Localization of Anticoagulantly Active Heparan Sulfate Proteoglycans in Vascular Endothelium: Antithrombin Binding on Cultured Endothelial Cells and Perfused Rat Aorta

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Abstract. We have studied the interaction of 125I-antithrombin (125I-AT) with microvascular endothelial cells (RFPEC) to localize the cellular site of anticoagulantly active heparan sulfate proteoglycans (HSPG). The radiolabeled protease inhibitor bound specifically to the above HSPG with a $K_d$ of $\sim 50$ nM. Confluent monolayer RFPEC cultures exhibited a linear increase in the amount of AT bound per cell for up to 16 d, whereas suspension RFPEC cultures possessed a constant number of protease inhibitor binding sites per cell for up to 5 d. These results suggest that monolayer RFPEC cultures secrete anticoagulantly active HSPG, which then accumulate in the extracellular matrix. This hypothesis was confirmed by quantitative light and EM level autoradiography which demonstrated that the AT binding sites are predominantly located in the extracellular matrix with only small quantities of protease inhibitor complexed to the cell surface.

We have also pinpointed the in vivo position of anticoagulantly active HSPG within the blood vessel wall. Rat aortas were perfused, in situ, with 125I-AT, and bound labeled protease inhibitor was localized by light and EM autoradiography. The anticoagulantly active HSPG were concentrated immediately beneath the aortic and vasa vasorum endothelium with only a very small extent of labeling noted on the luminal surface of the endothelial cells. Based upon the above data, we propose a model whereby luminal and abluminal anticoagulantly active HSPG regulate coagulation mechanism activity.

The biologic activities of blood coagulation enzymes are regulated by protease inhibitors of the serpin superfamily, particularly antithrombin (AT). This protease inhibitor is a 58,000-kD protein that inactivates its target enzymes, such as thrombin, by forming covalent complexes with them. Heparin binds AT, and induces a conformational change in the protein that augments the rate of formation of enzyme–inhibitor complexes by several orders of magnitude (Rosenberg and Damus, 1973).

Damus et al. (1973) proposed that the non-thrombogenic properties of blood vessels are due, in part, to anticoagulantly active heparin-like components on the luminal surface of endothelial cells. This hypothesis is supported by several independent lines of evidence. Heparan sulfate glycosaminoglycans with heparin-like activity have been extracted from bovine microvascular capillary endothelial cells and from aortic endothelium (Marcum et al. 1983; Marcum and Rosenberg, 1984). Cultured cloned or noncloned bovine ma-

1. Abbreviations used in this paper: AT, antithrombin; HSPG, heparan sulfate proteoglycans; IMDM, Iscove's modified Dulbecco's media; RFPEC, rat epididymal fat pad endothelial cells; SVC, surface-connected vesicle.
Thus, it seems likely that endothelial cells, in vivo, are endowed with anticoagulantly active HSPG whose glycosaminoglycan chains would interact with blood AT, and thereby regulate the activity of the coagulation system. The potential abluminal position of these potent anticoagulant substances has been assessed indirectly both in vivo and in vitro. Carlson and co-workers have infused AT into rabbit and humans and have observed a three-compartment distribution of the protease inhibitor between plasma, the vessel wall and an extravascular compartment (Carlson et al., 1984, 1985). Hatton and collaborators used intact as well as deendothelialized rabbit aorta segments to study AT binding to the vascular endothelium and to the subendothelial basement membrane. (Hatton et al., 1986, 1988). Flavobacterium heparitinase treatment of the exposed subendothelium reduced the AT binding and also seemed to deplete the subendothelium of small proteoglycan-rich granules and basement membrane, as shown by transmission EM.

We have attempted to visualize the position of the anticoagulantly active HSPG in cultured rat microvascular endothelial cells as well as in the intact rat aorta by examining the binding of $^{125}$I-AT with light and EM level autoradiography. This subset of proteoglycans bear carbohydrate chains with regions of defined monosaccharide sequence. Our results indicate that small amounts of the anticoagulantly active HSPG are present on the luminal surface of cultured endothelial cells as well as blood vessels but that much larger quantities of anticoagulant active proteoglycan are found in the extracellular matrix as well as bound to the abluminal cell membrane of blood vessels. Based upon these data, we propose that both luminal and abluminal sources of anticoagulantly active HSPG may regulate coagulation system activity under different conditions.

**Materials and Methods**

**Materials**

Purified human AT was purchased from Cutter Biological (Berkeley, CA). Flavobacterium heparitinase was purified as previously described (Marcum et al., 1984). Chondroitinase ABC and dextran sulfate were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium heparin from porcine intestinal mucosa was provided by Diosynth, Inc. (Chicago, IL).

**Cells in Culture**

Microvascular endothelial cells were originally isolated from rat epididymal fat pad, and then cloned from single cells as previously described (Marcum and Rosenberg, 1985). These cloned cells spontaneously transformed into a stable cell line that was used for this study (RFPEC). Monolayer cultures were grown in M199 medium containing 10% FCS (Gibco Laboratories, Grand Island, NY), 100 U/ml penicillin, and 100 μg/ml of streptomycin in a 5% CO$_2$ 95% air humidified atmosphere at 37°C. Cells in suspension were cultured in S-MEM (Joklik modified) medium (Gibco Laboratories) containing 20% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 1% BME 100 times vitamins (Sigma Chemical Co.), amino acids at the concentration used in standard M199 medium, and 1.06 g/liter Pluronic F68 (BASF Wyandotte Corp., Wyandotte, MI). The cells were grown in siliconized glass Erlenmeyer flasks, and kept under constant rotatory agitation on a shaker at 250 rpm for up to 5 d at 37°C. Chinese hamster ovary cells were a gift from M. Krieger (Massachusetts Institute of Technology). Bovine smooth muscle cells were isolated by enzymatic dispersion of bovine aortic media (Fritz et al., 1985).

**Isolated Rat Aorta**

Male Sprague-Dawley rats (300–400 g) were anesthetized by intramuscular injection of 80 mg of Nembutal (sodium pentabarbital)/kg animal weight. The arterial and venous trunks were carefully exposed, and then detached from the underlying tissue. All lumbar veins, as well as the inferior vena cava and dorsal aorta at the renal veins, and the common iliac artery were ligated. A 20 gauge butterfly needle was flushed, filled with Iscove’s modified Dulbecco’s media (IMDM) (Gibco Laboratories), inserted into the dorsal aorta just above the branch of the common iliac artery, and held in place with a double ligature. The dorsal aorta and inferior vena cava were scissed just beneath the ligature, and the aorta gently flushed with IMDM. The ligatures surrounding the lumbar veins and common iliac artery were cut, and the blood vessel preparation removed. For study of damaged aorta, the vessel was crushed repeatedly along its length with a pair of hemostats after dissection and following flushing with IMDM to remove the blood.

The aortic preparation was washed for 15 min with IMDM media containing 50 μg/ml BSA and 1% Nutridoma SP (Boehringer Mannheim Biochemicals, Indianapolis, IN) until the perfusate was clear. At this point, 3 ml of $^{125}$I-AT (5–10 nM $^{125}$I-AT, 2–4 × 10$^7$ cpm/ml), or $^{125}$I-BSA (6 nM $^{125}$I-BSA, 3 × 10$^7$ cpm/ml) was flushed through the aorta, for 3 h at a flow rate of 30 ml/h. These conditions were judged to be adequate with respect to oxygen and nutrient supply as evidenced by the clear maintenance of cellular integrity at the EM level. Oxygenation of the perfusate was achieved by bubbling air from a pasteur pipette every 1–2 min. As a further negative control vessels were perfused for 1 h with purified Flavobacterium heparitinase at a final concentration of 89 nM to remove heparan sulfates, washed for 1 min with IMDM containing 1% BSA and 1% Nutridoma SP and then perfused with $^{125}$I-AT as described above. After perfusion, free or loosely bound radioactive protein was removed by washing for 30 min with IMDM at a flow rate of 60 ml/h (which was found to cause no mechanical damage to the endothelium [Fig. 6 A]).

**Iodination of Proteins**

AT was labeled with $^{125}$I-NaI (15 mCi/μg, Amersham Corp., Arlington Heights, IL), using the chloramine T method (Greenwood et al., 1963). To protect the heparin binding site of the protease inhibitor during the labeling procedure, AT was iodinated in the presence of a 10-fold molar excess of affinity-fractionated heparin octasaccharide (Atha et al., 1987). Heparin octasaccharide was subsequently separated from $^{125}$I-AT by gel filtration on a Sephade G-75 (Pharmacia Fine Chemicals, Piscataway, NJ) column equilibrated in 50 mM sodium phosphate, pH 7.2 containing 1 M NaCl. Radiolabeled protease inhibitor was then desalted by gel filtration on a Sephadex G-25 column equilibrated in 50 mM sodium phosphate, pH 7.2 containing 0.15 M NaCl, and 20 μg/ml BSA. The $^{125}$I-AT had a specific activity of 8 × 10$^6$ cpm/μg, and was able to bind with avidity to high-molecular weight heparin (data not shown). For control experiments, BSA was iodinated by the above method to a specific activity of 7 × 10$^6$ cpm/μg.

**Binding of $^{125}$I-AT to Endothelial Cells in Culture**

**Cells in Monolayer.** The binding assay on monolayer cultures was performed on cells grown in 96-well microtiter plates for 7 d or more. The cells reached confluence after 3–5 d. Cell monolayers were pre-incubated for 1 h at 37°C with media containing 1% Nutridoma SP rather than serum, to eliminate serum-borne AT. The cells were then incubated with $^{125}$I-AT diluted in PBS containing 1% Nutridoma SP and 50 μg/ml BSA, with or without a 100-fold excess of unlabeled AT for 1 h at 4°C. The unbound ligand was eliminated by washing five times with PBS containing 100 μg/ml BSA, and the cells were removed from the wells by using cotton swabs. The swabs were then counted in a gamma counter (LKB Instruments, Gaithersburg, MD). Cell numbers were estimated by trypanstaining control wells, and then counting cells on a Coulter counter (Coulter Electronics, Hialeah, FL). All determinations were performed in triplicate.

**Cells in Suspension.** Cells grown in suspension were harvested by centrifugation (800 g, 1 min), and then resuspended in PBS containing 1% Nutridoma SP and 50 μg/ml BSA. Roughly 0.5–1 × 10$^6$ cells were incubated in siliconized Eppendorf tubes with 0.2 ml of $^{125}$I-AT with or without competing unlabeled AT for 1 h at 37°C with agitation. After eliminating free or loosely bound ligand by washing four times with 1 ml of PBS containing 100 μg/ml BSA, the bound labeled protease inhibitor, and the
numbers of cells in the pellet were estimated. All determinations were performed in duplicate.

The values of bound 125I-AT were normalized for 50,000 cells, and nonspecific binding was subtracted. Nonspecific binding was assessed by residual counts in the presence of a 100-fold excess of unlabeled AT, and accounted for 25-30% of the total bound counts.

Preparation of Cells and Tissue for Light and Electron Microscopy

All fixation steps were carried out with 2.5% glutaraldehyde in PBS for 1 h at 4°C. Cell suspensions and cell monolayers were processed differently because suspension cells were pelleted (800 g for 5 min) before fixation, whereas monolayer cells were fixed directly on the dish. After fixation, the cells were washed three times in Sabatini's solution (PBS with 6.8% sucrose). At this point, the cell pellets were cut into small (1 mm³) cubes. All samples were then postfixed with 1% osmium tetroxide for an additional hour, followed by three washes in Sabatini's solution. The samples were then dehydrated by treatment with propylene oxide (15 min for pellets (Epon-EOH for monolayers), a 1:1 Epon-propylene oxide mix (1 h), and three changes in pure Epon (3 h, 3 h, and overnight). Samples of monolayers suitable for sectioning were prepared by inverting Beem capsules full of resin. Polymerization was at 64°C overnight. After embedment, monolayer samples could be snapped off the dish, and cut en face; pelleted cells and aorta may be cut directly.

Semithin (250 nm) sections, slightly oblique to the plane of the dish from monolayer cell preparations or transverse sections of aorta, were cut for light-microscopic autoradiography. The obliquity of the section plane allowed cells from all levels within the culture to be examined in the same section. A microtome (Ultracut; Reichert Scientific Instruments, Buffalo, NY) with a sapphire knife (LKB Instruments) was used, allowing very large (6 mm) sections to be cut. Sections were dried on glass slides, stained with toluidine blue, and dried.

Serial ultrathin (60 nm) sections, true to the plane of the dish, were cut from monolayer samples, from cell pellets, and from selected regions of the aorta for EM. Similar non-skeletal sections, with no defined orientation, were cut from pelleted samples. All sections for EM were cut with a microtome (MT2; Sorvall Instruments Div., Newton, CT) with a diamond knife (Du Pont Co., Wilmington, DE). Sections for EM were mounted on 300-mesh grids, sections for EM autoradiography were lifted in a 3-mm Nichrome loop, and dried onto parafilm coated glass slides that were then carbon coated.

Autoradiographic Method

The light and EM level autoradiographic methods are essentially identical, except that Kodak NTB2 and Ilford L4 emulsions, respectively, were used. This resulted in individual silver grain sizes between 0.2 and 0.3 μm.

The mounted slides were dipped in emulsion (1:10 dilution in water for EM, 1:1 dilution in water for light microscopy, 40°C in total darkness, dried, and stored at 4°C. At varying times (<1 wk for light autoradiography, 1-6 wk for EM level autoradiography), slides were developed (Ilford D19 [light microscopy], Microdol X [EM], 2 min, 20°C); washed; fixed (4 min Kodak rapid fix, 20°C); and prepared for microscopy. For light microscopy, sections were counter stained with buffered toluidine blue (0.1%) mounted in Permount (Fisher Scientific, P.A. Lab, NJ) and cover slips were added. For EM, the method of Saltzberger and Bachmann (1972) was used. Sections were stripped from the slide in distilled water and dried onto grids; the mounting film was dissolved with butyl acetate and double stained with 2% uranyl acetate (7 min) and 1% lead citrate (3 min). All light-microscopic examinations were conducted with a Zeiss photomicroscope III. All EM-level examinations were carried out with a Phillips EM 300.

Quantification of AT Label in Cultures

Qualitative observations show that the monolayer cultures of RFPEC cells cannot be treated as a random sample, since differentiation of cellular form is apparent throughout the macroscopic depth of the culture. Given that the size of the culture is too great to sample completely by EM, it was necessary to select a region of cultured cells in which quantitation would prove most useful. To this end, samples were taken close to the dish surface, where AT labeling was most intense, cellular and matrix components were relatively random, and differentiation of cellular structure and label localization were easily analyzed. Furthermore, sections close to the dish surface showed development of an extensive array of surface-connected vesicles (SCV) which revealed a high degree of AT binding.

A large number of micrographs (70) were taken at random from sections representative of monolayer cultures close to the dish surface, as well as from random sections through pellets of suspension cultures. The AT attachment to the extracellular matrix, SCV, cellular perimeter, and background count in the free space were quantified by counting silver grains on micrographs and using a Microplan (Cambridge Instruments, Cambridge, MA) to measure the area of each compartment directly from the same micrograph.

The number of silver grains in each compartment of the monolayer culture was corrected for background by subtracting the count found in free space normalized for the area of the relevant compartment and then expressed as a percentage of the total number of labeling sites within the whole sectioned sample.

In suspension culture the extent of definable matrix and SCV is negligible (1.2 and 4.7% of the area found in monolayer culture, respectively). Thus, no attempt was made to quantify binding in these cultures. However, the number of counts per unit length of cell perimeter was determined to compare the AT binding directly with that seen in the monolayer cultures.

Results

Binding of AT to Endothelial Cells

The binding of AT to various cell types was evaluated with 125I-labeled protease inhibitor. This interaction was observed to be highly cell type related, as shown by the complexing of 125I-AT with cell types known to synthesize anticoagulantly active heparan sulfate such as rat fat-pad endothelial cells (RFPEC), but not with those only able to produce anticoagulantly inactive glycosaminoglycans such as Chinese hamster ovary cells or bovine smooth muscle cells (Table I). The binding of 125I-AT to RFPEC was demonstrated to be highly specific by competition studies using a 100-fold molar excess of unlabeled protease inhibitor (defined as specific binding) (Table II). The complexing of radiolabelled protease inhibitor to RFPEC is due to interactions with anticoagulantly active heparan sulfate since specific binding of 125I-AT is greatly decreased by addition of soluble heparin but not by admixture of dextran sulfate, another sulfated macromolecule devoid of anticoagulant activity (Table II). Furthermore, the preincubation of RFPEC with purified Flavobacterium heparitinase for 1 h at 37°C dramatically suppressed AT binding (Table II), whereas treatment with chondroitinase ABC had no effect on this parameter (data not shown).

The incubation of increasing concentrations of 125I-AT

### Table I. Cell Type Specificity of AT Binding

| Cells                  | Bound at 50,000 cells | % Binding |
|------------------------|-----------------------|-----------|
| RFPEC                  | 2,174 ± 50            | 100       |
| Bovine smooth muscle cells | 145 ± 24             | 7         |
| CHO cells              | 53 ± 7                | 2         |

Cell monolayers were kept in culture for 14 days prior to the incubation with 125I-AT. 125I-AT (0.5-9.0 nM, 2 × 10⁶ cpm/ml) was incubated for 1 h at 4°C on cell monolayers.
Table II. Specificity of 125I-AT Binding on RFPEC

| Treatment | % Binding |
|-----------|-----------|
| None      | 100       |
| AT (1 μM) | 28        |
| Heparitinase (0.5 U/ml) + PMSF (0.1 mM) | 8 |
| Heparin (10 μg/ml = 1.7 U/ml) | 2 |
| Dextran sulfate (10 μg/ml) | 105 |

125I-AT (0.5-1.0 nM) was incubated for 1 h at 4°C either alone or in the presence of cold AT, heparin, or dextran sulfate. Heparitinase was incubated on the cells for 1 h at 37°C before 125I-AT was added.

with RFPEC monolayer cultures revealed that the protease inhibitor binds to this cell type. Under our experimental conditions, the high-affinity binding sites for AT have a Kd of ~50 nM, a value comparable to that for heparin (Jordan et al., 1982), and the number of binding sites per cell is of the order of 106 (Fig. 1). Note, however, that the saturation curve of AT binding to these monolayer cultures does not reach a true plateau, indicating the presence of additional binding sites with much lower affinities for the protease inhibitor.

To ascertain whether AT bound to RFPEC is internalized, we examined the fate of radiolabeled protease inhibitor complexed to the above cell surface at 37°C. After 3 h of incubation, the amount of specifically bound 125I-AT reached a plateau at 3,880 ± 395 cpm/50,000 cells. The addition of a 100-fold molar excess of unlabeled AT at 2 h decreased the specific binding of radiolabeled protease inhibitor from 82% at 2 h to 9% at 3 h, and 2% at 4 h. Similarly, the admixture of a 100-fold molar excess of unlabeled AT at 3 h reduced the specific binding of radiolabeled protease inhibitor from 100% at 3 h to 11% at 4 h. These experiments indicate that significant amounts of AT are not internalized by RFPEC under the above conditions.

We have also monitored the evolution of the AT binding capacity of monolayer RFPEC cultures as a function of time in culture. A linear increase in the binding of protease inhibitor per cell with time in culture was noted (Fig. 2). The augmentation in this parameter could reflect either an accumulation of the anticoagulantly active HSPG on the cell surface or a constant secretion and accumulation of the biologically active proteoglycans in the extracellular matrix. That the number of AT binding sites per cell increases linearly for up to 16 d in culture suggests a steady-state secretion of the anticoagulantly active proteoglycans into the extracellular space.

This hypothesis was tested by establishing suspension RFPEC cultures where the accumulation of extracellular matrix is minimized, and then determining the capacity of the above cells to bind AT as a function of time in suspension culture. The suspension RFPEC cultures were initially trypsinized to remove anticoagulantly active HSPG, which abolished the interaction with added 125I-AT, and protease inhibitor binding per cell was then measured as a function of time in culture (Fig. 3). The data show the reappearance of detectable binding of 125I-AT after 1 h in culture with a peaking of complexed protease inhibitor per cell at 12 h. During the following 12 h in culture, the binding of 125I-AT per cell diminishes to half of that observed with confluent monolayer cultures, and then remains constant for up to 5 d. Throughout this period of time, the cells remain viable and go through at least one cycle of division.

The appearance of anticoagulantly active HSPG on the surface of RFPEC after short periods of time demonstrates the extremely rapid synthesis and/or transport of this specific proteoglycan. The decrease of AT binding per cell in suspension cultures noted between 12 and 24 h after trypsin treatment may reflect the fact that synthesis and exposure of anticoagulantly active HSPG at the cell surface initially occurs more rapidly than its release to the media. Once the synthesis and release of anticoagulantly active HSPG appear to have attained steady state values, the AT binding capacity of
Figure 3. 125I-AT binding on RFPEC suspension cultures. 125I-AT (0.4 nM) was incubated with suspension RFPEC cultures for various times. After trypsin treatment (t = 0), 125I-AT is rapidly restored, peaks at 12 h and stabilizes for 4 d.

The suspension RFPEC cultures becomes stable for a prolonged period of time in contrast to confluent monolayer RFPEC cultures of the same cell type. This observation could mean that anticoagulantly active HSPG do not accumulate on the cell surface of confluent monolayer RFPEC cultures as a function of time in culture, but rather would be rapidly secreted and accumulated in the extracellular matrix. The above conclusion is based upon the assumption that the synthetic activity of suspension and monolayer cells are roughly equivalent. Therefore, we have attempted to localize AT binding sites by morphologic means in both confluent monolayer and suspension RFPEC cultures. This approach was taken to visualize anticoagulantly active HSPG on the cell surface and within the extracellular matrix, as well as to ascertain whether monolayer cultures accumulate HSPG on the cell surface to a greater extent than cells grown in suspension.

Localization of AT Binding to Cultured Endothelial Cells and Perfused Aorta

125I-AT binds to RFPEC cultures, and its location may be ascertained by light-microscopic and EM level autoradiography. The light-microscopic examination of oblique sections through postconfluent monolayer cultures of RFPEC show clear differentiation of cell form. The cells nearest the dish surface are irregular in shape and often appear somewhat stellate. Moving away from the dish surface, additional sections indicate that cells form whorls or hollow spheres (Fig. 4 A). Within whorls, free isodiametric cells with no apparent specialization are evident. In electron micrographs, the whorl structures, in which the outermost cells appear columnar in shape, appear to be surrounded by collagen fibrils. Basal lamina are noted between cells, and the collagen matrix (Fig. 4 B). It should be noted that given the extreme thinness of sections for EM (60–80 nm) sections may pass over or under cells, which gives the appearance of subconfluence.

The above cultures were also studied by autoradiography at the light-microscopic level, which revealed a clear concentration of labeling for AT around the whorl structures (Fig. 5 A). The labeled protease inhibitor was observed in increased amounts near the dish surface, but little or none is noted on cells in free space within the whorls. Similar studies at the EM level support these observations. When fine details of the relative labeling within the cultures are resolved qualitatively, the morphologic evidence indicates that labeled AT is particularly associated with basal lamina/basement membrane, as is clearly defined around the whorls (Fig. 5 B, and inset). When cells are cut immediately adjacent to the dish surface (below the nucleus), they show extensive vacuolation, which frequently appears to be connected to the cell surface, and to contain nondifferentiated filamentous material (SCV). The material being extruded from the SCV vacuoles shows significant labeling for AT (Fig. 5 D). It is frequently possible to visualize matrix within SCV, which suggests that protease inhibitor binding may be due to complexing with these adhesive macromolecules. The entire cell perimeter is mainly free of label and associated matrix (Fig. 5 D).

The suspension RFPEC cultures appear relatively homogenous by light and EM, showing none of the pattern of differentiation seen in the monolayer cultures. The SCV were noted but were much less prominent (4.7% of that found in the equivalent sampled area) than in the confluent monolayer RFPEC cultures. However, some cells formed clusters, and extracellular matrix is present at the cellular interfaces, which constitute the primary localization of 125I-AT (Fig. 5 C). Such matrix is rarely detectable, and only constitutes 1.7% of that formed in an equivalent area of monolayer cultures.

Quantitation of AT label in monolayer cultures close to the dish surface defined the level of binding to the cell surface, to the extruded matrix outside the cells as well as within the SCV. It is clear that most (92%) of the count is associated with the extracellular matrix and SCV and only 8% may be attributed to the cell surface binding. This cell surface binding is expressed in relation to the measured membrane perimeter in the cell monolayer (0.03 silver grain per µm) as compared with the surface of suspension cells (0.006 silver grains per µm). The amount of count on the membrane of monolayer cultures is thus calculated to be five times that seen on suspension cultures labeled under the same conditions. This observation may be due to the different conditions under which monolayer and suspension cultures are maintained or to the close apposition of monolayer but not suspension cultures to the dish surface where secreted HSPG can be trapped.

The total area of SCV in monolayer cells constitutes 5% of the scored cellular area (2.5% total culture area examined) but contains 43% of the count, presumably due to the AT binding capacity of the newly secreted HSPG which is partially retained in that compartment. This possibility is reinforced by the fact that heavily labeled matrix components are frequently definable within the SCV.

The extracellular matrix binds 48% of the labeled AT, although it constitutes only 6.2% of the total culture area. It should be noted that it is difficult to define the limits of the

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2. We have failed to detect AT bound to RFPEC with specific anti-AT antibody populations. This observation suggests that antibody binding to the protease inhibitor destabilizes the polysaccharide-protein complex, and thereby induces release of AT.
matrix compartment precisely due to its diffuse and discontinuous nature, so that we estimate that the figure of 6.2% is high, possibly by as much as an order of magnitude. While it was not possible to derive quantitative figures for matrix binding in suspension cells due to its relative paucity, when matrix was apparent, labeling was intense in this compartment (Fig. 5 C). A control was executed to ensure that the labeling was specific and not due to nonspecific trapping of AT. To this end cultures were incubated with a 100-fold excess of cold AT, which effectively removed all labeling.

We also attempted to visualize the in vivo position of anticoagulantly active HSPG by perfusing normal and damaged rat aorta for 1 to 3 h with 125I-AT, and then determining the specific localization of labeled protease inhibitor as outlined above. The duration of perfusion had no obvious quantitative effect on the extent of labeling observed. Thus, the saturation of specific binding sites with labeled protease inhibitor probably occurs in <1 h. It is readily apparent that 125I-AT is bound to the aortic subendothelium. This finding is particularly evident with light microscopy, which shows that only small quantities of labeled protease inhibitor are found within the smooth muscle or connective tissue of the aorta, or bound to the luminal side of the endothelium (Fig. 6 A). This localization of AT binding is more clearly defined at the EM level, which demonstrates that labeled protease inhibitor is clearly present beneath the endothelial cells (Fig. 6 B). Quantitation of the above data reveal that <1% of the bound 125I-AT is associated with the luminal face of the endothelium, which is just above background values. For example, only 3 luminal counts were present in an experiment, versus 530 abluminal counts. The labeled protease inhibitor is also noted to bind in regions around small capillaries, peripheral to the aortic muscle layer. This observation is clearly depicted at the light microscopic level (Fig. 6 C), but the position of the 125I-AT is best defined at the EM level to be in the subendothelium, associated with the basal lamina/basement membrane rather than collagen fibers (Fig. 6 D). The above experiment was repeated by perfusing the aorta with 125I-BSA, which produced no detectable labeling in any locale. This latter observation demonstrates that the binding of 125I-AT within the aorta is not caused by nonspecific interactions or trapping of labeled protease inhibitor. To ensure that the 125I-AT interacts specifically with HSPG, the vasculature was perfused with Flavobacterium heparitinase before perfusion with 125I-AT. This treatment effectively prevented any binding of the labeled inhibitor to all regions of the tissue including the subendothelium, and left the endothelial layer intact.

We finally tried to mimic vascular injury by crushing the aorta before perfusion with 125I-AT, and then determined the position of the labeled protease inhibitor following tissue damage. The above manipulation dramatically altered the morphology and pattern of tissue labeling. The loss of endothelial cells was consistently evident, and light autoradiography showed an extremely intense concentration of 125I-AT apparently bound to the subendothelium. In the damaged aorta, the labeled protease inhibitor appeared as a solid line of dense silver (Fig. 6 E), whereas in the normal blood vessel, the 125I-AT is bound in particulate form. The EM level autoradiography revealed an increased accumulation of 125I-AT in the subendothelium with bound protease inhibitor also associated with capillaries, aortic muscular wall connective tissue, and extra-aortic connective tissue. The extensive complexing of 125I-AT with the subendothelial layer in the damaged aorta suggests that the low level of label on the luminal face of endothelial cells in the normal vessel is not caused by a shearing effect of the perfusate on the bound protein.

Discussion

Endothelial cell HSPG are members of the family of cell surface and extracellular matrix proteoglycans. In general, HSPG are either inserted into the cell membrane via a core protein hydrophobic domain or glycolipid anchor, loosely bound to the cell surface via interactions with glycosaminoglycan sidechains, or localized within the extracellular matrix because of complexing to collagen, fibronectin, laminin, and other structural glycoproteins (Hayman et al., 1982; Hook et al., 1984; Fransson et al., 1986; Ishibara et al., 1987). The cell surface associated and matrix associated proteoglycans have no discernible core protein hydrophobic domains, and are readily replaceable from the cell surface with heparin. Cells synthesize HSPG whose hydrophobic domains may be variably cleaved by a specific plasminemmal protease which allows the proteoglycan to become associated with the cell surface or the extracellular matrix (Brandan et al., 1989). Alternatively, cells also produce secretory HSPG with no hydrophobic domains which may be relatively resistant to the action of pronase and accumulate predominantly in the basement membrane (Oohira et al., 1983; Pejler et al., 1987).

The present investigation was initiated to define the position of anticoagulantly active HSPG on cultured endothelial cells as well as whole aortic preparations. This unique subset of HSPG bear carbohydrate chains with regions of defined monosaccharide sequence. Previous investigations summarized in the Introduction suggest that anticoagulantly active HSPG are produced by endothelial cells and are able to interact with blood AT (Marcum et al., 1986a,b; Stern et al., 1985). The localization of this specific subset of proteoglycans was attempted by visualizing the binding of 125I-AT with light and EMs level autoradiography. The first published studies to pinpoint the position of vessel wall HSPGs were reported by Simonescu and coworkers (Simonescu et al., 1981). These investigators used in vivo and in situ binding of cationic ferritin which could be eliminated with Flavobacterium heparinase to demonstrate by EM that the various classes of HSPG were concentrated on the fenestral diaphragms, and, to a much smaller extent, luminal plasmalemna of visceral capillary endothelium. Subsequent

Figure 4. Light and electron micrographs of monolayer RFPEC cultures. (A) Light micrograph of confluent RFPEC monolayer cultures thin sections (0.5 μm) suggesting that three-dimensional whorls of cells (as demarcated by lines) are clearly present. Bar, 10 μm. (B) Electron micrograph of whorl structure seen in A. Endothelial cells with columnar shape line the lumen, around which some pseudopodia are evident (open arrow). Collagen fibers (closed arrows) are associated with the basal layer. Bar, 1 μm.

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studies have generally confirmed the above conclusions (Clowes et al., 1984; Hatton et al., 1988).

We initially used a direct binding assay for 125I-AT, in conjunction with a pretreatment with purified Flavobacterium heparinase, to establish the presence of anticoagulantly active HSPG on the surface of cloned confluent monolayer RFPEC cultures. The results obtained are in excellent agreement with prior data which show that heparan sulfates isolated from the same cells accelerate thrombin-AT interactions as well as possess the critical monosaccharide sequence to bind protease inhibitor (Marcum et al., 1985). We subsequently measured the amount of AT bound by confluent monolayer RFPEC cultures as a function of time in culture, and showed that the number of protease inhibitor binding sites per cell increase in a linear manner for at least 16 d. Marcum et al. have observed a similar time dependent augmentation in the anticoagulant activity and cell surface heparan sulfates (Marcum et al., 1986a; Marcum, J., personal communication). These data suggest that the anticoagulant active HSPG synthesized by endothelial cells may be secreted into the extracellular matrix where they accumulate in substantial amounts. This possibility was tested by examining the behavior of suspension cultures of the same rodent microvascular endothelial cells which would have little opportunity to develop an elaborate extracellular matrix. Under these conditions, RFPEC did not exhibit any time dependent accumulation of radiolabeled protease inhibitor binding sites.

The above results prompted us to visualize the location of 125I-AT binding to confluent monolayer and suspension RFPEC cultures using both light and EM level autoradiography. In all cases cited, the binding of labeled AT was judged to be specific since it could not be duplicated with radiolabeled BSA but could be competed with unlabeled protease inhibitor. The extracellular matrix of RFPEC is defined morphologically as basement membranes which surround whorls or hollow spheres of cells; collagen fibrils, which are seen about the whorls; and a poorly defined matrix within SCV, and at the surface of the plastic culture dish. The most obvious subcellular compartments of monolayer cultures that label positively for AT are extracellular matrix associated with the basement membrane. The binding of radiolabeled protease inhibitor is also abundant in the matrix contained within or extruded from SCV. It is of interest to note that other investigators have observed that basement membrane contain heparan sulfate (Hay, 1981; Hedman et al., 1982). Therefore, it is not surprising that the anticoagulantly active HSPG are preferentially found within these locales. By contrast, little AT binding is observed on the suspension RFPEC, where the predominant labeling detected occurs at the occasional junction of two cells that may possess small amounts of extracellular matrix. In confluent monolayer RFPEC culture ~8% of the 125I-AT is associated with the cell surface. In suspension RFPEC cultures only ~1.7% of the radiolabelled protease inhibitor is complexed with this region.

We note with interest that the above estimate of cell surface anticoagulantly active HSPG is in reasonable accord with recent biochemical data collected by us for RFPEC. The rodent microvascular endothelial cells in monolayer culture have been radiolabeled for 24 h with 35S, and HSPG have been isolated from the cells and surrounding matrix by column separation techniques including AT affinity chromatography (unpublished data). The results indicate that only ~5-7% of the anticoagulantly active HSPG are endowed with hydrophobic domains as judged by retention on octyl-Sepharose, but that both types of anticoagulantly active HSPG species possess similar structures as judged by the molecular sizes and peptide maps of the core proteins (preliminary results). Given that the anticoagulantly active HSPG with no hydrophobic domains would be expected to continuously accumulate within extracellular matrix as the anticoagulantly active HSPG with hydrophobic domains is degraded, it is not surprising that only a small percentage of the AT binding sites synthesized over an 8-d period would constitute an integral component of the cell membrane.

To visualize the in vivo location of the anticoagulantly active HSPG, we perfused rat aorta with 125I-AT, and then determined the position of radiolabeled protease inhibitor binding sites as outlined above. The data revealed a highly significant interaction of 125I-AT with the aortic and vasa vasorum (microvascular) endothelial cell basement membranes, with only small amounts of protease inhibitor binding observed on the luminal surface of endothelial cells or in the surrounding smooth muscle cell connective tissues. Based upon the above data, we suggest that the overlying endothelial cells produce anticoagulantly active HSPG, the proteoglycans are initially positioned within the cell membranes due to their hydrophobic domains, the protein cores are then cleaved by the plasmalemmal proteases and the liberated macromolecules accumulate within the extracellular matrix. Alternately, we might hypothesize that endothelial cells synthesize a class of secretory anticoagulantly active HSPG that might also accumulate predominantly in the basement membrane. Indeed, this latter type of proteoglycan has been isolated from Reichert's membrane, and is structurally similar to the heparin proteoglycan in that it is resistant to cleavage by pronase (Pejler et al., 1987). The two models are consistent with the known synthesis of anticoagulantly active HSPG by cultured endothelial cells and anticoagulantly inactive HSPG by cultured smooth muscle cells as well as the previous isolation of anticoagulantly active HSPG from aortic intima and anticoagulantly inactive HSPG from aortic media (Marcum and Rosenberg, 1984).

Figure 5. Light and EM level autoradiographs of confluent monolayer and suspension RFPEC cultures. (A) Light microscopic autoradiographs of 125I-AT in which grains clearly delineate the periphery of whorl structures (arrows) in the basal layer. Bar, 10 μm. (B) Electron microscopic autoradiographs of interstitial collagen fibrils within confluent monolayer RFPEC culture showing a high density of label apparently associated with the obliquely cut basal lamina (arrows). Bar, 1 μm. (Inset) Area selected to show abundantly labeled, tangentially cut basal lamina (arrows) associated with the cell surface. Bar, 1 μm. (C) Matrix labeling of 125I-AT is mainly observed at the cell–cell interfaces of the suspension RFPEC cultures. Bar, 1 μm. (D) A cell from a monolayer RFPEC culture cut below the nucleus near the dish surface. Extensive 125I-AT labeling is seen within the surface connected vesicles, which are shown to contain extracellular matrix (arrow). Bar, 1 μm.
Figure 6. Light and electron micrographs as well as light and EM level autoradiographs of perfused normal and damaged rat aorta. (A) Light microscopic autoradiograph of rat aorta after perfusion with $^{125}$I-AT. The intense labeling is noted in the basement membrane beneath the endothelial cells (arrows). Bar, 10 $\mu$m. (B) EM level autoradiograph of rat aorta after perfusion with $^{125}$I-AT. The labeling is clearly evident beneath the endothelial cells. Bar, 1.0 $\mu$m. (C) Light microscopic autoradiograph of rat aorta after perfusion with $^{125}$I-AT. The intense labeling is noted around the small capillaries within the adjacent microcirculation, in association with the basal lamina and...
The minimal levels of ³¹¹-I-AT in the smooth muscle connective tissue could be due to the production of anticoagulantly active HSPG by fibroblasts (Marcum et al., 1986b).

The above localization of anticoagulantly active HSPG suggests that previous studies which measured the extent of AT binding to glycosaminoglycans of cultured endothelial cells or aortic explants need to be reinterpreted (Sterl et al., 1985; Marcum et al., 1986a). It would appear that the degree of AT binding per cell reflects not only the small amounts of cell surface anticoagulantly active HSPG but also access to the much larger quantities of the same material on the basolateral surface of these cells. Schleef and co-workers (Schleef et al., 1986a) have reported a similar situation for type I plasminogen activator inhibitor, which is mainly present on the basolateral surface of human umbilical vein endothelial cells, but is accessible to exogenously added tissue type plasminogen activator.

Two possible scenarios can be constructed whereby anticoagulantly active HSPG might regulate the coagulation cascade. On the one hand, the small amounts of luminal anticoagulantly active HSPG could be critically placed to bind plasma AT, accelerate the action of the protease inhibitor, and thereby regulate hemostatic mechanism activity at the blood-vessel wall interface. The much larger quantities of abluminal anticoagulantly active HSPG would serve as a potential reservoir that could be brought into play with extensive damage to the overlying endothelium. On the other hand, the substantial amounts of the anticoagulantly active HSPG that accumulate on the abluminal surface of endothelial cells could also act to modulate the ambient function of the coagulation cascade. Plasma AT should have relatively free access to this locale as suggested by numerous studies which document the extraordinary permeability of the endothelial cell layer (Simonescu, 1983). The presence of this subendothelial concentration of anticoagulantly active HSPG would also explain kinetic radiotracer studies that suggest that a significant amount of labeled protease inhibitor is located in a unique extravascular compartment (Carlson et al., 1985). The interaction of coagulation enzymes with AT bound to subendothelial anticoagulantly active HSPG could constitute the heparin-like, macromolecule-dependent acceleration of protease inhibitor action observed in animal models outlined above. This would imply that inhibition of coagulation enzymes is quite active within subendothelial regions, and would expand our present notions of the critical biologic surfaces which are in contact with the hemostatic mechanism. It seems quite likely that both scenarios outlined above could be functional under in vivo conditions.

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not with the bundles of collagen (open arrows). L, lumen. Bar, 10 μm. (D) EM level autoradiograph of rat aorta after perfusion with ³¹¹-I-AT. The intense labeling is seen around the correct junction (closed arrows) of a small capillary in the adjacent microcirculation, though not in the associated bundles of collagen fibers (open arrows). Bar, 1 μm. (E) Light microscopic autoradiograph of a rat aorta crushed before perfusion with ³¹¹-I-AT. The subendothelial labeling becomes extremely intense, and the labeling is also seen in the connective tissue surrounding the aortic smooth muscle cells (arrows). Bar, 10 μm. (F) EM level autoradiograph of a rat aorta crushed before infusion with ³¹¹-I-AT. The intense labeling of the basement membrane is readily apparent. Endothelial cells have been removed by damage. Bar, 1.0 μm.

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