Cell Surface Heparan Sulfate Proteoglycans from Human Vascular Endothelial Cells

CORE PROTEIN CHARACTERIZATION AND ANTITHROMBIN III BINDING PROPERTIES*

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Human aortic endothelial cells (HAEC) and human umbilical vein endothelial cells (HUVEC) were labeled with $^{35}$S-OH for 48 h. The membrane-associated proteoglycans were solubilized from these monolayers with detergent and purified by ion-exchange chromatography on Mono Q, incorporation in liposomes, and gel filtration. The liposome-intercalated proteoglycans were $^{125}$I-labeled and treated with heparitinase before SDS-polyacrylamide gel electrophoresis. Radiolabeled proteins with apparent molecular masses of 150, 60, 46, 35, and 30 kDa (HAEC) and 180, 130, 62, 43, and 35 kDa (HUVEC) were detected by autoradiography. Further characterization by affinity chromatography on immobilized monoclonal antibodies and by Northern blot analysis provided evidence for the expression of syndecan, glypican, and fibroglycan in human endothelial cells. Most of the heparan sulfate which accumulated in the subendothelial matrix was implanted on a 400-kDa core protein. This protein was immunologically related to perlecan and bound to fibronectin. Binding studies on immobilized antithrombin III suggested that all membrane-associated heparan sulfate proteoglycan forms had the capacity to bind to antithrombin III but that high affinity binding was more typical for glypican. Most of the proteoglycans isolated from the extracellular matrix also bound only with low affinity to antithrombin III. These results imply that glypican may specifically contribute to the antithrombotic properties of the vascular wall.

Heparan sulfate proteoglycans are found almost ubiquitously on the cell surface and in the extracellular matrix of mammalian cells. A wide range of biological functions, including growth control, cell adhesion, and anticoagulant activity,

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1The abbreviations used are: GPI, glycosyl phosphatidylinositol; HAEC, human aortic endothelial cells; HUVEC, human umbilical vein endothelial cells; GdnHCl, guanidine chloride; mAb, monoclonal antibody; PBS, phosphate-buffered saline; HS, heparan sulfate; CS, chondroitin sulfate; PG, proteoglycan; SDS, sodium dodecyl sulfate; Mops, 3-(N-morpholino)propanesulfonic acid; kb, kilobase(s).

have been ascribed to the heparan sulfate proteoglycans (for review see Kjellén and Lindahl, 1991). The heparan sulfate chains play a crucial role in most of the biological properties of these proteoglycans. Heparan sulfate can bind to extracellular matrix components such as laminin, fibronectin, collagen, and thrombospondin (for review see Jackson et al., 1991), is essential for the binding and signal transduction of growth factors (Rapraeger et al., 1991; Yayon et al., 1991), and anchors several enzymes, including lipoprotein lipase to the cell layer (Saxena et al., 1990). The most extensively studied interaction of heparin and heparan sulfate, however, is that with antithrombin III. High affinity binding to this proteinase inhibitor which results in an anticoagulant effect depends on the presence of a specific pentasaccharide sequence in these glycosaminoglycans (Thunberg et al., 1982). The structural requirements for the interactions of heparan sulfate with other proteins are not known but seem less stringent (Bengtsson et al., 1980).

The core proteins, on the other hand, target the glycosaminoglycans to their strategic positions on the cell surface and in the extracellular matrix and determine their accumulation and turnover at these sites. Several core proteins, which constitute different gene products, have been described. Lung fibroblasts for example synthesize a 400-kDa heparan sulfate proteoglycan core protein which binds to fibronectin and accumulates in the extracellular matrix (Heremans et al., 1990). This proteoglycan is related to perlecan, the proteoglycan which accumulates in basement membranes, but is not related to the cell surface-associated heparan sulfate proteoglycans of these cells which are characterized by core proteins of 125 kDa, 80 kDa (syndecan), 64 kDa (glypican), 48 kDa (fibroglycan) and 35 kDa (Lories et al., 1989). Gliptican and its associated heparan sulfate, which are membrane-linked through a GPI-1-anchor structure, are cleared from the cell surface by shedding, whereas all other cell surface proteoglycans appear subject to endocytosis (David et al., 1990).

Taking into account the structural heterogeneity of the core proteins and the multiplicity of functions ascribed to the heparan sulfate chains, the question arises whether functionally distinct heparan sulfate chains may occur on different core proteins. In this respect the endothelial cell system is of particular interest, since endothelial proteoglycans appear involved in specific biological functions which include the formation of atherosclerotic plaques and the propagation of tumor growth and metastasis.
anchorage and transcytosis of lipoprotein lipase (Shimada et al., 1981; Saxena et al., 1991), the binding of basic fibroblast growth factor, possibly as a "supply" for the high affinity fibroblast growth factor receptor (Sakseka et al., 1988), and an antithrombotic activity (Marcum et al., 1986). Recently, two distinct membrane-associated heparan sulfate proteoglycans that bind antithrombin III have been isolated from cloned rat microvascular endothelial cells (Kojima et al., 1992a, 1992b), whereas earlier studies on venous and aortic vascular endothelial cells had identified a heparan sulfate proteoglycan with a core protein of 350 kDa which accumulates in the extracellular matrix (Kinsella and Wight, 1988; Saku and Furthmayr, 1989; Lindblom et al., 1989). We have extended these studies and report the isolation and characterization of several distinct heparan sulfate proteoglycans from human aortic and umbilical vein endothelial cells. Besides the previously described matrix-associated heparan sulfate proteoglycan, we identified an important heparan sulfate fraction which could associate with artificial liposomes. These heparan sulfate chains were carried by several different hydrophobic core proteins that were related to the membrane-associated core proteins previously isolated from human lung fibroblasts. Binding studies on immobilized antithrombin III suggested that all membrane-associated heparan sulfate proteoglycans, to some extent, can bind antithrombin III with high affinity but that glypicans may bind preferentially. Like most of the cell surface proteoglycans, the heparan sulfate proteoglycan that accumulated in the subendothelial matrix displayed a low binding affinity for antithrombin III.

**MATERIALS AND METHODS**

**Cell Culture—**Human aortic endothelial cells HAEC (AG09979, Institute for Medical Research, Camden, NJ) were grown on gelatin (0.1%)-coated dishes in Medium 199 (Flow Laboratories) supplemented with 20% (v/v) fetal calf serum, 50 μg/ml of heparin, and 50 μg/ml of ECGF (Sigma).

Human umbilical vein endothelial cells (HUVEC) were prepared according to the procedure of Jaffe et al. (1973). The cells were grown on tissue-culture flasks coated with gelatin (0.1%) in Medium 199 containing 2 mM L-glutamine, 100 units/ml of penicillin, 100 μg/ml of streptomycin, 20 mM Heps, 10% fetal calf serum, and 10% human serum. As a control for the endothelial origin of the cultured cells, a preparation was assayed for von Willebrand Factor-related antigen using an immunofluorescence assay as described by Heremans et al. (1973). The cells were cultivated for a maximum of two passages. Confluent cultures (∼4 × 10⁶ cells/cm²) were labeled for 48 h with 5 μCi (1.8 × 10⁸ Bq) of carrier-free H235S04 (Du Pont-New England Nuclear)/mL of culture medium (25 μl of medium/175-cm² culture flask).

**Isolation of the Cell Surface Proteoglycans—**Confluent layers (∼3 × 10⁶ cells/experiment) were rinsed three times with cold phosphate-buffered saline (PBS). Rinsed endothelial cells were extracted with Triton X-100 buffer (0.5% Triton X-100, 10 mM Tris/Cl, pH 8.0, 160 mM NaCl) containing 50 mM 6-aminohexanoic acid, 10 mM EDTA, 5 mM N-ethylmaleimide, 5 mM benzamidine, 1 mM phenylmethanesulfonyl fluoride, and 1 μg/ml of pepstatin A as proteinase inhibitors (Lories et al., 1986). The detergent extract was cleared by centrifugation (10,000 x g; 60 min), concentrated by adsorption on DEAE-Trisacryl M, and eluted in urea buffer (0.5% Triton X-100, 50 mM Tris/Cl, pH 9) containing 750 mM NaCl. The eluate was submitted to ion-exchange chromatography on a Mono Q HR 5/5 column (Pharmacia LKB Biotechnology Inc.). After eluting the bulk of the bound proteins with a 0–0.6 M linear NaCl gradient (18 mM/ml in urea buffer (Lories et al., 1987), the retained proteoglycans were rinsed with urea buffer containing 50 mM octyl glucoside instead of Triton X-100, eluted in 4 mM GdnHCl buffer (see below) containing 750 mM octyl glucoside and proteinase inhibitors, and incorporated into lipid vesicles as described before (Lories et al., 1987). The proteoglycan/liposome mixture was fractionated on a (1 × 100 cm) Sepharose CLAB column in 4 mM GdnHCl buffer (4 mM GdnHCl, 100 mM 6-aminohexanoic acid, 10 mM EDTA, 10 mM N-ethylmaleimide, 5 mM benzamidine, 50 μg/ml of bovine serum albumin, 10 μg/ml of heparin, 10 μg/ml of chondroitin sulfate, and 50 mM sodium acetate, pH 5.8) at a flow rate of 3 ml/h, to separate hydrophobic liposome-associated proteoglycans from nonhydrophobic proteoglycans and free glycosaminoglycan chains. Excluded liposome-proteoglycan complexes were dissociated by addition of Triton X-100 (0.5% (v/v) final concentration) and rechromatographed at a flow rate of 3 ml/h on a (1 × 100 cm) Sepharose CL4B column in 4 mM GdnHCl buffer containing 0.5% (v/v) Triton X-100 (Lories et al., 1987).

**Isolation of the Matrix-associated HSPG—**The heparan sulfate proteoglycans from the extracellular matrix that remained attached to the culture flasks after extraction of the cell layers with detergent buffer were solubilized in 6 M GdnHCl and purified by C5c density gradient centrifugation in 4 M GdnHCl, ion-exchange chromatography on Mono Q in urea buffer, and gel filtration over Sepharose CL4B in 4 M GdnHCl buffer, as described by Heremans et al. (1988).

**Glycosaminoglycan Identification—**SO4²⁻ incorporated in heparan sulfate and chondroitin sulfate was measured by cetylpyridinium chloride precipitation of samples degraded by thermostabilin, chondroitinase ABC, and nitrous acid, pH 1.5, and control samples, as described by Lories et al. (1986).

**Radiolabeling and Immunopurification of the Proteoglycans—**Puriﬁcation of the surface and subendothelial heparan sulfate and chondroitin sulfate proteoglycans from HUVEC was performed as described by Lories et al. (1987). For treatment with phos-photadipositol-speciﬁc phospholipase C (Boehringer Mannheim), the proteoglycan samples were dissolved in 50 mM octyl glucoside, 10 mM EDTA, 50 mM Tris/Cl, pH 7.4, and incubated with 50 milliunits of enzyme for 3 h at 37°C.

**Gel Electrophoresis and Western Blotting—**Gel electrophoresis in 4–10% SDS-polyacrylamide gradient gels was done as described by Lories et al. (1987). Electrophoresis in SDS-agarose gels (4% Nusieve agarose, Pharmacia Fine Chemicals, Upsala, Sweden) was performed in buffer containing 90 mM Tris, 90 mM borate, 2 mM EDTA, 0.1% SDS, pH 8.0, for 3 h at 50 V.

In blotting experiments, the separated materials were electrotransferred from the gel to a Zeta-probe membrane (Bio-Rad) (15 h, 0.5 V). After transfer and inactivation for 1 h in PBS with 0.5% casein and 600 mM NaCl, the membrane was incubated with the appropriate monoclonal antibodies in PBS with 0.5% casein. The membrane was then rinsed three times in PBS containing 0.5% casein and 600 mM NaCl and further incubated for 1 h with alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulins (Promega), diluted 1/1000 in PBS which contained 0.5% casein. After rinsing, which contained 0.5% Tween 20, and two washes in 1 mM MgCl2, 50 mM bicinecarbonate, pH 9.5, the membrane was incubated with the chemiluminescent substrate 3-(2'-spiroadiamanate)-4-methoxy-3-(3'-phospholyloxy)phenyl-1,2-dioxethane disodium salt (AMPPD) (Tropix, Bedford, MA) (10 μl of AMPPD in 1 ml of bicinecarbonate buffer) for 5 min and submitted to autoradiography.

**Monoclonal Antibodies—**The monoclonal antibodies (mAb) used for these experiments were all raised against proteoglycans isolated from normal human fetal lung fibroblasts.

-mAbs 2E9, 6G12, and S1 are directed against the core proteins of different cell surface HSPG at lung and fibroblast (Lories et al., 1989). The mAbs 4B1, 9C9, 3H8, and 7H9, in contrast, all recognize the same matrix-associated HSPG (Heremans et al., 1989).

**Fibronectin Binding Assay—**The fibronectin binding assay was performed as described by Heremans et al. (1988). A 10-μg aliquot of purified plasma fibronectin (GIBCO-BRL) was submitted to SDS-electrophoresis to separate the fibronectin from possible contaminants in the sample and electoblotted to a nitrocellulose membrane. The membrane was incubated in PBS, 0.5% casein and incubated overnight at 4°C with heparitinase-digested 35S-labeled heparan sulfate proteoglycan from the subendothelial matrix. After rinsing, the membrane was submitted to autoradiography to localize any bound proteoglycan core and stained with polyclonal rabbit anti-fibronectin and peroxidase-conjugated swine anti-rabbit antibodies (Dakopatts, Glostrup, Denmark) to detect the proteoglycan core protein.
al. (1974) and covalently linked to CNBr-activated Sepharose CL-4B (3 mg/ml) in the presence of acetylated heparin. Free heparan sulfate chains, prepared by β-elimination of proteoglycans isolated from the culture medium of mouse mammary epithelial cells and known to contain the 3-O-sulfated glucosamine residues that mark the antithrombin-binding region (Fejler and David, 1987), were used to test the binding properties of the immobilized antithrombin. The 35S-labeled glycosaminoglycans were applied to the antithrombin column (5 ml of gel) in 50 mM NaCl in assay buffer (10 mM Tris/HC1, pH 7.4, containing 10 μg/ml of acetylated bovine serum albumin and 0.1% Triton X-100). After an overnight incubation at 4 °C, the column was washed with 50 mM NaCl in assay buffer (10 column volumes) and eluted at a flow rate of 9 ml/h with a 0.05-2 M NaCl gradient (15 mM/ml) in assay buffer. Materials eluting above 0.4 M NaCl were concentrated, reapplied (after dialysis) to the column, and eluted as before. Nearly 95% of this "high affinity" glycosaminoglycan fraction eluted above 0.4 M NaCl, indicating that the binding and elution properties of these materials were specific.

35S-labeled endothelial proteoglycan samples (representing the isolate from ~3 x 10^7 cells, and containing ~1.8 μg of HS or the equivalent of ~46 pmol of HS chains) were fractionated on the antithrombin column as described above. Alternatively, the radioiodinated cell surface proteoglycans (from ~3 x 10^7 cells) or the 35S-labeled proteoglycans from 100 ml of conditioned culture medium were immunopurified, dissolved in 300 μl of 50 mM NaCl in assay buffer, and mixed with 300 μg of the immobilized antithrombin. After an overnight incubation at 4 °C, the beads were washed and stepwise eluted using 4 column volumes of, successively, 0.05, 0.2, 0.4, and 2 M NaCl in assay buffer. When tested in this assay, more than 90% of the free HS chains (from mouse mammary epithelial cells) with high affinity for antithrombin III were eluted in the 2 M step. In both binding assays the immobilized antithrombin was present in 5-10 x 10^5-fold molar excess over the amount of HS added.

Isolation of RNA and Northern Blot Analysis—The poly(A)^+ RNA from ~3 x 10^7 cultured human umbilical vein endothelial cells was extracted in 5 M guanidine isothiocyanate and isolated by phenol/ chloroform extraction in the presence of sodium acetate (0.1 M), two isopropanol precipitations, and oligo(dT)-cellulose chromatography as described before (Marynen et al., 1989). Aliquots of 3 μg of denatured poly(A)^+ RNA/lane were submitted to electrophoresis in 1.3% agarose gels containing 0.2 M Mops, 0.05 M sodium acetate, pH 7.0, 10 mM EDTA, and 6% formaldehyde and further analyzed as described by David et al. (1990).

RESULTS

Isolation of the Membrane-associated and Matrix-associated Proteoglycans—The membrane-associated heparan sulfate proteoglycans from confluent human aortic (HAEC) and human umbilical vein endothelial cells (HUVEC) were metabolically labeled with 35SO_4^− for 48 h, extracted with Triton X-100 buffer, purified by ion-exchange chromatography on Mono Q, and incorporated into liposomes as indicated under "Materials and Methods," and finally fractionated over Sepharose CL-4B in 4 M GdnHCl buffer without detergent (Fig. 1). For both cell lines three distinct 35S-labeled peaks were obtained: excluded materials, materials eluting with Kav~0.33, and a peak with Kav~0.58. All the fractions of these eluates were analyzed for their [35S]HS and [35S]CS contents. In HAEC the excluded fractions represented approximately 40% of the total [35S]HS recovered from the Mono Q column. The majority of the extracted [35S]CS (85%), in contrast, was included in the column. To separate liposome-incorporated materials from excluded high molecular weight proteoglycans, the V0 fractions (indicated by the bar in Fig. 1) were pooled, subjected to Triton X-100, and eluted over Sepharose CL-4B in 4 M GdnHCl buffer containing Triton X-100 (Fig. 2). Nearly 65% of the [35S]sulfate which originally eluted in the void volume was included after detergent treatment (Kav ~0.33). These fractions, which represented ~26% of the detergent-extracted [35S]HS, were defined as membrane-associated hydrophobic proteoglycans. The recoveries of 35S-label at each step of the purification procedure and the heparan sulfate compositions of the fractions of a typical experiment in HAEC were listed in Table 1.

The extracellular matrix, still attached to the culture flask after extraction with detergent, was scraped in PBS and extracted in 4 M GdnHCl. Heparan sulfate proteoglycans were purified from this extract by CsCl density gradient ultracentrifugation. Nearly 70% of the [35S]labeled molecules were recovered in a peak with buoyant density of 1.32 g/ml. These fractions, which contained the bulk of the [35S]HS, were further purified by ion-exchange chromatography on Mono Q (eluting near 0.8 M NaCl) and gel filtration over Sepharose

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**Fig. 1.** Purification of the hydrophobic HSPG from vascular endothelial cells. Hydrophobic proteoglycans were extracted from the endothelial cell layer with detergent and purified by ion-exchange chromatography on Mono Q. The 35S-containing fractions were pooled, incorporated into liposomes, and submitted to gel filtration over Sepharose CL-4B in 4 M GdnHCl buffer. Each collected fraction was analyzed for its total [35S]HS ([••••]), [35S]HS (C-O) and [35S]-CS ([••••]) content. HAEC (data shown) and HUVEC (not shown) yielded similar results.

**Fig. 2.** Purification of the hydrophobic HSPG from vascular endothelial cells. Proteoglycans coeluting with the liposomes (indicated by the bar in fig. 1.) were pooled, supplemented with Triton X-100 (0.5%), and rechromatographed on Sepharose CL-4B in 4 M GdnHCl buffer containing Triton X-100. The first peak, not affected by the detergent, consisted of CSPG.

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*Endothelial Heparan Sulfate Proteoglycans*
prepare CL-4B in 4 M GdnHCl buffer (eluting with \( K_w < 0.1 \)) (not shown). From the recovery at this stage it was calculated that up to 30% of the heparan sulfate proteoglycans could be extracted from the cell layer with detergent, whereas nearly 70% were associated with the extracellular matrix.

Similar results were obtained for HUVEC (not shown). Based on the yield of \( ^{35} \text{S} \)HS at the final step and on the specific activity of the \( \text{SO}_4^{2-} \) in the culture medium (\( 1.1 \times 10^7 \) dpm/\( \mu \text{mol} \)), we calculated that \( \sim 1.8 \mu \text{g} \) of HS were recovered as membrane-associated proteoglycan from \( 3 \times 10^7 \) HUVEC.

**Characterization of the Matrix-Associated Heparan Sulfate Proteoglycans**—The heparan sulfate proteoglycans isolated from the HAE C or HUVEC matrix were labeled with \( ^{125} \text{I} \), and heparitinase-treated and nonnontreated iodinated samples were submitted to SDS-agarose electrophoresis, followed by transfer to a Zeta-probe membrane and autoradiography. Nonreduced digested samples showed a band of \( M_r \sim 400,000 \). Treating the samples with 100 mM \( \beta \)-mercaptoethanol (10 min at 100 \( ^\circ \)C in the presence of 1% SDS) decreased the mobility of this band (Fig. 3).

Immunostaining of a Western blot of unreduced matrix heparan sulfate proteoglycans, using a mixture of monoclonal antibodies raised against the perlecan-related matrix HSPG of human lung fibroblasts (Heremans et al., 1989), visualized the same high molecular weight band as detected by autoradiography (Fig. 4).

Further evidence for similarities between the matrix-associated HSPG from human fibroblasts and vascular endothelial cells was found by functional analysis. In prior studies we had identified a high affinity interaction between fibronectin and the core protein of the HSPG which is associated with the extracellular matrix of human lung fibroblasts (Heremans et al., 1990). To test for similar properties of the core of the matrix-associated HSPG in endothelial cell cultures, human plasma fibronectin was fractionated on a 4% NuSieve agarose gel and electrotransferred to a nitrocellulose membrane. After inactivation with casein, the membrane was incubated with heparitinase-digested \( ^{125} \text{I} \)-labeled HSPG from HUVEC were incubated with (+) or without (−) heparitinase, submitted to SDS-polyacrylamide gradient gel electrophoresis under reducing (R) or nonreducing (UR) conditions (\( 5 \times 10^2 \) cpm/lane), and detected by autoradiography. Similar results were obtained for HAE C (not shown).

**Characterization of the Membrane-Associated Heparan Sulfate Proteoglycans**—To identify the core proteins of the hydrophobic proteoglycans from vascular endothelial cells, \( ^{125} \text{I} \)-labeled proteoglycan samples were subjected to SDS-polyacrylamide gel electrophoresis and autoradiography. The intact proteoglycans migrated as a broad band in the high molecular weight region of the gel. In both HAE C (Fig. 6A) and HUVEC (Fig. 6B) multiple bands could be visualized after digestion with heparitinase. These bands represented
In fibroblasts, glypican is anchored to the plasma membrane via a GPI, whereas fibroglycan and syndecan are components with a transmembrane orientation. To investigate the possibility of membrane insertion through a GPI anchor in endothelial cells, the $^{125}$I-labeled samples from HUVEC were fractionated on immobilized mAb S1 and on mAb 2E9, and aliquots of the immunopurified proteoglycans were treated with phosphatidylinositol-specific phospholipase C. Phospholipase C-treated and nontreated samples were ultracentrifuged on a sucrose gradient (Andres et al., 1989). As a result of phospholipase C treatment, the ratio of liposome-incorporated to nontargeted mAb S1-purified proteoglycans was reduced from 1 to 0.3, while this proportion remained constant for proteoglycans purified on mAb 2E9. This indicated that at least a fraction of the mAb S1-bound membrane HSPG had a GPI-anchor.

Mab S1 reacted also with a HSPG from the culture medium conditioned by HUVEC (Fig. 8). The core protein of this nonhydrophobic medium proteoglycan was slightly smaller than the core protein of the hydrophobic cell surface-associated proteoglycan which reacted with the same antibody (Fig. 8). Similar results had been obtained previously for membrane and medium-associated glypican in human lung fibroblasts (David et al., 1990), and similar experiments with proteoglycans isolated from HAEc were in accordance with these findings (results not shown).

Finally, the expression of cell surface proteoglycans in human vascular endothelial cells was also studied at the transcriptional level. Northern blots of poly(A)$^+$ RNA from HUVEC were hybridized with $^{32}P$-labeled probes coding for fibroglycan, glypican, syndecan, and the 35-kDa HSPG core protein of human lung fibroblasts and submitted to autoradiography. The poly(A)$^+$ RNA contained a glypican RNA of ~3.7 kb, whereas the human syndecan probe detected a major ~2.5-kb mRNA. The fibroglycan probe and the 35-kDa HSPG core protein probe revealed mRNA bands of respectively ~2.3 and ~2.6 kb (Fig. 9). These results confirmed the expression of syndecan, glypican, and fibroglycan in vascular endothelial cells and suggested that the endothelial and fibroblastic proteoglycans which yielded a 35-kDa core were also identical or related to each other.

**Antithrombin III Binding Properties of the Endothelial HSPG**—Since we were not able to isolate sufficient amounts of $^{35}$S-labeled materials from the different hydrophobic HSPG forms to test for antithrombin III binding of the free HS chains, unfractionated preparations of hydrophobic proteoglycans (from ~3 × 10$^7$ HUVEC) were labeled with $^{125}$I and...
Endothelial Heparan Sulfate Proteoglycans

- Heparitinase
- Chondroitinase ABC

5- Mr x 10^{-3}

-200

-97

-68

-43

-28

-18

26

18

4.40

2.37

1.37

U

6C12

6G12

2E9

FIG. 7. Immunopurification of fibroglycan and syndecan from vascular endothelial cells. Purified and ^{125}I-labeled hydrophobic HSPG were incubated with mAb 2E9 or mAb 6G12 which had been immobilized on CNBr-activated Sepharose. Bound proteoglycans were eluted with 4 M GdnHCl. Equal amounts of nontreated, heparitinase-, and/or chondroitinase ABC-treated samples were analyzed by SDS-polyacrylamide gel electrophoresis (10^3 cpm/lane) and detected by autoradiography.

FIG. 8. Immunodetection of glypican in vascular endothelial cells. Purified hydrophobic proteoglycans (C) and proteoglycans isolated from the culture medium (M) of HUVEC were digested with heparitinase and fractionated by SDS-polyacrylamide gel electrophoresis. After electrotransfer to a Zeta-probe membrane, glypican was immunostained with mAb S1. The undigested immunoreactive materials migrated as broad smears and transferred poorly from the gels (not shown).

applied to a 10^4-fold molar excess of immobilized antithrombin III in a buffer of low ionic strength (0.05 M NaCl). After washing the antithrombin column with incubation buffer, ~30% of the applied sample remained bound to the column.

These materials were eluted with a NaCl gradient (Fig. 10), and equal amounts of radioactive HSPG from the initial sample (S), from the fall-through (FT), from the rinses (R), and from proteoglycans eluting below 0.4 M NaCl (pool I) and above 0.4 M NaCl (pool II) were digested with heparitinase and chondroitinase ABC and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography (Fig. 11). Compared with the original sample (S), the nonbound fractions (FT, R) were depleted, and the bound fractions (I and II) enriched in a 62-kDa core protein, suggesting that this proteoglycan had a higher density of antithrombin III-binding chains.

In an alternative assay the different ^{125}I-labeled membrane-associated proteoglycans (from ~3 x 10^7 cells) were affinity-purified on the mAbs S1, 2E9, and 6G12 and were individually incubated with an at least 5 x 10^3-fold molar excess of immobilized antithrombin III. After this incubation the beads were washed and stepwise eluted as described under "Materials and Methods." The radioactivity eluting with the different NaCl concentrations was counted and expressed as a percentage of the total eluted label (Table II). Only small fractions of the individual hydrophobic proteoglycans were tightly bound to the protease inhibitor and eluted with salt concentrations above 0.4 M. Reapplication to fresh beads confirmed the lack of high affinity binding of the fall-through fractions (not shown). Heparitinase digestion reduced the antithrombin III binding of the different hydrophobic proteoglycan forms, suggesting that this binding was mediated by heparan sulfate chains. Whether the residual antithrombin III binding was due to a nonspecific binding of the core protein, or due to the glycosaminoglycan stubs that remained attached to the core protein after digestion, was not further investigated. Compared with the proteoglycans purified on mAb 6G12 (fibroglycan) and mAb 2E9 (syndecan and the
HSPG which yields the 130-kDa core protein), the relative size of the high-affinity fraction (2 M elution) of the mAb S1-purified proteoglycans (glypican) was twice as large, again suggesting that glypican was more densely substituted with antithrombin III-binding heparan sulfate chains than the other proteoglycan forms. The \(^{125}\)I-labeled HSPG isolated from the endothelial extracellular matrix (perlecan) was also tested for its antithrombin III binding capacity. Compared with the hydrophobic proteoglycans, a higher proportion of the total matrix-associated HSPG bound to the antithrombin III beads at low ionic strength (0.2–0.4 M elution), whereas only a small portion was eluted with 2 M NaCl.

For glypican and for the matrix-associated HSPG, which both also accumulate in the endothelial culture medium, we were able to isolate sufficient \(^{35}\)SO\(_4^{2-}\)-labeled material to test the antithrombin III binding of the medium forms of these proteoglycans. In comparison with the corresponding iodinated fractions, a larger proportion of the \(^{35}\)SO\(_4^{2-}\)-labeled samples displayed high binding affinity (Table II). Such a result could reflect variations in glycanation within a single proteoglycan species, e.g. as a result of synthetic differences in the number and length of the heparan sulfate chains implanted on the cores or as a result of a partial degradation of the chains. However, the results of these experiments suggested that, on average, the matrix-associated HSPG had a weaker affinity for antithrombin III than glypican.

**DISCUSSION**

Several investigators have shown the presence of heparan sulfate on the endothelial cell surface as well as its function in anticoagulation (Marcum *et al.*, 1986), but little is known about the core protein structures that anchor this carbohydrate to the cell membrane.

We found that an important fraction, equivalent to \(\sim\)one-third of the detergent-extractable or \(\sim\)10% of the total endothelial heparan sulfate could be intercalated into liposomes. In primary or early-passage endothelial cells from umbilical veins as well as in long term (population doublings > 23) cultivated aortic endothelial cells, these membrane-associated glycosaminoglycans were carried by several distinct core proteins. Based on immunological cross-reactivities and Northern blot analysis, three, possibly four, of these proteoglycans were identified as syndecan, fibroglycan, and glypican, and possibly the cell surface HSPG with the 35-kDa core which were previously isolated from human lung fibroblasts (Lories *et al.*, 1989; Lories *et al.*, 1992). At least two of these, fibroglycan and syndecan, are proteins with a transmembrane orientation (Marynen *et al.*, 1989; Saunders *et al.*, 1989). Fibroglycan, which is abundant in fibroblasts but difficult to detect in human epithelial cells (Lories *et al.*, 1992) appears to be a major endothelial proteoglycan. Syndecan, which carries both CS and HS chains, is a relatively minor component in endothelial cells, and in that respect also, endothelial cells resemble fibroblasts rather than epithelial cells (Lories *et al.*, 1992).

It is unlikely, however, that the HSPG identified here in primary HUVECs cultures represent proteoglycans from contaminating fibroblasts or smooth muscle cells, since >99% of
the cells contained von Willebrand factor-related antigen. Glypican, in contrast, is produced by several epithelial and fibroblastic cell types, is attached to the cell membrane by a GPI-anchor, and is rapidly shed to the culture medium where it accumulates (David et al., 1990; Lories et al., 1992). Endothelial cells also express a glypican-related proteoglycan which loses its hydrophobic properties after phospholipase C treatment. The significance of this GPI-anchor for proteoglycan function is not clear (see also below), but it may be at the basis of a differential processing of the cell surface proteoglycans as, of all membrane-associated proteoglycans, only glypican was detected in the culture media of endothelial cells (not shown) and of fibroblasts (David et al., 1990).

Earlier reports on vascular endothelial cells seem to have failed to detect all or most of these cell surface proteoglycans. Lindblom et al. (1989), while suspecting the existence of membrane-associated HSPG in endothelial cells, did not detect heparan sulfate proteoglycan species with "small" protein cores. However, Griessmacher et al. (1987) isolated a HSPG with a core protein of 55–60 kDa from the culture medium of HUVEC which was also found in the cellular membrane pellet and probably represents glypican. Kinsella et al. (1988), on the other hand, isolated a HSPG with a core protein of 50 kDa from bovine aorta endothelial cells which could be the equivalent of fibroglycan. Kojima et al. (1992b), finally, identified HSPGs with core proteins of ~50 and ~30 kDa in cloned rat microvascular endothelial cells. The first was identified as the rat homolog of syndecan. The second represented a novel syndecan- and fibroglycan-related proteoglycan and is probably the rat homolog of the cell surface HSPG with the 35-kDa core that was found here in human endothelial cells and which has recently been cloned from human lung fibroblasts (David et al., 1992). In contrast, nearly all investigators reported the production of HSPG with large cores that, in retrospect, seem related to perlecian (Lindblom et al., 1989; Oohira et al., 1983; Kinsella and Wight, 1988; Marcum et al., 1986; Saku and Jurthmayr, 1989). This proteoglycan, which was also detected here, is indeed a major proteoglycan in endothelial cells and accumulates mainly in the matrix where it accounts for nearly 70% of all HS present in the monolayer. The reasons for the discrepancies between these studies, including our own, are not all clear. It is possible that differences exist between micro- and macrovascular endothelial cells and between primary and established cultures. Direct extraction of the cells with 4 M GdnHCl and detergent may be a reason why some of the prior investigations have failed to detect membrane-associated HSPG in endothelial cells, as under these conditions the hydrophobic HSPG would not be well resolved from the bulk of the coextracted matrix HSPG, neither by density gradient ultracentrifugation nor by gel filtration (Lories et al., 1986). On the other hand, the power, but also a potential weakness of the detergent extraction, ion-exchange, and liposome incorporation isolation procedure used here might be that in the case of the HSPG it tends to select for intact molecules that have retained both their membrane insertion domains and their glycanated domains, providing for samples that are highly enriched in membrane components but in which the less stable forms may be underrepresented. The judicious use of proteinase inhibitors during the extraction and isolation is therefore a potentially important aspect of the procedure.

It has been suggested that endothelial HSPG are at least in part responsible for the anticoagulant properties of the blood vessel wall (Colburn and Buonassisi, 1982, Marcum and Rosenburg, 1984). In most studies the binding of antithrombin III to the endothelial heparan sulfate, or the opposite, has been used to illustrate this anticoagulant activity. Histochemically, the bulk of the tissue antithrombin III-binding proteoglycans has been mapped to the basement membrane (De Agostini et al., 1990). On the other hand, Marcum et al. (1986) reported that a small fraction of the endothelial heparan sulfate displayed high affinity for antithrombin III and that this fraction was responsible for 99% of the total anticoagulant activity associated with the cellular glycosaminoglycans. These cellular anticoagulant heparan sulfate chains were associated with hydrophobic core proteins and were suggested to be part of the cell surface HSPGs. These results were recently substantiated by Kojima et al. (1992a), who reported that antithrombin III-binding HSPGs from an established rat microvascular endothelial cell line represented about 5% of the HSPG and who, based on the results of peptide mapping and sequencing experiments, identified these HSPGs as cell surface proteoglycans. The results of our binding studies suggest that an important fraction of the endothelial antithrombin III-binding heparan sulfate is indeed associated with the HSPG from the extracellular matrix, but the average binding affinity of these chains may be low, since the binding of most of the matrix proteoglycans was broken by moderate salt concentrations. In our hands also the membrane-associated HSPGs had the capacity to bind to antithrombin III.
and all the identified forms bound antithrombin to a certain extent. As a whole our results are consistent with the data of Kojima et al. (1992a) in microvascular endothelial cells, who established that active and inactive HSPGs (in terms of antithrombin III binding) yielded similar peptide patterns. However, the HSPG which in HUVEC more typically displayed high affinity binding for antithrombin III was glypicain, a proteoglycan which was not recovered or formally identified among the antithrombin-binding proteoglycans from microvascular endothelial cells. This suggests that glypicain, somewhat more often than other proteoglycans, contains the specific pentasaccharide sequence required for high affinity binding to antithrombin III. If these antithrombin III binding sequences are randomly distributed among the heparan sulfate chains and the analysis of the prevalence of these specific pentasaccharide sequences in different fractions.

Ultimately, the finding that in polarized cell types all glycosyl phosphatidylinositole-anchored proteins appear to be restricted to the apical surface (Rodriguez-Boulan and Nelson, 1989) may prove more relevant and significant for individual proteoglycan functions than small to moderate differences in antithrombin-binding activities. If the apical targeting rule is also true for glypicain, this could mean that glypicain becomes concentrated at the luminal side of the endothelium where it could act as a “first line” physiological anticoagulant, even if chains with anticoagulant activity were not restricted to glypcain. Then, the shedding of glypicain, if it also occurs in vivo, may result in the release of a soluble anticoagulant from the endothelial cell surface into the blood stream. As proposed by de Agostini et al. (1990) the small amounts of luminal anticoagulant HSPG may be critically placed to bind plasma antithrombin, accelerate the action of the protease inhibitor, and thereby regulate the ability of hemostatic mechanisms at the blood vessel wall interface. The possibly larger quantities of abluminal anticoagulant HSPG could serve as a reservoir or backup system that may be brought into play when extensive damage occurs to the overlying endothelium. This may extend to other proteoglycan functions as well. Lipoprotein lipase is bound to the vascular endothelial cell surface through heparin-like glycosaminoglycans (Shimada et al., 1981) and depends on a saturable transport system which requires HSPG for passage across the endothelial cells (Saxena et al. 1991). Since lipoprotein lipase bound to heart cell cultures can be released by the action of phosphatidylinositol-specific phospholipase C (Chajek-Shaul, 1989), an apically exposed glypicain would also be a candidate for binding lipoprotein lipase to the endothelial luminal cell surface.

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REFERENCES


