Sulfated Proteoglycans Synthesized by Vascular Endothelial Cells in Culture*

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Metabolically labeled proteoglycans were isolated both from the culture medium and from the cell layer of cultured bovine aortic endothelial cells. Proteoglycans were fractionated by sequential gel filtration on Sepharose CL-4B, DEAE-cellulose column chromatography, and CsCl density gradient centrifugation. The culture medium contained two distinct proteoglycans: a large proteoglycan (K<sub>x</sub> = 0.07) of low buoyant density containing heparan sulfate side chains (M<sub>r</sub> = 36,000) and a smaller proteoglycan (K<sub>x</sub> = 0.45) of high density containing chondroitin sulfate chains (M<sub>r</sub> = 20,000). The chondroitin sulfate proteoglycan fraction contained a small amount (less than 10%) of dermatan sulfate.

A very similar low density heparan sulfate proteoglycan was extracted from the cell layer with 2% sodium dodecyl sulfate in the presence of enzyme inhibitors. In addition, there was a high density proteoglycan of small size (K<sub>x</sub> = 0.43) containing heparan sulfate side chains (M<sub>r</sub> = 20,000) in the cell layer. Analyses of proteoglycans synthesized by cultured human umbilical vein endothelial cells gave similar results, except that these cells produced more dermatan sulfate and unidentified oversulfated chondroitin sulfate chains.

Morphologically atypical endothelial cells contained reduced levels of the large heparan sulfate proteoglycan.

Both indirect immunofluorescence and direct immunoelectron microscopy revealed that the basement membrane-like matrix under monolayers of bovine endothelial cells reacted with antibodies against the basement membrane proteoglycan isolated from a base-ment membrane-producing tumor. By electron microscopy, this material was shown to consist of a fine filamentous meshwork containing discrete 10-20-nm diameter ruthenium red positive granules resembling those present in basement membranes of intact arteries.

Vascular tissues contain two predominant cell types: endothelial cells and smooth muscle cells. Both cell types have been shown to produce glycosaminoglycans as well as other connective tissue components in culture (1-6), and the major proteoglycans isolated from a number of blood vessels including bovine (7, 8) and human (9) aorta have been characterized. Since vessel proteoglycans have been implicated in a variety of vascular functions, such as a maintenance of tissue integrity, permeability of macromolecules and hemostasis, as well as in the pathogenesis of vascular disease (see Ref. 10 for a review), their study is of considerable interest. Initial progress in this regard has been made by Kramer et al. (11) who have studied the synthesis of proteoglycans by bovine endothelial cells and their degradation by B16 melanoma cells.

It has recently been shown that heparan sulfate proteoglycan is a component of basement membranes (12) and is produced by PYS-2 cells, a cell line that may be useful as an in vitro model of basement membrane production (13). Endothelial cells are known to synthesize and secrete material that morphologically resembles basement membranes, and previous studies have demonstrated that endothelial cells synthesize heparan sulfate (3) and a heparan sulfate proteoglycan (11).

In this study, we demonstrate that bovine aortic endothelial cells in culture synthesize and secrete a large low buoyant density proteoglycan into the culture medium. In addition, a smaller proteoglycan with a density greater than that of the cell layer was detected in the culture medium. We also demonstrate that the proteoglycan produced by the endothelial cells resembles both those of more normal endothelial monolayers and the proteoglycans present in basement membranes of intact arteries.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Metabolic Labeling**—Adult bovine aortic endothelial cells (14) were provided by Dr. Stephen Schwartz, University of Washington. The cells, which had undergone about 20 cumulative population doublings (passage 9-13), were cultured in Waymouth's medium supplemented with 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 16% fetal calf serum (Reheis Chemical Co., Phoenix, AZ). Human umbilical vein endothelial cells (15) were supplied by Dr. John Harlan, University of Washington, and cultured in 5% fetal calf serum containing Waymouth's medium. Confluent monolayer cultures were labeled with 25 μCi/ml of carrier-free Na<sub>2</sub>35SO<sub>4</sub> and 5 μCi/ml of n-[6-3H]glucosamine (30.3 Ci/mmol, Amersham Corp.) in the same culture medium.

**Processing of Culture Medium and Cell Layer**—After labeling, the medium was harvested in the presence of enzyme inhibitors (2 mM phenylmethylsulfonyl fluoride, 10 mM N-ethylmaleimide, and 20 mM EDTA) at 0°C. The cell layer was washed with PBS, and the wash was combined with the medium. After centrifugation at 400 × g for 10 min to remove cellular debris, solid SDS was added to the supernatant to a final concentration of 2%. The SDS solution was chromatographed on a column (1.6 × 50 cm) of Sephadex G-25 equilibrated with 0.1% SDS containing 20 mM Tris-HCl, pH 7.5, 0.2

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1 The abbreviations used are: PBS, phosphate-buffered saline, SDS, sodium dodecyl sulfate.
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mm phenylmethylsulfonyl fluoride, 2 mM EDTA, and 1 mM n-ethylmaleimide to remove the free radioactive precursors. The polymer fraction was analyzed by gel filtration on a Sepharose CL-4B column (1.3 X 78 cm) as described previously (13).

Proteoglycans in the cell layer were solubilized with 2% SDS containing 50 mM Tris-HCl, pH 7.5, 2 mM phenylmethylsulfonyl fluoride, 20 mM EDTA, and 10 mM N-ethylmaleimide (13) or by pronase digestion, 0.1 M e-amino-caproic acid, 5 mM benzamidine hydrochloride, and 0.1 mM peptatin A (Peninsula Laboratories, Inc., San Carlos, CA) were added. Under the conditions used, at most 1% of the total labeled material remained unextracted. After gel filtration on Sephadex G-25, the extract was analyzed by gel chromatography on Sepharose CL-4B as described above.

Purification of Proteoglycans—The sulfated materials, obtained by gel filtration on Sepharose CL-4B, were pooled separately. As carriers, heparan sulfate from bovine kidney, chondroitin 6-sulfate from shark cartilage, and dermatan sulfate from hog skin (Seikagaku Kogyo Co., Tokyo) were added to the pooled fraction to a final concentration of 30 µg each/ml. In order to determine the relative amounts of glycosaminoglycans in the pooled fractions, the sulfated material was precipitated from an aliquot by adding 3 volumes of 95% ethanol containing 1.3% potassium acetate. Glycosaminoglycans were solubilized in 50 mM Tris-HCl, pH 7.5, 0.2% Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, and 1 mM N-ethylmaleimide at 4°C and then against 4 M urea, 50 mM Tris-HCl, pH 7.5, containing 0.2% Triton X-100 and protease inhibitors. The dialysate was chromatographed on a column (0.7 X 11 cm) of DEAE-cellulose with an NaCl gradient ranging from 0 to 0.7 M in the same buffer (total 100 ml) at 4°C. The proteoglycans were precipitated from the peak fractions by adding 3 volumes of 95% ethanol containing 1.3% potassium acetate at 0°C. The proteoglycans thus precipitated were analyzed by ultracentrifugation in a CsCl density gradient under dissociative conditions (13).

Characterization of Proteoglycans—An aliquot (10,000 cpm of 35S) of the DAE-purified proteoglycan was precipitated with ethanol as described above and dissolved in 150 µl of 0.2 M NaCl, 50 mM Tris-HCl, pH 7.5, containing 0.2% Triton X-100 and 1 mM sodium azide. The solution was chromatographed in the same buffer on a column (0.7 X 28 cm) of Sepharose CL-6B at room temperature. Another aliquot (20,000 cpm) was dissolved in 280 µl of 0.2 NaCl and kept at room temperature overnight to release glycosaminoglycans from the protein core. After adding 14 µl of 4 N HCl and 6 µl of 10% Triton X-100, one-half of the solution was chromatographed on the same column, and the remainder, 3 volumes of 95% ethanol, 1.3% potassium acetate, was added to precipitate glycosaminoglycans. The glycosaminoglycans thus precipitated were dissolved in 150 µl of 1.8 M acetic acid containing 1.8% sodium nitrite and kept for 80 min at room temperature (19) to digest N-sulfated hexosamine-containing glycosaminoglycans. The digest was then applied directly to the same column.

Preparation of Subendothelial Matrix—Subendothelial matrix was prepared by the method of Kramer et al. (11) with minor modifications. After labeling the cultures, the cell layer was washed once with PBS and then rinsed twice with 5 mM Tris-HCl, pH 7.5, at room temperature. The cell layer was incubated in this hypotonic buffer at 37°C for 10 min and then treated twice for 30 s with 0.5% Nonidet P-40 in 25 mM Tris-HCl, pH 7.5, containing 20 mM EDTA, 10 mM N-ethylmaleimide, 2 mM phenylmethylsulfonyl fluoride, 0.1 M e-amino-caproic acid, 5 mM benzamidine-HCl, and 0.1 mM peptatin A at room temperature. Solid SDS was added to the detergent extract to a final concentration of 2%. The isolated matrix was washed with PBS and treated with 2% SDS buffer containing cyanide inhibitors as described above.

Radioimmunoprecipitates—DNA in the cell layer was extracted by the method of Burton (20) using DNA type I from calf thymus (Sigma) as a standard after the cell layer was digested with pronase (free of nuclease, Calbiochem-Behring) (21). Treatment of heparan sulfate proteoglycan with bacterial collagenase (form III, Advance Biological, Lymbrook, NY) was performed essentially under the same conditions described previously (13).

Indirect Immunofluorescence—Rabbit anti-basement membrane proteoglycan antibodies (23) were provided by John Hassell, National Institute of Dental Research. This preparation did not react either with laminin or with Type IV collagen. Indirect immunofluorescence experiments were carried out as described previously (13). Some samples were pretreated with PBS containing 0.05% Triton X-100 for 1 min before fixation with paraformaldehyde.

Electron Microscopic Studies—Monolayers were initially fixed at room temperature in Zamboni's fixative which contains a mixture of saturated picric acid in 2% paraformaldehyde (24) followed by extensive washing on a rotary shaker with cold phosphate-buffered saline. The cultures were then incubated with 125I-labeled anti-proteoheparan sulfate antibodies for 1 h at room temperature (13). Antibodies were iodinated as previously described (13). The specific activity of labeled antibodies was 16 µCi/µg. The final dilution of the antibodies was 1:100. As control, rabbit IgG (33 µg in 10 µl PBS) was labeled with NaI251 in an identical fashion. The monolayers were then washed with PBS, fixed for 1 h in 0.1 M cacodylate-buffered 3% glutaraldehyde, pH 7.3, containing 0.2% ruthenium red for 1 h at room temperature (25). The monolayers were rinsed with 0.1 M cacodylate-buffered 1% osmium tetroxide, pH 7.3, containing 0.05% ruthenium red for 30 min. After a brief rinse with distilled water, the monolayers were dehydrated and embedded in epoxy resin (25). Thin sections were cut with diamond knives, double stained with uranyl acetate and lead citrate, and examined in either a JEOL 100B or an AEI 801 electron microscope.

For electron microscopic autoradiography, thin sections were placed on bare copper grids, coated with lifford L-4 emulsion, and processed as previously described (13).

RESULTS

Accumulation of 35S-labeled Materials—Fig. 1 shows the amount of 35S-labeled macromolecules obtained either from the culture medium or from an SDS extract of the cell layer of aortic endothelial cells labeled with [35S]sulfate for various periods of time. The secretion of 35S-labeled material into the culture medium was linear with time up to at least 47 h under the conditions used. The rate of accumulation of labeled material in the cell layer first increased rapidly for about 10 h and then more slowly. In most experiments, monolayer cultures in 75-cm² flasks (containing 200 µg of DNA) were labeled for 24 h. About 70% of the labeled material was found in the culture medium and about 30% was in the cell layer. The labeled material in the cell layer and in the culture medium was analyzed for glycosaminoglycan composition. Heparan sulfate accounted for about 80% of the radioactivity in the cell layer, while chondroitin sulfates were the major glycosaminoglycans (about 65% of the labeled material) in the culture medium. These observations are consistent with those of other investigators (1, 3).

Gel Filtration of Sepharose CL-4B—The 35S-labeled macromolecules, either in the extract of the cell layer or in the conditioned medium, were chromatographed separately on Sephadex G-25. The radioactivity in the polymer fraction was measured.

Fig. 1. Accumulation of 35S-labeled material in the cell layer or in the culture medium of aortic endothelial cells. After labeling, the culture medium and an SDS extract of the cell layer were chromatographed separately on Sephadex G-25. The radioactivity in the polymer fraction was measured.
culture medium, were analyzed by column chromatography on Sepharose CL-4B (Fig. 2). The extract was separated into three peaks: a sharp peak \((K_{av} = 0.07, \text{ designated as C-I})\), a somewhat broad retarded peak \((K_{av} = 0.43, \text{ C-II})\), and a broad peak eluting near the total volume \((K_{av} = 0.87, \text{ C-III})\). In the culture medium, there was a sharp peak eluting near the void volume \((K_{av} = 0.06, \text{ M-I})\) and a doublet with \(K_{av} = 0.45 \text{ (M-II)}\) and 0.63 \text{ (M-III)}\). This elution profile of sulfated materials remained essentially unchanged regardless of the doubling number of the cells used (ranging from primary cultures to 46 doublings).

**DEAE-cellulose Column Chromatography**—After dialysis against urea buffer, the peak fractions from the molecular sieve column were subjected to DEAE-cellulose column chromatography in the presence of 0.2% Triton X-100 to further purify the sulfated macromolecules (Fig. 3). The recovery of C-III after dialysis was less than 60%. Indeed, when the sulfated material was precipitated from C-III by adding 95% ethanol, 1.3% potassium acetate and analyzed by gel filtration on Sepharose CL-6B, as described under “Experimental Procedures,” the average molecular weight of C-III was estimated to be 7500. Since the elution position on Sepharose CL-6B was not changed by alkali treatment, C-III appeared to contain protein-free glycosaminoglycans, apparently a degradation product of proteoglycans. Therefore, this fraction was not analyzed further.

The labeled material in C-I, C-II, or M-I was eluted as a single peak, with a small shoulder eluting at a higher concentration of NaCl (Fig. 3). The majority of the labeled material in both M-II and in M-III was eluted at the position corresponding to the small shoulder present in the other fractions. In each sample, the recovery of the radioactivity after dialysis and chromatography was at least 80%. However, chromatography on DEAE-cellulose in the absence of Triton X-100 resulted in a poor recovery of sulfated material (less than 30%), especially in the case of C-I and M-I.
Characterization of Sulfated Macromolecules—When DEAE-purified C-I was chromatographed on Sepharose CL-6B, it was excluded from the column (Fig. 1a). Since C-I was slightly retarded from Sepharose CL-4B (Fig. 1), the molecular weight of this component was estimated to be about 1 x 10^6 (22). After treatment with alkali, C-I was eluted from Sepharose CL-6B as a broad peak with Kav = 0.59, indicating that the sulfated glycosaminoglycan was linked to a protein core. The apparent molecular weight of the glycosaminoglycan was 36,000, based on the elution position of chondroitin sulfates of known molecular weight (27). Since the glycosaminoglycan was degraded into small molecules by treatment with nitrous acid, it was identified as heparan sulfate. Analyses of M-I gave results closely similar to analyses of C-I (Fig. 1c). C-II was eluted from the column as a peak with Kav = 0.26, while alkali-treated C-II eluted with Kav = 0.50 (Fig. 1d). The overall and glycosaminoglycan chain molecular weights were estimated to be 130,000 and 20,000, respectively, based on the elution positions (12, 27). This glycosaminoglycan was also identified as heparan sulfate by nitrous acid treatment (23). However, only a small amount (at most 5%) of the glycosaminoglycan was sensitive to degradation with nitrous acid. The elution position of M-III was not changed by alkali treatment (Fig. 1e), suggesting that this fraction contains glycosaminoglycan single chains. The majority of this fraction was also resistant to degradation with nitrous acid.

The glycosaminoglycan moiety of each proteoglycan was further characterized by digestion either with chondroitinase ABC or with chondroitinase AC. As expected, the majority of the glycosaminoglycan in C-I, C-II, and M-I was resistant to the enzymatic degradation (Table I). On the other hand, M-II was shown to contain chondroitin 4-sulfate (47% of total radioactivity), chondroitin 6-sulfate (33%), and a small amount (12%) of dermatan sulfate and unidentified oversulfated chondroitin sulfates.

When DEAE-cellulose-purified C-I was centrifuged in a CsCl density gradient under dissociative conditions, about 60% of the labeled material was recovered in the top fraction (Fig. 5a, 4a, p = 1.35). A very small amount (about 5% or less) migrated to the bottom fraction (DI, p = 1.60). M-I showed a sedimentation pattern closely similar to that of C-I (Fig. 5e). On the other hand, another heparan sulfate proteoglycan, C-II, migrated to the bottom fraction (Fig. 5b). M-II was distributed more broadly in the CsCl density gradient (Fig. 5d).

The proteoglycan synthesized by the Engelbreth-Holm-Swarm sarcoma has a high density as measured by CsCl density gradient centrifugation (22), while the large heparan sulfate proteoglycan synthesized by bovine aortic endothelial cells has a low buoyant density. In general, proteoglycans with a higher ratio of protein to glycosaminoglycan have a lower buoyant density. In order to examine the possi-

![Fig. 5. CsCl density gradient centrifugation of the DEAE-cellulose-purified proteoglycans (Fig. 3). Four fractions (D1-D4) were collected and measured both for radioactivity and for density.](image)

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**Table I**

Glycosaminoglycan composition of DEAE-cellulose-purified proteoglycan fractions

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Heparan sulfate&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Chondroitin 4-sulfate</th>
<th>Chondroitin 6-sulfate</th>
<th>Other&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-I</td>
<td>100 (96)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>0</td>
</tr>
<tr>
<td>C-II</td>
<td>95 (94)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>&lt;4</td>
<td>&lt;4</td>
<td>0</td>
</tr>
<tr>
<td>M-I</td>
<td>98 (95)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td>0</td>
</tr>
<tr>
<td>M-II</td>
<td>&lt;10 (5)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>47</td>
<td>33</td>
<td>12</td>
</tr>
</tbody>
</table>

<sup>1</sup> Chondroitinase ABC-resistant radioactivity.
<sup>2</sup> This fraction contains dermatan sulfate and unidentified oversulfated chondroitin sulfates.
<sup>3</sup> Nitrous acid-sensitive radioactivity (fractions 45-59 in Fig. 4, a-c).
<sup>4</sup> Nitrous acid-sensitive radioactivity (fractions 50-57 in Fig. 4d).

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**Fig. 6.** Sepharose CL-2B gel chromatography in SDS buffer of the large proteoglycan obtained from the subendothelial matrix of bovine endothelial cells. The proteoglycan (20,000 cpm) was dissolved in 1 ml of 0.1 M 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid, pH 7.4, containing 10 mM N-ethylmaleimide, 0.2 mM phenylmethylsulfonyl fluoride, 5 mM CaCl<sub>2</sub>, 50 μg of bovine serum albumin, and 5 μg of collagenase, or in 1 ml of 50 mM Tris-HCl, pH 7.5, containing 3% ethanol, 50 μg of bovine serum albumin, and 50 μg of pronase, or in 1 ml of 0.2 N NaOH. After incubation either for 2 h at 37 °C for enzymatic digestion or for 16 h at room temperature after alkali treatment, the labeled material was precipitated with ethanol/potassium acetate. The precipitate was redisolved in 150 μl of 2% SDS, 50 mM Tris-HCl, pH 7.5, and chromatographed on a column (0.7 × 28 cm) of Sepharose CL-2B in 0.1% SDS buffer at room temperature. Another aliquot of the proteoglycan was dissolved in 150 μl of 2% SDS, 50 mM Tris-HCl, pH 7.5, containing 8 mg/ml of dithiothreitol (DTT). After heating for 3 min, the solution was chromatographed on the same column in 0.1% SDS buffer containing 1 mg/ml of dithiothreitol. Fractions of 0.14 ml were collected. V<sub>D</sub>, void volume; V<sub>T</sub>, total volume.
ility that the proteoglycan is linked by disulfide bonds to other proteins, such as collagen (28), the proteoglycan obtained from the subendothelial matrix of aortic endothelial cells after Nonidet P-40 extraction was chromatographed on Sepharose CL-4B in an SDS buffer either after reduction with dithiothreitol or after digestion with purified bacterial collagenase. The elution position ($K_w = 0.37$) of the proteoglycan was not changed either by collagenase digestion or by reduction with dithiothreitol (Fig. 6). The sample treated with pronase or with alkali was eluted later from the column ($K_w = 0.73$), indicating that degradation had occurred.

Proteoglycans of Cultured Human Umbilical Vein Endothelial Cells—Analyses of proteoglycans synthesized by human umbilical vein endothelial cells in culture gave results similar to those with bovine aortic endothelial cells. The elution profiles on Sepharose CL-4B of sulfated material obtained from the SDS extract of the cell layer and from the culture medium are shown in Fig. 7. Since the labeled components associated with the cell layer accounted for at most 15% of the total radioactivity, extensive studies of sulfated peaks in the cell layer extract could not be done, except for the peak eluting near the void volume. This peak contained predominantly a heparan sulfate proteoglycan. The average molecular weight of its heparan sulfate side chains was estimated to be 33,000. The first peak obtained from the culture medium was shown to be a low density proteoglycan containing heparan sulfate side chains with $M_r \sim 33,000$. The next peak ($K_w = 0.37$) was identified as a chondroitin sulfate-dermatan sulfate proteoglycan. The average molecular weight of its glycosaminoglycan side chains was estimated to be 25,000. The relative amounts of isomeric chondroitin sulfates were determined using the DEAE-cellulose-purified component. The amount of dermatan sulfate (the difference between chondroitinase AC II-resistant radioactivity and chondroitinase ABC-resistant material) was significantly higher (56% of total radioactivity) than that of the corresponding molecule, M-II, synthesized by bovine aortic endothelial cells (see Table 1). Additionally, paper chromatography of the chondroitinase ABC digest revealed that about 20% of the total radioactivity migrated to the position expected for an unsaturated oversul-

![Fig. 7. Sepharose CL-4B gel chromatography in SDS buffer of SDS extracts of the cell layer (a) and the culture medium of human umbilical vein endothelial cells (b). Confluent cultures were labeled with $[^35]S$]sulfate for 24 h. Horizontal bars indicate fractions which were pooled for further analyses.](https://www.jbc.org/content/263/22/10301/F1.large.jpg)

![Fig. 8. Indirect immunofluorescence of the cultures of bovine aortic endothelial cells stained with anti-basement membrane proteoglycan antiserum (b) and of the Triton X-100-treated cultures stained with the same antiserum (d). a and c are phase-contrast photomicrographs of the same fields shown in b and d, respectively. Magnification, ×1300.](https://www.jbc.org/content/263/22/10301/F2.large.jpg)
**Endothelial Cell Proteoglycans**

**FIG. 9.** Electron micrographs of intercellular matrix deposited by bovine aortic endothelial cells. *a,* this electron microscopic autoradiograph was prepared by incubating endothelial cells with 125I-labeled anti-basement membrane proteoglycan antiserum. The grains are confined to the area of the intercellular matrix that contains the small ruthenium red positive granules embedded in a filamentous matrix. Magnification, × 37,500. *b,* electron micrograph of a longitudinal section through the amorphous material deposited beneath the endothelial cells. The monolayers were fixed in the presence of ruthenium red. Numerous small (10-20 nm in diameter) ruthenium red positive granules are associated with the fine filamentous material. Magnification, × 75,000.

**FIG. 10.** Light micrographs of normal monolayer cultures (*a*) and morphologically atypical cultures (*b*) of bovine aortic endothelial cells. Magnification, × 260.

**Proteoglycans Synthesized by Atypical Cultures of Bovine Aortic Endothelial Cells—**Bovine aortic endothelial cells usually form a typical monolayer, but sometimes they develop an atypical morphology. In such cultures, cells are elongated and tend to grow in a mycelial pattern (Fig. 10). This pattern of growth has been referred to as sprouting (14, 15, 30). Sprouting cultures contained more DNA (253 µg/75-cm² flask) than typical monolayer cultures (200 µg/75-cm² flask).

Proteoglycans were isolated from the cell layer and from the culture medium of sprouting cultures and chromatographed on Sepharose CL-4B (Fig. 11). The elution pattern of the labeled material in the medium (Fig. 11a) was similar to that of a typical culture (see Fig. 2b), although the amount of the radioactivity in the culture medium was only 69% (based on the amount of DNA) of the typical culture. These findings suggest that, although the synthetic or secretory rate of proteoglycans decreases in association with the morphological change, the types of proteoglycans synthesized by the endothelial cells remain unchanged. However, the pattern of proteoglycans in the cell layer extract (Fig. 11a) differed significantly from that of a typical culture (see Fig. 2a). The amount of the large heparan sulfate proteoglycan was markedly reduced. Instead, there appeared a new sulfated peak (*K*ₐ = 0.57), and the amount of the small protein-free glycosaminoglycan (*K*ₐ = 0.90, corresponding to C-III in Fig. 2a)
increased. Further analyses revealed that the new peak with $K_w = 0.57$ contained mainly (83%) heparan sulfate single chains with an average molecular weight of 22,000. The molecular weight of this glycosaminoglycan is lower than that of the heparan sulfate side chain of C-I (36,000), but similar to that of the heparan sulfate side chain of C-II (20,000).

**DISCUSSION**

Vascular endothelial cells have been shown to produce a variety of extracellular components, including collagen (31), fibronectin (32), laminin (33), sulfated glycoproteins (34), thrombospondin (35), and glycosaminoglycans (1, 3). In this study, we have isolated two heparan sulfate proteoglycans with different molecular weights from the cell layer of cultured bovine aortic endothelial cells: a large heparan sulfate proteoglycan with a low buoyant density and a smaller one with a higher buoyant density. Furthermore, our ultrastructural studies demonstrate that this matrix material contained small ruthenium red positive granules which are similar to those granules containing heparan sulfate identified in basement membranes in various organs in vivo (29, 36–38). Kramer et al. (11) have also isolated a high molecular weight proteoglycan with similar characteristics from cultured bovine aortic endothelial cells. In addition, we found that endothelial cells secrete into the medium a large proteoheparan sulfate of low buoyant density and a high buoyant density chondroitin sulfate-decamannan sulfate proteoglycan.

Heparan sulfate proteoglycans occur in various basement membranes (11–13, 23) as well as at the cell surface of several cell types (39–41). These proteoglycans in basement membranes may play a role in the regulation of permeability by creation of a charge barrier (42, 43) and may also have antithrombotic properties (44). Cells such as fibroblasts may utilize heparan sulfate proteoglycans in attachment to the extracellular matrix (41, 45). We have demonstrated that antibodies against the basement membrane proteoglycan isolated from Engelbreth-Holm-Swarm sarcoma (23) reacted with the extracellular matrix beneath the monolayer of bovine aortic endothelial cells (Figs. 7 and 8); this matrix contained predominantly the large heparan sulfate proteoglycan. The Engelbreth-Holm-Swarm sarcoma proteoglycan has a smaller overall size ($M_w \sim 750,000$), larger heparan sulfate side chains ($M_w \sim 70,000$), and a higher buoyant density (23) than that produced by the vascular endothelial cells described here. The heparan sulfate proteoglycan isolated either from cultures of PYS-2 cells (13) or from glomerular basement membrane (12) is smaller both in overall size and in glycosaminoglycan side chain length, but higher in buoyant density.

In general, proteoglycans with higher protein content have a low buoyant density. Parthasarathy and Spiro (28) suggested that proteoglycans in the glomerular basement membrane were linked by disulfide bonds to collagen. If this were the case for the endothelial cell proteoglycan, its density would be low. However, as shown in Fig. 6, neither reduction with dithiothreitol nor digestion with collagenase changed the elution position of the proteoglycan on Sepharose CL-2B. Alternatively, it is well known that proteoglycans in micelles of lipids or detergents have a low buoyant density in CsCl (46). Our procedures for isolation and purification of proteoglycans included two detergents, SDS for extraction and Triton X-100 for DEAE-cellulose column chromatography. Although it is unlikely that the DEAB-cellulose-purified proteoglycan contained lipid, this possibility cannot be totally excluded. Similarly, micelles may have formed with the detergents used, accounting for the low buoyant density. When the major heparan sulfate proteoglycan in the culture medium of PYS-2 cells was purified by the same method used here and centrifuged in a CsCl density gradient, the proteoglycan was recovered in a high density fraction. Nevertheless, there is a possibility that the vascular proteoglycan has a more hydrophobic site in the molecule and, therefore, binds detergents more strongly. The ability of arterial heparan sulfate proteoglycans to form a complex with lipoproteins (47, 48) could represent a similar phenomenon.

The significance of a smaller heparan sulfate proteoglycan present in the cell layer of bovine aortic endothelial cells (C-II in Fig. 2) is puzzling. This proteoglycan had structural properties similar to those of the proteoglycan isolated from glomerular basement membrane (12). It is uncertain whether this class of proteoglycan represents a distinct second population of proteoheparan sulfate or is merely a breakdown product of the larger proteoheparan sulfate. Pulse-chase experiments using normal monolayer cultures revealed that these cells degraded heparan sulfate proteoglycans. The half-life of the [35S]sulfate-labeled material in the cell layer was less than 4 h. Active degradation of heparan sulfate proteoglycans was also observed in the cultures of mouse epithelial cells (49).

Experiments with atypical cultures (Figs. 10 and 11) also suggest that significant degradation of the proteoheparan sulfate takes place in these cultures. Although cells in sprouting cultures synthesized and secreted the same species of proteoglycans into the culture medium (Fig. 11a) as those synthesized by cells with a more normal morphology (Fig. 26), the proportion of the large heparan sulfate proteoglycan in the cell layer was decreased (Fig. 11d). It is possible that the large proteoglycan is degraded first into the smaller heparan sulfate proteoglycan and then into heparan sulfate single chains (the peak with $K_w = 0.57$ in Fig. 11a) since the glycosaminoglycan side chains of the smaller proteoglycan have an average molecular weight similar to that of the single chain heparan sulfate. The heparan sulfate single chains may be further degraded into smaller chains with $M_w \sim 7,500$ and finally into compounds that are removed by Sephadex G-25 chromatography.

Heparan sulfate has been shown to interact specifically with other extracellular matrix components, such as fibronectin-collagen complex of the pericellular matrix (50). Therefore, the degradation of heparan sulfate proteoglycans is likely to result in considerable reorganization of the extracellular matrix. Such changes in the extracellular matrix may be involved in the changes in morphology and in growth patterns observed in Fig. 10. However, our findings do not exclude other possibilities such as changes in the types or amounts of material that is actually being synthesized by cells in sprouting cultures.

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