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Aortic Endothelial Cell Proteoheparan Sulfate

I. Isolation and Characterization of Plasmamembrane-Associated and Extracellular Species

RUPRECHT KELLER, MD, PhD, JEREMIAH E. SILBERT, MD, HEINZ FURTHMAYR, MD, and JOSEPH A. MADRI, MD, PhD

From the Department of Pathology, Yale University School of Medicine, The Boston Veterans Administration Outpatient Clinic, and the Department of Medicine, Harvard Medical School

Proteoheparan sulfate biosynthesis was studied in cultured bovine aortic endothelial cells by means of pulse and pulse-chase experiments and subcellular fractionations. Three proteoheparan sulfate species were found in the medium. The major species, which the authors have called HS I, appeared in the medium only after an initial lag period and was also found associated with the plasma membrane. The other two (HS II and HS III) appeared in small amounts in the medium at early time points. At later times these were not readily observed because the large amounts of HS I present in the medium. The major medium species, HS I, appeared to be composed of approximately four heparan sulfate chains of approximately 35,000 daltons and a core protein of approximately 55,000 daltons apparent molecular weight. HS I appeared to be homogeneous by gel filtration on Sepharose CL 2B and 6B and elution from DEAE Sephacel, electrophoresis on Nu-Sieve agarose, and CsCl density centrifugation. After digestion with heparinase the core protein appeared to be homogeneous by S-200 Sephacel chromatography. HS I was also found associated with plasma membrane fractions of the cultured bovine aortic endothelial cells, and antisera raised against it stained epithelial and endothelial cells in patterns consistent with a cell surface localization. Of the other two species found in the medium, one (HS II) also appeared to be a component of the cell layer. This species appeared to contain approximately four heparan sulfate chains of approximately 20,000 daltons apparent molecular weight. Antisera raised against a similar molecule produced by HR 9 cell cultures stained basement membranes intensely, supporting the subcellular matrix localization of this molecule. The third species (HS III) was detected in culture medium only and apparently contained two heparan sulfate chains of approximately 20,000 daltons apparent molecular weight. These results support the concept of multiple endothelial cell proteoheparan sulfate species which exhibit differences in structure and localization and possibly diverse specialized functions. (Am J Pathol 1987, 128:286–298)

ENDOTHELIAL CELLS (ECs) are polar cells that provide a nonthrombogenic surface for the vascular system and serve as highly specialized metabolic units in the various vascular beds extending from the large arteries to parenchymal capillary beds.[1] ECs are also in intimate contact with the underlying matrix and are thought to synthesize and interact with matrix molecules in a dynamic fashion.[2–4] One notable group of molecules, the proteoheparan sulfates, appear to be present in the matrix as well as on the EC surface.[5–7] Various species of these molecules appear to participate in matrix composition and organization and have been implicated in a variety of vascular functions, including maintenance and modulation of hemostasis, control of vascular smooth muscle cell proliferation, and maintenance of permeability.[8–11] This diversity of localization and function is strongly suggestive of complex structure/function relationships that may originate from distinct species of proteoheparan sulfates—the products of several unique genes.

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Dr. Keller's present address is Central Laboratories, University Clinic of RWTH Aachen, Neues Klinikum 51 Aachen, West Germany.

Address reprint requests to Dr. Joseph A. Madri, Pathology Department, Yale University School of Medicine, 310 Cedar Street, New Haven, CT 06510.

Alternatively, this diversity could be explained by the modification of a single large-molecular-weight precursor with the various tissue species being the products of one gene. Because the biosynthesis of proteoheparan sulfates and their transport kinetics are complex, involving a great number of incompletely understood processing reactions, it has been difficult to distinguish between these two hypotheses.[12-15]

In this report we describe three proteoheparan sulfate species produced by cultured bovine calf aortic ECs and their transport kinetics. The data suggest that the three forms of proteoheparan sulfate isolated and characterized are not derived from each other, but are unique species with specific transport kinetics.

Materials and Methods

Carrier-free $Na_2{}^{35}SO_4$, carrier-free ^{125}I, ^{3}H-AMP, and UDP-^{14}C-Gal were purchased from New England Nuclear (Boston, Mass). Heparinase, heparitinase, and chondroitinase ABC were purchased from Miles Laboratories (Chicago, Ill). Purified bacterial collagenase was purchased from Biofactures (Lynbrook, NY) and was further purified by gel filtration with G-200 Sephadex. This enzyme exhibited collagenase activity but no other detectable proteolytic activities. DEAE-Sephacel, Sephadex G-25, G-50, Sephacryl S-200, and Sepharose CL-2B, 4B, and 6B were purchased from Pharmacia (Uppsala, Sweden). NuSieve agarose was obtained from FMC (Rockland, Me). Low-molecular-weight protein standards were obtained from BioRad Laboratories (Richmond, Calif). Glycosaminoglycan standards having molecular weights of 35,000, 19,000, 17,000, and 13,000 were obtained as a generous gift from Dr. Helmut W. Stuhlsatz, Technical University, Aachen, West Germany, as previously described.[16] Radioactivity was measured with a Beckman liquid scintillation spectrometer LS 230 or a Beckman Gamma 4000 counting spectrometer. Amino acids and amino sugars were analyzed on a Biotronic LC 6000 E amino acid analyzer after hydrolysis in 3 M HCl at 105 C for 15 hours.

Nitrous acid degradation was performed according to Shively and Conrad.[17] Radioiodination with ^{125}I was performed by the chloramine T method.[18] Two-dimensional peptide mapping was performed as described for the collagens by means of trypsin digestion of radiolabeled materials followed by high-voltage electrophoresis and chromatography on 13255 Eastman Chromagram cellulose plates, Eastman Kodak Company, Rochester, NY.[19]

Bovine calf aortic endothelial cells were isolated and cultured as previously described.[20] The culture medium used was minimal essential medium (MEM) supplemented with 20% heat-inactivated fetal calf serum, penicillin, streptomycin, and glutamine. In pulse and pulse-chase experiments sulfate-deficient MEM purchased from Irving Scientific (Santa Ana, Calif) were used. All tissue culture studies were performed at 37 C in a 5.0% CO_2 humidified incubator.

Pulse and Pulse-Chase Labeling Studies

For pulse-labeling experiments the cells were plated in 9.6-sq cm Petri dishes (Costar) and grown to confluency (~250,000 cells/dish). Cultures were then incubated with media containing 0.08 mM sulfate for 1 hour and then incubated with 4.0 ml of this medium containing 100 μCi ^{35}S-sulfate/ml for up to 8 hours. At various incubation times 0.5-ml aliquots were removed and stored frozen at −70 C until further analyses were performed.

For pulse-chase studies, cells were plated and cultured as described above. Following a 2-hour incubation with ^{35}S-sulfate-containing medium, cultures were washed ten times with ice-cold phosphate-buffered saline (PBS) containing 1 mM $MgSO_4$ and then incubated with 4.0 ml of this buffer. Media were removed and replaced with fresh media at several time points.

All medium samples were desalted on a Sephadex G-25 column (1 × 20 cm) with 0.5 M NH_4HCO_3, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA buffer. After lyophilization all samples were digested with 0.5 units of chondroitinase ABC in 50 mM Tris-HCl, 0.1 M NaCl, 1 mM PMSF, 1 mM EDTA, pH 7.5. Samples were chromatographed on a Sepharose CL-6B column (0.5 × 60 cm) with 0.13 M Tris-HCl, 1 mM PMSF, 10 mM N-ethylmaleimide (NEM), 1 mM EDTA, pH 7.2. Fractions of 200 μl were collected and analyzed for radioactivity by means of a liquid scintillation counter.

In other experiments in which the subendothelial matrix was analyzed, the cells were removed from T-75 culture flasks (Costar) nonenzymatically with 0.5 mM EGTA after incubation with 50 μCi/ml ^{35}S-sulfate (0.08 mM SO_4) for 24 hours. The matrix material remaining on the flasks was digested with 1 unit of chondroitinase ABC, solubilized in 2.0% 3-(3-cholamidopropyl)-dimethylammoniol 1-propane sulfate (CHAPS), and aliquots were chromatographed on Sepharose CL-6B as described.

Preparation and Analysis of Subcellular Membrane Fractions

For the preparation of subcellular fractions, a protocol describing the isolation of Golgi membranes

from hepatocytes was used with several modifications.[21] Four confluent T-75 flasks of the cells (approximately 8.0×10^6 cells) were first incubated with 10 ml each of medium (0.08 mM sulfate) for 1.0 hour. Following removal of these media, 5.0 ml/flask of medium containing 10 μCi ^{35}S-sulfate/ml was added. Cultures were then incubated for 10 minutes, 40 minutes, 5 hours, and 24 hours; and the media were removed. The flasks were then cooled to 4 C and washed ten times with 10 ml of ice-cold PBS containing 0.5 mM $MgSO_4$ and 0.5 mM $CaCl_2$ (until less than 1000 cpm ^{35}S-sulfate appeared in the final wash). The cells were then removed nonenzymatically by incubation with 2 ml/flask of 0.5 mM EGTA in PBS at room temperature for 10 minutes. After this, an additional 5 ml/flask of ice-cold PBS containing 0.5 mM $MgSO_4$ and 0.5 mM $CaCl_2$ was added, and the cells were centrifuged at 700*g* for 3 minutes. The cell pellet was then washed twice in this buffer.

The cell pellet was then suspended in 1.5 ml of ice-cold 0.25 M sucrose and sonicated with a Polytron (Brinkman Instruments, NY) three times for 30 seconds with 2-minute intervals at 4 C. The homogenate was then centrifuged at 10,000*g* at 4 C for 45 minutes, and the supernatant collected and diluted with 2.5 ml of 0.25 M sucrose. This supernatant was then centrifuged at 100,000*g* at 4 C for 45 minutes and the pellet suspended in 100 μl of water. This material was applied to the top of a discontinuous sucrose gradient consisting of 0.8 ml of 53% sucrose, 0.2 ml of 44% sucrose, 0.7 ml of 39% sucrose, 0.5 ml of 37% sucrose, 0.5 ml of 31% sucrose, and 1.2 ml of 18% sucrose. The gradient was centrifuged at 35,000 rpm (91,000*g*) in a Beckman SW 60 rotor at 4 C for 45 minutes. Fractions of 160 μl, obtained by puncture of the bottom of the tube, were assayed for radioactivity, 5′-nucleotidase, and galactosyl transferase as previously described.[22]

Analysis of Proteoheparan Sulfates

For analytic DEAE-Sephacel chromatography, proteoheparan ^{35}S-sulfate samples were adjusted to 0.130 M Tris-HCl, 7.0 M urea, 0.1% CHAPS, 1 mM PMSF, 10 mM NEM, 1 mM EDTA, pH 7.2, and chromatographed on a DEAE-Sephacel column (0.5×1 cm) equilibrated in the same buffer to which a linear gradient of 0.0–1.0 M NaCl (total volume of 10 ml) was applied. Logarithmic (concave) gradients were generated by means of a gradient maker containing 10.0 ml of the above elution buffer and 5.0 ml of the elution buffer plus 0.5 M NaCl. Fractions of 300 μl were collected and analyzed for radioactivity.

Alkali digestion of ^{35}S-proteoglycans was accomplished by incubation in 0.5 M NaOH at 4 C for 12 hours.[13] Digestion of ^{35}S-proteoglycans with a mixture of heparinase and heparitinase (5 units each) was performed in 0.05 M Tris-HCl, 0.1 M NaCl, 5 mM $CaCl_2$, 1 mM PMSF, 10 mM NEM, pH 7.3, at 37 C for 2 hours. Material from both digests were analyzed by means of Sepharose CL-2B, CL-4B, and CL-6B chromatography in 0.13 M Tris-HCl, 1 mM PMSF, 10 mM NEM, 1 mM EDTA, pH 7.3. Fractions of 200 μl were collected and analyzed for radioactivity.

Cesium chloride density gradient centrifugation was performed in a Beckman L5-65 centrifuge by means of a Ti 50 rotor. Samples of HS I were dissolved in 9 ml of CsCl, density 1.45, 0.1 M Na (CH_3COO), pH 6.5, and centrifuged at 160,000*g* for 60 hours. One-milliliter samples were then collected and analyzed for radioactivity.

Chromatography of HS I core protein on Sephacryl S-200 was performed on a 0.5×60-cm column with a 0.13 M Tris-HCl, 0.1% sodium dodecyl sulfate (SDS), 1 mM PMSF, 10 mM NEM, 1 mM EDTA, pH 7.2, elution buffer.

Preparation of Medium Proteoheparan Sulfate HS I

Endothelial cells were plated and grown to confluency in roller bottles (Falcon #3029) (1750 sq cm/bottle, approximately 2×10^8 cells), each containing 250 ml of medium. At 72 hours after confluency all media were collected and stored frozen at −70 C until used. For use as a radioactive tracer during the preparation, medium was harvested from 10^7 cells after a 24-hour incubation with 10 ml of sulfate-deficient MEM containing 20% heat-inactivated fetal calf serum with 100 μCi ^{35}S-sulfate/ml. This material was desalted on a Sephadex G-50 column (2.5×30 cm) in 0.5 M NH_4HCO_3, 1 mM PMSF, 1 mM EDTA, lyophilized, and combined with the unlabeled medium from the roller bottle cultures. The combined media were adjusted to 1 mM PMSF, 2 mM EDTA, 10 mM NEM, and incubated with 5 units chondroitinase ABC at 37 C for 1 hour. The material was then concentrated 1 : 10 by distillation under reduced pressure and was chromatographed on a Sepharose CL-6B column (5.0×70 cm). Fractions of 10.0 ml were collected and assayed for radioactivity and 280 nm absorbance. The V_o material (Fractions 30–45) was pooled, precipitated in 80% ethanol at 4 C for 15 hours, and harvested by centrifugation at 10,000*g*. The precipitate was then solubilized in the chromatography buffer and rechromatographed on the same Sepharose CL-6B column. The V_o material was then ethanol-precipitated and solubilized in 0.13 M Tris-HCl, 7.0 M urea, 0.1% CHAPS, 1 mM PMSF, 10 mM

NEM, 1 mM EDTA, pH 7.2, and chromatographed on a DEAE-Sephacel column (2 × 10 cm) in the same buffer with a linear gradient of 0.0–1.0 M NaCl (50 ml + 50 ml). Fractions of 4.0 ml were collected and assayed for radioactivity and 280 nm absorbance. The gradient fractions that contained radioactivity and were nitrous acid, heparinase, and heparitinase sensitive (0.5 M NaCl) were pooled and precipitated in 80% ethanol. The precipitate was then solubilized in 0.1 M sodium acetate, 7.0 M urea, 0.1% CHAPS, 1 mM PMSF, 10 mN NEM, 1 mM EDTA, pH 6.0, and chromatographed on a DEAE-Sephacel column (2 × 10 cm) in the same buffer and a linear gradient of 0.0 to 1.0 M NaCl (50 ml + 50 ml). Fractions of 4.0 ml were collected and assayed for radioactivity and for protein at 280 nm. Fractions that contained radioactivity and were nitrous acid, heparinase, and heparitinase sensitive (0.5 M NaCl) were pooled and precipitated in 80% ethanol, washed twice in 50% ethanol, and stored at −70 C.

Antiserum Production

Rabbit antisera to the bovine aortic endothelial cell medium proteoheparan sulfate HS I were produced by means of standard procedures. Briefly, 0.2 mg of HS I dissolved in 0.5 ml of PBS was mixed with an equal volume of complete Freund's adjuvant (Difco Laboratories, Detroit, Mich) and injected intradermally. Two weeks later an identical dose was administered, and the animals were bled under anesthesia 2 weeks after this last injection. Sera were collected and stored at −70 C. Antisera to a matrix proteoheparan sulfate isolated from HR 9 cells was produced in a fashion similar to that described elsewhere.[24,25] ELISA were performed for assessment of titers. Microtiter plates (Falcon #3912) were coated with 5 μg of proteoheparan sulfate/well in 100 μl PBS. All washing and incubation procedures (with the exception of the final incubation with substrate) were performed with 1.0% bovine serum albumin/PBS solution.[26] Antibodies were isolated from antisera by affinity chromatography on HS I–Sepharose CL-2B and Protein A–Sepharose CL-2B columns.

Immunoabsorption

Bovine aortic endothelial cells (BAECs) (~8 × 10^6 cells) were incubated with 50 μCi ^{35}S-sulfate/ml of media for 4 hours. The medium was desalted on Sephadex G-50 and lyophilized. This material was solubilized in 0.1 M sodium phosphate, pH 8.0, and 10 μl (13,500 cpm) was incubated with 100 μl of the HS I antisera for 24 hours. The mixture was run over a Protein A–Sepharose column (1.0 ml). The column was then rinsed with 25 ml of 0.1 M sodium phosphate, 0.1% CHAPS, pH 8.0, until no radioactivity was detected in the eluate. The bound material was eluted with 5 ml of 0.1 M sodium citrate, 0.1% CHAPS. This material was lyophilized, resuspended in 100 μl of 7 M urea, 2% SDS, 0.1 M Tris/HCl, 1 mM PMSF, 10 mM NEM, 1 mM EDTA, pH 7.3, and chromatographed on a Sepharose CL-4B column (0.5 × 60 cm) in 0.13 M Tris/HCl, 0.1% SDS, 1 mM PMSF, 10 mM NEM, 1 mM EDTA, pH 7.2.

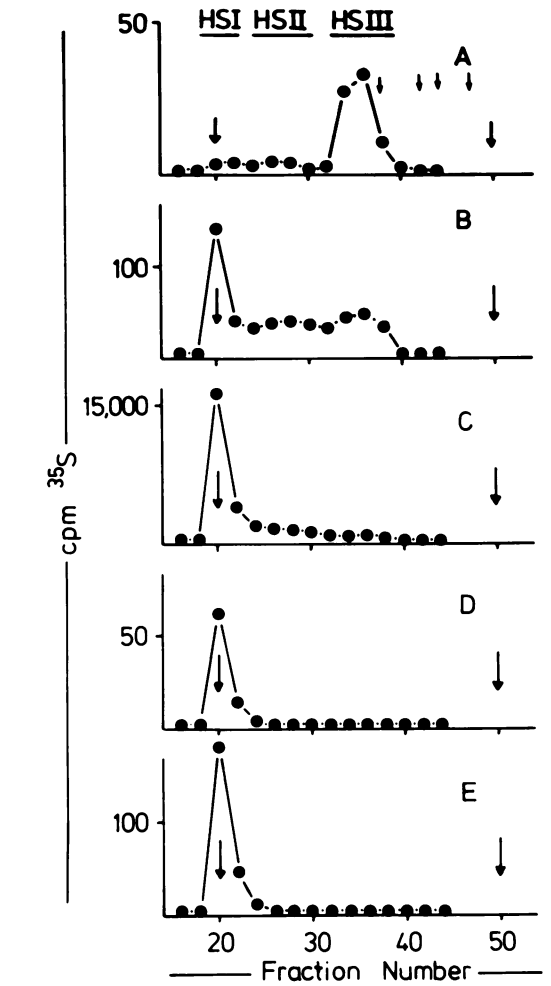
Immunofluorescence

Indirect immunofluorescence of tissue culture dishes and tissues was performed as previously described.[27] Photomicrographs were taken with a Zeiss research fluorescence microscope equipped with epiillumination and an automatic Olympus PM 10AD camera and Ektachrome ASA 400 film.

Results

Medium Proteoheparan Sulfate Biosynthesis—Pulse and Pulse-Chase Studies

Endothelial cells apparently produce three species of heparinase, heparitinase, and nitrous acid sensitive proteoglycans, which we have termed HS I, HS II, and HS III, which appeared in the culture medium (Figures 1A, B, C, and F). In order to avoid confusion with chondroitin sulfate proteoglycans, all the samples were treated with chondroitinase ABC prior to chromatography (see Discussion). Elution under dissociative conditions (4 M guanidine hydrochloride, 1% Triton X-100) did not alter the chromatographic patterns of these moieties (data not shown). While total proteoheparan ^{35}S-sulfate increased linearly during the incubation time (data not shown), the proportion of the ^{35}S-sulfate-labeled components varied. During early pulse periods (2–30 minutes) a single, well-defined proteoheparan sulfate species (HS III) was noted, eluting at Fraction 34–38, $K_{av} = 0.53$ (Figure 1A). During longer pulse periods (40–60 minutes) two other proteoheparan sulfate species were observed: one eluting as a peak in the V_o fraction (HS I) and the other (HS II) eluting between the positions of HS I and HS III (Figure 1B). HS II appeared to be less well defined than the other two proteoheparan sulfate species. At longer pulse times (2–8 hours) HS I became the predominant medium ^{35}S-sulfate-labeled species (90% at 8 hours), obscuring the presence of the smaller amounts of HS II and HS III (Figures 1C, D,



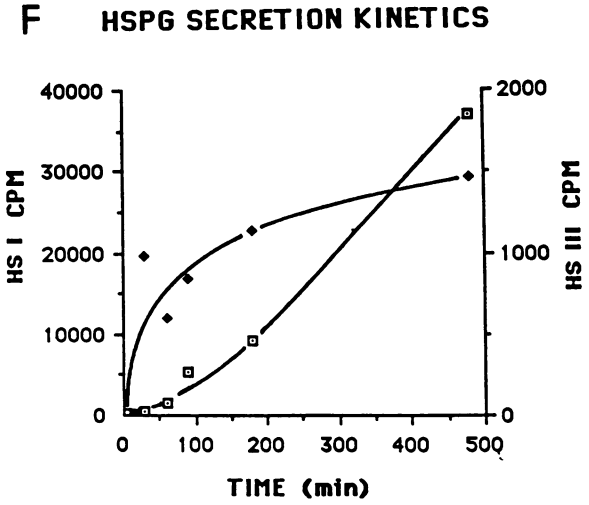


Figure 1—Sepharose CL-6B chromatography of chondroitinase ABC digested BAEC medium proteoglycans. Cells were incubated with ^{35}S-sulfate-containing medium, and aliquots were removed after 30 minutes, (**A**), 40 minutes (**B**) and 8 hours (**C**). In other experiments cells were incubated with ^{35}S-sulfate-containing medium for 1 hour, after which the cultures were chased with nonradioactive sulfate-containing medium for 30 minutes (**D**) and for 7.5 hours thereafter (**E**). All samples were desalted, digested with chondroitinase ABC, and chromatographed on a 0.5 × 60 cm column that had been calibrated with a series of glycosaminoglycans of known sizes (35,000, 19,000, 17,000, and 13,000). *Large arrows* denote V_o and V_t, respectively, whereas the *small arrows* denote the elution positions of the GAG standards. The horizontal bars denote the elution positions of HS I, HS II, and HS III. Figure 1F is a representative plot of the rates of accumulation of HS I and HS III during an 8-hour pulse period at 30-, 60-, 90-, 180-, and 480-min pulse times. *Open squares*, HS I; *closed diamonds*, HS III; *CPM*, CPM/fraction.

and E). As illustrated in Figure 1F, the kinetics of secretion of HS I and HS III were markedly different. HS I appeared in the medium after an initial delay but in much greater amounts than HS III (and HS II, data not shown), which appeared early but was present at low levels and accumulated at much lower rates.

In order to determine whether the observed changes in the chromatographic patterns of the ^{35}S-sulfate-labeled medium materials were due to breakdown or degradation of higher molecular weight components or to differences in release kinetics of individual components from the endothelial cell layer to the medium, a series of pulse-chase experiments was performed. The cells were incubated with ^{35}S-sulfate for 1 hour and then chased with nonradioactive media for various times (Figures 1D and E). No detectable radioactive lower molecular weight material was observed in any of the chase periods, which suggested that the lower molecular weight substances (HS II and HS III) were not derived from the higher molecular weight component (HS I).

Characterization of the BAEC Medium Proteoheparan Sulfate Species

In addition to the kinetic studies described above, the medium proteohaparan sulfate species were further characterized by chromatographic methods and by chemical and enzymatic treatments. HS I, HS II, and HS III were prepared for analyses from medium fractions of 1-hour incubations of the cells with ^{35}S-sulfate-containing media by Sepharose CL-6B chromatography.

Peak fractions (of Figure 1) were chromatographed on DEAE-Sephacel in 0.13 M Tris-HCl, 7 M urea, 0.1% CHAPS, 1 mM PMSF, 10 mM NEM, 1 mM EDTA, pH 7.2, with a linear gradient of 0.0–1.0 M NaCl. As observed in Figures 2A and B, HS I and HS II eluted at the same ionic strength (approximately 0.5 M NaCl) while HS III (Figure 2C) eluted as a broad peak between 0.15 and 0.4 M NaCl. HS I and HS II were both completely degradable with HNO_2, but HS III peak was only partially degradable with HNO_2. HS III was then chromatographed with a concave gradient of 0.0–0.5 M NaCl, resulting in two peaks (Figure 2D). The material in the first of these peaks was not degraded with HNO_2 and did not yield

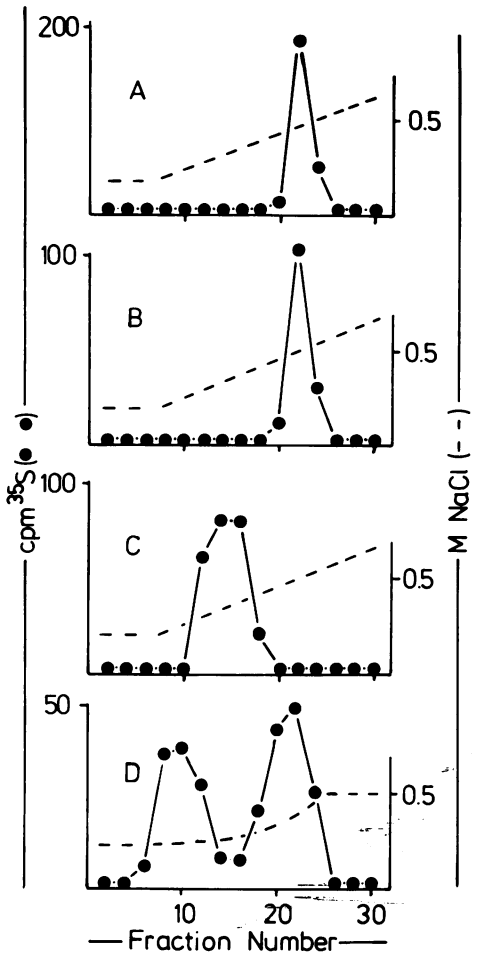


Figure 2—DEAE-Sephracel cnromatography of BAEC medium proteoheparan sulfates. Medium proteoheparan sulfates from a 1-hour pulse with ^{35}S-sulfate-containing medium were separated as depicted in Figure 1, applied to 0.5 × 1-cm columns, and eluted with linear (**A–C**) or logarithmic (**D**) gradients as described in materials and Methods. **A**, HS I, **B**, HS II; **C** and **D**, HS III.

high-molecular-weight material after alkali digestion, which indicated that it was not a proteoheparan sulfate. The material in the second peak, eluting later in the gradient (0.35 M NaCl), was degradable with HNO_2 and heparinase, which indicated that it was proteoheparan sulfate. This material was used for further studies.

HS I, HS II, and HS III were also chromatographed on Sepharose CL-4B and 6B before and after alkali digestion in order to ascertain the size and number of glycosaminoglycan chains for each species (Figure 3). Calibration of the columns was accomplished by using standards derived from corneal keratan sulfates as described by Stuhlsatz et al.[6] Following alkali digestion, the resultant glycosaminoglycan chains were estimated to be 35,000 daltons for HS I (using Sepharose CL-4B) and 20,000 daltons for HS II and HS III, respectively (using Sepharose CL-6B) (Figure 3,

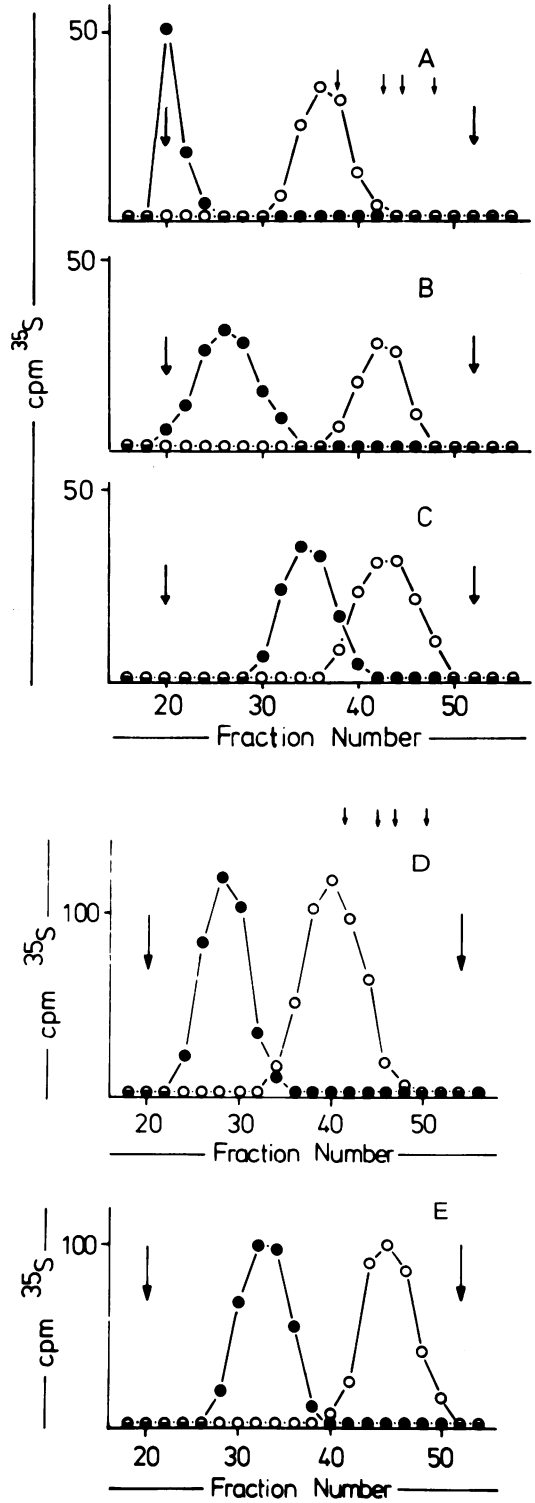


Figure 3—Sepharose CL-6B and 4B chromatography of BAEC medium proteoheparan sulfates before and after alkali digestion. Medium proteoheparan sulfates isolated from 1-hour pulsed cultures were isolated as described in Figure 1. The three species HS I (**A** and **D**), HS II (**B** and **E**), and HS III (**C**) were then run on 0.5 × 60-cm columns as described in Figure 1 before (*closed circles*) and after (*open circles*) alkali digestion. *Large arrows* denote V_o and V_t, respectively, and the *small arrows* denote the elution positions of the glycosaminoglycan standards of 35,000, 19,000, 17,000, and 13,000 as described in Figure 1. **A–C**, Sepharose CL-6B; **D** and **E**, Sepharose CL-4B.

open circles). After alkali digestion there was a definite decrease in the elution position of approximately fourfold for HS I and HS II and twofold for HS III, indicating a number of four glycosaminoglycan chains for HS I and HS II and two glycosaminoglycan chains for HS III.

Location and Subcellular Fractionation of BAEC Proteoheparan Sulfate Species

Because the pulse, pulse-chase and structural data supported the concept of at least three distinct BAEC proteoheparan sulfate species, we attempted to determine their localization using biochemical and morphologic techniques.

Confluent cultures of the cells were incubated with ^{35}S-sulfate-containing media for various times, ranging from 10 minutes to 24 hours, and the medium was decanted. The cells were lifted nonenzymatically by means of EGTA. The material remaining on the culture dish was considered to represent a "matrix" fraction. The medium, cellular, and matrix fractions were then analyzed separately. In assessing the matrix material a 24-hour incubation was performed to allow for adequate amounts of label to be incorporated. Matrix material was digested with chondroitinase ABC and then solubilized with 2% CHAPS; after this no further radioactive material could be extracted with 4.0 M guanidine hydrochloride. This material was then chromatographed on a Sepharose CL-6B column. Figure 4 (upper panel) shows the comparison of this material (closed circles) with the profile observed for HS I, the major medium proteoheparan sulfate obtained from the same incubation (open circles). The elution profile of medium-derived HS II, which could be isolated in modest amounts from culture medium after a 60-minute pulse (see Figure 1), indicates that the matrix-derived proteopheparan sulfate was highly enriched for HS II-like material as compared with the medium fraction from the same incubation. These data are consistent with the notion that HS II is a major proteohaparan sulfate matrix component produced by the cells in culture.

For the analysis of the cell-associated proteoheparan sulfate species subcellular fractionations of microsomal preparations of ^{35}S-sulfate pulse-labeled cells were performed. After discontinuous sucrose density gradient centrifugation two subcellular sulfate pools were observed: one appeared at 44% sucrose and did not accumulate label at longer incubation times; the other, which appeared at the interface between 18% and 31% sucrose was observed to accumulate label with time (Figure 5). These fractions were examined for their content of 5′-nucleotidase activity

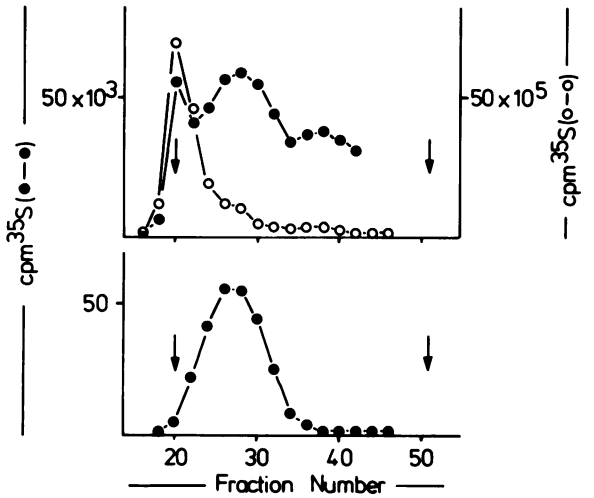


Figure 4—Sepharose CL-6B chromatography of chondroitinase ABC digested proteoglycans from the medium and the subendothelial matrix. **Top**—Cultures were incubated with ^{35}S-sulfate-containing medium for 24 hours. The medium was then removed, treated with chondroitinase ABC, and chromatographed on a 0.5 × 60-cm column with 0.5 M NH_4HCO_3 as eluant (*open circles*). Cells were removed with EGTA, and the remaining material was treated with chondroitinase ABC, solubilized with 2.0% CHAPS and chromatographed as above (*closed circles*). Fractions beyond #42 contain contaminating free sulfate and are not shown. **Bottom**—For comparison, an elution profile of a sample of the medium proteoheparan sulfate HS II is given. *Arrows* denote V_o and V_t, respectively.

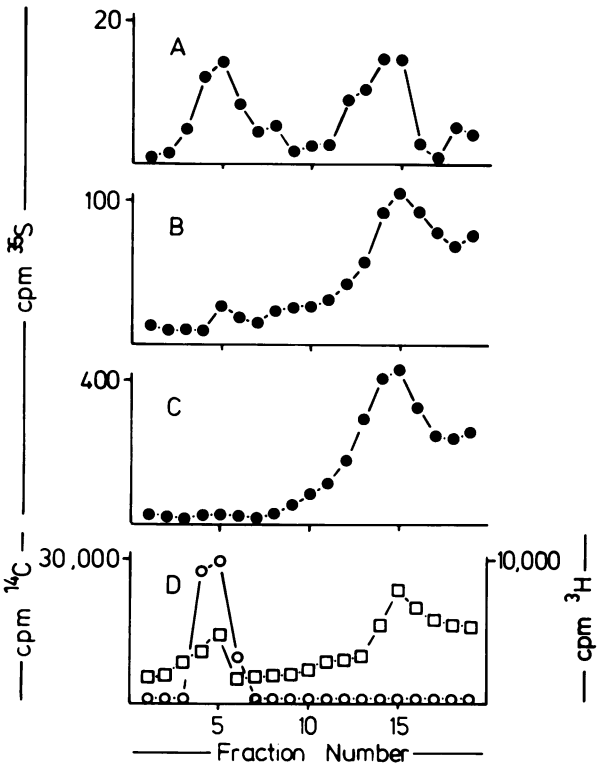


Figure 5—Sucrose density gradient centrifugation of BAEC subcellular fragments. Cells incubated with ^{35}S-sulfate for 10 minutes, 40 minutes, and 5 hours were washed, removed with EGTA, and sonicated. A 10,000–100,000*g* pellet was then applied to the top of a discontinuous sucrose density gradient. After centrifugation at 100,000*g*, fractions were assayed for ^{35}S for 10 minutes (**A**), 40 minutes (**B**), 5 hours (**C**), or (**D**) 5′-nucleotidase (*squares*) and galactosyltransferase (*circles*).

(a marker enzyme for plasma membranes) and galactosyl transferase activity (a marker enzyme for Golgi membranes). The low-density fractions contained 75% of the 5′-nucleotidase activity and only very low levels of galactosyl transferase activity. In contrast, approximately 95% of the galactosyl transferase activity was found associated with the high-density fractions, which contained low amounts of the 5′-nucleotidase activity. Furthermore, more than 90% of the labeled macromolecular material from the low-density membranes was found to be proteoheparan ^{35}S-sulfate as determined by chondroitinase ABC resistance and heparinase and heparitinase sensitivity (data not shown). In addition, this material was found to coelute with HS I isolated from medium fractions when chromatographed on Sepharose CL-2B (Figure 6).

The localization of cell surface- and matrix-associated proteoheparan sulfates (HS I and HS II, respectively) was also evaluated by indirect immunofluorescence microscopy by means of antisera raised against the HS I proteoheparan sulfate isolated from BAEC culture media and antisera raised against a matrix-associated proteoheparan sulfate isolated from HR 9 cells.[24] As illustrated in Figure 7, antibodies to HS I react with epithelial and endothelial cells of the bovine cornea. Because of the small amount of HS II obtainable from EC cultures, it has not been possible to isolate sufficient material for raising antibodies in rabbits. We have, however, obtained an antiserum to HS II-like material isolated from HR-9 cells,[24] which exhibits cross-reactivity with a proteoheparan sulfate from BAEC culture medium (T. Saku and H. Furthmayr, unpublished observations). When these antibodies were used in immunofluorescence studies, HS II was apparently localized in the basement membrane underlying the cells, in addition to a speckled, irregular pattern in the corneal stroma. When cross-sections of bovine aorta were stained, HS I was found to be associated with ECs. In contrast, HS II was noted to be localized beneath the ECs in a linear pattern. When mechanically denuded aortas were stained, no HS I was found on the acellular subendothelial matrix, but HS II was present in this location (data not shown). The antisera raised against HS I were also used for immunoprecipitation studies. Cells were incubated with ^{35}S-sulfate for 4 hours, and the medium was desalted on Sephadex G-50. After this, 13,500 cpm of this material was incubated with HS I antisera for 24 hours; then the mixture was passed over a Protein A-Sepharose column. The column bound 2870 cpm, which could be eluted at acidic pH. Because 62% of the labeled material was susceptible to chondroitinase ABC, we calculated that 56% of the chondroitinase-resistent material had been immunoabsorbed. The bound material was examined by chromatography on Sepharose CL-4B. As shown in Figure 7i, immunoabsorption of the labeled EC medium selected a substance out of the proteoglycan mixture (open circles), which eluted in the position of HS I (closed circles). This material was completely degraded by a heparinase/heparitinase mixture (data not shown), which suggested the absence of chondroitin sulfate moieties on HS I.

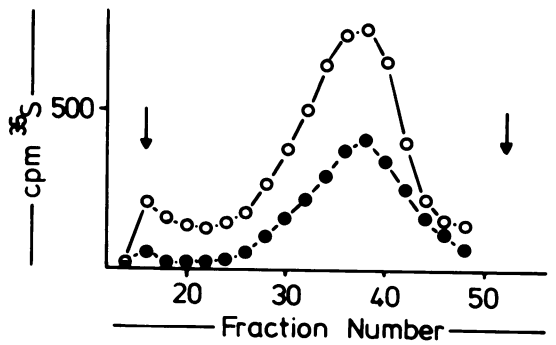


Figure 6—Sepharose CL-2B chromatography of plasmalemmal membrane proteoheparan sulfate and medium proteoheparan sulfate HS I. Medium HS I isolated as described in Figure 1 (*closed circles*) and the plasmalemmal membrane-associated proteoheparan sulfate isolated from a 5-hour pulse sucrose density gradient run as described in Figure 5 (*open circles*) were chromatographed separately on a 0.5 × 60-cm column in Tris-HCl running buffer containing 0.1% SDS. *Arrows* denote V_o and V_t, respectively.

Isolation and Partial Characterization of BAEC Proteoheparan Sulfate–HS I

In order to obtain preparative amounts of BAEC culture HS I, roller bottle cultures were used to harvest large amounts of medium. Purification of HS I involved chondroitinase ABC digestion and two cycles of chromatography on Sepharose CL-6B followed by two cycles of chromatography on DEAE-Sephacel at different pH as described in the Methods section. Figures 8 and 9 illustrate the gel filtration and ion exchange chromatography steps. As noted, the final product eluted as a single peak from the last DEAE-Sephacel column. When run on NuSieve Agarose gel electrophoresis this material appeared to migrate as a single band.[24] In addition, this material was examined by means of CsCl density gradient centrifugation at an average density of 1.45. The proteoglycan (HS I) appeared as a broad peak with enrichment at a buoyant density of approximately 1.5 (Figure 10). No enrichment of ^{125}I label was observed at the low-density fractions, which indicated very little contamination with proteins. This material was used as the immunogen for antisera production, for

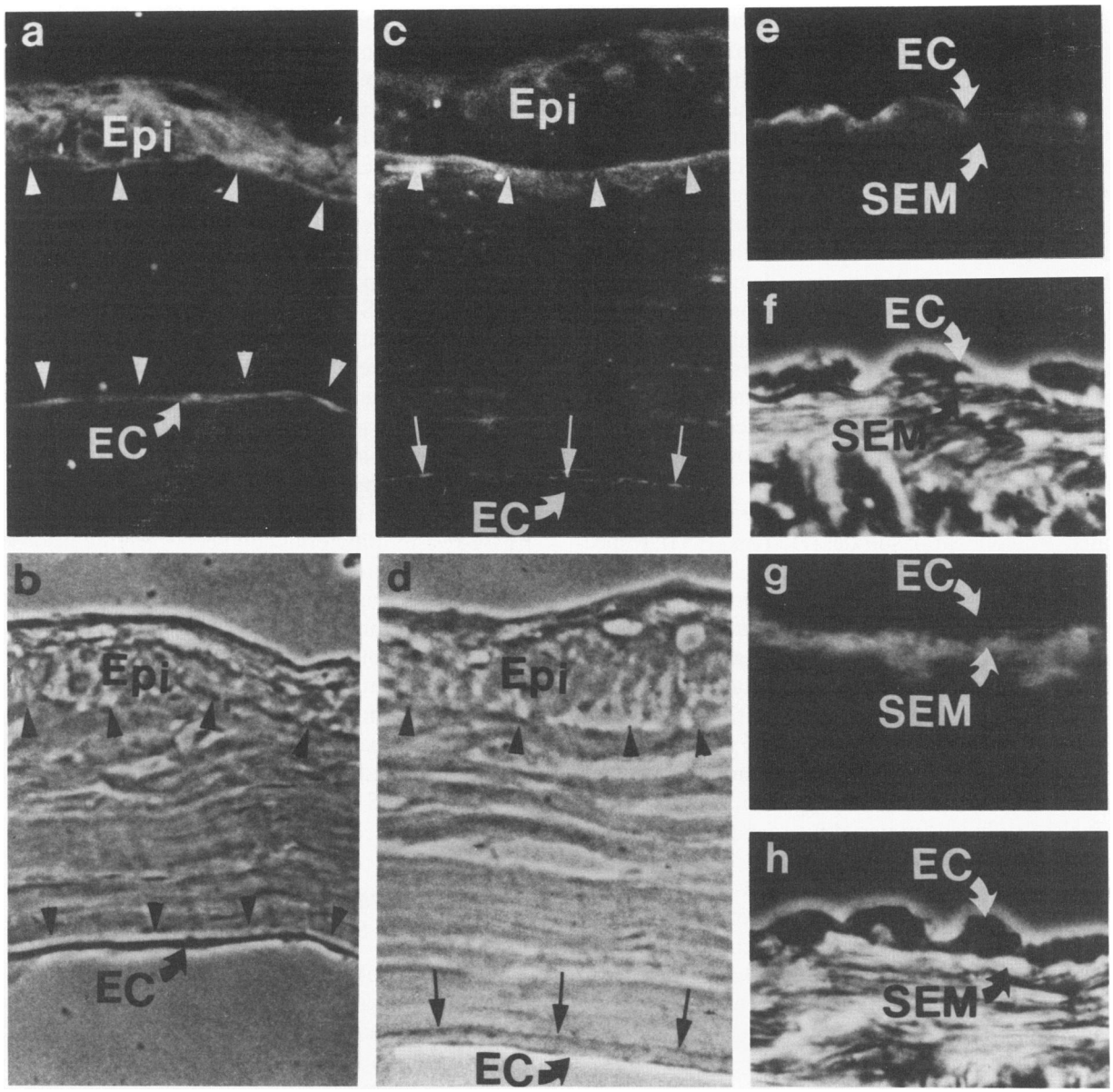


Figure 7—Indirect immunofluorescence of murine cornea and bovine aorta stained with antisera raised against the BAEC plasmalemmal membrane-associated proteoheparan sulfate HS I and a basement membrane proteoheparan sulfate isolated from HR 9 cells similar to BAEC HS II. **a**—Cross-section of cornea stained with HS I antisera illustrating intense fluorescence of epithelial (*Epi*) and endothelial (*EC*) cells and a lack of matrix staining. **b**—Phase micrograph of the above section revealing the epithelial (*Epi*) and endothelial (*EC*) cells as well as their respective basement membrane regions (*arrowheads*). **c**—Cross-section of cornea stained with HR 9 HS II antisera, illustrating linear fluorescence of Descemet's membrane (*arrows*) and the epithelial basement membrane (*arrowheads*) as well as a speckled fluorescence throughout the stroma. **d**—Phase micrograph of the above section revealing the epithelial (*Epi*) and endothelial (*EC*) cells and their respective basement membranes (*arrowheads*). **e**—Cross-section of aorta stained with HS I antisera illustrating endothelial cell (*EC*) fluorescence. Removal of the endothelial cells resulted in a loss of fluorescence (data not shown). **f**—Phase micrograph of the above section revealing the lining endothelial cells (*EC*) and the underlying subendothelial matrix (*SEM*). **g**—Cross-section of aorta stained with HR 9 HS II antisera illustrating fluorescence of the subendothelial matrix (*SEM*). Removal of the endothelial cells did not appreciably affect the fluorescence pattern (data not shown). **h**—Phase micrograph of the above section revealing the endothelial cells (*EC*) and the underlying subendothelial matrix (*SEM*). (Original magnification, ×400)

preparation of core protein, subsequent peptide mapping, and amino acid and amino-sugar analyses.

For estimation of the size of the HS I core protein, a chloramine T ^{125}I-labeled preparation was digested with a 1:1 mixture of heparinase and heparitinase and chromatographed on a S-200 column (Figure 11). A single peak was observed that eluted in a position of approximately 55,000 daltons when compared with elution profiles of protein standards. Although digestion with this mixture of enzymes is more effective than with heparinase alone, it may still not remove the glycosaminoglycans quantitatively, and the mo-

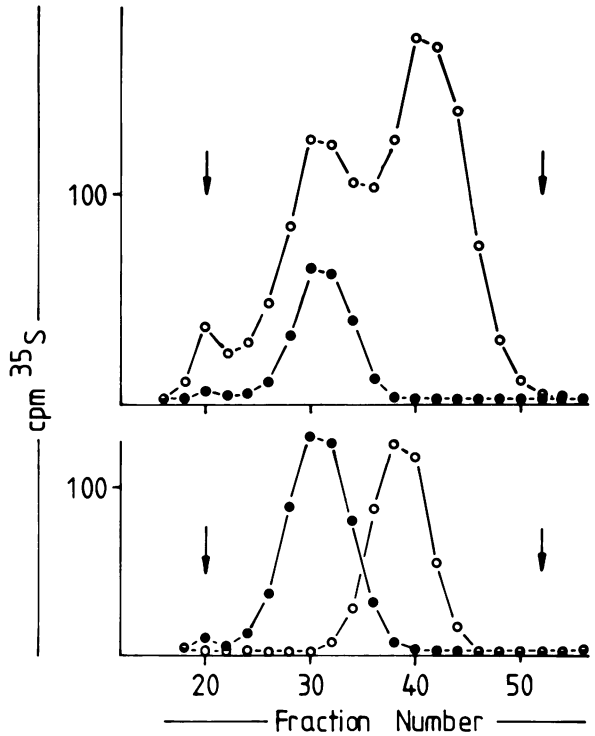


Figure 7i—Sepharose CL-4B chromatography of endothelial cell medium proteoglycans immunoabsorbed and eluted from HS I antisera. Desalted endothelial cell medium proteoglycans (13,500 cpm) from a 4-hour incubation were chromatographed on Sepharose CL-4B (0.5 × 60 cm) in Tris/HCl running buffer containing 0.1% SDS before (*open circles, upper figure*) and after absorption and elution from Protein A-Sepharose-bound HS I antisera (*closed circles, upper figure*). For comparison, the elution profiles of medium proteoheparan sulfates HS I (*closed circles, lower figure*) and HS II (*open circles, lower figure*) on Sepharose CL-4B from a 60 min ^{35}S-sulfate pulse are given. Arrows indicate V_o and V_t.

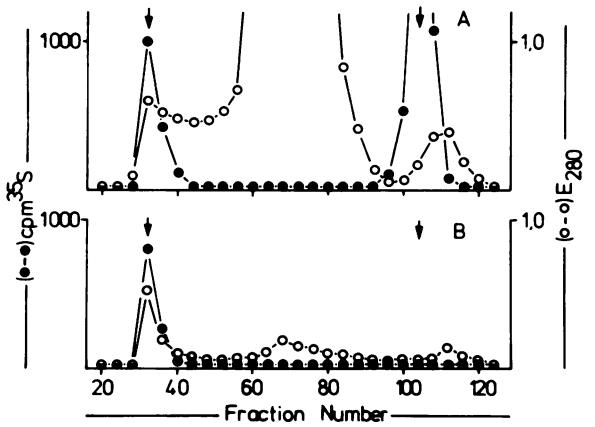


Figure 8—Preparative Sepharose CL-6B chromatography of BAEC medium proteoheparan sulfate HS I. **A**—Medium from roller bottle cultures was desalted, digested with chondroitinase ABC, concentrated, and run on a 5.0 × 70-cm column as described in the Methods. The void peak (containing HS I) was collected. **B**—The void peak was concentrated, rerun on the same column, and collected again. *Arrows* denote V_o and V_t, respectively. *Open circles*, $OD_{280\ nm}$; *closed circles*, cpm^{35}S.

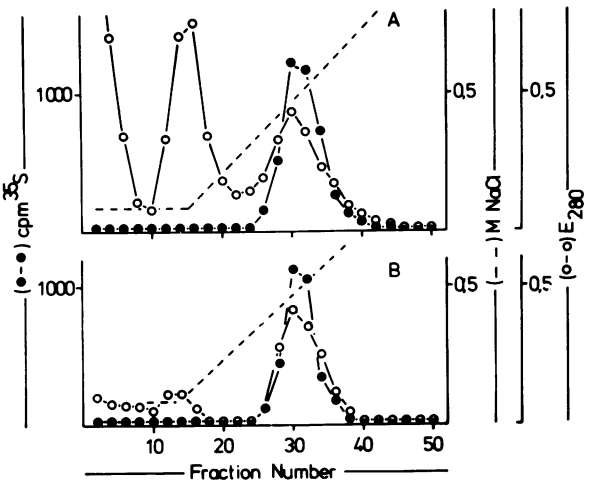


Figure 9—Preparative DEAE-Sephacel chromatography of BAEC medium proteoheparan sulfate HS I. **A**—The void peak collected from the second Sepharose CL-6B preparative column (Figure 8) was equilibrated in DEAE buffer, pH 7.2, applied to the column, and eluted with a linear gradient as described in Materials and Methods. The material eluting at 0.5 M NaCl was pooled. **B**—The material pooled in **A** was equilibrated in DEAE buffer, pH 6.0, applied to the column, and eluted with a linear gradient as described in Materials and Methods. The material eluting at 0.5 M NaCl was collected and used for further characterization and antisera production. *Closed circles*, ^{35}S; *open circles*, $OD_{280\ nm}$; ----, NaCl gradient.

lecular weight value obtained may still be too high. With this limitation, a value of approximately 195,000 daltons could be tentatively given for the molecular weight of HS I. Reduction and alkylation did not alter the elution positions of any of the endothelial proteoheparan sulfates.

Amino acid and amino sugar analyses of HS I (Table 1) are consistent with the findings of four glycosaminoglycan chains per core protein as determined by chromatography before and after alkali digestion. From this, assuming 100 as an average amino acid molecular weight, we estimated the ratio of protein to glycosaminoglycan to be approximately 1 : 3.

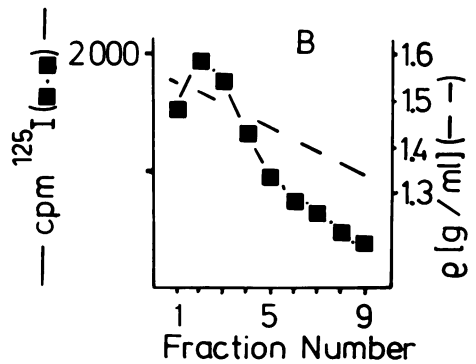


Figure 10—CsCl density gradient centrifugation of BAEC medium proteoheparan sulfate HS I. Chloramine T ^{125}I-labeled HS I was dissolved in CsCl (density, 1.45, 0.1 M Na[CH_3CO], pH 6.5, and centrifuged at 160,000*g* for 60 hours.

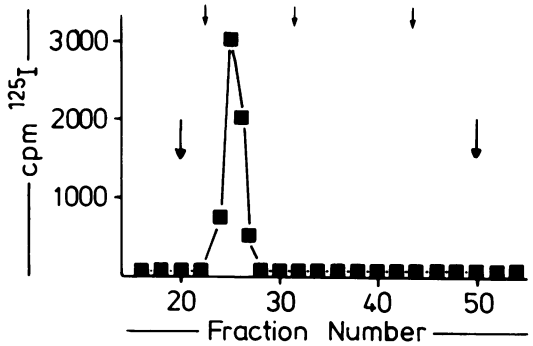


Figure 11—Sephacryl S-200 chromatography of ^{125}I-labeled BAEC medium proteoheparan sulfate HS I core protein. Radiolabeled (^{125}I) HS I was digested with a mixture of heparinase and heparitinase and applied to a 0.5 × 60-cm column. A single peak was noted. *Large arrows* denote V_o and V_t, respectively. *Small arrows* denote the elution positions of protein standards (66,000, 45,000, and 31,000).

Discussion

ECs have been shown to produce a variety of proteoheparan sulfate species in culture.[5-7] Whether these are the products of different genes or result from the processing of a single precursor is still not clear. Our data suggest that BAECs in culture produce at least three medium proteoheparan sulfates, which differ in molecular weight and in behavior on DEAE ion exchange resin. Pulse and pulse-chase experiments indicate that these proteoheparan sulfates can be grouped into two different populations according to their transportation kinetics from the cell layer to the medium. The predominant proteoheparan sulfate (HS I) appears in the medium only after a lag period (data not shown). This suggests that HS I is enriched in an intermediate pool (such as the plasmalemmal membrane) prior to its release into the medium. Supporting this concept was the finding of a proteoheparan sulfate of identical size and characteristics associated with plasmalemmal fractions of the cells. In addition, antisera raised against HS I stained ECs and selected tissues in patterns consistent with a plasmalemmal membrane/cell surface localization.

Table 1—Amino Acid and Aminosugar Composition of HS I

Amino acid	Mol/1000 mol
Asp	87
Thr	72
Ser	145
Glu	143
Gly	148
Ala	86
Val	48
Met	6
Ile	27
Leu	65
Tyr	25
Phe	30
Lys	48
His	30
Arg	40
Hex-N	546

While these data are consistent with the notion that HS I is released from the cell surface, it may not reflect an *in vivo* biologic phenomenon and could be explained by shedding of plasma membrane. Indeed, when proteoheparan sulfate from aortic EC organ cultures were examined, virtually no HS I was found in the medium (unpublished observations). Our interpretation of these findings is that the release of cell surface proteoheparan sulfate HS I is due to shedding and that it may represent a peculiarity of the tissue culture system. HS I was found to have four side chains of an estimated molecular weight of 35,000 daltons and a core protein of an estimated molecular weight of 55,000 daltons. On the basis of these data, an overall molecular weight of 195,000 could be calculated. This molecular weight is considerably smaller than the value reported earlier for the proteoheparan sulfate species isolated from the matrix of the EHS tumor (core protein of 100,000 and overall molecular weight of 400,000).[13] The differences could be due to differences in tissue and/or cellular source. However, other values (10,000 and 300,000) have been reported by another group for these proteoheparan sulfate species from the EHS tumor.[28] BHK cells were reported to make three cell-associated proteoglycans with core proteins of 65, 85, and 120 kd,[29] and rat liver membranes reportedly contain a cell surface proteoheparan sulfate with a molecular weight for the core protein between 17 and 40 kd.[30] In more recent work Rapraeger et al described a trypsin-released proteoheparan sulfate from the cell surface of mouse mammary epithelial cells, and this species had a core protein size of 53 kd.[31] Interestingly, this material also appears to contain chondroitin sulfate chains. These data indicate that plasma membrane proteoheparan sulfate core protein size probably is different from proteoheparan sulfates of the matrix and that it may also be variable, depending upon the cellular sources.

In contrast to HS I, the second group of proteoheparan sulfates (HS II and HS III) appear in the medium at early time points and are present in the medium at low levels during all pulse periods studied. However, they were not found in the medium during the chase period. HS II was found to be the major proteoheparan sulfate component in the subendothelial matrix after removal of the cells. Similar EC matrix-associated proteoheparan sulfate species have been found by other investigators and elution profiles

on Sepharose CL-4B and 6B are in good agreement with our data.[32,33] Furthermore, BAEC HS II has structural characteristics similar to a matrix-associated proteoheparan sulfate isolated from HR 9 cells, with four side chains of an estimated size of 20,000 daltons and elution patterns on Sepharose CL-4 and 6B and DEAE-Sephacel.[24] Additionally, a proteoheparan sulfate with the same properties as HS II was found to be liberated by collagenase digestion of the subendothelial matrix of aortic organ cultures after short incubation with ^{35}S-sulfate (unpublished observations). HS III—having two side chains with an estimated molecular weight of 20,000 daltons and unique elution pattern on DEAE-Sephacel—was the major labeled medium product during short incubations with ^{35}S-sulfate. It was only found in small amounts in the subendothelial matrix and in association with the plasmalemmal membrane. The function of this minor secreted proteoheparan sulfate of cultured BAECs is not yet clearly understood; however, it appears identical to the major secreted proteoheparan sulfate observed in aortic organ cultures maintained with pulsatile medium flow (unpublished observations). Our findings are in agreement with the recently published data of Kinsella and Wight, who have also found three proteoheparan sulfate species in the medium and cell layer fractions of cultured aortic ECs having similar glycosaminoglycan chain sizes and sensitivities to chondroitinase ABC and nitrous acid.[34] Our data support the concept that cultured BAECs produce at least three distinct species of proteoheparan sulfates having different transport kinetics: the plasmalemmal membrane-associated form (HS I) is shed into the culture medium in large amounts after an initial delay; the matrix-associated form (HS II) and the medium form (HS III) appear earlier and are therefore not derived from the larger HS I.

Recently, Rapraeger et al described a hybrid cell surface proteoglycan, isolated from cultured mammary gland epithelial cells, bearing both chondroitin sulfate and heparin sulfate glycosaminoglycans.[31] The existence of a similar molecule cannot be excluded in our study because we employed a chondroitinase ABC digestion prior to our analyses. However, the data of Kinsella and Wight and our immunoabsorption data obtained from non-chondroitinase-treated material indicate that the high molecular weight medium heparan sulfate proteoglycan isolated from confluent endothelial cell monolayer cultures is essentially completely sensitive to nitrous acid, heparinase, and heparitinase, which indicates an absence of chondroitin sulfate.[34] The functions of these moieties *in vitro* and their relevance *in vivo* can only be speculated upon at this time but could include participation in cell–cell and cell–matrix interactions, modulation of the clotting cascade, influence on matrix organization, and modulation of vascular smooth muscle cell proliferation and migration.[10,11,28]

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