Isolation and Characterization of Proteoglycans Synthesized by Cultured Mesangial Cells*

Eishin Yaoita, Kayoko Oguri, Eiko Okayama, Katsutoshi Kawasaki, Sentaro Kobayashi, Itaru Kihara, and Minoru Okayama

From the Clinical Research Institute, National Nagoya Hospital, 4-1-1 Sannomaru, Nakaku, Nagoya 460 and the Department of Pathology, Institute of Nephrology, Niigata University School of Medicine, Niigata 951, Japan

Rat mesangial cells selected by long-term culture of glomeruli exhibited a hill and valley appearance in the confluent state and were stained with antibodies against vimentin and desmin, suggesting that they are smooth muscle-like mesangial cells. The glycoconjugates produced by the cells were metabolically labeled with [35S]sulfate and [3H]glucosamine and extracted with 4 M guanidine HCl containing 0.5% Triton X-100. The radiolabeled glycoconjugates were separated on DEAE-Sephacel and compared with those synthesized by glomeruli labeled in the same conditions. Of the three major sulfated glycoconjugates, sulfated glycoprotein (17% of the total [35S]-labeled macromolecules), heparin sulfate proteoglycan (35%), and chondroitin sulfate proteoglycan (30%) synthesized by glomeruli, the cultured mesangial cells synthesized mainly chondroitin sulfate proteoglycan (more than 90%). After purification by CaCl₂ density-gradient centrifugation, the chondroitin sulfate proteoglycan from the cell layer was separated on Bio-Gel A-5m into three molecular species with estimated Mᵦ values of 230,000, 150,000, and 40,000-10,000, whereas that released into the medium consisted of a single species with an Mᵦ of 135,000. In the β-elimination reaction, the former two larger proteoglycans released chondroitin sulfate chains with Mᵦ of an apparent 30,000 and the latter from the medium released the glycosaminoglycan chains with an Mᵦ of 36,000. The Mᵦ of the smallest proteoglycan from the cell layer was not significantly changed after β-elimination, indicating that this species had only a small peptide, if any. Analysis with chondroitinase AC-II and ABC demonstrated that all the chondroitin sulfate proteoglycans were homogenous in chain length, demonstrating that they consist of sialoglycoconjugates and sulfated glycoconjugates (1-4). Of the sulfated glycoconjugates, heparin sulfate is present in the basement membrane as the predominant sulfated glycosaminoglycan (5-8) and plays a role in creating the normal restrictive permeability properties of the basement membrane via interactions with plasma proteins (9, 10). This glycosaminoglycan is also present in the mesangial matrix (7, 8). However, no information is available on whether the heparin sulfates that have these different localizations in the glomerulus have distinct isomeric structures and are present as distinct proteoglycan species. Chondroitin sulfate appears to be enriched in the mesangial matrix (7, 8), but its role is unknown. Another glomerular sulfated glycoconjugate, entactin, a sulfated glycoprotein with an Mᵦ of 158,000, which is located in the basement membrane, has been proposed to anchor the epithelial cells to the basement membrane (11).

Previous studies in our laboratory have indicated that freshly isolated glomeruli synthesize similar levels of three molecular species of sulfated glycoconjugates in vitro, i.e. sulfated glycoproteins probably including entactin, heparan sulfate proteoglycan, and chondroitin sulfate proteoglycan (12). Accumulated results indicate that the relative ratios of these macromolecules synthesized varies depending on the systems studied, such as an in vitro incubation system (12-15), a kidney perfusion system (16, 17), or an in vivo labeling system (14, 15, 18). There is now widespread agreement that basement membranes are extracellular matrices formed by epithelial and/or endothelial cells (19). Thus, although there is no direct evidence, it appears reasonable to consider that the heparan sulfate proteoglycan(s) of the glomerular basement membrane is predominantly synthesized by epithelial and endothelial cells. However, the question of which cell type(s) in the glomerulus synthesizes the glomerular chondroitin sulfate proteoglycan still remains unanswered.

The main purpose of this study was to provide direct evidence that glomerular chondroitin sulfate proteoglycan (14, 16, 20) is synthesized by mesangial cells. For this, chondroitin sulfate proteoglycan produced by cultured mesangial cells was compared with that synthesized by freshly isolated glomeruli under the same incubation conditions. The mesangial cells...
were isolated by long-term culture of rat glomeruli as described previously (22). For determination of the purity of the mesangial cell culture, changes in cell types that occurred during this long-term culture were followed by double immunofluorescence staining with various antibodies against polypeptides of intermediate-sized filaments. The mesangial cell culture thus obtained was labeled with $^{[35]S}$ sulfate or $[^3H]$ glucosamine, and radiolabeled proteoglycans were isolated and characterized. The results indicated that the cultured mesangial cells synthesized predominantly chondroitin sulfate proteoglycan consisting of two large molecular species associated with the cell layer and a single species released into the medium, all of which were similar in their $M_r$ values and the isomeric structures of their chondroitin sulfate chains to those of the species synthesized by the glomeruli in vitro. This clearly indicates that most, if not all, of the glomerular chondroitin sulfate proteoglycan is synthesized by mesangial cells. In addition to this proteoglycan, the cells also synthesized hyaluronic acid at a similar level to the chondroitin sulfate proteoglycan and a small, but significant, amount of heparan sulfate proteoglycan. A preliminary report of part of this work has been presented in a communication (22).

**EXPERIMENTAL PROCEDURES**

**Materials**—Female Fisher 344 rats (8-12 weeks old) bred in our institution were used. Mouse monoclonal antibodies against rat Thy-1, rat Ia antigen, and rat leukocyte-common antigen were obtained from Sera-Lab Ltd. (Sussex, United Kingdom). Anti-vimentin was from Boehringer Mannheim. Rabbit antiserum against human cytokeratin was from DAKO Co. (Santa Barbara, CA), and desmin from Bio-Science Products AG (Emmenbruck, Switzerland). Rabbit antiserum against human von Willebrand factor was kindly provided by Dr. M. Handa of Keio University. Authentic glycosaminoglycans, hyaluronic acid from human umbilical cord, chondroitin sulfate A from whale cartilage and chondroitin sulfate C from shark cartilage, authentic unsaturated disaccharides, 2-acetamido-2-deoxy-3-O-(β-D-glucopyranosyluronic acid)-D-galactose (ΔDi-OS), 2-acetamido-2-deoxy-3-O-(β-D-glucopyranosyluronic acid)-4-O-sulfod-galactose (ΔDi-4S), and 2-acetamido-2-deoxy-3-O-(β-D-glucopyranosyluronic acid)-6-O-sulfod-galactose (ΔDi-6S), and the enzymes hyaluronidase from Streptomyces hyaluronicus, chondroitinase AC-II from Arthrobacter actinomyces, and chondroitinase ABC from Arthrobacter ulei were obtained from Seikagaku Kogyo Co. (Tokyo, Japan). Chondroitin 4-sulfate proteoglycan (M, 136,000) and its glycosaminoglycan side chain (M, 98,000) and two chondroitin 6-sulfate proteoglycans (M, 46,000 and 16,000) and their glycosaminoglycan side chains (M, 14,400 and 10,900) prepared from human platelets (23) and rabbit bone marrow (24), respectively, were used as standard molecules for estimation of $M_r$ values of proteoglycans and their glycosaminoglycan side chains. Over-sulfated unsaturated disaccharide, 2-acetamido-2-deoxy-3-O-(β-D-glucopyranosyluronic acid)-4,6-O-disulfod-galactose (ΔDi-DisG) was a gift from Dr. O. Habuchi of Aichi Kyiku University. It was prepared by digestion of chondroitin sulfate E from squid cartilage with chondroitinase ABC (25). D-[6-$^3$H]glucosamine (19 Ci/mmol) was purchased from Du Pont-New England Nuclear and $^{[35]S}$ sulfate (carrier-free) from Radioisotope Association (Tokyo). D-[6-$^3$H]glucosamine (19 Ci/mmol) was purchased from Du Pont-New England Nuclear and $^{[35]S}$ sulfate (carrier-free) from Radioisotope Association (Tokyo).

**Cell Culture**—Mesangial cell cultures were prepared as described previously (21). Briefly, kidneys were excised and the cortices were cut away from the medullae. Glomeruli were isolated by serial sieving from the medullae. Glomeruli were isolated by serial sieving cut away from the medullae. Glomeruli were isolated by serial sieving.

1 The abbreviations used are: ΔDi-OS, 2-acetamido-2-deoxy-3-O-(β-D-glucopyranosyluronic acid)-D-galactose; ΔDi-4S, 2-acetamido-2-deoxy-3-O-(β-D-glucopyranosyluronic acid)-4-O-sulfod-galactose; ΔDi-6S, 2-acetamido-2-deoxy-3-O-(β-D-glucopyranosyluronic acid)-6-O-sulfod-galactose; ΔDi-DisG, 2-acetamido-2-deoxy-3-O-(β-D-glucopyranosyluronic acid)-4,6-O-disulfod-galactose; US, urea-soluble.

**Preparation of Protein-free Glycosaminoglycans**—Radiolabeled proteoglycan samples were mixed with 200 μg each of chondroitin sulfate A and chondroitin sulfate C, and then 3 volumes of 95% ethanol, 1.3% potassium acetate, 0.27 mM EDTA was added and the mixture was placed in ice-water for 2 h to coprecipitate the radiolabeled proteoglycans with the carrier glycosaminoglycans. The precipitated material was washed twice with ethanol solution, dissolved in 0.2 M NaOH containing 1 M sodium borohydride, and incubated for 12 h at room temperature. The reaction was terminated by adjusting the pH to 5.5 with 4 M acetic acid. Macromolecules in the reaction mixture were then precipitated with ethanol solution, and digested extensively with Pronase. The resulting glycosaminoglycans were again precipitated, washed with ethanol solution, and dissolved in an appropriate buffer.

**Enzymatic Treatments**—Samples were digested with Pronase (1 mg/ml) in 2 mM CaCl₂, 0.1 M Tris-HCl, pH 8.0, at 50°C for 72 h.
under toluene with further addition of 0.5 mg of enzyme at 24 h intervals. Digestion with chondroitinase AC-II, ABC (25), or hyaluronidase (27) was carried out as described previously.

**Chemical Analysis**—Protein concentrations were measured by the method of Lowry et al. (28). Hexuronic acid was measured by the method of Bitter and Muir (29). DNA was assayed by the method of Hinegardner (30) with dAMP as a standard.

**RESULTS**

**Selection of Mesangial Cells by Long-term Culture of Rat Glomeruli**—Isolated rat glomeruli were mostly decapsulated and few tubular fragments were present in the preparation (Fig. 1A). Most of the cells that grew out first from the glomeruli had a cobblestone-like polygonal shape (Fig. 1B). Stellate cells began to overlap the polygonal cells on day 10 (Fig. 1C) and had increased in number by day 15 (Fig. 1D). The polygonal cells disappeared and the stellate cells predominated on day 20 (Fig. 1E). On day 21, the cells were subcultured. Four weeks after subculture, the cells had become confluent and had a hill and valley appearance (Fig. 1F).

To characterize the cell types growing out during long-term culture of glomeruli, we examined cultures at different stages by immunofluorescence studies using antibodies against cytoskeletal intermediate-sized filaments, cytokeratin, vimentin, and desmin, which are known to be present in epithelial cells, mesenchymal-derived cells, and smooth muscle cells, respectively. Double staining of the primary culture with anti-cytokeratin and anti-vimentin showed that the cells growing out from glomeruli after culture for 7 days consisted of three types of cells (Fig. 2, A and B). Most of the cobblestone-like cells that predominated on day 7 of culture gave positive reaction with anti-cytokeratin and weakly positive reaction with anti-vimentin (large arrowheads in Fig. 2, A and B). Other polygonal cells scarcely reacted with anti-cytokeratin but gave a strong positive reaction with anti-vimentin (small arrowheads). Neither of the cell types of cobblestone-like cells gave a positive reaction with antibody against desmin or von Willebrand factor (21). The stellate cells, that grew out later stained intensely with anti-vimentin but not at all with anti-cytokeratin (arrows). These stellate, anti-vimentin positive cells had increased in number on day 11 (Fig. 2, C and D). These cells were also stained with anti-desmin and anti-Thy-1 (21). On day 20 of primary culture, no anti-cytokeratin-reactive cells were observed (Fig. 2E) and only the stellate cells that reacted with anti-vimentin were presented (Fig. 2F). On day 21, these stellate cells were subcultured. When doubly stained with anti-vimentin and anti-desmin on day 14 after subculture, all the cells reacted with both antibodies (Fig. 2, G and H). From these results, and the previous finding of the coexistence of these two intermediate filaments in mesangial cells in situ (31), we conclude that the subcultured cells consisted of smooth muscle-like mesangial cells.

**Extraction and Isolation of Proteoglycans**—Freshly isolated glomeruli and confluent cultures of mesangial cells were labeled by incubation with [35S]sulfate or [3H]glucosamine for 24 h, a period in which the culture glomeruli exhibited linear incorporation of [3H]glucosamine into macromolecules (12). Almost all the radiolabeled macromolecules associated with the tissue or the cell layer could be extracted with guanidine-Triton solution containing proteinase inhibitors. On equilibration of the extracts with urea-Triton solution, more than 95% of the 35S- and 3H-labeled guanidine-Triton X-100 soluble macromolecules were collected in the soluble fractions (Table I).

![Figure 1](http://example.com/) **Fig. 1.** Phase contrast micrographs of cells growing out during long-term culture of rat glomeruli. Isolated rat glomeruli explanted into a culture dish (A). Cells growing out from glomeruli cultured for 5 (B), 10 (C), 15 (D), and 20 (E) days. Cells cultured for 4 weeks after subculture on day 21 of primary culture (F). G, glomeruli. Bar, 100 μm.
**Fig. 2.** Indirect double immunofluorescence micrographs of cells growing out during long-term culture of glomeruli with antibodies against cytoskeletal intermediate-sized filaments. Cultures were stained with anti-cytokeratin on day 7 (A), 11 (C), and 20 (E) of primary culture of glomeruli. The same cultures were also stained doubly by anti-vimentin (B, D, and F). Cells cultured for 2 weeks after subculture were stained with anti-desmin (G) and anti-vimentin (H). Three types of cells (large arrowsheads, small arrowsheads, and arrows) were distinguishable on day 7 of primary culture. The subculture consisted of one type of cells. Bars, 100 μm.

**Table I**

Incorporation of $^{35}$S-sulfate and $[^3H]$glucosamine into glycoconjugates synthesized by cultured rat mesangial cells and glomeruli

Mesangial cells in the confluent state (2–3 x 10$^5$ cells/21-cm$^2$ dish) and freshly isolated glomeruli (2 x 10$^4$ glomeruli/ml) were labeled with 50 μCi of $^{35}$S-sulfate or $[^3H]$glucosamine/ml for 24 h. Labeled macromolecules were prepared from the cells or tissues and media as described under "Experimental Procedures." The DNA contents of mesangial cells were measured by the method of Hinegardner (30) after removal of salts from the samples. Figures in parentheses represent percentages.

<table>
<thead>
<tr>
<th>Cells or tissues</th>
<th>Medium</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesangial cells</td>
<td>$^{35}$S$^a$ 887 (52)</td>
<td>820 (48)</td>
</tr>
<tr>
<td>Glomeruli</td>
<td>$^{35}$S$^b$ 2840 (76)</td>
<td>902 (24)</td>
</tr>
</tbody>
</table>

$^a$ cpm x 10$^{-3}$/mol DNA.

$^b$ cpm x 10$^{-7}$/10$^4$ glomeruli.

The radiolabeled urea soluble (US) fractions and the macromolecular fractions from the medium were each subjected to ion-exchange chromatography on DEAE-Sephacel in the presence (the former samples) or the absence (latter samples) of urea-Triton X-100. On elution of materials with a linear gradient of NaCl, $^{35}$S-labeled macromolecules of both the US fraction and medium fraction, from the culture of glomeruli, were separated into three radioactive peaks with approximately 80% of column recoveries; 17% in T-SGP, and 17% in M-SGP, 40% in T-HSPG, and 30% in M-HSPG, and 28% in T-CSPG, and 34% in M-CSPG (Fig. 3, A and B). $^{35}$S-Labeled materials in the T-SGP and M-SGP fractions were previously shown to be sulfated glycoproteins and those of the T-HSPG and M-HSPG fractions to be heparan sulfate proteoglycans (12). The radiolabeled materials in the T-CSPG and M-CSPG fractions were subsequently demonstrated to consist of proteoglycans with highly sulfated chondroitin sulfate (20). On chromatography of the $^{35}$S-labeled US fraction from the cell layer and medium fraction, of the mesangial cell culture, 82%
Fig. 3. Ion-exchange chromatography on DEAE-Sephacel of \(^{35}S\)- and \(^{3}H\)-labeled macromolecules synthesized by glomeruli and mesangial cells. The \(^{35}S\)- and \(^{3}H\)-labeled US fractions from glomerular tissue and the cell layer and the medium macromolecular fractions were each applied to a column (1 × 7 cm) of DEAE-Sephacel as described in the text. Materials were eluted with 400 ml of a linear gradient of 20 mM to 1 M NaCl in the presence (for the US fractions) or absence (for the medium fractions) of 7 M urea and 0.5% Triton X-100. The flow rate was 8 ml/h and fractions of 2.1 ml were collected. Elution profiles of the \(^{35}S\)-labeled US fraction from the tissue (A) and the medium fraction (B) of the culture of glomeruli, the samples from the cell layer (C) and medium (D) of a mesangial cell culture, and those of the \(^{3}H\)-labeled US fraction from the cell layer (E) and the medium fraction (F) of the mesangial cell culture.

Identification of Hyaluronic Acid—When the \(^{3}H\)-labeled materials in fractions C-HA and M-HA were chromatographed on a column of Bio-Gel A-5m, 49 and 68% of the \(^{3}H\) radioactivities of the respective samples were excluded from the column, and the remaining radioactivities were retarded on the column (data not shown). The \(^{3}H\)-labeled materials collected in these four fractions were analyzed by gel chromatography on Bio-Gel P-6 with authentic hyaluronic acid before and after digestion with hyaluronidase (Fig. 4). Before digestion, authentic hyaluronic acid and all the \(^{3}H\)-labeled samples except the fraction of the M-HA sample retarded on Bio-Gel A-5m were excluded from the column. Digestion of authentic hyaluronic acid with the enzyme resulted in generation of three compounds eluted in fractions a, b, and c (Fig. 4A), which were calculated to be tetra-, tri-, and di-repeating disaccharides having unsaturated hexuronate at their nonreducing ends, respectively, on the basis of their molarities of hexuronate and unsaturated hexuronate. Digestion of \(^{3}H\)-labeled materials with a large M, from the C-HA sample (fraction excluded from Bio-Gel A-5m) yielded the three oligosaccharides (Fig. 4B). On digestion of \(^{3}H\)-labeled mate-rial with the small M, from the C-HA sample (retarded fraction on the column), 64% of the radioactivity was susceptible to the enzyme but the remaining material was resistant (Fig. 4C). About 80% of the material with a large M, of the M-HA sample was susceptible to digestion with hyaluronidase, the remaining being resistant (Fig. 4D). The M-HA-derived material of small M, was separated on Bio-Gel P-6 into two fractions, one in the void volume fraction (35% of the sample) and the other \(K_p 0.16 (65\%)\) (Fig. 4E). This was unexpected because the M-HA sample had been excluded from a Bio-Gel P-6 column in the step for removal of unincorporated \(^{3}H\] of the former sample and 89% of the latter was eluted as a single sharp peak, at a slightly lower NaCl concentration than the materials in fractions T-CSPG and M-CSPG (C-PG and M-PG in Fig. 3, C and D) and less than 5% of radiolabeled materials were eluted in the fractions corresponding to fractions T- and M-HSPG. As shown in Fig. 3, E and F, 18% of the \(^{3}H\)-labeled cell layer-derived sample and 28% of the material were excluded from the column, and the remaining radioactivities were retarded in the fractions corresponding to fractions C-HA and M-HA, accounting for 12 and 19%, respectively, of the total samples, were mostly hyaluronic acid as described above.

Fig. 4. Gel chromatography on Bio-Gel P-6 of digests of \(^{3}H\)-labeled hyaluronic acid with or without treatment with S. hyaluronitieus hyaluronidase. \(^{3}H\)-Labeled C-HA- and M-HA, fractions were desalted against water and freeze-dried. The resulting material was dissolved in 4 M guanidine-HCl, 50 mM Tris-HCl, pH 7.3, and applied to a column of Bio-Gel A-5m (data not shown). The combined fractions of materials excluded from and retarded in the column, respectively, were desalted and supplemented with 400 \(\mu\)g of hyaluronic acid as a carrier. Each sample was divided into two portions, one of which was treated with hyaluronidase. The samples were then applied to a column (1 × 50 cm) of Bio-Gel P-6 in 0.5 M ammonium acetate, pH 5.5. The flow rate was 3.6 ml/h and fractions of 0.6 ml were collected. Elution profiles: untreated (O) and hyaluronidase treated (●) authentic hyaluronic acid (A); untreated and hyaluronidase-treated \(^{3}H\)-labeled materials in the void volume fraction (B) and retarded fraction (C) on Bio-Gel A-5m chromatography of the C-HA fraction, \(^{3}H\)-labeled materials in the void volume fraction (D) and the retarded fraction (E) on the Bio-Gel A-5m column of the M-HA fraction. The right upper panel shows the calibration curve obtained from the elution positions of octa- (a), hexa- (b), and tetra- (c) saccharides.
Some macromolecule(s) in the labeling medium. Possibly on the first Bio-Gel P-6 column developed with 20 mM NaCl, 20 mM Tris-HCl, pH 7.5, the ³H-labeled small materials were coeluted with some macromolecule(s) in the medium and were separated into void volume and retarded fractions on Bio-Gel A-5m chromatography in 4 M guanidine HCl solution (see "Experimental Procedures"). From a calibration curve obtained for the elution positions of the hyaluronate-derived unsaturated oligosaccharides (calibration curve in Fig. 4), the material retarded on the column was calculated to consist of 10–12 monosaccharides. After digestion with the enzyme, approximately 90% of the excluded material and 74% of the retarded material were converted to octa-, hexa-, and tetrasaccharides. In addition to the hyaluronic acids eluted in these hyaluronate-enriched fractions, this glycosaminoglycan was eluted in the sulfated proteoglycan fractions C-PG and M-PG and all the materials in these fractions had a large hydrodynamic size and so were excluded from the Bio-Gel A-5m as described below. The distributions of the ³H-labeled hyaluronic acids recovered in these fractions are summarized in Table II.

Characterization of Sulfated Proteoglycans Synthesized by Cultured Mesangial Cells—Equal amounts of radioactivities of ³⁵S- and ³H-labeled materials in fractions C-PG or M-PG were mixed (referred as ³⁵S/³H-labeled samples) and subjected to CsCl density-gradient centrifugation in guanidine-Triton solution (Fig. 5). The ³H-labeled proteoglycans mostly sedimented to the bottom six fractions with small tailing, but significant amounts of ³H-labeled materials were recovered at densities of 1.41–1.23 g/ml as a radioactive peak. The ³H-labeled materials of these middle fractions were identified mainly as hyaluronic acid with a large Mₐ as described below, indicating that significant amounts of hyaluronic acid were coeluted with sulfated proteoglycans from DEAE-Sephacel.

The bottom and middle fractions were pooled together and analyzed by gel filtration on Bio-Gel A-5m under a dissociative condition. ³⁵S/³H-Labeled proteoglycan derived from the cell layer was eluted from the column as two sharp peaks at partition coefficients, Kₐ values, of 0.10 and 0.19 and as a broad peak from KD 0.41–0.86 (Fig. 6A). The higher ratio of ³H to ³⁵S of the KD 0.10 component than of the KD 0.19 component was apparently due to hyaluronic acid excluded from the column. ³⁵S/³H-Labeled proteoglycan from the medium eluted as a single sharp peak at KD 0.21, and some ³H-labeled material excluded from the column (Fig. 6B) was also identified as hyaluronic acid. On the basis of a calibration curve with chondroitin sulfate proteoglycans (inset in Fig. 6B), the Mₐ of the components with KD values of 0.10 and 0.19 from the cell layer and the sulfated proteoglycan derived from the medium with a KD of 0.21 were estimated to be approximately 230,000, 150,000, and 135,000, respectively. The Mₐ of the ³⁵S/³H-labeled material eluted from KD 0.41–0.85 was estimated to be approximately 40,000–10,000. This small sulfated proteoglycan was found only in the cell layer but not in the medium.

Identification of Glycosaminoglycans—The ³⁵S/³H-labeled proteoglycans in fractions C-PG1 and M-PG were treated with alkaline borohydride to release glycosaminoglycans, and the released glycosaminoglycans were analyzed by gel chromatography on Bio-Gel A-5m. Of the ³H-labeled glycosaminoglycan released from the C-PG1 sample, 88% was coeluted from the column with the ³⁵S-labeled one at KD 0.38, and the remainder was eluted in the void volume without ³⁵S radioactivity (Fig. 7A). Of the ³H-labeled glycosaminoglycan from the M-PG sample, 86% was coeluted with ³⁵S-labeled one as a single sharp peak at KD 0.33 and the remainder was excluded from the column without ³⁵S radioactivity (Fig. 7B). All the ³H-labeled glycosaminoglycans excluded from the column were susceptible to hyaluronidase, being converted to the three oligosaccharides as described above (data not shown), indicating that they are all hyaluronic acid. These hyaluronic acids in fractions C-PG and M-PG would not come from fractions C-HA and M-HA on DEAE-Sephacel chromatography because only small amounts of hyaluronic acids were detected in the fractions eluted between the C-HA and C-PG.

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**Table II**

Distribution of ³H-labeled hyaluronic acids in various fractions

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Large hyaluronic acid</th>
<th>Small hyaluronic acid</th>
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<tbody>
<tr>
<td>C-HA</td>
<td>10.0 %</td>
<td>ND*</td>
</tr>
<tr>
<td>C-PG</td>
<td>10.5 %</td>
<td>ND*</td>
</tr>
<tr>
<td>M-HA</td>
<td>16.2 %</td>
<td>ND*</td>
</tr>
<tr>
<td>M-PG</td>
<td>16.2 %</td>
<td>ND*</td>
</tr>
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* ND, not detected.

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**Fig. 5.** CsCl density-gradient centrifugation of radiolabeled sulfated proteoglycans. Equal amounts of radioactivities of the ³⁵S- and ³H-labeled proteoglycans in fractions C-PG and M-PG were mixed, dialyzed against water, and freeze-dried. The resulting materials were dissolved in guanidine-Triton solution. Solid CsCl was added to give an initial density of 1.44 g/ml. A density gradient was formed by centrifugation at 170,000 × g for 72 h at 15 °C. The radioactivities of ³⁵S (○) and ³H (▲) in aliquots of each fraction were measured. Dashed lines show the densities of the fractions. The sedimentation patterns of the ³⁵S/³H-labeled C-PG fraction (A) and M-PG fraction (B) are shown.
FIG. 6. Gel chromatography on Bio-Gel A-5m of $^{35}$S/$^3$H-labeled proteoglycans. The $^{35}$S/$^3$H-labeled proteoglycan fractions obtained by CsCl density-gradient centrifugation were digested against water, freeze-dried, and dissolved in guanidine-Triton solution. The sample was applied to a column (1 × 114 cm) of Bio-Gel A-5m. The flow rate was 3.2 ml/h and fractions of 1.63 ml were collected. The radioactivities of $^{35}$S (●) and $^3$H (○) of aliquots of each fraction were measured. The elution profiles are for $^{35}$S/$^3$H-labeled proteoglycans from the cell layer (A) and the medium (B). The large arrows show the void volume and the total volume of the column. The inset in B shows the calibration curve obtained by plotting $K_D$ versus log $M_r$, of standard chondroitin sulfate proteoglycans. The arrows, 1–3, represent chondroitin 4-sulfate proteoglycan with a $M_r$ of 136,000 and chondroitin 6-sulfate proteoglycan with a $M_r$ of 46,000 and 19,000, respectively. The $M_r$ values were estimated by the sedimentation-equilibrium centrifugation method (23, 24). The arrows, a–c, indicated the elution positions of individual sulfated proteoglycan components.

fractions or the M-HA and M-PG fractions, representing the complete separation of these hyaluronic acids. On the basis of a calibration curve (inset in Fig. 7B), the $M_r$ values of $^{35}$S/$^3$H-labeled glycosaminoglycans derived from the C-PGl and M-PG proteoglycans were estimated to be approximately 30,000 and 36,000, respectively. The $M_r$ of the $^{35}$S/$^3$H-labeled small proteoglycan was not significantly changed before and after treatment with alkaline borohydride (data not shown), indicating that it contained only a small peptide(s), if any.

For examination of the isomeric structures of the $^{35}$S/$^3$H-labeled sulfated glycosaminoglycans obtained, the samples were first digested with chondroitinase AC-II and analyzed by gel chromatography on Bio-Gel P-6. Before digestion, the radiolabeled materials in all the samples were excluded from the gel (data not shown). The digest of the C-PGl-derived glycosaminoglycan separated into four molecular species on the column (Fig. 8A): enzyme-resistant sulfated glycosaminoglycan eluted in fraction R (accounting for 5 and 7% of the applied $^{35}$S and $^3$H, respectively), sulfated oligosaccharides, the chondroitinase AC-II-resistant portions of the glycosaminoglycan chains, emerging in broad peaks in fraction a (20% of $^{35}$S and 22% of $^3$H) and unsaturated disaccharides eluted in fractions b and c (57 and 56% of the $^{35}$S and $^3$H, and 14 and 7% of those radioactivities, respectively). The elution profile of the digest of M-PG-derived sulfated glycosaminoglycan with the enzyme was very similar to that from the C-PGl-derived one (Fig. 8B), giving fraction R (accounting for 9 and 10% of the applied $^{35}$S and $^3$H, respectively), fraction a (26% each of $^{35}$S and $^3$H) and fractions b and c (53 and 54%, and 10 and 5% of $^{35}$S and $^3$H, respectively). All the radioactivities eluted in fractions R were also resistant to chondroitinase ABC-digestion but depolymerized on treatment with nitrous acid (data not shown), indicating that they were heparan sulfate.

Paper chromatograms of the chondroitinase AC-II-digests with authentic unsaturated disaccharides showed that about 72 and 63% of the $^{35}$S- and $^3$H-labeled C-PGl-derived sulfated glycosaminoglycan and 63 and 60% of the M-PG-derived material, respectively, were depolymerized by digestion with the enzyme and comigrated with ADi-diS$_6$, ADi-diS$_5$, and ADi-diS$_4$, and the remainders were retained at the origin (Fig. 9, B and D). The same amounts of the radioactivities as recovered in fractions b and c on Bio-Gel P-6 were comigrated with ADi-diS$_6$, ADi-diS$_5$, and ADi-diS$_4$. On digestion with chondroitin ABC of the $^{35}$S- and $^3$H-labeled sulfated oligosaccharides recovered in fractions a on the Bio-Gel P-6 column (see Fig. 8), all the radiolabeled materials were further digested and comigrated with ADi-diS$_6$, ADi-diS$_5$, ADi-diS$_4$, and ADi-diS$_3$, ADi-diS$_2$, and ADi-diS$_1$ (Fig. 9, C and E). The relative radioactivities of $^{35}$S and $^3$H comigrating with these
unsaturated disaccharides are summarized in Table III. These results indicate that the sulfated glycosaminoglycan chains of both the C-PGl- and M-PG-proteoglycans consist of four repeating disaccharides, glucurono-N-acetylgalactosamine (chondroitinase AC-II and ABC susceptible unit), and iduronosyl-N-acetylgalactosamine (chondroitinase AC-II susceptible unit), both of which have sulfate groups at position 4 or 6 of their hexosamine moieties. The sulfated glycosaminoglycan derived from the C-PG1 proteoglycan (A) and the M-PG proteoglycan (B). Fraction R, containing 35S/3H-labeled glycosaminoglycans resistant to the enzyme and fraction a containing unsaturated sulfated oligosaccharides produced by enzyme treatment, respectively, were collected for further study. Fractions b and c contained unsaturated disaccharides consisting mostly of ADi-4S and ADi-diSr, respectively, as described previously (20). The arrows show the void volume and the total volume of the column.

DISCUSSION

Cultured mesangial cells were previously shown to have many similarities to vascular smooth muscle cells, such as exhibiting multilayered growth with a hill and valley appearance (32, 33), containing smooth muscle myosin and contracting in response to angiotensin II (34). We found previously that rat mesangial cells selected by long-term culture of glomeruli could be distinguished from aortic smooth muscle cells by the differences in their reactivities with antibodies against Thy-1 and desmin and differences in the size of their actin fibrils (21). Moreover, in the present study we found that mesangial cells stained doubly with antibodies against vimentin and desmin (Fig. 2, G and H) which generally form light. B and D, chromatographic patterns of digests with chondroitinase AC-II of radiolabeled sulfated glycosaminoglycans derived from C-PG1 and M-PG, respectively. C and E, profiles of digests with chondroitinase ABC of fractions a in A and B of Fig. 8.

FIG. 8. Gel chromatography of digests by chondroitinase AC-II of radiolabeled sulfated glycosaminoglycans on Bio-Gel P-6. 35S/3H-labeled glycosaminoglycan fractions from Bio-Gel A-5m were dialyzed against water and freeze-dried. The resulting material was dissolved in water and divided into two portions, one of which was treated with chondroitinase AC-II. Aliquots of the resulting samples were then applied to a column (1.101 cm) of Bio-Gel P-6 previously equilibrated with 0.5 M ammonium acetate, pH 5.5. Material was eluted with the same solution at a flow rate of 4 ml/h, and fractions of 1.4 ml were collected. The radioactivities of 35S (O) and 3H (●) in aliquots of each fraction were measured. Elution profiles are for digests of the sulfated glycosaminoglycan samples from the C-PGl proteoglycan (A) and the M-PG proteoglycan (B). Fraction R, containing 35S/3H-labeled glycosaminoglycans resistant to the enzyme and fraction a containing unsaturated sulfated oligosaccharides produced by enzyme treatment, respectively, were collected for further study. Fractions b and c contained unsaturated disaccharides consisting mostly of ADi-4S and ADi-diSr, respectively, as described previously (20). The arrows show the void volume and the total volume of the column.

FIG. 9. Paper chromatography of digests of radiolabeled glycosaminoglycans with chondroitinase AC-II and of unsaturated sulfated oligosaccharides with chondroitinase ABC. Fractions a in Fig. 8 were desalted by gel filtration on a column (1.5 × 80 cm) of Cellulofine GCL-25m in water and evaporated by a Vapor Mix S-10. The samples were then digested with chondroitinase ABC. These digests and aliquots of the same chondroitinase AC-II digests as analyzed by gel chromatography on Bio-Gel P-6 (see Fig. 8) were chromatographed on paper in 1-butanol, acetic acid, 1 NH,OH, 2:3:1 (v/v), for 30 h at room temperature. The distributions of radioactivities of 35S (open bars) and 3H (closed bars) were determined by counting 1-cm segments of the paper strip. A, paper chromatogram of authentic unsaturated disaccharides, ADi-diSa (a), ADi-6S (b), ADi-4S (c), and ADi-OS (d), as internal markers, detected by ultraviolet light. B and D, chromatographic patterns of digests with chondroitinase AC-II of radiolabeled sulfated glycosaminoglycans derived from C-PG1 and M-PG, respectively. C and E, profiles of digests with chondroitinase ABC of fractions a in A and B of Fig. 8.
The percentages of individual compounds were calculated from analytical values obtained by gel chromatography on Bio-Gel P-6 after digestion with chondroitinase AC-II (Fig. 8) and paper chromatography after digestion with chondroitinases AC-II and ABC (Fig. 9). The letter R represents molecules resistant to digestion with the two enzymes.

<table>
<thead>
<tr>
<th>Glycosaminoglycan</th>
<th>Chondroitinase AC-II-susceptible</th>
<th>Chondroitinase ABC-susceptible</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\Delta$Di-d$_{35}$S</td>
<td>$\Delta$Di-4S</td>
</tr>
<tr>
<td>C-PG1-derived GAG</td>
<td>5.1</td>
<td>14.1</td>
</tr>
<tr>
<td>$^3$H</td>
<td>6.8</td>
<td>6.5</td>
</tr>
<tr>
<td>$^3$H/$^3$H</td>
<td>0.8</td>
<td>2.2</td>
</tr>
<tr>
<td>M-PG-derived GAG</td>
<td>8.5</td>
<td>10.0</td>
</tr>
<tr>
<td>$^3$H</td>
<td>12.5</td>
<td>5.4</td>
</tr>
<tr>
<td>$^3$H/$^3$H</td>
<td>0.7</td>
<td>1.9</td>
</tr>
</tbody>
</table>

The glycosaminoglycan chains of all chondroitin sulfate proteoglycans synthesized by rat glomeruli in vitro were previously shown to consist of characteristic repeating units, glucuronomyl-$N$-acytylgalactosamine (chondroitinase AC-II susceptible sequences) and iduronosyl-$N$-acytylgalactosamine (chondroitinase ABC-susceptible sequences), both of which have sulfate groups at position 4 or positions 4 and 6 of their galactosamine (20). This type of chondroitin sulfate, namely chondroitin sulfate H, has been found in the notochord of hagfish at the chemical level (39). The present study demonstrated that in the chondroitin sulfates from all four proteoglycans synthesized by mesangial cells 60-72% of the glycosaminoglycan chains were susceptible to chondroitinase AC-II, and 20-26% were susceptible to chondroitinase ABC, both of which have sulfate groups at position 4 (53-57% in the chondroitinase AC-II-susceptible sequences and 17-23% in the chondroitinase ABC-susceptible sequences) or at positions 4 and 6 (10-14% in the former and about 3% in the latter) of their galactosamine moieties. The finding that all the chondroitinase ABC-susceptible sulfated oligosaccharides were produced by digestion with chondroitinase AC-II of the glycosaminoglycans indicates that all these chondroitin sulfate chains contain these two sequences in the same chains. Furthermore, no detectable amount of 6-sulfated repeating disaccharide was present in any of the glycosaminoglycan chains, suggesting that during their synthesis sulfation at position 6 of the di-sulfated galactosamine moieties might occur after sulfation at position 4. The contents of 6-sulfated $N$-acetylglactosamine moieties in both the chondroitinase AC-II-susceptible and the chondroitinase ABC-susceptible sequences of the glycosaminoglycans synthesized by the mesangial cells were significantly lower than those of the glomerular chondroitin sulfates. This may indicate that long-term culture of glomeruli for selection of mesangial cells is associated with significant decrease in activity(ies) for sulfation at position 6 for production of chondroitin sulfate H, probably from chondroitin sulfate B. However, all the chondroitin sulfates produced contained significant amounts of these characteristic disaccharide units in their chains and were very similar in isomeric structures to those synthesized by glomeruli (20). All the results described above for sulfated proteoglycans clearly show that most, if not all, of the glo-
merial chondroitin sulfate proteoglycans are synthesized by mesangial cells.

In addition to these sulfated proteoglycans, the cultured mesangial cells synthesized hyaluronic acid at a similar level to chondroitin sulfate proteoglycan. Unexpectedly, the hyaluronic acids from both the cell layer and the medium were separated by DEAE-Sephasel chromatography into two fractions, i.e., fractions C-HA and C-PG, and fractions M-HA and M-PG. The hyaluronic acids of the hyaluronate-enriched fractions, C-HA and M-HA, were further separated on a Bio-Gel A-5m column into two fractions, excluded and retarded fractions. All the retarded hyaluronic acid in the C-HA fraction was excluded from a Bio-Gel P-6 column, while about 60% of the M-HA hyaluronic acid was eluted at Kᵅ 0.18 and was calculated to consist of 10–12 monosaccharides. On the other hand, all the hyaluronic acid contained in the sulfated proteoglycan fractions, C-PG and M-PG, had a large hydrodynamic size and was excluded from the Bio-Gel A-5m column. The finding that the oligohyaluronate was present only in the medium contrasted with the finding that the small sulfated proteoglycan was present only in the cell layer. Probably the oligohyaluronate is produced by degradation of the larger hyaluronic acids, and if so, this degradation probably occurs in the extracellular compartment or on the cell surface.

The small hyaluronate has not previously been reported as a metabolic product in cultures. Interestingly, the number of constitutive monosaccharides of this oligohyaluroante was approximately the same with the minimum number of monosaccharides of oligohyaluronate required to inhibit hyaluronate-involved aggregate formation with cartilage proteoglycan and link protein in cultures of Swann rat chondrosarcoma reported by Kinura et al. (40). They found that the ability of oligohyaluronates consisting of less than 32 monosaccharides to form aggregate(s) decreased linearly with decrease in the number of monosaccharides and that oligohyaluronate containing 10 monosaccharides showed no ability to form aggregates. Furthermore, the findings that the hyaluronic acids in the sulfated proteoglycan fractions C-PG and M-PG were separated from those of the hyaluronate-enriched fractions, C-HA and M-HA, on DEAE-Sephasel and that they all had a large hydrodynamic size, suggests that the former might be coeluted as complexes probably with the large chondroitin sulfate H proteoglycans in fractions C-PG and M-PG. In this connection, it is interesting that the chondroitin sulfate B proteoglycan synthesized by cultured vascular smooth muscle cells, which exhibit many similarities with mesangial cells in culture, as described above, has the ability to bind hyaluronic acid (39). Thus, the hyaluronic acid synthesized by mesangial cells is probably involved in formation of the mesangial matrix, as has been demonstrated for cartilage (40, 41), and the selective release of the oligohyaluronate into the medium may be, therefore, attributed to loss of the ability to form the matrix with other macromolecules such as the chondroitin sulfate proteoglycans described here.

REFERENCES
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