pH 7.0, to prevent self-association during centrifugation (Huang *et al.*, 1982). The apparent M_r of the proteoglycan was dependent on the solute concentration, as shown in Fig. 6. In accordance with Heinegård & Axelsson (1977), the extrapolated M_r of the proteoglycan was determined to be approx. 136 000.

The amino acid and hexosamine compositions of the proteoglycan were analysed after hydrolysis of the material with 6 M-HCl and with 3 M-HCl respectively. A

Table 2. Amino acid composition of the purified proteoglycan from human platelets

Residue	Amino acid composition	
	(residue/1000 residues)*	(mol of residue/mol)†
Asx	127.6	26.3 (26)
Thr	33.0	6.8 (7)
Ser	153.4	27.9 (28)
Glx	131.5	27.1 (27)
Gly	129.5	26.6 (27)
Ala	37.2	7.7 (8)
Cys	4.8	1.0 (1)
Val	21.9	4.5 (4)
Met	13.0	2.7 (3)
Ile	24.2	5.0 (5)
Leu	99.3	20.4 (20)
Tyr	22.3	4.6 (5)
Phe	48.3	9.9 (10)
Lys	26.2	5.4 (5)
His	19.6	4.0 (4)
Arg	59.0	12.1 (12)
Pro	67.3	14.2 (14)
Total	1000	(206)

* No correction was made for the destruction of amino acids during HCl hydrolysis.

† The numbers of amino acid residues are calculated on the basis of the M_r (136 000) of the proteoglycan. Values in parentheses are nearest integers.

notable feature of the amino acid composition was the high contents of serine, glycine, glutamic acid, aspartic acid and leucine, which together constituted 64% of the total amino acid content (Table 2). Galactosamine was found as the sole hexosamine, with glucosamine being absent (results not shown). The hexuronate content was nearly equal to the galactosamine content, and was almost equal to the *N*-acetylgalactosamine content

Table 3. Chemical composition of the purified proteoglycan from human platelets

Component	Composition (%)
Glucuronic acid	30
<i>N</i> -Acetylgalactosamine	32
Sulphate*	14
Protein†	15

* Taken from analysis with the Morgan–Elson reaction of Δ Di-OS produced by digestion of galactosaminoglycan with chondroitinase AC-II plus chondro-6-sulphatase and chondro-4-sulphatase (see details in the text).

† Taken from analysis of amino acid composition.

Fig. 7. Gel filtration on Bio-Gel A-0.5m of the glycosaminoglycan of the purified proteoglycan

The purified proteoglycan was treated overnight with 0.3 M-NaOH/1 M-NaBH₄ at room temperature to release glycosaminoglycan. The reaction mixture was then neutralized with acetic acid, and small molecules were removed by ethanol precipitation treatment. The sample was dissolved in 1 ml of 1 M-NaCl/50 mM-Tris/HCl buffer, pH 7.3, and applied to a column (0.95 cm × 50 cm) of Bio-Gel A-0.5m. Material was eluted at a flow rate of 2.5 ml/h, and 0.5 ml fractions were collected. The inset shows the calibration curve obtained by plotting K_D (abscissa) versus M_r (plotted on a logarithmic scale) of heparins of known M_r : I (22000), II (13000), III (9400), IV (7300) and V (5700). The arrow indicates the K_D of the platelet glycosaminoglycan.

determined by the borate-catalysed Morgan–Elson reaction of Δ Di-OS, which was produced by digestion with a combination of chondroitinase AC-II plus chondro-4-sulphatase and chondro-6-sulphatase of the galactosaminoglycan from the same proteoglycan sample (see below). On the basis of these analytical data, together with the fact that all the Δ Di-OS was produced from Δ Di-4S by digestion with the sulphatase as described

Fig. 8. Two-dimensional electrophoresis on a cellulose acetate membrane of the glycosaminoglycan of the purified proteoglycan

Two-dimensional electrophoresis of the glycosaminoglycan from Bio-Gel A-0.5m was carried out on a cellulose acetate membrane in 0.1 M-pyridine/0.47 M-formic acid, pH 3.0, at 1 mA/cm for 1.2 h for the first dimension, and in 0.1 M-barium acetate, pH 8.0, at 1 mA/cm for 4.5 h in the second. The standard glycosaminoglycans used as external references were hyaluronic acid (HA), chondroitin sulphate A (CS-A), dermatan sulphate (DS), chondroitin sulphate C (CS-C), heparan sulphate (HS) and heparin (HP).

below, the purified proteoglycan was calculated to contain 30% glucuronic acid, 32% *N*-acetylgalactosamine, 14% sulphate and 15% protein (Table 3).

The galactosaminoglycan chains were released from the proteoglycan by alkaline-borohydride treatment, and the resulting sample was applied to a column of Bio-Gel A-0.5m. The galactosaminoglycan was eluted close to the void volume of the column with K_D 0.18 (Fig. 7). The column was previously calibrated with heparins of known M_r (Fig. 7 inset), which gave a calibration curve similar to that obtained by Wasteson (1971) on Sephadex G-200 (results not shown). On the basis of this calibration curve, the apparent M_r of the galactosaminoglycan was assessed to be approx. 28000. On electrophoresis, the galactosaminoglycan migrated as a single compact spot without any tailing, and coincided in position with chondroitin sulphate A and chondroitin sulphate C, used as references (Fig. 8). Thus the galactosaminoglycan side chains were chondroitin sulphate of very homogeneous charge density.

The molecular species of chondroitin sulphate was determined by digesting galactosaminoglycan samples (equivalent to 0.2 μ mol of hexuronate each) with the multienzyme systems described in the Materials and methods section and measuring the *N*-acetylgalactosamine content of the produced Δ Di-OS by the Morgan–Elson reaction (Saito *et al.*, 1968). The *N*-acetylgalactosamine contents of the digests were calculated as 0 μ mol for the digest with chondroitinase AC-II plus chondro-6-

Fig. 9 Gel filtration on Bio-Gel A-15m of the upper fraction from the CsCl density gradient (Fig. 3)

The upper fraction was dialysed against water at 0 °C, and freeze-dried. The residue was dissolved in 1 M-NaCl/50 mM-Tris/HCl buffer, pH 7.3, and applied to a column as described in Fig. 4 legend. The arrows show the void volume and the total volume of the column.

sulphatase, 0.21 μ mol for that with chondroitinase AC-II plus chondro-6-sulphatase plus chondro-4-sulphatase, and 0.20 μ mol for that with chondroitinase ABC plus the two chondrosulphatases. These data showed that the chondroitin sulphate in the purified proteoglycan was composed of only 4-sulphated disaccharide units and did not contain a significant amount of 6-sulphated, non-sulphated or iduronic acid-containing disaccharide units. Thus the chondroitin sulphate in human platelets was concluded to be fully sulphated chondroitin 4-sulphate.

Minor components

Finally, we examined characteristics of the minor components (see Scheme 1). When the proteoglycan in the upper fraction from the CsCl density gradient was applied to a column of Bio-Gel A-15m as described above, it was eluted from the column at the same position (K_D 0.22) as that of the proteoglycan in the bottom fraction (Fig. 9). This fraction was treated by alkaline borohydride to release glycosaminoglycan chains. The glycosaminoglycan obtained was eluted as a sharp symmetrical peak at K_D 0.18 from a column of Bio-Gel A-0.5m (Fig. 1a) and migrated as a single compact spot similar to that derived from the major proteoglycan on electrophoresis (Fig. 10a inset). On the other hand, the

Fig. 10. Gel filtration on Bio-Gel A-0.5m and two-dimensional electrophoresis on cellulose acetate membrane of the glycosaminoglycans of the proteoglycans contained in the upper fraction from the CsCl-density-gradient centrifugation and the retarded fraction on Bio-Gel A-15m

The proteoglycans contained in the upper fraction from the CsCl-density-gradient centrifugation (see Fig. 3) and the retarded fraction on Bio-Gel A-15m (see Fig. 4) were desalted by the ethanol precipitation treatment. Samples were then treated with 0.3 M-NaOH/1 M-NaBH₄. The resultant glycosaminoglycans were divided into two portions and were analysed by Bio-Gel A-0.5m chromatography and two-dimensional electrophoresis as described in Fig. 7 legend and Fig. 8 legend respectively. The profiles of glycosaminoglycans in the upper fraction (a) and the retarded fraction (b) are shown. The arrows indicate the void volume and the total volume of the column. External standards of the electrophoretograms (insets) are the same as described in Fig. 8 legend.

glycosaminoglycan released from the retarded fraction on Bio-Gel A-15m (see Fig. 4) was eluted from Bio-Gel A-0.5m as a rather broad peak (Fig. 10b), and showed a single spot with a slight tailing on electrophoresis (Fig. 10b inset), indicating that it consists of components of more heterogeneous molecular mass and/or charge density. Analysis of the two glycosaminoglycans by the multienzyme systems described above showed that both glycosaminoglycans were composed of chondroitin 4-sulphates very similar to that derived from the major proteoglycan described above (results not shown).

DISCUSSION

The platelets used here were isolated within 12 h after obtaining blood from donors, and were washed thoroughly to remove contaminating plasma components, since plasma contains a considerable amount of low-sulphated chondroitin 4-sulphate (5.8 μg of hexuronate/ml of plasma; Nakanishi *et al.*, 1984). The platelet fraction contained a few other cells, such as erythrocytes and leucocytes (Table 1), but erythrocytes do not contain chondroitin sulphate and the number of leucocytes was negligible. It is therefore reasonable to consider that almost all of the galactosaminoglycan in the fraction was derived from the platelets. The platelets contained galactosaminoglycan equivalent to 530 μg of hexuronate/ 10^{11} platelets.

The present study indicates that the galactosaminoglycan is present in a form of proteoglycan, in which the galactosaminoglycan chains are linked to a core protein through alkali-labile bonds. Most of the proteoglycan was collected in the urea-soluble fraction, and was successfully purified from that fraction in an intact form with a yield of approx. 47% of the total proteoglycan by DEAE-Sephacel chromatography, CsCl-density-gradient centrifugation and then gel filtration on Bio-Gel A-15m (see Scheme 1). When the major proteoglycan obtained on the gel was rechromatographed on DEAE-Sephacel, hexuronate-containing material was eluted from the column as a single sharp peak at 0.5 M-NaCl concentration coinciding with protein having an absorption maximum at 275 nm, indicating that the proteoglycan is reasonably pure.

The apparent M_r of the purified proteoglycan was determined to be approx. 136000 by sedimentation-equilibrium centrifugation under conditions similar to those used by Huang *et al.* (1982). This was considerably larger than those determined by Barber *et al.* (1972) (M_r 59000) and Huang *et al.* (1982) (M_r 53000). Barber *et al.* (1972) isolated the proteoglycan from freshly separated platelets, but purified it in the absence of proteinase inhibitors, and Huang *et al.* (1982) used out-dated frozen platelet-rich plasma as starting material. We recently found that, even when most plasma proteins have been denatured in 8 M-urea solution, human plasma still contains high proteolytic activity that degrades the core peptide of the plasma proteoglycan (M. Okayama, K. Oguri & H. Nakanishi, unpublished work). Thus it is very likely that in the studies by previous workers proteolysis of the proteoglycan occurred during storage of the platelets in the plasma or purification of the proteoglycan in the absence of proteinase inhibitors. The M_r of the proteoglycan identified as the C1q inhibitor was estimated to be 175000 on the basis of its partition coefficient on Sepharose CL-2B chromatography

(Silvestri *et al.*, 1981). However, the elution position of the C1q-inhibitor proteoglycan from Sepharose CL-4B, which was also examined in that study, appeared to correspond to that of the proteoglycan described in the present work.

The galactosaminoglycan released by alkaline-borohydride treatment of this proteoglycan was converted stoichiometrically into 4-sulphated unsaturated disaccharide by digestion with chondroitinase AC-II, indicating that it was fully sulphated chondroitin 4-sulphate. The M_r of the chondroitin sulphate was estimated to be approx. 28000 by gel chromatography on Bio-Gel A-0.5m. It is well known that plasma and leucocytes are other main blood constituents containing chondroitin sulphates. However, the plasma component has been reported to consist mainly of low-sulphate chondroitin 4-sulphate (Taniguchi *et al.*, 1974; Juvani *et al.*, 1975; Hata *et al.*, 1978; Nakanishi *et al.*, 1984) with an M_r of 17000 (Juvani *et al.*, 1975) or 8000 (Hata *et al.*, 1978). The chondroitin sulphate synthesized *in vitro* by human leucocytes has also been demonstrated to be low-sulphated chondroitin 4-sulphate (Olsson, 1968; Hata *et al.*, 1978). Our earlier finding (Oguri *et al.*, 1984) indicated that the M_r of the low-sulphated chondroitin 4-sulphate contained in the human leukaemic myeloid cells is much smaller (9000–12000) than that of the platelet chondroitin 4-sulphate described in the present paper. All these results indicate that the platelet chondroitin sulphate is distinctively different from those of plasma and leucocytes. However, it was very similar to the galactosaminoglycan of the C1q-inhibitor proteoglycan, which has been reported to be fully sulphated chondroitin 4-sulphate with an M_r larger than 22000 (Silvestri *et al.*, 1981). This evidence, together with the fact that the platelet proteoglycan described in the present paper is very similar to that of the C1q inhibitor, suggests that the C1q inhibitor is derived from platelets. It is therefore likely that the proteoglycan of platelets acts as a C1q inhibitor in addition to being a carrier of platelet factor 4 (Barber *et al.*, 1972; Huang *et al.*, 1982).

The minor components separated from the major proteoglycan during the purification steps were found to be chondroitin 4-sulphate proteoglycans. The M_r values of the proteoglycan and its galactosaminoglycan in the upper fraction in the CsCl density gradient were similar to those of the major proteoglycan. However, it could not be elucidated why the minor component has the lower buoyant density. Another minor proteoglycan in the retarded fraction on Bio-Gel A-15m gel filtration was shown to have smaller, rather heterogeneous, galactosaminoglycan chains, indicating that the M_r of the proteoglycan reflected the length of its galactosaminoglycan chains. It is still uncertain whether these minor components are subclasses of platelet proteoglycans with different localizations, i.e. on the platelet surface and in the α -granule (Ward & Packham, 1979), or whether they are separate biosynthetic and/or degradation products.

This work was supported in part by a Grant-in-Aid for Cancer Research and a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan and by a grant from the Ishida Foundation, Japan.

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Received 8 May 1985/30 July 1985; accepted 2 September 1985