Isolation and Characterization of Chondroitin 6-Sulfate Proteoglycans Present in the Extracellular Matrix of Rabbit Bone Marrow

By Eiko Okayama, Kayoko Oguri, Toshihiko Kondo, and Minoru Okayama

The authors' previous studies showed that the glycosaminoglycans present in rabbit bone marrow were composed of chondroitin 6-sulfate (79%) and hyaluronic acid (16%). Immunohistochemically the chondroitin 6-sulfate was demonstrated to be in bone marrow matrix constructing hematopoietic microenvironment. In this study the authors isolated and characterized these glycosaminoglycans in their macromolecular form (i.e., proteoglycans). Bone marrow of 3-month-old rabbits was defatted with organic solvents containing proteinase inhibitors at −20°C, and proteoglycans were extracted from the defatted tissue with 4 mol/L guanidine HCl containing the proteinase inhibitors. After extensive dialysis of the extract against 7 mol/L urea, more than 90% of hexuronate-containing materials was recovered in the urea-soluble fraction. The proteoglycans were purified from the urea-soluble fraction by diethyl aminomethyl (DEAE)-Sephacel chromatography, CsCl density-gradient centrifugation, and Bio-Gel A-5m gel filtration, then were rechromatographed on DEAE-Sephacel and on Bio-Gel A-5m. The proteoglycans were separated into three molecular species with different mol wts that were assessed to be 46,000, 16,000, and 8,300 by sedimentation-equilibrium centrifugation. Amino acid analyses of these proteoglycans revealed that serine and glycine accounted for approximately 60% of the total amino acids common to the three proteoglycans. The glycosaminoglycan side chains of these proteoglycans were converted stoichiometrically into unsaturated 6-sulfated disaccharide by digestion with chondroitinase AC-II, indicating that they were fully sulfated chondroitin 6-sulfate. Their apparent mol wts were estimated by gel filtration on Bio-Gel A-0.5m to be 10,900, 14,400, and 7,700. Computation of these results, taken together with their biochemical composition, revealed that the largest proteoglycan, PG-II, consisted of four chains of the chondroitin 6-sulfate and the core peptide with mol wt of approximately 4,000. The smaller two proteoglycan subpopulations, PG-I and PG-III, consisted of a single glycosaminoglycan chain linked to a small peptide with mol wts of approximately 500 and 1,000, respectively.

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isolated and characterized the proteoglycans containing the chondroitin 6-sulfate present in rabbit bone marrow.

**MATERIALS AND METHODS**

**Materials.** Three-month-old female albino rabbits (1.8 kg body weight) were obtained from a local animal supplier. The following materials were purchased from Seikagaku Kogyo Co, Tokyo: chondroitinase ABC, chondroitinase AC-II, chondro-4-sulfatase, chondro-6-sulfatase, hyaluronidase from *Streptomyces hyalurolyticus*, glycosaminoglycan standards for cellulose acetate membrane electrophoresis, chondroitin sulfate A, chondroitin sulfate C, dermatan sulfate, hyaluronic acid, heparan sulfate and heparin, and external trophoresis, chondroitin sulfate A, chondroitin sulfate C, dermatan glycosaminoglycan standards for cellulose acetate membrane electrophoresis, streptomyces hyalurolyticus, collagease was obtained from Worthington (Freehold, NJ), and was purified to obtain chondroitinase AC-II, chondro-4-sulfatase, chondro-6-sulfatase from P-L Biochemicals Inc (Milwaukee). Collagenase was obtained from Worthington (Freehold, NJ), and was purified to remove other proteinases as described previously.27

**Extraction of macromolecules.** The procedure used for extraction and fractionation of macromolecules from rabbit bone marrow is summarized in Fig 1. Both ends of rabbit femora and tibiae (five rabbits in each experiment) were cut off, and the bone marrows were washed out with phosphate-buffered saline (PBS). The marrow tissues were defatted by successive treatments twice each at −20°C with 150 mL of vol acetone, ether, chloroform/methanol (2/1, vol/vol), and acetone, containing 10 mmol/L EDTA, 10 mmol/L N-ethylmaleimide, 1 mmol/mL phenylmethylsulfonyl fluoride, and 0.036 mmol/L pepstatin A as proteinase inhibitors.27 The acetone-dried powder was weighed and then suspended in 150 mL of 4 mol/L guanidine HCl/50 mmol/L sodium acetate, pH 6.0, containing the same proteinase inhibitors. The suspension was stirred at 4°C for 24 hours and centrifuged at 14,000 g for 40 minutes. The supernatant was collected, and the residue was resuspended in 100 mL of the extraction buffer, stirred at 4°C for another 24 hours, and then centrifuged. The residue (guanidine HCl-residual fraction, Fig 1) was suspended in 50 mL of water and stored at −20°C until use. The combined supernatant was dialyzed against seven 3 L vol of 20 mmol/L NaCl/7 mol/L urea/20 mmol/L Tris HCl, pH 7.3, at 4°C. A precipitate appeared during this dialysis, was collected by centrifugation at 8,000 g for 20 minutes, was washed with 50 mL of urea solution, and was dissolved in 100 mL of extraction buffer (urea-insoluble fraction, Fig 1). The supernatants were combined (urea-insoluble fraction, Fig 1).

**Analytical methods.** Protein was measured by the method of Lowry et al28 with bovine serum albumin (BSA) as a standard. Hexuronate was determined by the carbazole method of Bitter and Muir,29 with glucuronolactone as a standard. Before the determination, proteineous contaminants were removed from the samples as described previously.28 The molecular species of chondroitin sulfates were determined as described by Saito et al30; the borate-catalyzed Morgan-Elson reaction,31 with Di-O-S as a standard, after digestion of the samples with an appropriate combination of chondroitinase ABC, chondroitinase AC-II, chondro-4-sulfatase, and chondro-6-sulfatase, or paper chromatography, as described previously.32

**Analytical ultracentrifugation.** Sedimentation-equilibrium studies were performed by the meniscus-depletion method33 with a Beckman model E analytical ultracentrifuge at a rotor temperature of 20.0°C (for PG-I, see the text), 19.3°C (PG-II), or 20.2°C (PG-III) and speed of 48,000 rev/min (for PG-I), 24,000 rev/min (PG-II), or 40,000 rev/min (PG-III). Proteoglycans were used at concentrations of 0.2, 0.5, and 1.0 mg/mL in 1 mol/L NaCl/20 mmol/L sodium phosphate buffer, pH 7.0. The mol wt was calculated by using a value of 0.54 cm2/g for the partial specific volume of the proteoglycan samples.

**Amino acid and hexosamine analyses.** Purified proteoglycans were hydrolyzed in sealed evacuated tubes with 6 mol/L HCl at 110°C for 16 h for amino acid analysis and with 3 mol/L HCl at 100°C for 16 h for hexosamine analysis. Analyses were carried out in an automatic amino acid analyzer (Hitachi model 835) by the method of Spackman et al.34

**Cellulose acetate membrane electrophoresis.** Two-dimensional electrophoresis of glycosaminoglycans was carried out on a cellulose acetate membrane (10 × 10 cm) by the method of Hata and Nagai35 in 0.1 mol/L pyridine/0.47 mol/L formic acid, pH 3.0, at 1 mA/cm for one hour in the first dimension; and in the second dimension in 0.1 mol/L barium acetate, pH 8.0, at 1 mA/cm for 4.5 hours.

**Enzyme digestion.** Samples were digested with chondroitinase ABC, chondroitinase AC-II, chondro-4-sulfatase, and chondro-6-sulfatase, as described previously.35 Samples were digested with pronase in 0.1 mol/L Tris HCl, pH 8.0, at 50°C for 48 hours with further addition of the same amount of enzyme after the first 24 hours. Samples were digested with collagenase in 10 mmol/L CaCl2/0.1 mol/L Tris HCl, pH 7.3, at 37°C for 16 hours, with papain in 15 mmol/L cysteine/6 mmol/L EDTA/0.3 mol/L sodium acetate, pH 6.0, at 37°C for 24 hours, and with hyaluronidase in 75 mmol/L NaCl/20 mmol/L sodium acetate, pH 6.0 at 37°C for two hours.

**RESULTS**

**Extraction of proteoglycans.** Whole bone marrow of the femora and tibiae of 3-month-old rabbits were used. The tissues from five rabbits were usually combined from one experiment (Fig 1). Since bone marrow contains much lipid that interferes with the extraction and purification of proteoglycans, the material was extracted extensively with acetone, ether, chloroform/methanol, and acetone. To avoid proteolysis during extraction, these procedures were carried out at −20°C in the presence of proteinase inhibitors. The mean

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**Fig 1.** Extraction and fractionation of proteoglycans of rabbit bone marrow.
dry weight of the defatted tissue obtained from five rabbits each in four experiments was 2.7 ± 0.15 g. The defatted tissue (2.5 to 3.1 g) was extracted with 150 mL of 4 mol/L guanidine HCl/50 mmol/L sodium acetate, pH 6.0, containing the same proteinase inhibitors. To ensure complete extraction, the guanidine HCl-insoluble residue was reextracted with 100 mL of the same solution. Approximately 95% of hexuronate was recovered in the guanidine HCl-soluble fraction. Extensive dialysis of the guanidine HCl-extract against 7 mol/L urea solution resulted in the appearance of a substantial amount of insoluble material that was removed by centrifugation. The precipitate was washed with urea solution and dissolved in 50 mL of guanidine HCl solution. The protein and hexuronate contents of the three fractions (guanidine HCl-residual, urea-soluble, and urea-insoluble fractions) were determined, using small portions of these fractions. More than 90% of the tissue hexuronate was collected in the urea-soluble fraction, and proteoglycans were further purified from this fraction. This fraction contained 637 mg protein, which accounted for approximately 71% of the total tissue protein.

Purification of proteoglycans. All the steps described below were carried out at 4°C except dialysis, which was done on ice. The urea-soluble fraction obtained from five rabbits (containing 6.32 mg hexuronate and 637 mg protein) was applied to a column of diethyl aminoethyl (DEAE)-Sephacel and washed with the equilibrating buffer solution. Bound material was eluted stepwise with 0.35 mol/L, 1 mol/L, 1.5 mol/L, and 2 mol/L NaCl/7 mol/L urea/20 mmol/L Tris HCl, pH 7.3, and then with 0.5 mol/L NaOH. Hexuronate and protein in the eluate were monitored (Fig 2). No detectable amount of hexuronate or protein was eluted with 1.5 mol/L or 2 mol/L NaCl (data not shown). Proteoglycans were eluted with 0.35 mol/L, 1 mol/L NaCl, and 0.5 mol/L NaOH and were freed from a large amount of protein. The fractions indicated by bars were pooled, and the hexuronate and protein contents of these fractions were determined. The total recovery of hexuronate was 86%, with recoveries of 16%, 58%, and 13% in the fractions eluted with 0.35 mol/L, 1 mol/L NaCl, and 0.5 mol/L NaOH, respectively. As described above, the material containing hexuronate eluted with 0.5 mol/L NaOH was not eluted with 1.5 mol/L or 2 mol/L NaCl in urea solution and thus was assumed to bind irreversibly to DEAE-Sephacel and to be eluted from the column only after degradation by exposure to NaOH. This material, therefore, was not investigated further in this study. The 0.35 mol/L NaCl fraction contained a large amount of protein (44% of the total protein) and so was further purified by rechromatography on DEAE-Sephacel with a linear gradient of NaOH. On this chromatography the proteoglycan was eluted as a single peak, freed from approximately 95% of the protein (inset in Fig 2). The material that was detected by monitoring absorbance at 280 nm and coeluted with proteoglycan had an absorbance maximum at 260 nm and was thought to be nucleic acid. When the 0.35 mol/L NaCl fraction was dialyzed extensively against water at 0°C for concentration by freeze drying before being applied to the second DEAE-Sephacel column, all the hyaluronic acid in this fraction was selectively precipitated as described below (as the water-insoluble fraction). The reason for this selective precipitation of the hyaluronic acid is unknown.

The proteoglycan fractions eluted with 0.35 mol/L NaCl and 1 mol/L NaCl were dialyzed against water and freeze dried. The resulting material was dissolved in guanidine HCl solution, solid CsCl was added, and the material was centrifuged. Approximately 90% of the proteoglycan of each sample was collected in the bottom five fractions, with

Fig 2. DEAE-Sephacel column chromatography of the urea-soluble fraction. The urea-soluble fraction was applied to a column (2.2 × 25 cm) of DEAE-Sephacel previously equilibrated with 20 mmol/L NaCl/7 mol/L urea/20 mmol/L Tris HCl, pH 7.3. Materials were eluted stepwise with 20 mmol/L, 0.35 mol/L and 1 mol/L NaCl in the equilibrating buffer, and then with 0.5 mol/L NaOH. The flow rate was 44 mL/h, and fractions of 17.5 mL were collected. The absorbance at 280 nm (•) and hexuronate content (○) of the fractions were monitored. The fractions of 0.35 mol/L NaCl eluate, shown by a thin bar, were pooled, dialyzed against water, freeze dried, and dissolved in 20 mmol/L NaCl/20 mmol/L Tris HCl, pH 7.3, and then rechromatographed on DEAE-Sephacel with a linear gradient of 20 mmol/L to 1 mol/L NaCl/20 mmol/L Tris HCl, pH 7.3, to remove contaminating protein (inset). The fractions, shown by thick bars, were pooled as the 0.35 mol/L and 1 mol/L NaCl fractions for further purification.
material having an absorbance maximum at 260 nm (probably nucleic acid), whereas contaminating protein was removed in the top half (Fig 3). The fractions containing proteoglycan were pooled as shown in the figure, dialyzed against water, and concentrated by freeze drying. The resulting residue was dissolved in 1 mol/L NaCl/50 mmol/L Tris HCl, pH 7.3, and applied to a column of Bio-Gel A-5m (Fig 4). The proteoglycan derived from the 0.35 mol/L NaCl fraction was eluted from the column with a partition coefficient, K_P, of 0.70 as a sharp peak. The proteoglycans from the 1 mol/L NaCl fraction separated into two peaks with K_P 0.43 and 0.62. These three proteoglycan fractions (I, II, and III) were pooled, adjusted to 20 mmol/L NaCl/20 mmol/L Tris HCl, pH 7.3, and applied to a column of DEAE-Sephacel equilibrated with the same buffer solution (Fig 5).

Materials were eluted with a linear gradient of 20 mmol/L to 1 mol/L NaCl/20 mmol/L Tris HCl, pH 7.3, without 7 mol/L urea. The proteoglycan in the three samples was eluted from the column as symmetrical peaks. Dashed lines show the densities of the three purified proteoglycan samples did not contain detectable amounts of material with absorbances at 280 nm and 260 nm. This indicates that these proteoglycan samples did not contain any significant amounts of aromatic amino acids and were not contaminated with nucleic acid. The authors, therefore, considered that all these three proteoglycan samples were reasonably purified. These proteoglycans were designated as PG-I, PG-II, and PG-III. The yields of PG-I, PG-II, and PG-III from 1 g of defatted tissue were determined to be approximately 300 μg, 680 μg, and 390 μg hexuronate, respectively.

**Characterization of proteoglycans.** The mol wts of the three purified proteoglycans were measured by sedimentation-equilibrium centrifugation in 1 mol/L NaCl/20 mmol/L phosphate buffer, pH 7.0 (Fig 7). The apparent mol wts of the proteoglycans were dependent on the solute concentration. By the method of Heinegard and Axelsson,37 the extrapolated mol wts of the proteoglycans, PG-I, PG-II, and PG-III, were determined to be approximately 8,300, 46,000, and 16,000, respectively.

The amino acid and hexosamine compositions of these proteoglycans were analyzed after hydrolysis of the samples.
galactosaminoglycans derived from the proteoglycan samples with a combination of chondroitinase AC-II, chondroitin-6-sulfatase, and chondroitin-4-sulfatase. On the basis of these analytical data, together with the fact that all the Di-OS was produced from Di-6S by digestion with the sulfatase as shown below, the three purified proteoglycans were calculated to have the chemical compositions shown in Table 2.

Since the amino acid compositions of these proteoglycans were very unusual, the authors examined the susceptibility of the core peptide of PG-II with the largest mol wt to proteinases, such as bacterial collagenase, pronase, and papain. Aliquots of PG-II were digested with the proteinases, and the digests were applied to a column of Bio-Gel A-5m (Fig 8). For comparison with the digests, intact PG-II and galactosaminoglycans released from PG-II by β-elimination reaction were also chromatographed on the same column. The

Fig 5. DEAE-Sepharose column chromatographies of the fractions I, II, and III from Bio-Gel A-5m. Fractions I, II, and III were dialyzed against water, freeze dried, dissolved in 20 mmol/L NaCl/20 mmol/L Tris HCl, pH 7.3, and applied to a column (0.7 x 15 cm) of DEAE-Sepharose previously equilibrated with the same buffer solution. Materials were eluted with 200 ml of a linear gradient of 20 mmol/L to 1 mol/L NaCl in the same buffer. The flow rate was 6 ml/h, and fractions of 2 ml were collected. The absorbance at 280 nm (A) and hexuronate content (C) of the fractions were measured. Bars show fractions pooled for further purification. (A) Fraction I; (B) Fraction II; (C) Fraction III from Bio-Gel A-5m.

with 6 mol/L HCl and with 3 mol/L HCl, respectively. A notable feature of the amino acid compositions of all three proteoglycans was their high contents of serine and glycine, which together constituted 59% to 65% of the total amino acid contents (Table 1). The ratios of serine to glycine in PG-I, PG-II, and PG-III were approximately 2/1, 1/1, and 3/2, respectively. Galactosamine was found as the only hexosamine in all three proteoglycans, with no glucosamine. The hexuronate contents of these proteoglycan samples were nearly equal to their N-acetylhexosamine contents, which were determined by the borate-catalyzed Morgan-Elson reaction of DAi-OS, which was produced by digestion of the galactosaminoglycans derived from the proteoglycan samples with a combination of chondroitinase AC-II, chondroitin-6-sulfatase, and chondroitin-4-sulfatase. On the basis of these analytical data, together with the fact that all the DAi-OS was produced from DAi-6S by digestion with the sulfatase as shown below, the three purified proteoglycans were calculated to have the chemical compositions shown in Table 2.

Fig 6. Rechromatography on Bio-Gel A-5m of fractions I', II', and III' from DEAE-Sepharose. Fractions I', II', and III' were dialyzed against water, freeze dried, dissolved in 1 mol/L NaCl/50 mmol/L Tris HCl, pH 7.3, and applied to a column (1.6 x 90 cm) of Bio-Gel A-5m. The flow rate was 6 ml/h, and 3 ml fractions were collected. The hexuronate content of the fractions was determined. Arrows show the void volume and the total volume of the column. Bars show fractions pooled for characterization as purified proteoglycans. (A) Fraction I'; (B) Fraction II'; (C) Fraction III' from DEAE-Sepharose.

Since the amino acid compositions of these proteoglycans were very unusual, the authors examined the susceptibility of the core peptide of PG-II with the largest mol wt to proteinases, such as bacterial collagenase, pronase, and papain. Aliquots of PG-II were digested with the proteinases, and the digests were applied to a column of Bio-Gel A-0.5m (Fig 8). For comparison with the digests, intact PG-II and galactosaminoglycans released from PG-II by β-elimination reaction were also chromatographed on the same column. The
eluted at the same digestion with bacterial collagenase was released by alkali-borohydride treatment can be seen in Fig 9. The elution profiles indicated that the galactosaminoglycan chains are linked to a peptide. The galactosaminoglycan chains of the three proteoglycans were released by alkali-borohydride treatment of the proteoglycans, and their apparent mol wts were estimated by gel chromatography on Bio-Gel A-0.5m. The galactosaminoglycans released from PG-I, PG-II, and PG-III were eluted as sharp, symmetrical peaks at KD 0.51, 0.44, and 0.39 (Fig 9). The column was previously calibrated with the three purified proteoglycans. The KD of PG-I, PG-II, and PG-III on the column were found to be 0.49, 0.15, and 0.38, respectively (Fig 10). The elution profiles indicated that the chain sizes of the three galactosaminoglycans were very homogeneous. The apparent mol wts of the galactosaminoglycans were estimated to be 7,700, 10,900, and 14,400, respectively, from the calibration curve. On electrophoresis the galactosaminoglycans of the three samples migrated as single compact spots without any tailing and coincided in position with chondroitin sulfates A and C, used as references (Fig 9). Thus all three galactosaminoglycans were chondroitin sulfate of very homogeneous charge density.

When the three chondroitin sulfate samples were digested with chondroitinase AC-II and the digests were subjected to paper chromatography, all the materials were found to be converted stoichiometrically to ΔDi-6S (Fig 11). This was also confirmed by the fact that the three galactosaminoglycans were completely converted into ΔDi-OS by digestion with chondroitinase AC-II plus chondroitinase A-0.5m and the ΔDi-OS produced by digestions was determined by the borate-catalyzed Morgan-Elson reaction. The results

| Table 1. Amino Acid and Hexosamine Compositions of the Purified Proteoglycans From Rabbit Bone Marrow |
|----------------|-----------|-----------|-----------|
| Residue  | PG-I | PG-II  | PG-III |
| Aax  | 59.4  | 72.1 (3) | 62.8  |
| Thr  | 18.1  | 24.6 (1) | 26.6  |
| Ser  | 426.7 | 332.5 (15) | 356.6 |
| Gln  | 69.2  | 63.0 (3) | 101.6 |
| Gly  | 213.0 | 320.3 (15) | 233.8 |
| Ala  | 40.8  | 33.8 (2) | 50.3  |
| Cys  | 3.2   | 2.8 (0)  | 3.3   |
| Val  | 28.4  | 2.8 (0)  | 28.0  |
| Met  | 6.0   | 2.3 (0)  | 5.7   |
| Ile  | 8.5   | 6.9 (0)  | 12.0  |
| Leu  | 28.8  | 54.6 (3) | 30.0  |
| Tyr  | 7.5   | 4.8 (0)  | 9.7   |
| Phe  | 11.4  | 20.6 (1) | 15.3  |
| Lys  | 10.7  | 9.1 (0)  | 13.7  |
| His  | 18.8  | 6.4 (0)  | 19.0  |
| Arg  | 18.1  | 7.6 (0)  | 6.0   |
| Pro  | 31.6  | 35.8 (2) | 27.6  |
| Total | 1,000 | 1,000 (45)| 1,000 |
| GalNH₂† | 3581.2 | 2269.5 | 3097.2 |

*No correction was made for the destruction of amino acids during HCl hydrolysis.
†Numbers of amino acid residues were calculated on the basis of the mol wt (46,000) of PG-II. Values in parentheses are nearest integers.
‡Numbers of hexosamine residues are expressed per 1,000 amino acid residues without correction for destruction during hydrolysis.

Table 2. Chemical Compositions of the Purified Proteoglycans From Rabbit Bone Marrow

<table>
<thead>
<tr>
<th>Component</th>
<th>PG-I</th>
<th>PG-II</th>
<th>PG-III</th>
</tr>
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<tbody>
<tr>
<td>Glucuronic acid</td>
<td>38</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>N-Acetylgalactosamine</td>
<td>41</td>
<td>40</td>
<td>41</td>
</tr>
<tr>
<td>Sulfate*</td>
<td>16</td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td>Peptide†</td>
<td>5</td>
<td>8</td>
<td>6</td>
</tr>
</tbody>
</table>

*Taken from analysis with the Morgan-Elson reaction of ΔDi-OS produced by digestion of galactosaminoglycans with chondroitinase AC-II plus chondroitinase A-0.5m (see details in the text).
†Taken from analysis of amino acid composition.
digests were treated with water). These fractions were treated with alkaline borohydride and then digested extensively with pronase. The material eluted from PG-I; (B) That from PG-II; (C) That from PG-III. by treatment with alkaline borohydride were applied to a column (1 x 113 cm) of Bio-Gel A-0.5m. The eluting system was the same as described for Fig 8. Arrows show the void volume and the total volume of the column. Molecular heterogeneity of these galactosaminoglycans was also examined by two-dimensional electrophoresis on cellulose acetate membrane. Galactosaminoglycans were detected by alcian-blue staining. Electrophoretic patterns are shown on the left hand of the individual elution profiles. The standard glycosaminoglycans used as external references were hyaluronic acid (HA), chondroitin sulfate A (CS-A), chondroitin sulfate C (CS-C), dermatan sulfate (DS), heparan sulfate (HS), and heparin (HP). (A) Galactosaminoglycan derived from PG-I; (B) That from PG-II; (C) That from PG-III.

clearly indicated that all three chondroitin sulfates did not contain a detectable amount of 4-sulfated repeating disaccharide unit in their chains (data not shown).

Identification of hyaluronic acid. Previously rabbit bone marrow was found to contain hyaluronic acid accounting for approximately 16% of the total glycosaminoglycan of the tissue. However, no hyaluronic acid was detected in any proteoglycan fractions obtained during purification of the proteoglycans from the urea-soluble fraction. The authors, therefore, tested for the presence of hyaluronic acid in other fractions obtained during the purification (ie, the guanidine HCl-residual fraction, the urea-insoluble fraction, and the water-insoluble fraction obtained on dialysis of the 0.35 mol/L NaCl fraction from first DEAE-Sephasel column against water). These fractions were treated with alkaline borohydride and then digested extensively with pronase. The digests were treated with 5% trichloroacetic acid at 0°C, and the insoluble material was removed by centrifugation. The combined supernatant was dialyzed against water, freeze dried, and dissolved in 1 mol/L NaCl/50 mmol/L Tris HCl, pH 7.3. Aliquots of the samples were applied to the column of Bio-Gel A-0.5m, and hexuronate in the eluate was monitored (Fig 12 A, B, and C). Peak fractions of hexuronate were pooled, and their hexuronate content was determined. The results indicated that the recoveries of hexuronate into the excluded fractions were 80%, 84%, and 24% for the samples derived from the guanidine HCl-residual, urea-insoluble, and water-insoluble fractions, respectively; 46% of hexuronate in the sample from the water-insoluble fraction was recovered in the much retarded fraction (Fig 12C).

The four samples were adjusted to 20 mmol/L sodium acetate, pH 6.0, digested with hyaluronidase, and applied to a column of Bio-Gel P-6. Approximately 40% of the material in the guanidine HCl-residual fraction and almost all the material in the urea-insoluble fraction and in the excluded fraction of the water-insoluble fraction were depolymerized by digestion with Streptomyces hyalurolyticus hyaluronidase (Fig 12 D, E, and F). These materials were thus identified as hyaluronic acid. The hexuronate-containing material in the retarded fraction from the water-insoluble fraction was not sensitive to the enzyme (data not shown).

Since the material eluted from the column had a similar K_d value to chondroitin 6-sulfate (see Figs 9 and 12C), it was...
1208
30 40 50 60
TUBE NUMBER

row tissues from femora and tibiae contained 2.44 mg hexuronate/g of defatted tissue, and approximately 90% of the hexuronate was recovered in the urea-soluble fraction. From this fraction the chondroitin 6-sulfates were isolated as three molecular forms of proteoglycans (PG-I, PG-II, and PG-III) in a total yield of approximately 56% (PG-I, 0.30 mg; PG-II, 0.68 mg; PG-III, 0.39 mg as hexuronate). They did not exhibit a detectable absorbance at 260 nm or 280 nm, and their chemical compositions showed that they contained 5% to 8% protein (Table 2). The mol wts of PG-I, PG-II, and PG-III were determined to be approximately 8,300, 46,000, and 16,000, respectively, by sedimentation-equilibrium centrifugation methods. Their mol wts, especially those of PG-I and PG-III, were much smaller than those of other interstitial chondroitin sulfate proteoglycans so far reported. The chemical compositions of the three were very similar, suggesting that the two smaller components were degradation considered to be a chondroitin sulfate. The data indicated that approximately 75% of the total hyaluronic acid present in the bone marrow was recovered in these fractions (ie, 21% in the guanidine HCl-residual fraction, 30% in the urea-insoluble fraction, and another 24% in the water-insoluble fraction), with no significant amount of hyaluronic acid in the soluble fractions.

DISCUSSION

The authors reported previously that the glycosaminoglycans present in rabbit bone marrow are composed of chondroitin 6-sulfate (79%) and hyaluronic acid (16%) and that the chondroitin sulfate is localized in the extracellular matrix by using a MoAb (3-B-3) specific to unsaturated 6-sulfated disaccharide generated at the nonreducing ends of chondroitin sulfate chains. The main object of this study was to isolate and characterize the chondroitin 6-sulfates of rabbit bone marrow as intact molecules and hyaluronic acid. The whole bone marrow tissues from femora and tibiae contained 2.44 mg hexuronate/g of defatted tissue, and approximately 90% of the hexuronate was recovered in the urea-soluble fraction. From this fraction the chondroitin 6-sulfates were isolated as three molecular forms of proteoglycans (PG-I, PG-II, and PG-III) in a total yield of approximately 56% (PG-I, 0.30 mg; PG-II, 0.68 mg; PG-III, 0.39 mg as hexuronate). They did not exhibit a detectable absorbance at 260 nm or 280 nm, and their chemical compositions showed that they contained 5% to 8% protein (Table 2). The mol wts of PG-I, PG-II, and PG-III were determined to be approximately 8,300, 46,000, and 16,000, respectively, by sedimentation-equilibrium centrifugation methods. Their mol wts, especially those of PG-I and PG-III, were much smaller than those of other interstitial chondroitin sulfate proteoglycans so far reported. The chemical compositions of the three were very similar, suggesting that the two smaller components were degradation

Fig 11. Paper chromatography of digests of the galactosaminoglycans with chondroitinase AC-II. The galactosaminoglycans eluted from a column of Bio-Gel A-0.5m (Fig 9) were precipitated with 70% ethanol containing 1% potassium acetate and 0.2 mmol/L EDTA and dissolved in a small volume of water. Samples (each containing 10 to 13 μg hexuronate) were digested with chondroitinase AC-II (0.06 unit) for 30 minutes at 37°C, and the digests were chromatographed with ΔDi-OS, ΔDi-4S, and ΔDi-6S as external references on Toyo No. 51A paper. The paper was then stained with silver nitrate. Lanes A, B, and C show the chromatographic patterns of digests of the samples derived from PG-I, PG-II, and PG-III, respectively. Lanes R show the chromatogram of the external references.
products of the core peptide of the largest component, PG-II, formed during the purification procedure. However, this possibility is unlikely for the following reasons. (1) Proteinase inhibitors were added to all solutions used for extraction and fractionation. (2) When the proteoglycans synthesized in vitro by freshly isolated rabbit bone marrow cells in the presence of [35S]sulfate and [3H]glucosamine were purified by a different method, which consisted of simplified procedures including extraction followed directly by CsCl density-gradient centrifugation to remove almost all proteins from a proteoglycan fraction under a dissociative condition and the continuous presence of proteinase inhibitors, the same three molecular species were isolated. These indicate that PG-I and PG-III did not arise during purification by degradation of PG-II but existed as these three molecular forms in bone marrow tissue.

All the three proteoglycans had very high contents of serine and glycine, accounting for approximately 60% of their total amino acid contents. On the basis of the chemical composition of PG-II and the mol wt of its chondroitin sulfate side chains described below, the core peptide of PG-II was calculated to be composed of 45 amino acid residues of nine amino acid species. There are some reports of proteoglycans with core peptides that have high contents of serine and glycine. Bourden et al. demonstrated that on the basis of the sequence of a core peptide cDNA of chondroitin sulfate proteoglycan produced by rat yolk-sac tumor cells, the core peptide is composed of 104 amino acids and consists of three regions, including a central region of 49 amino acids composed of alternating serine and glycine residues. It is noteworthy that the yolk sac is the first hematopoietic environment in embryonic development, where the blood islands are formed. It is therefore possible that these proteoglycans are characteristic of the matrix essential for hematopoiesis.

Unlike other proteoglycans rich in the serine and glycine contents, the largest proteoglycan, PG-II, was depolymerized by digestion with pronase and papain. However, the chondroitin sulfates of the three proteoglycans were released by alkaline borohydride treatment, indicating that the chondroitin sulfate chains are linked to serine residues of the core peptides through an alkali-labile bond. The chondroitin sulfates released from PG-I, PG-II, and PG-III were very homogeneous in both chain length and charge density, and their mol wt s were estimated as 7,700, 10,900, and 14,400, respectively. This result, together with the results on the chemical compositions and mol wt s of the proteoglycans, indicates that PG-II consisted of four chains of chondroitin sulfate and a core peptide of approximately mol wt 4,000, whereas the two smaller proteoglycans, PG-I and PG-III, consisted of a single chondroitin sulfate chain and a small peptide of approximate mol wt 500 and 1,000, respectively.

In previous studies the authors found that the bone marrow contained hyaluronic acid accounting for 16% of the total glycosaminoglycan of the tissue. In the extraction and fractionation procedures of proteoglycans adopted here, approximately 20% of the hyaluronic acid remained in the guanidine HCl-residual fraction. Furthermore, the hyaluronic acid extracted with guanidine HCl was mostly recovered in the insoluble fractions obtained during dialysis of the extract against 7 mol/L urea solution and during dialysis of the 0.35-mol/L NaCl fraction from DEAE-Sephadex against water. The hyaluronic acid in these insoluble fractions was therefore considered to form aggregates with another macromolecule(s) in the absence of an appropriate concentration of salts and to become insoluble. The hyaluronic acid in these fractions became soluble in water after successive treatment with alkaline borohydride and then with pronase, suggesting that bone marrow contains material(s) with specific affinity to bind hyaluronic acid at low-salt concentration and that the material may play an important role in formation of the bone marrow matrix, as shown in the case of formation of the matrix of cartilage.

In this study the cell types responsible for the synthesis of the chondroitin 6-sulfate proteoglycans and hyaluronic acid were not elucidated. But for the following reasons they are probably synthesized and secreted into the bone marrow matrix by nonhematopoietic cells (ie, stromal cells such as fibroblasts, adipocytes, endothelial cells, and/or reticular cells). (1) The stromal cells in a long-term bone marrow culture synthesize chondroitin sulfate proteoglycans, and the proteoglycans were involved in construction of the matrix in vitro, although the isomeric structure of their chondroitin sulfate side chains have not been determined in those studies. (2) The cytoplasmic granular sulfated glycosaminoglycans found in peripheral blood constituents such as platelets and leukocytes are predominantly chondroitin 4-sulfates regardless of animal species and have been demonstrated to be synthesized by their precursor cells such as megakaryocytes and granulocytes in bone marrow. In fact, the authors' preliminary study of the biosynthesis of sulfated glycosaminoglycans using rabbits labeled in vivo with [35S]sulfate demonstrated that (1) the bone marrow contained a significant amount of radiolabeled chondroitin 4-sulfate (about 9% of the total 35S-labeled glycosaminoglycans) and heparan sulfate-like molecules (about 10% of that) with the very high specific radioactivities to hexuronic acid as compared to that of chondroitin 6-sulfate; and (2) platelets and leukocytes of the animal contained those glycosaminoglycan species but not chondroitin 6-sulfate at all.

It is, however, still unclear at present whether the three chondroitin 6-sulfate proteoglycans are totally localized in the extracellular matrix or not, and if it is the case, their functional roles on hematopoiesis remain to be resolved. The most notable feature of these proteoglycans is their chondroitin sulfate side chains, which are composed solely of a 6-sulfated disaccharide unit. Such proteoglycans have not been identified in other adult mammalian tissues. Sampaio and Dietrich suggested that chondroitin 6-sulfate acts as a stimulant of cell division on the basis of their observation that the concentration of chondroitin 6-sulfate increases during bovine fetal development and decreases progressively to an undetectable level in most adult tissues. Since bone marrow, unlike most other tissues in adults, maintains continuous cell proliferation, the chondroitin 6-sulfate of bone marrow may be important in regulation of proliferation of hematopoietic cells. During preparation of this manuscript, Gordon et al. reported that glycosaminoglycans in bone marrow have a
role for compartmentalizing a hematopoietic growth factor (GM-CSF).

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Isolation and characterization of chondroitin 6-sulfate proteoglycans present in the extracellular matrix of rabbit bone marrow

E Okayama, K Oguri, T Kondo and M Okayama