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HEPARINLIKE MOLECULES WITH ANTICOAGULANT ACTIVITY ARE SYNTHESIZED BY CULTURED ENDOTHELIAL CELLS

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Cultured microvascular endothelial cells isolated from rat epididymal fat pads produce glycosaminoglycans that accelerate thrombin-antithrombin complex formation. The heparinlike nature of these macromolecules was established by complete destruction of their anticoagulant activity employing purified Flavobacterium heparinase. Only 15% of the biologic activity of these complex carbohydrates was expressed when the heparin binding domain on the protease inhibitor was chemically modified at the Trp 49 residue. The anticoagulantly active species contains disaccharides which constitute the unique antithrombin binding region of the mucopolysaccharide. Removal of the biologically active heparinlike components from endothelial cells with 0.05% trypsin suggests that these molecular species are present on the cell surface.

Marcum, et al. (1,2) have recently demonstrated that anticoagulantly active heparinlike molecules are intimately associated with vascular endothelial cells as well as being present within mast cells. In addition, these investigators have perfused the rat hindlimb preparation with purified thrombin and antithrombin and observed an acceleration of enzyme-inhibitor complex formation (3,4). Furthermore, Marcum, et al. (3,4) have also shown that this phenomenon is dependent upon heparinlike molecules that are bound to the endothelium which activate antithrombin in a manner similar to commercial heparin. However, the cellular origin of the biologically active molecules was not unambiguously established in the above studies. In the present communication, we provide the first conclusive evidence that microvascular endothelial cells synthesize heparinlike molecules which contain the unique elements of the antithrombin binding site of heparin and exhibit anticoagulant

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activity indistinguishable from that of commercially available mucopolysaccharide.

Methods and Materials

Endothelial cell isolation and culture: Endothelial cells were isolated from rat epididymal fat pads according to Wagner, et al. (5). Isolated microvascular cells were suspended in M199 (Earle salts) containing 20% fetal bovine sera (Gibco), 200 micrograms/ml of partially purified endothelial cell growth factor (6), ITS Premix (Collaborative), 100 units/ml penicillin, and 100 micrograms/ml streptomycin, plated on plastic Petri dishes which had been previously treated with a 1% solution of swine skin gelatin (Sigma, 300 Bloom) in 0.9% NaCl for 15 min at 37°, and incubated in a 5% CO_2, 95% air humidified atmosphere at 37° until confluent within 7-9 days. Dr. R. Hoover, Boston, MA, provided 2 x 10^6 endothelial cells from the rat epididymal fat pad which had been cloned from single cells utilizing cloning rings (7). These latter cellular elements as well as all postconfluent cells were maintained in M199 containing 10% fetal bovine serum, and antibiotics. Non-cloned and cloned endothelial cells at 10-20 and 30-40 population doublings, respectively, were utilized in experiments at 4 days postconfluence. Cells were subcultured at confluence employing 0.05% trypsin and 0.02% EDTA, pH 7.5 (Gibco).

Histology: Cultured endothelial cells were examined by light microscopy employing one micrometer-thick, Giemsa-stained, Epon-embedded sections (8). The presence of von Willebrand's protein in cultured endothelial cells was ascertained by immunofluorescence microscopy (1,9). Non-specific immunofluorescence was minimal as judged by controls utilizing smooth muscle cells.

Protein purification and quantification: Human thrombin and antithrombin were isolated from plasma and were judged homogeneous by sodium dodecylsulfate and polyacrylamide gel electrophoresis (10). In certain instances, the protease inhibitor was modified at Trp 49 with dimethyl (2-hydroxy-5-nitrobenzyl) sulfonium bromide (11). Bacterial heparinase was purified from Flavobacterium heparina provided by Dr. R. Langer, Boston, MA (3). The final product was homogeneous as judged by nonequilibrium pH gradient electrophoresis (12). The heparin cleaving activity of the purified bacterial enzyme was 3,650 units/mg. This preparation of Flavobacterium heparinase cleaved heparin and heparan sulfate but not chondroitin sulfate, dermatan sulfate, or hyaluronic acid (12). Protein concentrations were quantitated by the method of Lowry, et al. (13).

Heparinlike activity assay: Whole endothelial cells were isolated from culture dishes employing a rubber policeman. On occasion, cells were treated with 0.05% trypsin for 25 min at 37° to remove cell surface glycosaminoglycans. The residual cellular elements were pelleted by brief centrifugation, and the supernate containing the cell surface mucopolysaccharides, was decanted. Whole cells, the residual cell pellet, and the cell surface material were incubated sequentially with 1 mg/ml of papain (Sigma) and then 3 mg/ml of protease XIV (Sigma) for 48 hr at 37°. The samples were treated for 4 hr at 37° with chondroitin ABC lyase (Sigma) at a final concentration of 0.2 units/ml. Heparinlike activity was measured by quantitating the amount of thrombin-antithrombin complex generated within 10 sec in the presence of the endothelial cell mucopolysaccharide via a double antibody radioimmunoassay assay (1,14). Non-specific effects of contaminating polypeptides and polynucleotides which may mimic heparin activity by trapping ^{125}I-thrombin-antithrombin counts within the supernate of the radioimmunoassay incubation mixtures were negligible as judged by hirudin addition to thrombin prior to admixing antithrombin (1).

Chemical analysis of heparinlike molecules: [^{35}S]$NaSO_4$ (New England Nuclear) was added at 50 microcuries/ml to cloned endothelial cells at 2 days postconfluence. Cell surface mucopolysaccharides were harvested 48 hrs later, and cells were pelleted by centrifugation at 250 x g for 5 min. The supernate

was applied to Sephadex DEAE-A25 (1.6 cm x 70 cm) equilibrated with 0.15 M NaCl in 0.01 M Tris-HCl, pH 7.5. The column was eluted with a linear gradient consisting of the equilibrating buffer in the mixing chamber and 2 M NaCl, 0.01 M Tris-HCl, pH 7.5 in the reservoir. A single peak of radioactivity emerged at 0.8 M NaCl. The sample was incubated with 2 units of chondroitin ABC lyase for 4 h at 37° and then filtered at 8 ml/h on Sepharose 4B (1.1 cm x 120 cm) equilibrated with 0.15 M NaCl in 0.01 M Tris-HCl, pH 7.5. Undigested material with an average molecular weight of 30,000 (2), was affinity fractionated employing antithrombin temporarily immobilized on Concanavalin-A Sepharose (2). The bound material as well as the unbound fraction was deacetylated by hydrazanolysis (15). The samples were quantitatively cleaved to disaccharides containing anhydromannitol as the reducing end group by sequential high pH (3.9) and then low pH (1.5) nitrous acid treatments, followed by sodium borohydride reduction (16,17). The relative amounts of disaccharide types were estimated by HPLC ion exchange. The separation was conducted at a flow rate of 1 ml/min on a Whatman Particil PXS-1025 SAX column equilibrated with 40 mM potassium phosphate, pH 4.3 (17,18). Monosulfated disaccharides were eluted isocratically with the equilibrating buffer, and disulfated disaccharides were eluted with a convex gradient from 40 to 400 mM potassium phosphate, pH 4.3. The column was calibrated with disaccharides of known structure (17).

Results and Discussion

Microvascular endothelial cells were isolated from rat epididymal fat pads and maintained in culture as outlined above. Immunofluorescence microscopic analysis employing antisera directed against von Willebrand's protein, revealed that >95% of these cellular elements exhibited the perinuclear, punctuated cytoplasmic, and cellular matrix staining which is characteristic of endothelial cells (data not shown). Mast cells were not detected in these cultures employing one micrometer-thick, Giemsa-stained sections (8). Beaven and coworkers have observed that these latter cellular elements are not present in rat epididymal fat pad tissue.[1]

The amount of heparinlike activity from cultured endothelial cells was determined by quantitating the acceleration of thrombin-antithrombin complex formation in the presence of the cellular product (Table I). This procedure is more specific than previous assays for determining heparinlike activity, since thrombin-antithrombin complex formation is measured directly, rather than indirectly estimated via the residual enzymatic activity. The heparinlike nature of these samples was established utilizing purified Flavobacterium heparinase which completely abolished the biologic activity of these molecules (Table I).

[1]Personal communication.

TABLE I
Heparinlike activity from rat fat pad endothelial cells

	Heparinlike Activity* x 10^{-4} USP units/10^6 cells	
	Non-cloned	Cloned
Cell material + Native antithrombin	5.63±1.33 (n=4)	5.93±0.29 (n=3)
Flavobacterium heparinase-treated cell material + Native antithrombin	N.D.+ (n=4)	N.D.+ (n=3)
Cell material + Modified antithrombin		0.83±0.27 (n=3)
Cell surface material + Native antithrombin		5.11±1.13 (n=2)
Cell pellet material + Native antithrombin		0.27±0.08 (n=2)

*Mean±SE. Assay conducted in triplicate.

+None detected.

Cloned endothelial cells from rat epididymal fat pads were examined to confirm that these cellular elements produce anticoagulantly active material. Immunofluorescence detection of von Willebrand's factor utilizing an antiserum directed against this protein, revealed that ~100% of these cellular elements exhibited a pattern of cellular staining similar to that described above for non-cloned cells (data not shown). The amount of Flavobacterium heparinase-sensitive biological activity produced by the cloned cells was similar to that synthesized by the non-cloned cells (Table I). In addition, cultured bovine aortic smooth muscle cells (~17 x 10^6 cells) or 3T3 fibroblasts (~15 x 10^6 cells) did not exhibit any heparinlike potency. These latter cellular elements were utilized at 6 times the minimal number of endothelial cells (2.5 x 10^6 cells) necessary for detection of heparinlike activity. These data suggest that the biologically active mucopolysaccharide is specifically produced by endothelial cells and does not represent an artifact of the cell culture system.

To ascertain whether the heparinlike species from cultured endothelial cells activate antithrombin in a manner similar to commercial heparin, purified protease inhibitor was chemically modified at Trp 49 (~1.3 residues per molecule). The altered antithrombin interacts with thrombin at a normal rate in the absence of heparin, whereas the rate of enzyme neutralization is only minimally augmented in the presence of this mucopolysaccharide (19). The heparinlike activity of cloned endothelial cells was expressed to only a minor extent when modified antithrombin was substituted for the native protease inhibitor (Table I). It should be noted that the native and modified antithrombin exhibited identical immunoreactivities when complexed to thrombin.

Heparinlike molecules have been shown to be present on the surface of cultured endothelial cells (20). To ascertain the cellular location of the anticoagulantly active macromolecules, mucopolysaccharides were removed from the cell surface of cloned endothelial cells utilizing 0.05% trypsin. This enzymatic treatment is known to liberate glycosaminoglycans from the cell surface (21,22). The biologically active heparinlike molecules were almost completely removed from the surface of cultured endothelial cells utilizing the above enzymatic treatment with little activity remaining in the residual cellular pellet (Table I). Trypan blue exclusion of the trypsin-treated cells revealed that >95% of these cellular elements remained intact. These data suggest that anticoagulantly active heparinlike species are present on the surface of endothelial cells, possibly as an integral component of the membrane.

Commercial (mast cell) heparin has been shown to contain a unique set of tetrasaccharides which are responsible for the ability of the mucopolysaccharide to bind to antithrombin (17,23,24,25,26). These tetrasaccharides exhibit the following structures:

IdA ➤ GlcNAc-6-O-SO_3 ➤ GlcA ➤ GlcN-SO_3-3,6-O-SO_3

IdA ➤ GlcNAc-6-O-SO_3 ➤ GlcA ➤ GlcN-SO_3-3-O-SO_3

GlcA ➤ GlcNAc-6-O-SO_3 ➤ GlcA ➤ GlcN-SO_3-3,6-O-SO_3

Cloned endothelial cells were labeled with $[^{35}S]NaSO_4$ in order to determine whether heparinlike molecules from these cellular elements contain the four types of disaccharides which constitute the tetrasaccharide binding domain of the mucopolysaccharide. Glycosaminoglycans were isolated from the cell surface with 0.05% trypsin and affinity fractionated employing purified antithrombin temporarily immobilized on Concanavalin-A Sepharose. Approximately 15% of these molecular species bound to the protease inhibitor. A control mixture in which antithrombin was replaced by 0.15 M NaCl in 0.01 M Tris-HCl, pH 7.5, revealed that only 1.5% of the labeled mucopolysaccharides bound non-specifically to the matrix. The high affinity as well as the low affinity material were deacetylated and quantitatively cleaved to disaccharides by treatment with nitrous acid. HPLC ion exchange of the above samples revealed that the four disaccharides GlcA ➤ AMN-6-O-SO_3, IdA ➤ AMN-6-O-SO_3, GlcA ➤ AMN-3-O-SO_3, or GlcA ➤ AMN-3,6-O-SO_3 which comprise the antithrombin binding domain of the mucopolysaccharide were present in significantly greater quantities in the affinity fractionated species than in the depleted material (Table II). Furthermore, there was an augmentation of the common disaccharide species, IdA-2-O-SO_3 ➤ AMN-6-O-SO_3, within the low affinity material (Table II). These data suggest that antithrombin binding sites are present every 8 to 12 residues in the biologically active mucopolysaccharide. The number of binding sites for the protease inhibitor found in the cultured endothelial cell heparinlike molecules exceeds that observed in commercial heparin by 2 to 3 fold. It must be noted that the values obtained for the disaccharide composition of these mucopolysaccharides represents only those species which contained radiolabeled sulfate. The amount of non-sulfated disaccharides within the above populations, if any, was not determined.

In conclusion, we have demonstrated that cultured microvascular endothelial cells isolated from rat epididymal fat pads produce heparinlike molecules exhibiting anticoagulant activity. These macromolecules express their function via a biochemical mechanism of action identical to commercial

TABLE II
HPLC analysis of disaccharides from affinity fractionated and depleted heparinlike molecules

Disaccharide	Percentage	
	Affinity Fractionated	Depleted
GlcA ► AMN 6-O-SO_3	8*	4.5
IdA ► AMN 6-O-SO_3	7	4
IdA ► AMN 2-O-SO_3	6	8
GlcA ► AMN 3-O-SO_3	21	N.D.+
IdA ► AMN 2-O-SO_3 6-O-SO_3	48	80
GlcA ► AMN 3,6-O-SO_3	10	3.5

*This value has been corrected for losses incurred during high pH nitrous acid treatment of the original sample.

+None detected.

heparin and contain critical elements of the tetrasaccharide region important for the binding of heparin to antithrombin. Release of the biologically active mucopolysaccharide from endothelial cells with trypsin suggests that these molecules are on the cell surface. Based upon these data and our earlier studies (1-3), we propose that heparinlike molecules are produced specifically by endothelial cells and positioned on the luminal surface of the endothelium where circulating antithrombin can be bound and activated. Thus, the protease inhibitor would be critically placed to inhibit coagulation enzymes and thereby endow natural surfaces with antithrombotic properties. Alterations in the biosynthesis of heparinlike molecules by endothelial cells could result in arterial as well as venous thrombotic disease in humans.

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