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The Ultrastructual Characterization of the Antithrombin III Stationary Cofactor Found on Bovine Aortic Endothelium.

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THE ULTRASTRUCTUAL CHARACTERIZATION OF THE ANTITHROMBIN III STATIONARY COFACTOR FOUND ON BOVINE AORTIC ENDOTHELIUM.

By

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ABSTRACT

THE ULTRASTRUCTUAL CHARACTERIZATION OF THE ANTITHROMBIN III STATIONARY COFACTOR FOUND ON BOVINE AORTIC ENDOTHELIUM.

By

Margaret Ellen Hogan

A stationary cofactor for Antithrombin III (ATIII) exists on the surface of isolated bovine aortic endothelial cells. This cofactor is involved in the ATIII inactivation of the serine protease, thrombin. Previously, its characterization was limited to kinetic studies of the cell-bound and isolated cofactor, which demonstrated heparin-like acceleration of ATIII inactivation of various serine proteases. This research ultrastructurally characterizes the cofactor to provide morphological correlation with kinetic studies.

Endothelial cells grown on microculture beads were examined using ^{125}I radiolabelled ATIII. The cells showed specific binding of ATIII that was inhibited by: Preincubation of the cells with cold ATIII or a heparin-specific enzyme, and preincubation of labelled ATIII with heparin or thrombin solutions. Results were cosupported by immuno-gold labelling.

The cofactor resembled isolated heparin in activity and enzyme specific degradation, however, unlike isolated heparin, it bound and held ATIII to the cell surface.

To my parents, for all their love and giving me the chance to explore.
To Jill, for lots of support and friendship.
To Chris Knight, for the perfect scientific perspective.

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I would like to thank the Staff and Friends of the Center for Electron Optics for their help and encouragement. For without their unique blend of talents I would have surely completed this thesis a much less rounded individual.

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
Anti-AT	Antibodies against ATIII
ATIII	Antithrombin III
BAT	Bovine ATIII
BEI	Backscatter electron image
BEEM	Better Equipment for Electron Microscopy (vendor)
CS	Chondroitin sulfate
ddH_2O	Double distilled water
DME	Dulbecco's modification of Eagle's medium
DS	Dermatan sulfate
EDTA	Ethylenediamine tetraacetic acid
EM	Electron microscopy
FBS	Foetal bovine serum
GAG	Glycosaminoglycan
HA	Hyaluronic acid
HAT	Human ATIII
HCl	Hydrochloric acid
HEPES	Hydroxyethylpiperazine ethane sulphonic acid
Hep Sulf	Heparan sulfate
HS	Heparan sulfate
PA	Plasminogen activator
PB	Phosphate buffer
PBS	Phosphate buffered saline
PGI_2	Prostacyclin
PHG	Phosphate-HEPES-Glucose buffer
RT	Room temperature
SEI	Secondary electron image
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
Th	Thrombin
tPA	tissue Plasminogen activator
vWF	vonWillebrand Factor

INTRODUCTION

Endothelial cell involvement in maintaining blood fluidity encompasses many actions, some passive, some active. One endothelial cell characteristic, previously described and tested here, is that of providing a membrane surface instrumental in the Antithrombin III (ATIII) inactivation of circulating blood coagulation proteins. Though this compound has been described to be heparin-like in nature, no substantial proof has been given of its true identity, or of its relative concentration in the body. This nonthrombogenic function of the endothelium links the vascular wall to yet another facet in the control of unwanted thrombosis.

Heparin is not naturally found in the circulating blood in appreciable amounts. Yet, this endogenous glycosaminoglycan (GAG) (once concentrated) has been a key theraputic anticoagulant for over thirty years. Heparin is a linear, polymetric molecule that has an average molecular weight of 10,000 to 15,000, but ranges from 3,000 to 45,000. It is composed of repeating units of glucosamine and one of two uronic acids (iduronic or glucuronic acid) both in pyranose form (Johnson and Mulloy, 1976) (Figure 1). Its primary mode of action is as a catalyst for Antithrombin III (ATIII), a circulating serine protease inhibitor. Of the coagulation proteins affected, thrombin inactivation by ATIII is quantitatively the most important.

Heparin-mediated inactivation of the clotting factors include two

mechanisms. One involves its specific binding to ATIII through a critical tryptophan residue (Blackburn, et al. 1984) contained in a lysine block on the ATIII molecule. This binding produces a conformational change in the ATIII that makes its reactive site more accessible to the active center of the activated clotting factors (Lindahl and Hook, 1978) (Figure 2).

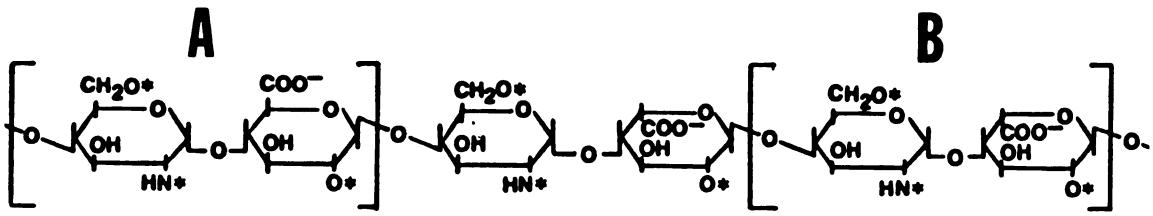


Figure 1. Structure of Heparin. Two forms of the repeating units of heparin. (A) Glucuronic acid and (B) Iduronic acid, the only difference between the two uronic acids is the location of the carboxyl group. Areas that can be sulfated are indicated by an asterisk (*). (After Johnson and Mulloy, 1976).

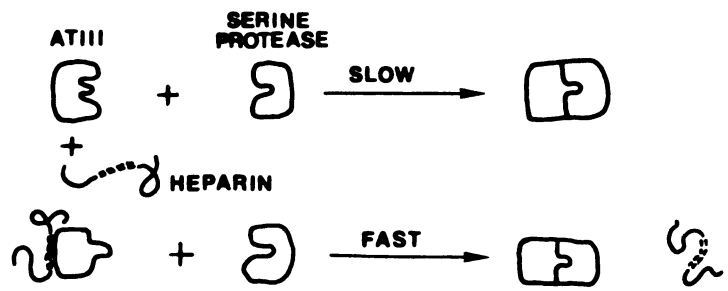


Figure 2. ATIII Interaction With Heparin. Acceleration of ATIII inhibition of a serine protease via a conformational change in ATIII by heparin binding. The ATIII binding site of heparin as shown by (===), is specific. (After Lindahl and Hook, 1978)

The second mechanism is the non-specific binding of heparin to circulating plasma proteins. This binding is due to heparin's high negative charge density. It is this characteristic that is often exploited in the purification of plasma factors IX, XI and thrombin. In the case of thrombin, heparin helps bring the ATIII molecule in closer proximity to the thrombin molecule, therefore facilitating the inactivation of thrombin by ATIII (Figure 3).

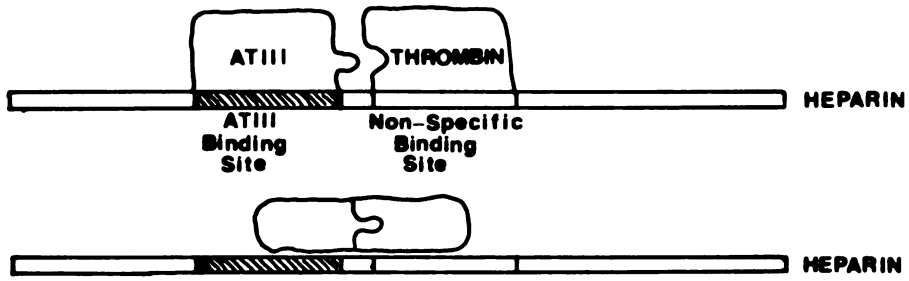


Figure 3. Interaction of Heparin, ATIII and Thrombin. Illustration of heparin-mediated ATIII inactivation of thrombin. (A) ATIII binds to a specific site on heparin, while thrombin binds to heparin non-specificly. The resulting closeness of the two molecules allow binding. (B) After binding, heparin is released from the ATIII-Thrombin complex.

The anionic density of heparin is an important factor in its anticoagulant activity. It has been shown that with a decrease in sulphation there is a related decrease in anticoagulant activity (Hurst, et al. 1979; Ayotte, et al. 1981; Riesenfield, et al. 1981). Also, the molecular weight of heparin has been shown to be related to anticoagulant activity. The larger molecules are found to promote a stronger inhibition of thrombin by antithrombin III (Andersson, et al. 1979; Laurent, et al. 1978). The ATIII-thrombin interaction is kinetically very slow. In the presence of heparin, this reaction is accelerated 200-fold.

Recently a heparin-like cofactor for ATIII was observed to be attached to the surface of isolated endothelial cells. It was shown to have ATIII binding capabilities as well as be involved in the ATIII inactivation of thrombin (Dryjski, et al., 1982, 1983). It was this cofactor that was examined in this current research using electron microscopy via the binding of ATIII.

My characterization involved three primary objectives: 1) the

binding characteristics of the cofactor, 2) the determination of the most likely GAG to be responsible for the activity, and 3) the localization / quantitation of the cofactor on the endothelial cell. These three characterizations were examined as follows.

Hatton, et al. (1978), demonstrated the binding characteristics of various endothelial GAGs bound to a substrate. One of their observations was the selective binding of heparin to ATIII. This selective binding was utilized in my experiments to tag the stationary cofactor. In this research, ATIII binding was quantitated on the endothelial cell, and compared with binding of ATIII complexed with various molecules (GAGs and thrombin).

To further characterize the cofactor, I employed treatment of the endothelium with various GAG-specific enzymes. The enzymes heparinase and heparitinase were used. Heparinase is specific for heparin and heparitins C and D, while heparitinase is specific for heparitins A and B (heparan sulfate). Heparitinase does not digest heparin, heparitin C or D. The objective of the research was to distinguish between the three most likely GAGs involved with the cofactor activity (heparin, dermatan sulfate and heparan sulfate).

Localization of the cofactor was approached with the use of electron microscopy (EM) combined with immunogold labelling. The previously described ATIII binding experiments were designed to give some insight to the actual location of the cofactor while providing the information for the binding study.

The examination of the endothelial cell - coagulation protein interaction through electron microscopic methods has not previously been widely utilized. The use of EM to demonstrate and localize this

interaction ultrastructurally made this approach invaluable to correlate previously examined kinetic experiments. The methods used in my study, autoradiography and immunogold cytochemistry, were used to probe the surface as well as the interior of the endothelial cell. In this way a number of characteristics were examined. The most important of these being: 1) the quantitation of binding sites per cell, 2) the condition and maturity of the cells tested, and 3) the binding characteristics of the cofactor itself.

Literature Review

The vascular endothelium plays a significant role in maintaining the fluidity of circulating blood. This ability is made possible by the varied functions of the endothelial cell which include; 1) formation of a relative, mechanical barrier between the blood components and the sub-endothelial matrix; 2) the synthesis or metabolism of mediators that regulate the interaction between the blood components and the vessel wall; 3) control of vascular repair through cell contraction, cell migration and proliferation, and thrombolysis; and 4) the maintenance of thromboresistance (Figure 4).

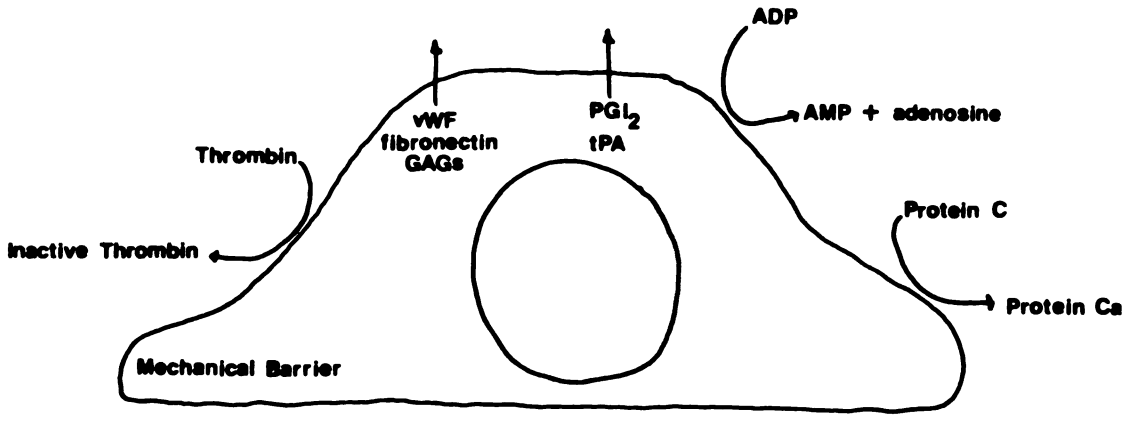


Figure 4. The Endothelial Cell Role in Maintaining Blood Fluidity.

The vascular endothelium is a single layer of cells that line all blood vessels in the body. They provide a physical barrier between the pro-coagulant factors in the blood (platelets, coagulation proteins) and the subcellular matrix. This matrix, when exposed, causes the induction of the hemostatic plug through direct platelet interaction and / or the activation of the coagulation cascade. Therefore, under physiologic conditions, the endothelium inhibits hemostasis and thrombosis, but when injured, promotes these two processes (Gimbrone, 1981).

Mediators synthesized or metabolized by the endothelium are primarily involved in platelet interaction, though some are active in mechanical regulation of vessel tone. The most important of these synthesized compounds are von Willebrand factor (vWF), fibronectin, and the glycosaminoglycans (GAGs). The von Willebrand factor is synthesized and released (Jaffe, et al. 1974) both into the circulation and subendothelially, where it is absorbed to exposed collagen (Sakariassen, et al., 1979). Following vascular injury, the role of vWF in hemostasis is to act as a cofactor in the adhesion of platelets to exposed subendothelial collagen, thereby promoting clot formation. Fibronectin synthesis and secretion is towards the subcellular matrix (Yamada and Olden, 1978; Mosher, et al. 1982) where it acts as a substrate for factor XIIIa (the fibrin stabilization factor) following vessel injury. This interaction causes crosslinking between fibronectin, fibrin or collagen (Mosher, et al., 1979), contributing to a stable, hemostatic plug. Endothelial production of GAGs primarily plays a passive role in the cell's non-thrombogenic nature. The proteoglycans found in greatest abundance are, in increasing

concentration, hyaluronic acid, chondroitin sulfate, dermatan sulfate (chondroitin sulfate B) and heparan sulfate (Buonassisi, 1973; Gardais, et al., 1973). Of those listed, only the sulphated GAGs dermatan sulfate and heparan sulfate have been shown to have appreciable anticoagulant activity (Hook, et al., 1984). Even this activity, however, requires approximately 70-times the concentration of each to equal that of heparin, a widely used anticoagulant (Thilo, et al. 1983). Their activity is believed to mimic the action of heparin.

Heparin is a natural, heterogenous GAG produced primarily in the mast cell, and is found circulating in the blood in only minute quantities. One of the anticoagulant effects of heparin is through its interaction with ATIII, a circulating anticoagulant protein. It catalyzes the rate at which ATIII neutralizes the proteolytic activities of several activated clotting factors in the intrinsic and common coagulation pathways (Bjornsson and Greenberg, 1985). Heparin can also have a direct effect on blood factors through electostatic binding that interfers with enzymes' procoagulant activities. It is believed that some of heparin's activity occurs while absorbed to the surface of the endothelium (Barzu, et al., 1985). It is in this relationship that the anticoagulant characteristics of the secreted GAGs are related to heparin, and, therefore, provide a non-thrombogenic function of the endothelial cell.

Upon injury, the endothelium undergoes a number of changes to resolve the damage and limit hemorrhage. These changes include endothelial cell contraction, cell migration and proliferation, and thrombolysis. With vascular injury there is a brief period of vasoconstriction, which in small vessels serves to reduce blood flow.

The formation of a hemostatic plug, via platelet interaction and blood coagulation factors then covers the site of injury. Migration and regeneration of the endothelial layer follows fibrin clot contraction, therby renewing the endothelial layer. At the same time, the hemostatic plug is being removed through fibrinolytic substances (tissue plasminogen activator (tPA)) secreted from the endothelial cell. The outcome is a repaired, non-thrombogenic cell layer and the removal of the hemostatic plug (Robbins, et al. 1984; Ogston, 1983).

Another function of the endothelium is that of thromboresistance. This characteristic involves both passive and active mechanisms. As stated before, the endothelial GAGs provide a surface that is passively non-thrombogenic. Active thromboresistance by the endothelium is maintained through several mechanisms, including the following: 1) synthesis and release of prostacyclin; 2) secretion of tPA; 3) degradation of ADP by membrane-bound ADPase; 4) uptake and degradation of vasoactive amines; 5) contribution of a cofactor (thrombomodulin) in the thrombin-dependant activation of Protein C; and 6) uptake, inactivation and clearance of thrombin (Thompson and Harker, 1983).

Prostacyclin (PGI_2) is a very potent inhibitor of platelet aggregation, it has also been shown to be a potent stimulator of cAMP accumulation (Hopkins and Gorman, 1981). Prostacyclin is produced through membrane arachadonic acid-ester conversion by first, cyclo-oxygenase then by prostacyclin synthetase. The resulting prostaglandin has at least two actions. One is to dilate vessels locally (therefore increase blood flow), and the other is to increase intra-platelet concentrations of cAMP and so depress platelet aggregation. PGI_2 is the most active platelet aggregation inhibitor,

and like most highly active substances, is short-lived in the system.

A number of different forms of plasminogen activator are synthesized and secreted from the endothelium, a fibrin-dependant (tPA) and fibrin-independant (urokinase-like) PA activity. All which show species, localization and activity differences (Todd, 1959; Laug, 1981; Levin and Loskutoff, 1982). Their function is to cleave plasminogen into the active, fibrinolytic enzyme, plasmin. Plasmin is not normally found in the blood, and requires the conversion of the zymogen plasminogen, via some activating factor, to become the active enzyme. The half-life of plasmin in the body was calculated to be 2.5 minutes (Matsuo, 1982). Plasmin acts on the insoluble fibrin of the hemostatic plug, converting it to a variety of soluble degradation products. Secretion of tPA by the endothelial cells has been linked to thrombin stimulation of the endothelium (Levin, et al., 1984), postulated to be stimulated by catacholamines (Cash, 1978), and can be stimulated by a number of drugs (Davidson, et al., 1972; Nilsson, 1978).

ADP regulation through endothelial ecto-ADPase is involved in the control of ADP-induced platelet aggregation (Cooper, et al. 1979). ADP is one of the secreted constituents of the platelet's dense granules, which is released upon activation. Degrading ADP by endothelial cell membrane-bound ADPases causes the production of AMP and adenosine, both potent inhibitors of platelet aggregation (Pearson, et al. 1978). This leads to a negative feedback system that helps maintain blood fluidity.

The removal of vasoactive amines by the endothelium maintains blood fluidity. The vasoactive amines are released by platelets and include 5-hydroxytryptamine (serotonin), histamine and epinephrine (Robbins, et al., 1984). Serotonin and histamine have action on smooth muscle and

are believed to be involved in blood vessel constriction. Epinephrine has been shown to increase the effect of ADP on platelet aggregation (Ardlie, et al., 1966).

Thrombomodulin is a receptor on the endothelial cell that binds thrombin, a serine protease. This binding results in a 20,000-fold increase in the conversion of Protein C to active Protein Ca (Esmon and Owen, 1981). Thus, this endothelial cell receptor has the ability to accelerate the rate of thrombin-dependant Protein C conversion. This in turn allows the active Protein C to inhibit the conversion of the zymogens, Factors V and VIII to their active forms, both of which are important as cofactors in the coagulation cascade (Rosenberg and Rosenberg, 1984). Another endothelial involvement in Protein C mediation is the synthesis and release of Protein S (Stern, et al., 1986). Protein S is a regulatory plasma protein, which is an essential portion of the Protein C anticoagulant pathway. It acts as a non-enzymatic cofactor which promotes binding of the activated Protein C to membrane surfaces. Once bound to a phospholipid surface, activated Protein C can effectively exert its anticoagulant function (Walker, 1984).

Thrombin circulates in the blood as the zymogen, prothrombin. Upon cleavage (of a single arginyl-isoleucine bond), the zymogen undergoes a change permitting the expression of enzymatic activity. Thrombin has several functions in thrombosis and hemostasis (Figure 5), and therefore, thrombin clearance from the circulation is essential to maintain blood fluidity (Fenton, et al. 1977).

Thrombin is modulated by the serine protease inhibitor ATIII. Antithrombin III forms a 1:1 stoichiometric complex with thrombin

rendering it enzymatically inactive (Abildgaard, 1969). Complex formation is greatly enhanced in the presence of heparin, resulting in a more rapid neutralization of thrombin.

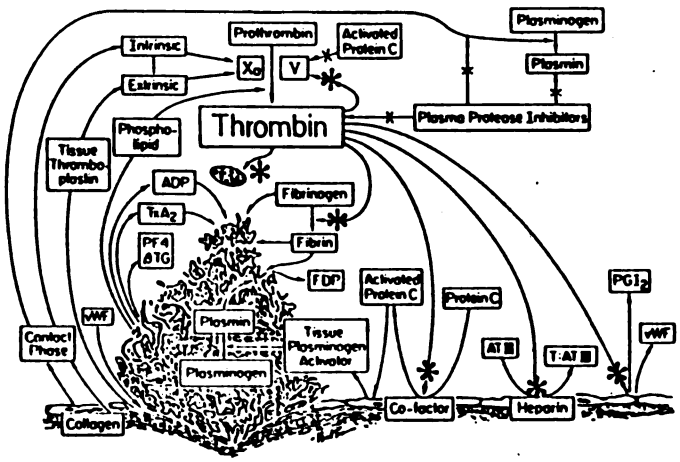


Figure 5. Thrombin Involement in Hemostasis and Thrombosis. Sites of thrombin activity are shown by the asterisk (*). Taken from Manual of Hemostasis and Thrombosis. Thompson, et al., 1983.

The endothelium has been noted for its ability to bind and inactivate thrombin (Dryjski, et al. 1983). It has been shown that the endothelium quickly removes thrombin from the circulation in rabbit (Lollar and Owen, 1980), bovine (Busch, et al., 1982) and human (Awbrey, et al., 1979) endothelial cell cultures. The identity of this thrombin receptor was attributed to two, possibly different, sites. The first, was thrombomodulin thrombin binding (Lollar, et al. 1982), and the other, was a specific thrombin receptor that upon binding inactivated the thrombin (Lollar and Owen, 1980; Busch, et al. 1982). The distinction was made when thrombin-ATIII complexes were produced upon contact with the endothelium (Busch, et al., 1982). This work was substantiated with perfusion studies by Marcum and coworkers in 1983 and 1984. What was found was a heparin-like, stationary cofactor for circulating ATIII. When ATIII bound to the surface cofactor, thrombin

binding was greatly increased and resulted in the liberation of an inactive thrombin-ATIII complex. Antagonists of the ATIII cofactor activity of heparin significantly reduced the capacity of the preparation to inhibit thrombin (Busch and Owen, 1982).

The possible presence of heparin attached to the vascular endothelium poses many questions. The objectives of this research was to distinguish the cofactor from the most likely GAGs involved in the activity, and to determine the nature of the cofactor in regards to its binding characteristics and the endothelial response to the cofactor manipulation.

MATERIALS AND METHODS

Endothelial Cell Isolation and Culture: Bovine aorta were obtained at a slaughter house, and immersed in ice-cold sterile Dulbecco's Modified Eagle Medium (DME) to transport. The cell isolation was carried out in a laminar flow hood using the following modifications of previously described isolation procedures (Booyse, et al., 1975; Gimbrone, 1976; Huey, 1985). The aortic endothelial cells were released from the intima by digesting with a solution of collagenase (36U/ml) dissolved in sterile DME at 37° C. The resulting cell suspensions were seeded into 100mm tissue culture plates and grown in a humidified, 37° C incubator with 10% CO_2. Once the primary endothelial cell cultures reached confluency, they were washed briefly in sterile PBS, and removed from the plates using the splitting buffer, 0.02% EDTA, 0.5% BSA in Phosphate-HEPES-Glucose (PHG) buffer (5 minutes at 37 ° C). The cells were pelleted at 1800 rpm for 5 minutes. The splitting buffer was removed, and the cells were divided into 100mm petri plates (no tissue culture surface) which contained 1.5ml of prepared microcarrier beads (Cytodex 1, Pharmacia). Media was changed every other day until the cells were confluent on the beads. See Appendix A for detailed protocol.

Microcarrier Bead Preparation: The microcarriers (Cytodex 1) were prepared by hydrating 1gm of beads in 50mls of 0.2% Diffco gelatin in double distilled water (ddH_2O), and then autoclaving the bead

slurry at 120° C, 15psi for 20 minutes (liquid cycle). The sterile beads were then distributed to petri plates in a concentration of 1.5ml of bead slurry for every 30mls of culture media - providing approximately 200,000 beads/plate and a surface area of 180cm^2 (Hogan, et al., 1987).

Iodination of Antithrombin III: Iodination of ATIII was by the Chloramine T method (Greenwood, et al., 1980). One mCi ^{125}I (ICN Biomedicals, Inc. 456 mCi/ml) was added to ATIII (5mgs/1.5mls PBS) and swirled. 250ul of Chloramine T (1mg/ml PBS) was then added dropwise to the protein mixture and allowed to react for 30 minutes on ice. To terminate the reaction, 250ul sodium metabisulfite (1mg/ml PBS) was added and the solution swirled. The calculated specific activities were, 1.3 x 10^9 cpm/mg for Bovine ATIII and 6.1 x 10^9 cpm/mg for Human ATIII. To remove unbound ^{125}I, the mixture (2.0mls) was passed through a column packed with Dowex 1, prepared as follows.

Dowex Column Preparation: Dowex 1 was suspended in 50:50 ethanol:acetone (45gm resin/100mls) for 1 hour. The suspension was filtered dry and resuspended in ddH_2O and mixed with a magnetic stirrer. Solid sodium acetate was added to give a final concentration of 1N. The Dowex was filtered dry, and the cake was placed into ddH_2O. Concentrated hydrochloric acid was added to obtain a final concentration of 3M (approximately a 1:4 dilution of the HCl stock). The resin was again filtered and washed with 0.5M HCl, followed with ddH_2O until the filtrate was neutral. The Dowex was then in the chloride ion form and ready to use.

Radioassay of GAG-ATIII Binding: The GAGs, heparan sulfate (HS, Miles Scientific Inc.), dermatan sulfate (DS, Sigma #C4259), hyaluronic

acid (HA, Sigma #H1751) and chondroitin sulfate (CS, Sigma #C8529) were prepared to a final concentration of 0.1mg/ml in Phosphate Buffer (PB). Heparin (H, Upjohn Co.) and thrombin (Th, DiaTech Inc. #303061) were diluted to 0.1U/ml in PB. Rabbit antibodies to ATIII (anti-ATIII) and purified ATIII (bovine (BAT, Sigma #A9141) and human (HAT, USDA isolate)) were diluted to 0.1mg/ml in PB. PB alone was used as a control. 50ul of each of the above solutions were allowed to incubate in a 96 well microtiter assay plate for 1 hour at room temperature. Each well was washed three times with PB (GAG binding was approximated by staining test wells with 3% alcian blue), and was blocked with 3% BSA followed by a final wash in PB. 100,000 cpm/well of ^{125}I-ATIII (bovine or human) in PB was added in a volume of 50ul, and was incubated 30 minutes at room temperature. Plates were washed three times with PB, dried, the wells removed and counted in a gamma counter.

ATIII Binding to Endothelial Cells: Into 1500ul microfuge tubes approximately 0.5mls of bead/cell suspension was placed. Each sample was washed twice with PHG to remove residual media. The buffer was removed, and half of the samples were put on ice and the other half left at room temperature. 300ul of each of the test solutions (see below) were added to the tubes, and all were incubated 30 minutes. The test solutions were removed, the samples washed twice with PHG and fixed in 2% glutaraldehyde in PB.

Treatments (incubated at both 4 and 27° C)	Volume
Labelled ATIII alone (diluted with PB)	150ul each
Labelled ATIII + Heparin (0.1U/ml)	150ul each
Labelled ATIII + Heparan Sulfate (0.1mg/ml)	150ul each
Labelled ATIII + Thrombin (0.1U/ml)	150ul each
ATIII (pre-incubation, 30 minutes) then labelled ATIII	300ul each
PB	300ul

Enzyme Treatment of Endothelial Cells: 0.5mls of the cell-covered beads were aliquoted into each of six 1500ul microfuge tubes. The cells were then washed two times with PHG to remove the media, the supernatants removed, and were incubated with the enzymes (Heparinase, 5U/reaction/500ul; Heparitinase, 5U/reaction/500ul; Miles Scientific, Inc.). After 30 minutes at 37° C, the incubation solution was removed, and the cells were washed two times with PHG. Dual incubations with radio-labelled ATIII were done at both room temperature and on ice. Again, after a 30 minute incubation the reaction mixture was taken off, the cells washed twice with PHG, and then were prepared for microscopy (Figure 6.).

Enzyme Treatment (30 minutes, 37° C)	^{125}I-ATIII Treatment (30 minutes)
Heparitinase	room temperature (27° C)
Heparitinase	4° C
Heparinase	room temperature (27° C)
Heparinase	4° C
PHG	room temperature (27° C)
PHG	4° C

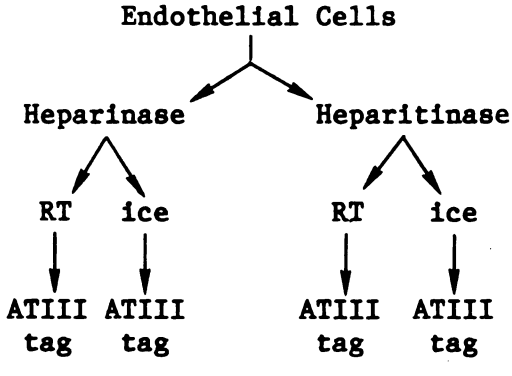


Figure 6. Enzyme Treatment Flowchart.

Colloidal Gold Labelling: Microcarrier beads cultured to confluency, were removed from the growth medium and pooled. The

suspension was washed with warm (37° C) PHG buffer, and aliquoted into individual reaction tubes. Supernatants were removed and discarded after the cells settled, and half of the samples were incubated with the ATIII reaction mixtures (as used in the ATIII Binding to Endothelial Cells Study), and the other half prefixed with 0.5% paraformaldehyde in PHG buffer (15 minutes, 37°C.), then reacted with the identical solutions. After the 30 minute incubation, the cells were washed then fixed in 0.5% paraformaldehyde (15 minutes, 27° C), washed again and blocked with 3% BSA. Anti-ATIII prepared in rabbits, was then added to the cell mixtures, and reacted 30 minutes at 37° C. After washing twice with PHG, Protein A-Gold (1:4 dilution) prepared according to Bendayan (1984a) was incubated with the cells (30 minutes, 37° C). The cells were again washed to remove unbound gold, and fixed in paraformaldehyde/glutaraldehyde, and prepared for electron microscopy (Bendayan, 1984b). Controls were prepared by leaving out one reagent (ATIII, anti-ATIII or both) and replacing it with PHG. Procedures remained the same.

Electron Microscopy Preparation: A common procedure was followed for both TEM and SEM preparation of the cell cultures (Figure 7).

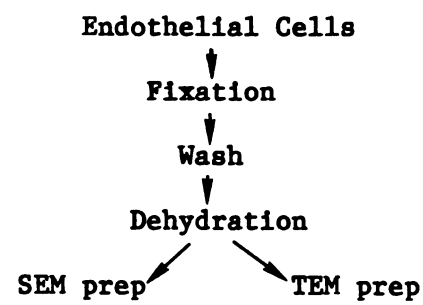


Figure 7. Common Sample Preparation for TEM and SEM.

Cell cultures were fixed in a 0.5% paraformaldehyde/1.0% glutaraldehyde

solution prepared in PHG buffer (1 hour, 4° C). Post-fixation was in 1.0% osmium tetroxide in PHG for 2 hours at room temperature. After fixation the samples were washed repeatedly with 50 mM HEPES buffer, then double distilled water to remove any glucose in the samples. Specimens were dehydrated through graded ethanol, then divided into two portions, one for SEM and the other for TEM preparation.

Transmission Electron Microscopy: Dehydrated samples were infiltrated with Spurrs-Mollenhauer resin (Klomparens, et al., 1986), after the following schedule:

Alcohol : Acetone	3:1	1 hour
Alcohol : Acetone	1:3	1 hour
Acetone	100%	1 hour
Acetone : Resin	3:1	3 hours
Acetone : Resin	1:1	3 hours
Acetone : Resin	1:3	3 hours
Resin	100%	overnight

Samples in 100% resin were placed under mild vacuum (20 psi) 1 hour to insure complete infiltration/exchange of the bead. The following day the cell-covered beads were added to the top of filled BEEM (Better Equipment for Electron Microscopy) capsules, and allowed to sink to the tip. Again the specimens were placed under vacuum (20 psi, 15 minutes), then polymerized in a 60° C oven for 48 hours. Blocks were sectioned with a diamond knife, stained with uranyl acetate and examined with a JEOL 100 CXII transmission electron microscope operated at 80 kV.

Scanning Electon Microscopy: Dehydrated samples were placed in porous baskets and critical point dried. The dried samples were then attached to aluminum stubs with sticky tape, and coated with carbon or gold. Carbon evaporation was done with a Ladd evaporator with a rotary stage (5nm of carbon), and gold was applied with a Film-Vac, Inc.

sputter coater (15nm of gold). Secondary electron images (SEI) and backscattered electron images (BEI) were both run at 15kV and were performed on the same JEOL 35CF scanning electron microscope.

Autoradiography: TEM- Dark gold sections (100nm) of labelled cells were cut and transfered to plastic-coated (0.5% collodion in amyl acetate) glass slides. The slides were then carbon coated (approx. 4nm) using a Ladd vacuum evaporator. Coated slides were dipped in Kodak emulsion NTB 2, diluted to obtain a gold reflection over the sections. Specimens were allowed to dry, and then stored, desiccated at 4^{o} C for exposure.

Light microscopy- 2 micrometer sections were collected on clean glass slides and then dipped into undiluted Kodak emulsion NTB 2. When dry, the slides were stored as were the slides with the thin sections.

Exposed emulsion development- After various exposure times, glass slides with the exposed emulsion were allowed to reach room temperature (from 4^{o} C), were developed in half-strength Kodak D19, for 2 minutes, fixed 2-3 minutes in full strength fixer, washed for 10 minutes and dried (Budd, 1971; Klomparens, et al., 1986).

For LM examination, slides were coverslipped and examined. For TEM, the collodion was floated off the slides using hydrofluoric acid. Grids were then placed on the area that contained sections, picked up, stained with uranyl acetate and examined.

Quantitation of ATIII Binding: Unstained autoradiographic thick sections (1 micrometer) were photographed and enlarged to a final magnification of 1000X. The number of exposed silver grains per cell section was determined, then each cell was cut out, pooled by treatment and weighed (to the 10^{-4} place) on a Mettler H15 balance. All weights

were compared to known standards (pre-determined by micrographing, enlarging (to 1000X), cutting out and weighing a grid of known area). All micrographs were printed on Kodak Polycontrast RCII, medium weight paper. Counts per cell slice was converted to counts per whole cell (based on an average endothelial cell size of $10um^3$).

Statistical Analyisis: Data was analized by using a Random Analysis of Variance, where the variance was tested for heterogeneity with a F-max test. Multiple comparisons were made using Dunnett's test for comparison of all treatments to a control, and Bon Ferroni's test for the comparison of treatments. See Appendix C for complete analysis.

RESULTS

The ATIII binding to substrate-attached GAGs had approximately the same order of affinity in both bovine and human ATIII, with only a change in order between Hyaluronic Acid (HA) and Dermatan Sulfate (DS) (Table 1).

Table 1. Radioassay of ATIII Binding to GAGs

	Human ATIII (mean cpm)	Bovine ATIII (mean cpm)
Heparan Sulfate	5601.3	2633.3
Dermatan Sulfate	5834.6	4538.6
Hyaluronic Acid	7971.6	4014.6
Chondroitin Sulfate	9118.6	4605.3
Heparin	9618.6	5060.0
Thrombin	12800.0	6493.3
anti-ATIII	11150.6	9180.0
Human ATIII	5663.0	2264.0
Bovine ATIII	5491.3	2803.6
Load Count per 50ul (cpm)	55895	73542

Note: Mean cpm based on N=6

The rank of the GAGs in regards to their ability to bind ATIII was as follows:

Bovine ATIII: H > CS > DS > HA > HS

Human ATIII: H > CS > HA > DS > HS

With both forms of ATIII, heparin bound to a substrate demonstrated the greatest ability to bind ATIII, where heparan sulfate was closest to a negative control in binding ATIII.

Attempts to localize radiolabelled ATIII binding to the surface of the endothelium using decreased temperature ($4^{o}C$) were unsuccessful. When comparing binding experiments done at RT and $4^{o}C$, no substantial difference was found. Essentially, all labelling was internalized. Autoradiographs for TEM were sparse in silver grains (Figure 8A), therefore quantitation of ATIII binding was made at the light microscope level (Figure 8B). The EM was then used to identify areas within the cell that tended to show the highest levels of binding. These areas were dispersed with no apparent organelle containment.

ATIII binding to endothelial cells measured by autoradiographic means showed a significant difference via Dunnett's test between the treatments and the control. When the treatments were compared to their ability to block ATIII binding to the cells, only ATIII pre-bound to thrombin and ATIII pre-bound to heparin showed any significant reduction in binding (via Bon Ferroni's test) to the cells (Table 2). The amount of thrombin reacted with the ATIII was far below saturation levels, this was to prevent precipitation of the complexed molecules following reaction.

Table 2. ATIII Binding Measured by LM Autoradiography

Binding at $4^{o}C$

Treatment	mean counts / cell	variance
Control	16.92	2.793
Heparin	23.45	8.789
Thrombin	24.41	14.933
Heparan Sulfate	34.52	8.544
ATIII	37.23	13.573

Binding at RT

Treatment	mean counts / cell	variance
Control	16.86	1.361

Figure 8. ATIII Binding Autoradiography. Demonstration of the radiolabelled ATIII bound to the endothelial cell. A) TEM of three labelled cells. Nuclei (Nu) and mitocondria (m) visible, as well as the exposed silver grains (arrows). Stained with uranyl acetate only. Bar = 1 micrometer. B) LM of the autoradiographs. B1 is a stained section (toluidine blue) to show nuclei (Nu) of the cells, and the bead (B). B2 is unstained autoradiograph section showing bead (B) surrounded by cells. Both bars = 10 micrometers.

Figure 9. Gold Labelling of ATIII Binding. TEM demonstration of gold labelling. A) Single cell with nucleus (Nu) present, showing common sparse labelling of gold (arrow). Portion of microculture bead present (asterisk). Bar = 1 micrometer. B) Enlargement of bead (B) surface showing labelled cell fragment (arrows). Bar = 1 micrometer.

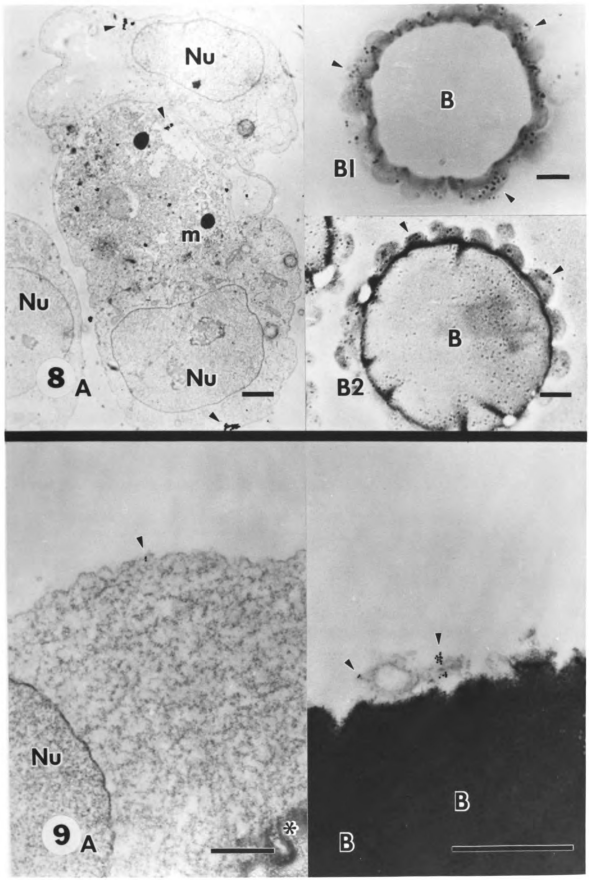

Nu
m
Nu
Nu
8 A
B
B1
B
B2
Nu
9 A
*
B
B

Table 2. ATIII Binding Measured by LM Autoradiography (cont.)

Binding at RT

Treatment	mean counts / cell	variance
Heparin	22.87	9.345
Thrombin	28.17	12.916
Heparan Sulfate	33.28	7.961
ATIII	36.30	14.938

Notes: See Appendix A for statistical analysis.
Mean counts per cell is taken to be equivalent to $10um^2$.
Mean counts based on N=10

Enzyme treatment of endothelial cells with heparinase and heparitinase followed by binding of labelled ATIII (Table 3) showed that endothelial cells enzymatically treated with heparinase showed a marked decrease in their ability to bind labelled ATIII. Cells treated with heparitinase, specific for heparan sulfate, showed no significant difference with cells having no pretreatment.

Table 3. Autoradiography of Enzyme Treatment

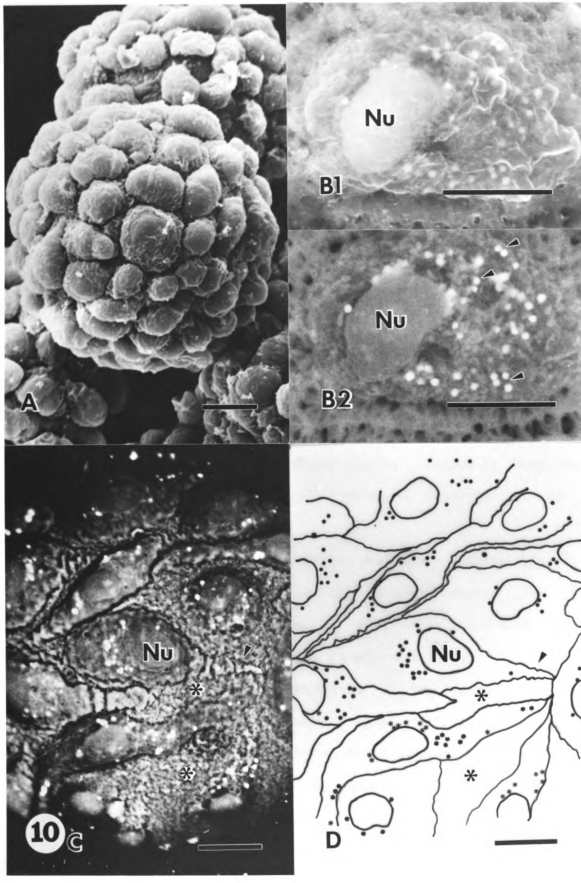
Treatment	mean counts / cell	variance
Contol	29.14	5.95
Heparitinase	28.26	5.42
Heparinase	23.64	2.61

Note: See Appendix A for statisical analysis.
Mean counts per cell is taken to be equivalent to $10um^2$.
Mean counts based on N=10

Pre-embedding gold labelling of the cofactor gave little information at the TEM level. The incidence of the cofactor on the cell surface was infrequent enough that examination produced few, if any, of the gold markers (Figure 9A). Pre-fixation of the cells did prevent internalization of the gold label. This was an improvement over the attempts to reduce internalization with a decrease in

temperature. All label found was either on the surface of the cells or within convolutions on the exposed microcarrier bead surface in close proximity to the cell (Figure 9B). At the SEM level, the gold labelled cofactor was evident and easily observed using backscatter electron detection (Figure 10B). When compared to the bead surface (Figures 10C and 10D), specific binding was demonstrated. Gold labelling was not quantified, and was used only as a confirmation of the ATIII binding ability of the endothelial cells.

Figure 10. SEM of Gold Labelling of ATIII Binding. Visualization of labelling using BEI. A) SEM of untreated microcarrier cell cultures showing one bead confluent with cells. Bar = 10 micrometers. B) Secondary (B1) and backscatter (B2) electron images of labelled cells. Nucleus (Nu) and gold particles (arrows) are evident. Bars = 5 micrometers. C) BEI of sub-confluent microcarrier bead showing cell specific binding. Nucleus (Nu) can be seen on micrograph as well as on line drawing (D). Exposed bead surfces (asterisks) and gold labeling (small stars) can be seen. Bars = 5 micrometers.


A
Nu
B1
Nu
B2
Nu
10
C
Nu
D

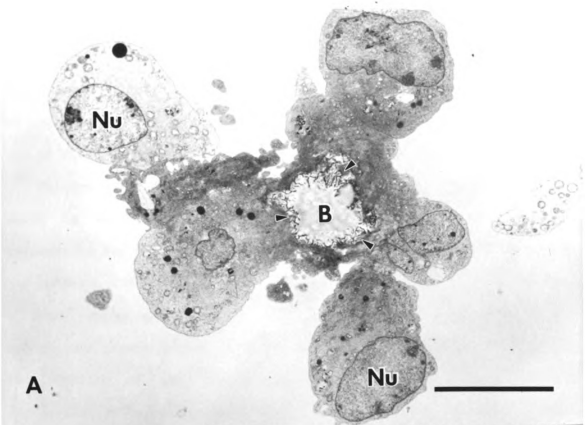
DISCUSSION

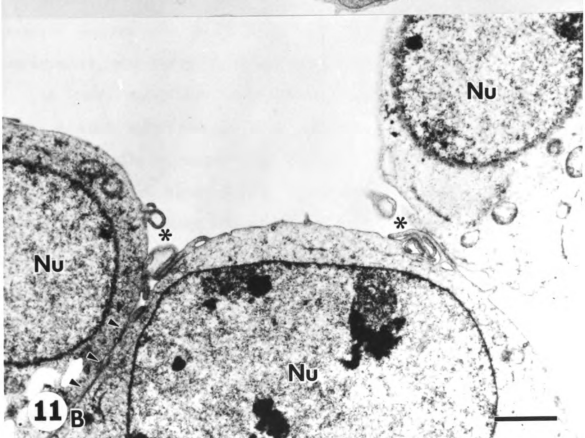
The ultrastructural examination of endothelial cell monolayers posed a difficult, technical problem. Monolayers are difficult to embed for TEM examination, especially if a minimum of cell disruption is desired. For this reason, I established method for the use of endothelial cells grown on sectionable cross-linked dextran beads for EM. These microcarrier beads provided an excellent growth matrix, as well as made correlation between SEM, TEM and LM possible. Another advantage was the ability to pool cell cultures, thus allowing proper population sampling, something that would not have been possible had the cells been grown on tissue culture plates. Figure 11 shows the cells grown on the beads (A), demonstrating a retention of cell morphology (Figure 11B).

To begin the characterization of the stationary cofactor, it was necessary to establish some idea as to its general nature. It had previously been described as able to bind ATIII, and with ATIII accelerate the inactivation of thrombin (Busch and Owen, 1982). In addition to this, its close association to the endothelial cell prompted its description as heparin-like. Taking this into consideration, it was then necessary to determine what possible GAGs, common to the endothelium, were possibly responsible for this cofactor activity.

Hatton, et al. 1978, ran a series of experiments that demonstrated

Figure 11. Microcarrier Cultures - TEM. Micrographs of cell grown on cross-linked dextran beads. A) TEM of cells on bead (B). Four cells can be seen. Nuclei (Nu) are distinct. Bar = 10 micrometers. B) Enlargement of endothelial cells showing intact morphology. Nuclei (Nu) have retained bilayer membranes. Cell-to-cell contact is allowed (arrows), and cell maintain fine cytoplasmic projections (asterisks). Bar = 1 micrometer.


Nu
B
Nu
A


Nu
Nu
Nu
11
B

ATIII-heparin specific binding, to the exclusion of the other GAGs tested. I repeated these experiments, with some modifications, and came to the same conclusions as Hatton's group. The ATIII binding experiments clearly showed that, with the exception of heparin, binding of the inhibitor to the GAGs was negligible. These results allowed the use of ATIII to probe the surface of the endothelial cell.

The next step was to determine if ATIII binding to the cofactor could be inhibited. It has been described that the most likely GAG responsible for the cofactor activity is heparan sulfate (Buonassisi and Colburn, 1982). They report, using ^{35}S labelling, finding protein - bound carbohydrate chains both at the cell surface and in the supernatant growth medium of rabbit endothelial cultures. They assume the identity of the GAG to be heparan sulfate via resistance to degradation by chondroitinases AC and ABC. It was for this reason heparan sulfate was included in the bulk of my characterization experiments, even though it showed reduced affinity for ATIII.

In the inhibition experiments, radiolabelled ATIII was pre-incubated with various test solutions, then incubated with the endothelial cells to assess the degree of ATIII blockage. Pilot experiments used cold ATIII pre-incubations, followed by cell incubation, then probing with radiolabelled antibodies to ATIII. This raised the question whether the decrease in binding observed, was due to inhibition of ATIII binding or if the antibody was hindered in its binding to ATIII. By eliminating the secondary labelling, a direct measure of binding could be determined.

Another problem arose due to the endothelial cell's predilection to endocytosis. This prompted the use of $4^{o}C$ incubations to try to hinder

the endocytosis of label into the cell. Even at this temperature, the cells maintained a level of endocytosis that essentually resulted in a surface devoid of label. Therefore, autoradiographic labelling was quantitated in the interior of the cells.

The results of the inhibition experiments showed that heparin, thrombin pre-incubation with ATIII could inhibit the binding of ATIII to the endothelial cell surface. Also, incubation of the endothelial cells with unlabbeled ATIII could also inhibit labbelled ATIII binding via cofactor saturatation. This was substantiated with incubations at both $4^{o}C$ and $27^{o}C$ (room temperature). Heparan sulfate showed no significant inhibition of ATIII binding. This tends to direct thought to a more heparin-like molecule as opposed to that of heparan sulfate.

To further characterize the cofactor and perhaps distinguish between its heparin or heparan sulfate nature, enzymatic digestion of the endothelial cell surface was performed. The enzymes used were specific for heparin and heparan sulfate cleavage. The enzymes, isolated from Flavobacterium heparinum, digest the molecules at different glucosyl linkages (Miles Scientific, Product Profile). This is based on the number of iduronic acids the molecules possess.

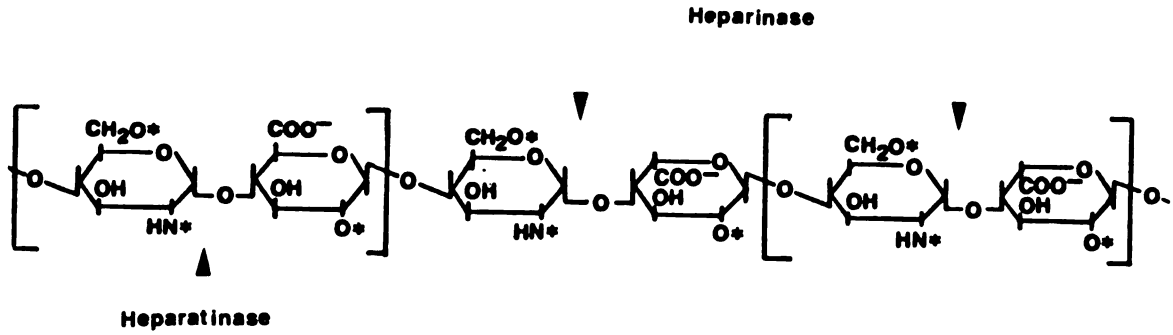


Figure. 12. Enzymatic Cleavage Points of Heparinase and Heparatinase. Arrows indicate sites of cleavage.

Heparin is rich in the iduronic acid form, while heparan sulfate is rich in the glucuronic acid form. Therefore, the enzymes are more specific for one or the other.

These experiments resulted in a marked decrease in the endothelial cell's ability to bind ATIII after digestion with the heparinase and not heparitinase. This points to a GAG rich in iduronic acid components.

The calculation of the number of binding sites per endothelial cell was based on the number of silver grains per one micrometer section in control tissue. This reflects approximately 300 to 350 binding sites, as determined by maximum ATIII binding on a cell 10 um^3. This calculation is much lower than that of Marcum, et al., 1986. Their estimation was of 58000 protease inhibitor binding sites per cell for a heparan sulfate-like cofactor on the surface of endothelial cells. This could either be due to the measuring of additional binding sites unmasked by the cofactor isolation, or that the age of the cells in question has a bearing on the material Marcum's group characterized. They used solubilization of the cell surface followed by affinity fractionization to identify a cofactor showing anticoagulant activity, specifically, the acceleration of ATIII inactivation of thrombin. This study employed cloned endothelial cells, that had under gone "less than 70 population doublings" (approximately 2 months old). These cells are essentially different than the endothelial cells used in my studies.

Goldsmith, et al., 1984 examined the change in endothelial cell properties and kinetics over time while being grown in tissue culture. They found that there was a gradual decline in prostacyclin release as soon as the first population doubling. Angiotensin converting factor

release also dropped off, both signs of a decrease in cellular response. When discussing cultured endothelial cell kinetics, it was found that the cells tended to arrest their growth upon subculturing, to the point that highly subcultured cells could be used for models of in vitro senescence. To parallel Marcum's cloned cell cultures (approximately 2 months old) to the cultures used in my experiments (approximately 2 weeks old) would be innappropriate unless only general comparisons were made.

Of interest was Marcum's finding that a high affinity (for ATIII) surface fraction contain the GlcA-AMN-3-O-SO_3 which represents the ATIII-binding region of heparin, which constitutes a structual marker for heparin. Also, the complexing of ATIII was completely eliminated by pre-treatment of the cells with a purified Flavobacterium heparinase (digests both heparin and heparan sulfate), substantiating the enzyme treatments performed in my experiments. These two observations again suggest a possible sub-population of heparin on the surface of the endothelial cell.

Though the cofactor has been shown to be similar to heparin in kinetic studies, one characteristic it does not share with the GAG is its ability to bind ATIII without subsequent release. Heparin only is in contact with ATIII long enough to conformationally change ATIII into a more accessible molecule, this binding does not endure. This is not the case of the surface cofactor. It has been shown to bind ATIII even after repeated washings.

These morphological findings, along with kinetic studies from other laboratories, may help in the understanding of the role the endothelial cell plays in the regulation and prevention of unwanted thrombosis.

SUMMARY

1) Only light pre-fixation of the cells could prevent endocytosis, as opposed to low temperature incubations
2) Heparin or thrombin incubated with ATIII could inhibit the ATIII's binding to the endothelial cell surface
3) ATIII incubated with heparan sulfate showed no decrease in its ability to bind to the endothelial cell surface
4) Digestion of the endothelial cell surface with heparinase destroyed the ability of the cell to bind ATIII
5) Digestion of the cells with heparitinase showed no significant change in ATIII binding to the cell
6) ATIII binding sites per cell is approximately 300 to 350 at maximun binding (based on one cell = 10 micrometers^3)
7) Unlike isolated heparin, this ATIII cofactor binds ATIII and can maintain union even after repeated washings of the cell surface

APPENDICES

APPENDIX A

Endothelial Cell Isolation and Culture

This section provides detailed description of the isolation procedure of the bovine aortic endothelial cells and their subsequent tissue culturing. Appendix B provides the formulations of the buffers used in these protocols.

A.1. Endothelial Cell Isolation

Bovine aorta were obtained at a slaughter house, and immersed in ice-cold, sterile DME for transport. The cell isolation was carried out in a laminar flow hood on a coated, absorbant mat. The aorta was rinsed out with sterile PBS throughout the entire procedure to prevent drying of the endothelium. The external fat and facia was then removed, being careful to leave the intercostal arteries intact (Figure 13A). These arteries were then tied off with suture close to the point of branching (Figures 13B and 13C). The small end of the aorta was then clamped off with a hemostat to produce a bag-like structure. To facilitate hanging the aorta during incubation, either a portion of the brachiocephalic artery can be left attached to the aorta (Figure 14A), or the aorta can be cut near the upper, larger opening providing a means of attaching it to a ring stand. In this instance, a plastic-coated clamp was ethanol sterilized, and the single prong was used to hang the aorta at an appropriate height that allowed free suspension.

The suspended aorta was then filled with warm DME to check for leaks. The color of the media provided a means to locate any leaks from sutures or cuts in the tissue. Once the aorta is leak-free, the

Figure 13. Isolated Aorta Preparation. A) Excess fat and facia is removed from the vessel. B) Tying off of the intercostal arteries close to the main vessel. C) Trimming of the tied off vellel and the suture cord.

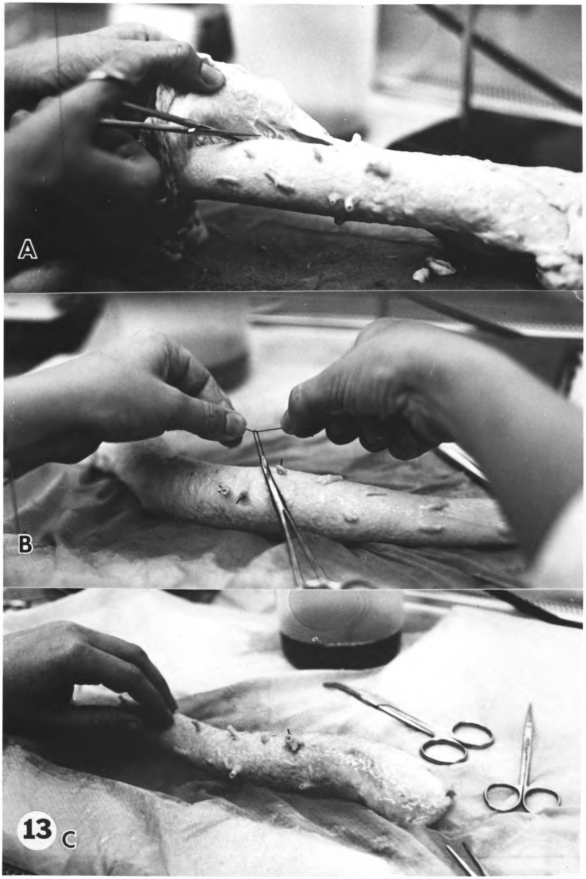
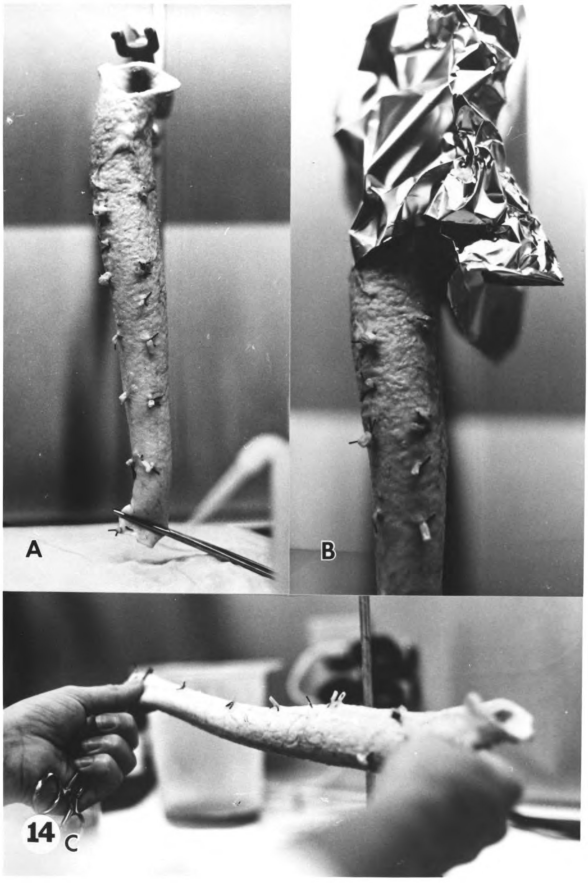

A
B
13 C

Figure 14. Digestion and Removal of Cells. A) Aorta is hung from a ring stand, producing a water-tight, blind tude. B) Warm collagenase mixture is added to the vessel, is covered with foil, and incubated. C) Aorta is drained, refilled with media and shaken to dislodge loosened cells.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B
14 C

Figure 15. Collection of Cells. A) After shaking, vessel is cut off at narrow (distal) end. B) and media is decanted into sterile centrifuge tubes.


A
15 B

DME was replaced with a collagenase mixture of 36U/ml (CLS, Cooper Biomedical, Lot 46N6783) in 37°C. DME. The aorta was covered with a sterile piece of foil, and was incubated for 20 minutes at 37°C. (Figure 14B). The collagenase mixture was then decanted from the aorta and discarded. The aorta was filled three-quarters full with DME culture media, the large end clamped shut, and was vigorously shaken for about 3 minutes (Figure 14C). The bottom (small end) was cleaned with ethanol, and cut near the clamp to allow decanting of the media into sterile centifugation tubes. Before decantation, the open end was wrapped with a disposable tissue to prevent contamination of the cell suspension from liquid on the surface of the vessel (Figures 15A and 15B). Media was added 4 more times with progressively rigorous agitation and rubbing to dislodge the attached cells. The tubes of media were spun in a swingout rotor at 1800 rpm for 5 minutes.

A.2. Endothelial Cell Tissue Culture

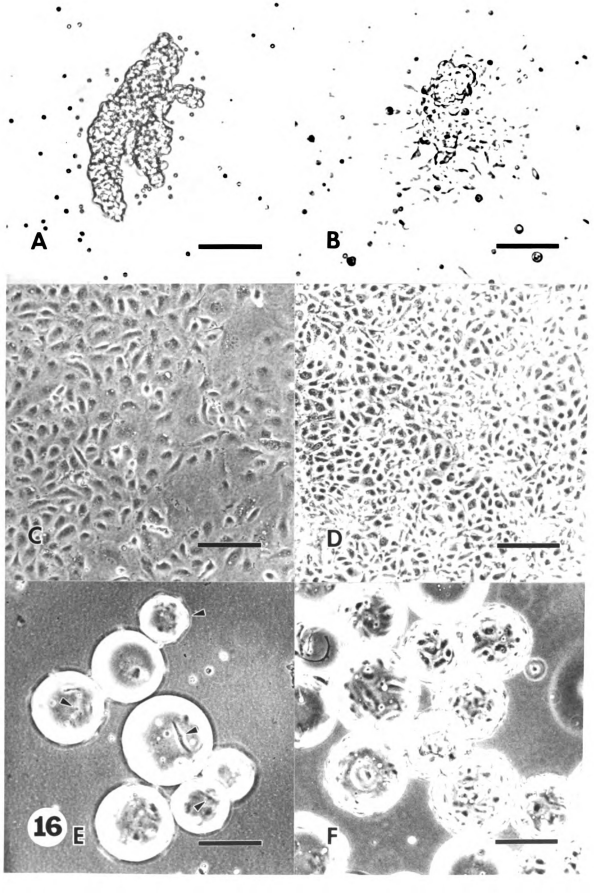
The resulting pellet was resuspended in fresh culture media and was used to seed 100mm tissue culture plates, that were placed in a humidified incubator at 37°C. and 10% CO_2. The media was changed at 6 hours and again at 24 hours to remove red blood cells and other debris. After this point, the media was changed every other day until the cells reached confluency (usually 8 to 10 days).

Detachment from the primary cell plates was facilitated by a breif wash with sterile, 37°C. PBS, followed by 0.02% EDTA, 0.5% BSA, PHG solution which was allowed to incubate on the plates 5 minutes at 37°C.

This incubation should not be exceeded due to possible cell damage by the EDTA. The loosened cells were gently washed off, pelleted as before and split into 100mm bacteriological petri plates in a ratio of 1:3.

To each of the plates microculture beads were added, gently swirled, placed in the incubator, and not moved again for at least 18 hours. The bead cultures were handled in the same manner as the primary cultures except extra care was required when changing media as not to draw off the beads with the media (Figure 16).

Figure 16. Endothelial Cell Tissue Culture. A) Endothelial cell sheet isolated from the aorta. 0 hours. B) Cell sheet after attachment to culture dish. Notice spreading of cells. 24 hours. C) Sub-confluent culture on tisue culture dish. Some cells still spread, others beginning to round up. 5 days. D) Typical "cobble-stone" appearance of confluent endothelial cultures. 8 days. E) Freashly seeded microcarrier beads. Cells just begining to attach and spread (arrows). 2 days. F) Confluent bead cultures. 8 days. All bars = 50 micrometers.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D
16
E
F

APPENDIX B

Formulations

This section provides the formulations of the buffers used in the experimental procedures.

DME Culture Media:
DME, with 4gm/liter glucose, pH 7.5
10% FBS
20mM HEPES, pH 7.5
2mM Glutamine
Penicillin 100000U/liter
Streptomycin 100mg/liter

PB (Phosphate buffer):
0.1 M monobasic sodium phosphate
0.1 M dibasic sodium phosphate
to give a pH of 7.5

PBS (Phosphate buffered saline):
0.8% Sodium chloride
0.1M Phosphate buffer, pH 7.5

PHG (Phosphate-HEPES-Glucose buffer):
0.1 M PBS, pH 7.5
15mM HEPES, pH 7.5
11mM Glucose

Splitting Buffer (Endothelial Cell Culture):
0.02% EDTA
0.5% BSA
in PHG buffer, approximately 5mls per 100mm plate

APPENDIX C

Statistical Analysis of Data

The purpose of this section is to provide the statistics used in the data supplied in the results section. All of the measurements as well as the formulas are included.

C.1. ATIII Binding Study - Autoradiography

Table 4. Count and Weight Conversions of ATII Binding Study

Treatments	Counts	Weight (gm)	Counts / $10um^2$	Mean	Treatment Total	Variance
Heparin, 4°C	181	.5810	25.71			
	131	.5502	19.65			
	152	.6278	19.98			
	114	.5005	18.79			
	109	.3455	26.04			
	61	.1867	26.97			
	140	.4676	24.71			
	165	.5808	23.45			
	92	.2953	25.71	23.45	211.01	8.789
Heparan Sulfate, RT	174	.3602	33.87			
	123	.2706	37.52			
	74	.1650	37.02			
	49	.1485	30.23			
	149	.3844	31.99			
	222	.4651	36.39			
	108	.3186	30.98			
	123	.3370	30.12			
	176	.5109	31.43	33.28	299.55	7.961
Heparin, RT	187	.6227	24.79			
	170	.6426	21.84			
	212	.6614	26.46			
	84	.3612	19.19			
	364	1.0829	27.74			
	205	.7380	22.93			
	63	.2785	18.67			
	175	.6755	21.38	22.87	183.00	9.345
Thrombin, RT	136	.5247	25.39			
	139	.3531	32.49			
	141	.5288	24.01			
	135	.4253	26.19			
	142	.3032	30.66			
	120	.2832	34.97			
	141	.3793	30.68			
	152	.5092	24.64			
	131	.4703	24.99			
	119	.3546	27.69	28.17	281.71	12.916

Table 4. (cont.)

Treatments	Counts	Weight (gm)	Counts / $10um^2$	Mean	Treatment Total	Variance
Thrombin, 4°C	93	.3581	21.35			
	87	.2828	25.39			
	27	.0934	23.86			
	60	.2298	21.55			
	123	.2939	34.54			
	56	.1938	23.85			
	74	.2834	21.55			
	82	.3018	22.43			
	46	.1509	25.16	24.41	219.68	14.933
Heparan Sulfate, 4°C	71	.1462	38.08			
	182	.4295	34.99			
	161	.4395	31.24			
	96	.2457	32.23			
	107	.2579	34.24			
	113	.2294	37.66			
	140	.3654	33.62			
	118	.3351	31.06			
	141	.2919	39.87			
	102	.2611	32.24	34.52	345.23	8.544
cold ATIII, 4°C	89	.4920	14.93			
	136	.5440	20.63			
	212	1.0347	16.91			
	121	.5941	16.81			
	161	.8726	15.15			
	139	.7075	16.22			
	149	.7450	16.51			
	96	.4329	18.30			
	64	.2874	18.38			
	100	.5370	15.38	16.92	169.22	2.793
cold ATIII, RT	98	.3979	21.33			
	56	.2307	20.03			
	58	.2337	20.48			
	101	.4555	18.30			
	83	.3419	20.04			
	77	.3211	19.79			
	96	.4070	19.47			
	90	.3888	19.11			
	124	.5332	19.19			
	129	.5960	16.86	19.46	194.56	1.361
ATIII*, RT	242	.5820	34.32			
	224	.5031	34.82			
	275	.7412	30.62			
	268	.7343	30.12			
	163	.3660	36.76			
	78	.1642	39.21			
	331	.6670	40.96			
	184	.3735	40.66			
	211	.4442	39.21	36.30	326.68	14.938

Table 4. (cont)

Treatments	Counts	Weight (gm)	Counts / 10um^2	Mean	Treatment Total	Variance
ATIII*, 4^{o}C	277	.3933	38.04			
	170	.4097	34.24			
	121	.1646	30.67			
	184	.3680	41.27			
	195	.4407	36.52			
	166	.2158	43.49			
	124	.1265	40.45			
	208	.2517	34.10			
	104	.1113	38.56			
	41	.0968	34.96	37.23	372.31	13.573

C.1.1. F-max test for heterogeneity of variances between treatments:

F= largest varience / smallest variance

= 14.938 / 1.361

= 10.977 = calculated F-max

For F-max tabled: a= total number of groups = 10
n= smallest number of observations = 8
p= probability level = 0.05

F-max(tabled)= 11.7

No significant heterogeneity of variance within all treatments

C.1.2. ATIII Binding at 4^{o}C.

Analysis of Variance:

1. Correction term $C=(\text{sum } X)^2$ / total observations

$=(1317.45)^2$ / 48
=1735674.5 / 48
=36159.885

2. Sums of Squares (Total) $SS_{To}=\text{sum}(X^2) - C$

=39414.636 - 36159.885
=3254.75

3. Sums of Squares (Treatment) $SS_{Tr}=\text{sum}(T^2 / r) - C$

=38952.781 - 36159.885
=2792.896

T= totals of each group
r= number of replicates

ANOVA Table:

Source	dF	SS	MS	F
treatment	4	2792.9	698.2	65.0
error	43	461.9	10.7	
total	47	3254.8	----	

4. F= MS(treatment) / MS(error)
   = 698.2 / 10.7
   = 65.0 = calculated F value

5. F(tabled)= 2.59 at 0.05 probability
   F(calc) > F(tabled) therefore reject H_o= all treatments are the same.

Multiple Comparison of Data:
Dunnett's test for comparison of all treatments to a control

Treatment	Mean
control	16.92
heparin	23.45
thrombin	24.41
hep sulf	34.52
ATIII	37.23

1. Dunnett's Critical Value
   Dunnett's t value x square root of (2MS(error) / r)

   =2.22 x 1.46
   =3.25

2. Comparison of Means

   Control:Heparin 6.53
   Control:Thrombin 7.49
   Control:Heparan Sulfate 17.60
   Control:ATIII 20.31

   -all show significant difference from control (difference of treatment means > Dunnitt's critical value)

Bon Ferroni's test for the comparison of treatments

1. Bon Ferroni's Critical Value
   Bon Ferroni's t value x square root of (2MS(error) / r)

   =2.30 x 1.46
   =3.36

2. Comparison of Means

ATIII:Heparan Sulfate	2.71	no significant difference
ATIII:Heparin	13.78	significant difference

C.1.3. ATIII Binding at RT

Analysis of Variance:

1. Correction term $C=(\text{sum } X)^2 / rt$

$=(1285.50)^2 / 46$
$=1652510.3 / 46$
$=35924.136$

2. Sums of Squares (Total) $SS_{To}=\text{sum}(X^2) - C$

$=38160.49 - 35924.136$
$=2236.35$

3. Sums of Squares (Treatment) $SS_{Tr}=\text{sum}(T^2 / r) - C$

$=37735.32 - 35924.136$
$=1811.18$

ANOVA Table:

Source	df	SS	MS	F
treatment	4	1811.2	452.8	43.66
error	41	425.2	10.3	
total	45	2236.4	----	

4. F= MS(treatment) / MS(error)
= 452.8 / 10.4
= 43.66 = calculated F value

5. F(tabled)= 2.59 at 0.05 probability
F(calc) > F(tabled) therefore reject H_o = all the treatments are the same

Multiple Comparison of Data:
Dunnett's test for comparison of all treatments to a control

Treatment	Mean
control	16.86
heparin	22.87
thrombin	28.17
hep sulf	33.28
ATIII	36.30

1. Dunnett's Critical Value
   Dunnett's t value x square root of (2MS(error) / r)

   =2.22 x 1.46
   =3.25

2. Comparison of Means

   Control:Heparin 6.01
   Control:Thrombin 11.31
   Control:Heparan Sulfate 16.42
   Control:ATIII 19.44

   -all show significant difference from control (difference of treatment means > Dunnett's critical value)

Bon Ferroni's test for comparison of treatments

1. Bon Ferroni's Critical Value
   Bon Ferroni's t value x square root of (2MS(error) / r)

   =2.30 x 1.46
   =3.36

2. Comparison of Means

   ATIII:Heparan Sulfate 3.02 no significant difference
   ATIII:Heparin 13.43 significant difference

C.2. Enzyme Treatment Study - Autoradiography

Table 5. Count and Weight Conversions of Enzyme Study

Treatments	Counts	Weight (gm)	$\frac{\text{Counts}}{10\text{um}^2}$	Mean	Treatment Total	Variance
No Enzyme	33	.1069	25.48			
	89	.2821	26.04			
	163	.4238	31.75			
	141	.3835	30.35			
	137	.3836	29.48			
	320	.7872	33.53			
	59	.1758	27.70			
	213	.5943	29.58			
	157	.4569	28.36	29.14	262.27	5.95
Heparitinase	316	.8208	31.77			
	245	.7697	26.27			
	157	.4286	30.23			
	228	.6475	29.06			
	143	.4433	26.62			
	155	.5058	25.41			
	158	.4123	31.63			

Table 5. (cont.)

Treatments	Counts	Weight (gm)	Counts / 10um^2	Mean	Treatment Total	Variance
Heparitinase	125	.3775	27.32			
	179	.5674	26.04	28.26	254.35	5.42
Heparinase	104	.3749	22.89			
	216	.7754	22.99			
	193	.6678	23.85			
	213	.7008	26.09			
	184	.6642	22.86			
	116	.3723	25.72			
	113	.3740	24.94			
	107	.3852	22.93			
	101	.3858	20.61	23.64	212.88	2.61

C.2.1. F-max test for heterogeneity of variances between treatments.

F= largaest variance / smallest variance

= 5.95 / 2.61

= 2.28 = calculated F-max

For F-max tabled: a= total number of groups = 3
n= smallest number of observations = 9
p= probability level = 0.05

F-max(tabled)= 5.34

No significant heterogeneity of variance

C.2.2. ATIII Binding with Enzyme Treatment.

Analysis of Variance:

1. Correction term $C=(\text{sum } X)^2 / \text{total observations}$

$=(729.5)^2 / 27$
$=532170.25 / 27$
$=19710.009$

2. Sums of Squares (Total) $SS_{To}=\text{sum}(X^2) - C$

$=19992.041 - 19710.009$
$=282.03$

3. Sums of Squares (Treatment) $SS_{Tr}=\text{sum}(T^2 / r) - C$

$=19866.374 - 19710.009$
$=156.37$

T= totals for each group
r= number of replicates

ANOVA Table:

Source	dF	SS	MS	F
treatment	2	156.4	78.2	15.04
error	24	125.6	5.2	
total	26	282.0	----	

4. F= MS(treatment) / MS(error)
   = 78.2 / 5.2
   = 15.04 = calculated F value

5. F(tabled)= 3.40 at 0.05 probability
   F(calc) > F(tabled) therfore reject H_o = all treatments are the same.

Multiple Comparison of Data:
Dunnett's test for comparison of all treatments to a control

Treatment	Mean
control	29.14
heparinase	23.64
heparitinase	28.26

1. Dunnett's Critical Value
   Dunnett's t value x square root of (2MS(error) / r)

   =2.01 x 1.08
   =2.16

2. Comparison of Means

Control:Heparinase	5.50	significant difference
Control:Heparitinase	0.88	no significant difference

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