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Raghid A. Kadi

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By

RAGHID A KADI

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ABSTRACT

SECRETION OF TISSUE-TYPE PLASMINOGEN ACTIVATOR AND PLASMINOGEN ACTIVATOR INHIBITOR BY CULTURED HUMAN ENDOTHELIAL CELLS: *THE EFFECT OF DIFFERENT pHs.*

By

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Increased fibrinolytic activity has been measured following exercise, venous occlusion, acidosis, and liver transplantation. One variable common to these conditions is a decrease in blood pH. To investigate the hypothesis that pH stimulates tissue plasminogen activator (t-PA), and plasminogen activator inhibitor 1 (PAI-1) release, confluent 5th passage human umbilical vein endothelial cells were exposed to pHs 6.0 to 8.5 M199 serum free culture media (CM). T-PA & PAI-1 (activity and antigen), and factor VIII related antigen (F VIII RAg), levels were measured after 0.5 4, 12, 17, and 24 hrs. The rate of increase was greatest between 4 & 17 hrs, with highest levels at 17 hr (t-PA activity 0 IU/ml, t-PA Ag 6.5$\pm$1.5 IU/ml, PAI-1 activity 1.5$\pm$0.4 IU/ml, PAI-1 Ag 26.5$\pm$4 IU/ml). The value at 24 hrs was not different from 17 hr. The measured increase in t-PA, PAI-1, & F VIII RAg with time was not affected by pHs 6.0 to 8.0. At pH 8.5 the level of all proteins was reduced. Cell viability at pH 8.5 was only 25% of normal and could account for the decrease. This in vitro study does not support the hypothesis that pH (6.0-8.0) affects the release of t-PA & PAI-1.

Dedicated to most loved
parents.

Your love and encouragement
have made this accomplishment.
And to
Ranya, my loving wife, for her
continuing support and help

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TABLE OF CONTENTS

LEST OF TABLES

TABLE OF FIGURES

Introduction

There are two major systems in the blood controlling the fluidity of this protein rich fluid. *The coagulation system*, which is responsible for fibrin formation, and the *fibrinolytic system* which brakes down fibrin and/or fibrinogen. Both systems are continuously working, at a very low rate, in a balance designed to keep blood circulating through an unobstructed vascular bed. The idea of a continuously working fibrinolytic system is supported by the finding of plasminogen activator activity in normal blood (1-2). The potential use of this fibrinolytic agent in the treatment of thrombolytic complications, has generated a great deal of interest in research in this area. The increased therapeutic application of these agents is the result of a better understanding of the biochemical mechanisms behind each step of the fibrinolytic system.

I. The Fibrinolytic System

Most of the components of the fibrinolytic enzyme system were identified between 1930 and 1950 (3). The primary fibrinolytic enzyme *plasmin*, is a serine protease capable of digesting fibrinogen and fibrin (4). Like most serine proteases, plasmin is not normally found in the

circulation. Plasminogen is the circulating zymogen from which plasmin is derived upon cleavage by highly specific serine proteases termed *Plasminogen Activators* (5). Although many enzymes can activate plasminogen to plasmin, only two, *urokinase* (6) and *tissue plasminogen activator* (7), are considered to be physiologically important.

A. Urokinase

Urokinase was first described by *Macfarlane and Pilling* as a fibrinolytic activity found in the urine (8). *Williams* then demonstrated that this activity was due to the presence of a plasminogen activator in the urine, that is now known as urokinase (9). Urokinase is a trypsin-like protease that can occur in two molecular forms , S1 (Mr 31,600) and S2 (Mr 54,000). The smaller form is probably a proteolytic degradation product of the larger molecular weight species (3). The half life of this enzyme in the blood following intravenous injection is approximately 10 min (10). Urokinase has been identified in human plasma (11,12,13), cultures of bovine endothelial cells (14), macrophages (15), kidney cell tissue culture (16), and other tissues. Human endothelial cells were once thought to release both tissue plasminogen activator (t-PA), and urokinase. Recent studies have shown that these cells do not release urokinase (17).

Urokinase has been used clinically as a fibrinolytic agent. Since the use of this enzyme causes a systemic activation of the fibrinolytic system, many different plasminogen activators are under development for clinical testing (5).

B. Tissue Plasminogen Activator

In 1947 Astrup and Permin reported an agent in animal tissue that could activate plasminogen (18). This factor was called *fibrokinase*. It is now known as *tissue plasminogen activator (t-PA)*. Highly purified enzyme has been obtained from pig heart (19), hog ovaries (20), human uterus (21), and melanoma cells (22,23).

Tissue plasminogen activator is a serine protease with a molecular weight of approximately 60,000 and is composed of two disulfide-linked polypeptide chains (3). The most important property of this protein is its very high affinity for fibrin (1,24). This characteristic is very important in the clinical use of the enzyme because it provides higher fibrinolytic activity at the site of fibrin formation with lower systemic fibrinolytic activity in comparison to urokinase (5). Immunocytochemical studies have identified t-PA in endothelial cells of both veins and arteries (25). However it could not be demonstrated in the endothelium of capillaries (26). Synthesis and secretion of t-PA by

cultured bovine and human endothelial cells has also been demonstrated (26,27,28). The vascular endothelium appears to be the principal source of t-PA in the plasma. Many studies have been done on the secretion of t-PA by different types of cells. Human endothelial cells derived from the umbilical vein are the most popular source of human cells because they are easy to obtain. Comparative studies suggested that t-PA secretion from these cells is several fold lower than from cells isolated from vena cava, large arteries, or microvasculature (29). However, a recent study by *Bartha et al.* showed that after 6-8 passages, the amount of t-PA secreted by umbilical vein human endothelial cells is comparable to the amount secreted by aortic endothelial cells (30).

The plasma t-PA level has been reported to increase following exercise, venous occlusion, stress, and many other clinical cases (1). These observations along with the important clinical use of the enzyme, made t-PA an area of concentration for many researchers during the last two decades. Understanding the mechanism of t-PA release and the factors that affect this release are important issues that require additional investigation.

II. Inhibitors of The Fibrinolytic System

Inhibitors are proteins that play an important role in controlling the fibrinolytic system. These proteins were first described in 1947 as substances found in the blood that can inhibit the fibrinolytic process (5). These proteins have been divided into two major groups: 1. Proteins that inhibit plasmin, called *antiplasmins*, and 2. proteins that inhibit plasminogen activators, called *antiactivators*.

A. Antiplasmins

Antiplasmins are protease inhibitors that inhibit plasmin. The rate of inhibition varies between the different inhibitors. This reaction depends on the presence and level of both plasmin and plasminogen, and on the type of plasminogen activator that has been used to activate plasminogen (31,32). *Alpha-2-antiplasmin* (alpha 2AP) is the major inhibitor of plasmin. The rate of interaction between plasmin and alpha 2AP is one of the fastest known protein-protein interaction (Rate constant = $30uM^{-1}sec^{-1}$)(5). Beside the instantaneous inhibition of plasminogen, alpha 2AP also interferes with the adsorption of plasminogen to fibrin and is crosslinked to fibrin by factor XIII (31). These three

functional properties of alpha 2AP make it the most specific and efficient inhibitor of plasmin. *Alpha 2-macroglobulin* is another inhibitor that binds plasmin, but does so at a slower rate and with less affinity (5).

B. Antiactivators

The inhibitors of the plasminogen activators are more important in the regulation of the fibrinolytic system, than the plasmin inhibitors. Since the most important plasminogen activator is t-PA, inhibitors of t-PA are the most physiologically important fibrinolytic inhibitors (33). These inhibitors have been divided into two groups: (A) The secondary inhibitors and (B) the primary inhibitors.

1. The Secondary Inhibitors

The proteolytic activity of t-PA like other serine proteases, is subject to inhibition by plasma serine protease inhibitors (serpins). These serpins serve as a suicide substrates (34). Part of these substances resemble the binding site of the original substrate; they bind to the active site of the enzyme blocking its functional activity. Tissue plasminogen activator is subject to this kind of reaction with *C1-esterase inhibitor, alpha 1-antitrypsin, alpha 2-antiplasmin, alpha 2-macroglubin and protease nexin.*

All of these serpins form a 1:1 complex with t-PA, with first-order rate constants between 10^0-10^5 M^{-1} sec^{-1} (35,36,37,38,39).

2. The Primary Inhibitors of t-PA

Because of its rapid ($K=10^8$ M^{-1} sec^{-1}) and specific inhibition of plasminogen activators, plasminogen activator inhibitor 1 (PAI-1) is the most important inhibitor or the primary inhibitor of t-PA (33). Another inhibitor in this group is *plasminogen activator inhibitor* 2 (PAI-2). This inhibitor, which is a specific plasminogen activator inhibitor found in the plasma during pregnancy, binds t-PA at a slower rate than PAI-1, and is considered to be of secondary importance(40).

PAI-1 was described by *Wiman et al.* as an inhibitory factor found in the serum (41). It has since been described by other investigators (39,42). PAI-1 is synthesized by megacaryocytes and is found in the alpha granules of platelets (43,44,45), vascular endothelial cells (46,47), cultured hepatocytes (48), and cultured hepatoma cell line (49). It has also been found in other cell lines such as smooth muscle cells (50). Most of these PAI-1 are immunologically related(51,52,53). PAI-1 is a protein (Mr 50,000) (54,55) that forms a 1:1 sodium dodocyl sulfate (NaDodSO4)-resistant complex of Mr 120,000 with t-PA (52).

It has been suggested that fibrin might play a role in the dissociation of this complex, and that this characteristic leads to elevated fibrinolytic activity at the site of the thrombus (24).

PAI-1 in platelets is stored in alpha granules and released during aggregation (56), so the concentration of the protein at the sight of the thrombus is relatively high. In vascular endothelial cells the situation is different in that it appears that the secretion of the protein reflects an immediate synthesis, not a release from cytoplasmic stores (33). This suggestion is supported by the finding of a several fold increase in the level of PAI-1 mRNA in TNF (*Tumor necrosis factor*) and LPS (*Lipopolysaccharide*) stimulated endothelial cells (57,58).

PAI-1 can be found in four different forms: free, latent, complexed with t-PA, and bound to extracellular matrix. These four forms differ in their capacity to inhibit t-PA (33). These four forms are:

a. Free active PAI-1: Less than 10% of PAI-1 in the blood is found in the free active form that can bind t-PA immediately (58).

b. Latent PAI-1 : Latent PAI-1 represents 90% of the total PAI-1 in the blood. This form can be converted

to the active form in vitro by protein denaturants (59,60). It can also be activated to the active form through binding to phospholipid membranes (61). This might be a very important mechanism for the controlling of PAI-1 activity in vivo.

c. PAI-1 complexed to t-PA : Because of the very high affinity of PAI-1 to t-PA, all free t-PA in the blood will be bound to free PAI-1 (33). This concept is very important in the therapeutical use of t-PA. As there will be no free active t-PA in the circulation unless all free PAI-1 is saturated with t-PA. Therefore the t-PA dose should always be calculated on this basis (33).

d. PAI-1 bound to extracellular matrix (ECM) or cell surface : PAI-1 is specifically bound to ECM in an active form and can be released after exposure to t-PA (50,61). This binding serves two functions :
(1) Protecting extracellular matrix from plasminogen activator induced degradation, and (2) serving as an extracellular pool of active PAI-1 (33). Also it has been suggested that almost all of the active PAI-1 is found in a storage pool on the surface of the endothelial cells, and that the reaction between t-PA and PAI-1 occurs mainly at that site (62,63).

III. The Regulation and Control of Fibrinolysis :

The regulation and control of fibrinolysis appears to occur at several levels :

A. Fibrin associated activation of plasminogen.

B. Plasmin inhibition by alpha 2-antiplasmin.

C. The regulation of t-PA synthesis and release.

D. PAI-1 release and activation.

The critical and accurate fibrinolytic activity level in the blood is due to the effects of all of these factors together. Each of these factors plays a different role in a given situations.

A. Fibrin associated activation of plasminogen

Several hypothesis have been proposed to explain the activation of plasmin on the surface of the clot. One of these hypothesis is that the plasminogen activators are found in the circulation bound to their inhibitors. This complex has a high affinity for fibrin; it accumulates on the fibrin surface along with plasminogen, and causes the activation of plasminogen to plasmin and the digestion of fibrin. Following fibrin digestion plasmin and the activator will bind to its specific inhibitor (24,64).

Another hypothesis suggested by *Sherry*, states that plasminogen is adsorbed to the polymerizing fibrin and converted to active enzyme by activators that diffuse into the thrombus (65). Plasmin would then exert its action in an environment free of inhibitors. *Ambrus and Markus* proposed that plasmin-plasmin inhibitor complexes are always in the circulation, and that plasmin will leave the inhibitor and bind to fibrin because it has a greater affinity for fibrin than for the inhibitor (66). *Allington and Sharp* suggested that the activators bind to fibrin and convert plasminogen in the thrombus to plasmin (67). This last hypothesis supports the currently accepted model of activation. This model is based on the high affinity of t-PA for fibrin and the fact that t-PA becomes more active in the presence of fibrin. The activator binds along with plasminogen to the surface of the fibrin clot. Plasminogen activation then occurs on the fibrin surface. When the fibrin concentration goes down, alpha 2-antiplasmin inhibits the free active plasmin molecules in the area (68).

B. Plasmin inhibition by 2-antiplasmin :

Alpha 2-antiplasmin not only forms a complex with plasmin, but also interacts weekly with the proenzyme plasminogen(3). About 30% of the alpha 2-antiplasmin in the circulation is complexed with plasminogen. The concentration of free alpha 2-antiplasmin in the plasma is

much higher than the concentration of free plasmin, which is usually close to zero. Since the affinity of plasmin for alpha 2-antiplasmin is very high, any new plasmin formed in the circulation will be inhibited. But when the amount of plasmin formed exceeds the amount of alpha 2-antiplasmin, free plasmin molecules will exist and local fibrinolytic activity will appear. Alpha 2 macroglobulin will inhibit plasminogen, but the rate of the reaction is too slow to prevent local fibrinolysis (69).

Fibrinogen can inhibit the reaction between alpha 2-antiplasmin and plasmin (3). When the level of fibrinogen is high, more free plasmin will form and the excess fibrinogen will be digested.

Since the plasmin formed at the site of the clot last much longer in the absence of alpha 2-antiplasmin, patients who lack this inhibitor, have a greater bleeding tendency (70).

C. The regulation of t-PA synthesis and release

1. t-PA biochemistry:

t-PA exists in two forms, Single and double chain t-PA. It is first secreted as a single polypeptide chain, and is then cleaved at the Arg275-Ile276 peptide bond to yield a

disulfide-linked two chains form. This form is the active form of t-PA (71), and it becomes even more active in the presence of fibrin (72). Even though single chain t-PA has been previously thought to have no fibrinolytic activity (73), a recent study showed that, this form of t-PA does have fibrinolytic activity, but the Vmax is five times less than the double chain t-PA (74).

2. Effectors that alter the t-PA level:

A variety of effectors have been reported to alter the synthesis and release of t-PA. Some of these effectors are natural and have been demonstrated in vivo, while others have only been demonstrated in vitro.

In vivo studies have shown that exhaustive physical exercise in a healthy subjects results in the release of large amounts of t-PA into the blood, while the urokinase level remains unaltered (75,76,77). The increase in t-PA level following maximum exercise is greater than the level of PAI-1. This increase ratio of t-PA to PAI-1 decreases the PAI-1 inhibitory capacity (78). Even though exercise results in an increase in the fibrinolytic activity, no systemic fibrinolysis could be detected by Speiser et al.(79). This finding might be due to the observation that the ability of t-PA to activate plasminogen to plasmin is greatly reduced in the absence of fibrin (5).

Venous occlusion, like exercise, increases plasmin-mediated fibrinolysis via an increased level of t-PA (79). *Epinephrine* has also been demonstrated to increase blood plasminogen activator activity (80).

Patients with diabetes mellitus have been reported to have high levels of both t-PA and PAI-1 antigen. However the fibrinolytic activity (FA) in these patients has been reported to be lower than healthy controls of the same age (81,82). This lower FA has been attributed to the inhibition of t-PA by the increased level of PAI-1. Patients who have liver transplants have also been reported to have high fibrinolytic activities (83).

These clinical examples that are associated with high t-PA levels, are also known to be associated with alteration in the blood pH. Exercise is known to increase the blood pCO2 and lactic acid levels (84), which will lead to a low pH (85,86,87). Diabetics are known to develop severe ketoacidosis (88). Recent studies have reported that the arterial blood pH of these patients goes down to as low as 7.1 +/- 0.12 (89,90). The finger capillary blood pH, from a maximally exercised arm, has been reported to be as low as 6.8 (91). Venous occlusion and liver transplantation also cause low blood pHs (83,92).

In vitro studies have been used to demonstrate the effect of a variety of substances and conditions on the rate of synthesis and secretion of both t-PA and PAI-1. *Glucocorticoids and androgenic steroids* have been reported to alter t-PA production in some cell lines. Dexamethasone decreases t-PA production by *human mammary carcinoma cell line MDA-MB-231* (93). However *HBL-100 mammary carcinoma* cells increase t-PA production with an associate accumulation of t-PA mRNA, in the presence of dexamethasone (94). In contrast, dexamethasone has no effect on the production of t-PA by cultured bovine endothelial cells (26). That suggests that the same effector might have different effects on different kinds of endothelial cells. Plasminogen activator activity has also been shown to be decreased in *HTC rat hepatoma cells* after incubation with glucocorticoids (95,96). This effect however, was the result of an increased secretion of PAI-1 (97).

Thrombin stimulates, in a dose and time dependent manner, t-PA secretion by cultured human endothelial cells. This increase in the release of t-PA seems to be due to protein synthesis rather than secretion of already made protein, because it was inhibited in the presence of cyclohexamide or actinomycin D (28,57).

Reduced secretion of t-PA by human endothelial cells after exposure to *interleukin*-1 (IL-1) has been reported

(98). *Basic fibroblast growth factor* stimulates the secretion of t-PA as well as PAI-1 from bovine endothelial cells (99). Some hormones have also been reported to have an effect on the secretion of t-PA. *Gonadotropins, follicle stimulating hormone, and leutenizing hormone,* induce more synthesis and release of t-PA by rat granulosa cells (100). *Histamine* causes an increase in the t-PA secretion from cultured endothelial cells (57).

D. PAI-1 release and activation :

PAI-1 is synthesized by the endothelial cells. The rate of synthesis and release can be altered by many agents. Dexamethasone has been reported to increase secretion of PAI-1 by *hepatoma* (49,101) and *fibrosarcoma* (102) cells. It has also been observed to increase the level of PAI-1 transcription in *HT1080 cells* (58). *Tumor necrosis factor* (TNF) and *lipopolysaccharide* (LPS) increase PAI-1 mRNA in endothelial cells, by increasing the level of protein transcription (103). An increased synthesis and release of PAI-1 was also the result of a thrombin effect on human umbilical vein endothelial cells (17,104,105).

Hypertriglyceridemia has also been demonstrated to associate with high PAI-1 levels (85). PAI-1 was also observed to correlate significantly with age, suggesting an age-dependent decrease in fibrinolytic activity (85).

In order to determine the net effect on the fibrinolytic system, and since many different individual factors are involved, it is critical to measure all relevant factors and their total effects on each proposed analysis to determine modulating factors.

IV. Summary

The fibrinolytic activity of the blood is regulated at different levels. Because of their major role in the regulation of fibrinolysis, t-PA and PAI-1 are considered as the most important regulators. The level of both enzymes in the blood is affected by many physiological conditions. Exercise, stress, venous occlusion, diabetes mellitus, and liver transplantation are all conditions accompanied by elevated levels of t-PA (1,75,76,77,78,79,81,82,83,106). The mechanism through which these conditions affect the secretion of these enzymes, is still not clear. Researchers who have studied these factors have concentrated on the effects of different hormones or other proteins on the secretion of these enzymes. However, the natural physiological changes that take place in vivo during these conditions, such as the changes in blood pH, pCO2, or pO2, have not been tested. The most common and noticeable alteration associated with in vivo changes in t-PA and PAI-1 is the blood pH. Exercise, stress, venous occlusion, diabetes mellitus, and liver transplantation are all cases that are accompanied with

decreased blood pH (83,85,86,88,89,90). In a study of five groups of rats, it was demonstrated that the lower the pH in the blood after a sudden death, the higher the fibrinolytic activity measured (107). The increased fibrinolytic activity has been observed during liver transplantation, which is another condition characterized by acidosis, bleeding, and high levels of fibrin fragments (83). All of these observations suggest that alteration of blood pH might affect the level of synthesis and secretion of t-PA and PAI-1 from the endothelial cells; the major site of both proteins synthesis.

This study was designed to evaluate the effects of different pHs (6.0 to 8.5) on the release of t-PA and PAI-1 from cultured human endothelial cells. Cells derived from the umbilical vain were grown in M 199 culture media at different pHs. The level of t-PA and PAI-1 released into the culture media at the different pHs were measured at various points during a 24 hr time period.

V. Tissue Plasminogen Activator Assay

There are two major types of assays for t-PA: (1) assays that measure the fibrinolytic activity, and (2) assays that measure the t-PA antigen.

A. Methods of measuring the fibrinolytic activity:

Many methods exist to determine the fibrinolytic activity. Most are based on the activation of a given amount of plasminogen, and measure the effects of the generated plasmin on different substrates. These methods measure only the free active plasminogen activators without measuring activators in activator-inhibitor complexes. Many of these methods are nonspecific in that they measure all plasminogen activators and are not specific for t-PA.

1. The fibrin plate assay:

The *fibrin plate assay* measures the total fibrinolytic activity in a euglobulin precipitate(108). In this method, a plasminogen rich fibrin gel is formed in a petri dish. The dissolved euglobulin precipitate (30 ul) is placed on the surface of the fibrin gel. The fibrin plate is then incubated for 17 hrs at 37°C. Plasminogen activators present in the sample will activate plasminogen to plasmin which will degrade the fibrin clot. The diameter of the lysed zone is then compared to the zone produced by t-PA standards. This assay does not detect t-PA activity below 0.5 IU/ml (13). The ^{125}*I-fibrin microtiter well assay* is a modified method of the fibrin plate assay that can detect two to three orders of magnitude less than that detected by the fibrin plate assay(109).

2. Titration methods for measuring t-PA activity:

A synthetic chromogenic plasmin substrate *H-D-Val-leu-Lys-pNA (S-2251)*, is frequently used to measure fibrinolytic activity. Plasmin generated during the incubation of the sample with excess plasminogen, cleaves the substrate yielding a color that can be measured at a wavelength at 405 nm(105,109,110). Since the presence of fibrin or detergents such as triton X-100 greatly increases the fibrinolytic activity of plasmin (30,112), a mixture of the two components is usually added to the assay mixture for amplification of signal (110,111).

Another plasmin substrate used in measuring the fibrinolytic activity is *Z-Lys-SBzl*. Plasmin cleaves this substrate and the resulting product forms a color compound with 5,5'-dithiobis(2-nitrobenzoic acid) [DTNB]. The color generated is measured at a wavelength 412nm. (113). This assay has been modified by Moonen et al. so it can be used, to differentiate between urokinase and t-PA (114). This modification is based on the fact that t-PA is much more active in the presence of fibrin, while fibrin does not affect the activity of urokinase.

Since all of these assays are based on the action of plasmin on various substrates, samples that contain antiplasmins cannot be measured with these methods. Therefore, in the presence of alpha 2AP, the fibrinolytic activity in the native plasma cannot be measured unless an excessive amount of plasminogen is added to the sample (115). Various methods have been developed to separate alpha 2-antiplasmin from plasma or to inactivate it. Among these are Lys-Sepharose adsorption of t-PA plasminogen (116), and euglobulin precipitation (117).

B. Methods of measuring t-PA antigen :

Tissue plasminogen activator antigen concentration is usually measured by immunological assays, using an antibody that is directed against t-PA antigen. These assays allow direct measurement of t-PA without any interference from urokinase. However they measure both free t-PA and t-PA in t-PA-PAI-1 complexes. This set of assays can be divided into two major groups:

1. Radioimmuno assays (Immunoradiometric assays)(RIA, IRMA):

In 1983 Prowse and Macgregor developed a radioimmuno assay that could detect 2ng t-PA/ml plasma (118). This assay is based on the incubation of the sample or the standards

with a rabbit anti-t-PA for 18 hrs. I-125 labeled t-PA is then added to the mixture and allowed to bind to the free anti-t-PA remaining from the first step. To remove the bound t-PA (labeled from free), donkey anti-rabbit IgG bound covalently to Sepharose is added. The mixture is then centrifuged to remove the sepharose and the radioactivity of the supernate is counted in a gamma counter. When t-PA Ag in the sample is low, there will be more free anti-t-PA left in the first step. This free anti t-PA will bind to the iodinated t-PA added in the second step. The number of counts reflects the amount of iodinated t-PA bound to the anti t-PA, and is therefore indirectly proportional to the concentration of t-PA in the sample. Another way of doing the immunoradiometric assay was developed by *D. Collen et al.*(119). This technique is called the *Two-site immunoradiometric assay*. After incubation of the samples in the wells of a microtiter plate coated with a rabbit antibody against t-PA, the amount of the bound t-PA is quantitated by the subsequent binding of 125 I-labeled aminospecific antibody.

2. Enzyme Linked Immuno Assay :

This method is based on the adsorption of the t-PA in the sample onto a microtiter plate coated with rabbit antihuman t-PA. The amount of t-PA bound is quantitated by

successive incubation with goat antibody against t-PA and then enzyme labeled rabbit antibody against goat IgG. The sensitivity of this assay is about 1 ng t-PA/ml.(120).

C. Methods for measuring the PAI-1 activity :

1. Reverse fibrin autography:

This method, developed by Erickson et al. in 1984 (121) is based on the fractionation of the samples by polyacrylamide gel electrophoresis in the presence of sodium dodocyl sulfate. The gel is then placed on another indicator film that consist of proteases incorporated with fibrin-agar. These proteases will cause the lysis of the fibrin unless a protease inhibitor is present. The inhibitory activity in the first gel will appear on the indicator film as lysis-resistant zone. The diameter of the zone reflects the amount of inhibitor in the sample.

2. Titration methods for PAI-1 activity:

The titration methods for measuring PAI-1 activity are based on the t-PA inhibitory capacity of PAI-1. In 1983 Emeis et al. reported the use of the substrate S-2251 in the detection of plasminogen activator inhibitors in the conditioned medium (CM) from cultured endothelial cells.

This method is based on measuring a known amount of t-PA activity using S-2251(see page 21 for method) in the presence and absence of the CM. The decrease in t-PA activity is directly proportional to the amount of inhibitors presented in the CM.(122).

In 1986 a modification of this method was presented by Speiser et al. In this method The samples were preincubated with a known amount of t-PA prior to the addition of the other assay reagents (123).

D. Method for measuring the PAI-1 Antigen :

ELISA techniques have been developed for the measurements of PAI-1 Ag. These techniques are basically the same as the one used for the t-PA measurement. The first antibody is referred to as the catcher antibody. To determine the amount of PAI-1, a second antibody, usually a goat anti PAI-1, is added to the microtiter plate followed by enzyme linked rabbit anti goat IgG. The color developed after an incubation with a chromogenic substrate for the enzyme linked on the anti IgG, is directly proportional to the level of Ag in the sample.

Procedures and Methods

I. Tissue culture techniques:

A. Isolating the cells from the umbilical cord:

Endothelial cells were isolated from human umbilical cord by the method of Jaffe et al. (124).

Sterile conditions were maintained throughout the isolation and culture techniques. All cords were immediately removed from the placenta, placed in cold cord buffer (0.14 M NaCl, 0.004 M KCl, 0.001 M Phosphate buffer, Ph 7.4, 0.011 M glucose), held at 4°C (melted ice), and were processed within 18 hrs. After the cord was inspected for tears or clamps marks, the umbilical vein was canulated from both ends, and washed with 60 ml of cold cord buffer. When all visual traces of blood were removed, 10 ml of 0.2% collagenase in cord buffer was injected into the vein and the cord was incubated in 37°C cord buffer for 15 min. After incubation, the cord was gently hand massaged to enhance removal of the endothelial cells. The collagenase solution containing the isolated cells was flushed into a sterile 50 ml Corning capped tube. To maximize recovery and to inhibit further action of the collagenase enzyme, the

vein was washed with 30 ml media M199 supplemented with 15% fetal calf serum (FCS) (the FCS inhibits the collagenase). The mixture was centrifuged at 300 X G for 10 min. The precipitated cells were suspended in 10 ml M199-plus culture media. M199-plus refers to M199 culture media supplemented with 15% FCS, penicillin (200 U/ml), streptomycin (200 ug/ml), L glutamine (2 mM), endothelial cells growth factor ECGF (8 ug/ml), sodium heparin (90 ug/ml), and 150 U/ml amphotericin-B (125,126,127). The cell suspension was then transferred to a 100 mm. plastic tissue culture plate (*Corning 25020-100*) previously coated with 1% gelatin. Culture plates were incubated at 37°C, 5% CO2 incubator.

Since endothelial cells adhere to the dish much faster than fibroblasts (124), the culture media was changed after 2 hrs of incubation to reduce possible contamination by fibroblast. Confluency was reached in 4-5 days. Based on the morphology appearance of the cells, these cultures appeared to be free of fibroblast contamination.

B. Transferring and growing of the isolated cells:

At confluency, the cells were passaged by removing the culture media and replacing with 3 ml PBS-EDTA [phosphate buffer saline, pH 7.4, with 2% EDTA (Ethylene Diamine Tetracetic Acid)]. After 10-15 min of incubation at 37°C, most of the endothelial cells (EC) had released from the

surface of the plate. The release of the cells from the surface of the culture dish was observed under light microscope to determine the end of the incubation period. The PBS-EDTA cell suspension was transferred to a sterile 50 ml plastic Corning centrifuging tube, and centrifuged for 10 min at 300 X G. After the precipitated cells were resuspended in media M199-plus, they were divided into 3 gelatin coated culture plates and incubated to confluency.

C. Testing the cells for the presence of F VIII RAg:

After 3 passages the cells were tested for factor VIII related antigen (F VIII RAg). The reason for waiting until the third passage before testing for F VIII RAg was to allow any contaminating fibroblast to overgrow the endothelial cells.

F VIII RAg is a marker used in identifying the endothelial cells (125). Fibroblasts do not contain F VIII RAg and therefore would be negative by our technique. Cells to be tested were grown on a gelatin coated cover slip in M199-plus culture media.

After removing the culture media, the cells were fixed in methanol 100% for 15 min, allowed to air dry, then washed 2 times with PBS (phosphate buffer saline, pH 7.4). A diluted goat anti F VIII RAg solution was added to the fixed

cells and incubated for 30 min. After washing 2 times with PBS, a diluted rabbit anti goat IgG-FITC conjugate was added. Following 30 min of incubation, the cells were washed 2 times with PBS to remove excess conjugate. The cells were viewed using a fluorescent scope with 495 nm filter.

Based on uniform fluorescents of the cells, our cultures appeared to be pure endothelial cells.

D. Testing the morphology of the isolated cells:

The cells used in this study grew as a confluent monolayer without a definable whirling pattern. The cells were homogeneous, closely opposed, large (30 X 50 um), and polygonal with an oval centrally located nucleus. This morphological appearance is characteristic of the endothelial cells, and is different from fibroblasts which grow close one to each other in parallel arrays with overlapping layers (125). Smooth muscles cells are similar to fibroblasts in size and pattern of growth (125).

E. Studying the cells with the scanning electron microscopy:

Cells used in this study were farther characterized by a scanning electron microscope (SEM) study. Cells examined

with SEM were grown on either a gelatin coated cover slip or gelatin coated 150 um micro bead carrier using the method of Hassouna et al. (128). The cover slip or the micro carriers were placed in the bottom of a nongelatin coated petri dish and the cells were allowed to grow for 5-6 days to insure confluency.

At confluency the cover slips were removed and the cells were fixed for 8 hrs in 0.2 M phosphate buffer, 1% glutaraldehyde, pH 7,4. After primary fixation, the cells were washed three times with phosphate buffer saline (PBS), pH 7.4, and postfixed with 0.2 M phosphate buffer, and 1% osmium tetroxide, for 2 hrs. Dehydration of cells was then achieved by treatment with a series of graded alcohols (20%, 40%, 60%, 80%, 90%, 100%, and a repeat 100%), the cells were allowed to set in each solution for 1 hr. The cells were then critical point dried in CO_2 at a temperature of 40°C and at a pressure of 1,350 lb/in^2. The cover slips were glued on aluminum stubs, gold coated, and studied under SEM.

After placing the microcarrier beads in a 50um pore basket, they were fixed and dehydrated using the same procedure that was used for the cover slips. After fixation, the beads were scattered on a glued surface stub, gold coated, and observed under the SEM.

F. Freezing of the living cells:

Cells from each passage were stored at -180°C (liquid nitrogen) for future reference. Cells to be frozen were removed from the culture plate using PBS-EDTA buffer and the normal procedure for passaging the cells. After centrifuging, the cells were suspended in media M199-plus containing 10% DMSO (dimethyl sulfoxide) in an average concentration of 120,000 cells/ml. 1.8 ml of this suspension was placed into Gibco freezing tubes (*Gibco 14072*), wrapped in cotton, and frozen at -70°C for 18-24 hrs before transferring to liquid nitrogen for long term storage (129).

Reculturing the frozen cells was achieved by quickly melting the frozen cells in a 37°C water bath. The suspension was then transferred to a 50 ml Corning sterile tube, diluted 3 times with culture media M199-plus, and centrifuged at 300 G for 10 min. After centrifuging the cells were suspended in 10 ml culture media M199-plus, transferred to gelatin coated petri dish, and incubated in 37°C incubator with 5% CO_2 to confluency.

II. Preparing and collecting the conditioned media:

The cells of two confluent plates were transferred to a 12-well culture plate (*Corning 25815*), and grow to confluency in M199-Plus culture media. At confluency, the cells were washed three times with PBS (pH 7.4), then 0.5 ml of experimental culture media (Ex-M) was added to each well. Ex-M refers to M199 culture media supplemented with penicillin (200 U/ml), streptomycin (200 ug/ml), L glutamine (2 mM), 50 U/ml amphotericin-B, and adjusted to a specific pH in the range of 6.0 to 8.5 as indicated in the experimental protocol). After addition of the Ex-M at the pH to be tested, the plates were incubated at 37^{o}C for time periods ranging from 0 to 24 hours.

In each test, six 12-well culture plates were started at the same time. The conditioned media (CM) from each plate was collected after different incubation times (1/2, 1, 4, 12, 17, and 24 hrs). In each plate the effects of six different ex-CM, with pHs of 6, 6.5, 7, 7.5, 8, and 8.5, on the endothelial cells, were tested in duplicate.

To ensure that the pH of the Ex-M at the end of incubation was not significantly different from starting pH, the pH of the post incubation Ex-M (conditioned media, CM) was measured, the CM was centrifuged at 300 X G for 10 min

to remove cell debris, made 0.01% with tween 80, put in two plastic vials, and stored at -80°C until tested.

Each experiment was run on two different cell lines and on two different occasions. The two different cell lines tested were: Cell line 1, cells that were started from a single umbilical vein, and cell line 2, cells from three different umbilical cords that had been pooled together at the third passage.

III. The cells viability test:

The number of viable cells was determined after 1, 4, 17, and 24 hrs of incubation in CMs at pH 6, 6.5, 7.5, 8, and 8.5 using trypan blue method (Sigma). This method is based on the principle that live cells will not take up certain dyes, whereas dead cells will.

After the cells had been incubated in Ex-M of different pHs and for different time, the cells were removed from the culture dish using PBS-EDTA buffer, centrifuged at 300 X G for 10 min, and resuspended in 10 ml PBS, pH 7.4. 0.2 ml of the cell suspension was added to 0.5 ml Trypan Blue (*Sigma*) and 0.3 ml PBS. After incubating at room temperature for 10 min, a hemocytometer was loaded with this mixture. The nonstained cells (viable cells) and stained cells (dead cells) were counted in the four corner squares. The

percentage of living cells in the culture suspension was determined by number of cells counted - number of living cells/total number of cells. The number of cells/ml cells suspension was determined by the formula: number of cells counted in the four corner squares of the hemocytometer / 0.4 * 1,000. The number of living cells, as well as the number of dead cells, was calculated for 1 ml suspension and for the whole tissue culture plate.

IV. Screening the samples for t-PA and PAI-1:

Four different assays were run on each sample: (1) t-PA activity assay, (2) PAI-1 activity assay, (3) t-PA antigen assay, and (4) PAI-1 antigen assay.

A. The t-PA activity assay:

The coloremetric assay by Moonen et al. (94) was used for the measurement of t-PA and PAI-1 activities in our samples.

This is a two steps assay involving: (A) Plasminogen activation, and (B) Determining the plasmin generated in the first step.

1. Plasminogen activation: All reagents in this step were diluted before the assay in a diluting buffer (0.1 M glycine-tris buffer, pH 8.5, containing 0.5 mg/ml bovine serum albumin (*Calbiochem*)). To each well of a 96-well flat-bottomed microtiter plate the following reagents were added: 5 ul sample/or standards, 5 ul of 0.4 ug/ul plasminogen (*American Diagnostica*), 5 ul of cyanogen bromide cleaved fibrinogen fragments (*prepared in our laboratory*), and 15 ul of the diluting buffer. The plate was then incubated at 37°C for 1 hr to allow the activation of plasminogen to plasmin.

The stock t-PA standard was made 40 IU/ml, saved at -80°C, and diluted prior to the assay to (2, 1.5, 1, 0.75, 0.5, and 0.25 IU/ml).

2. Determining the level of resulted plasmin: The reagent mixture for this step consists of 100 parts PBS (Phosphate Buffer Saline, 0.2 M Na-phosphate and 0.2 M NaCl, pH 7.5), one part triton X-100 (Merck) at 0.1% in water, one part 22 mM (in water) DTNB (5,5'Dithio-bis-(2-nitrobenzoic acid), (*Sigma*), one part Z-Lys-SBzl (20 mM in water), (*Sigma*). 250 ul of this mixture was added to each well after the first incubation, and the reaction allowed to proceed for 30 min.

The optical density (OD) was measured at 410 nm in a Dynatech MR 300 microplate spectrophotometer with instrument zero set on a blank prepared by adding 5 ul of ex-CM instead of samples in the first step. The blank was treated the same as the samples throughout the rest of the assay steps.

The average OD of duplicate standards was plotted against their known concentrations (0 to 2.0 IU/ml). An example of the resulted linear plot which has been used to determine the t-PA levels in the samples, is shown in figure 1.

B. The PAI-1 activity assay:

PAI-1 activity was quantified in the samples by measuring its ability to inactivate t-PA. 5 ul of 2 IU/ml t-PA was challenged with 5 ul of the sample in the absence of fibrinogen fragments. The mixture was incubated at 37°C for 20 min to allow the inhibitory activity in the samples to inhibit a known amount of t-PA. After incubation, 5 ul of 0.4 ug/ul plasminogen, 5 ul fibrinogen fragments, and 10 ul glycine tris buffer was added to the mixture. After this addition the assay was continued as in the t-PA activity procedure.

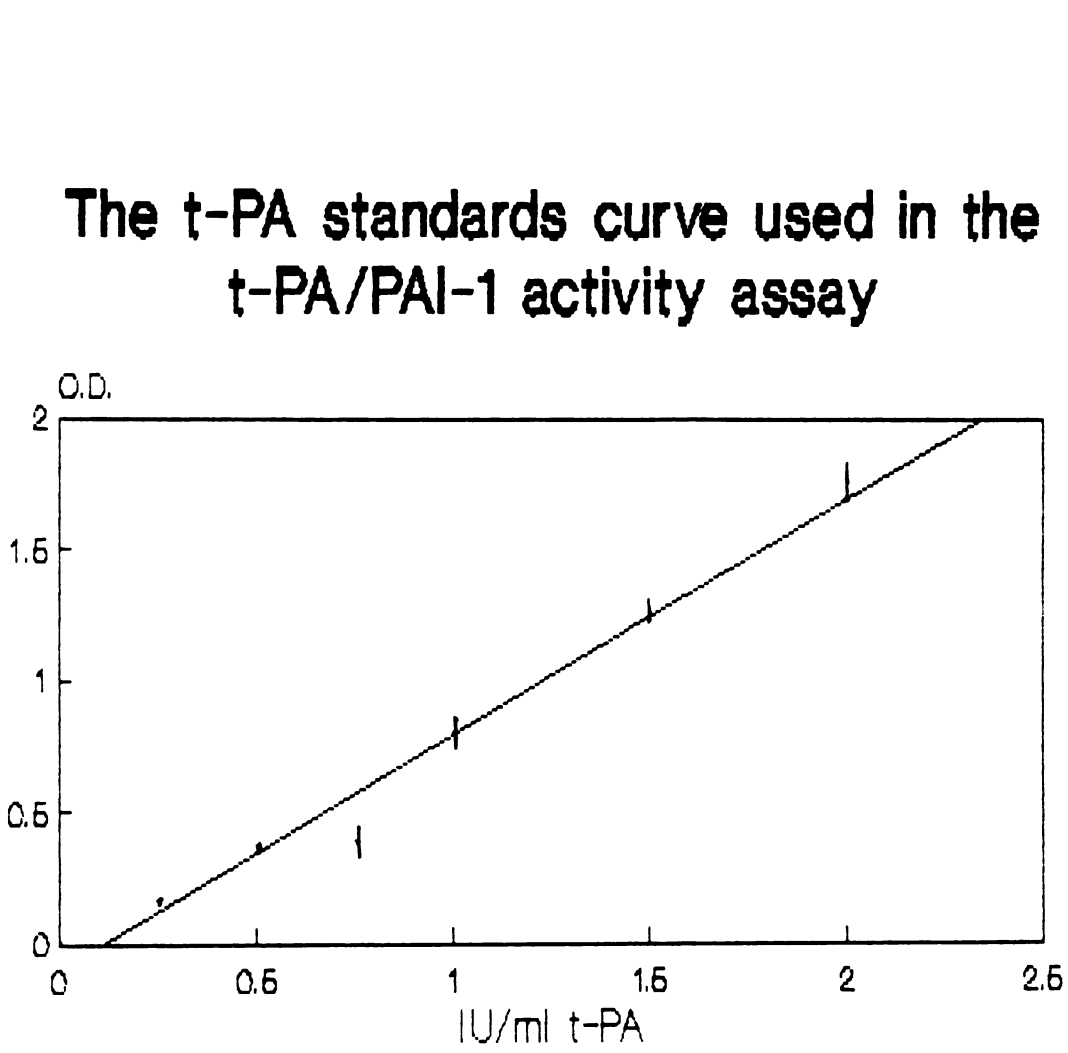

Figure 1: ***The t-PA standards curve produced in the t-PA/PAI-1 activity assay:*** t-PA standards were tested each time the assay was run. Standards were diluted prior to the assay, loaded to the microtiter wells in duplicate, and were treated the same as the rest of the samples. The resulted O.D.s were then plotted vs the concentration of each standard and the resulted linear curve was used for estimating the t-PA concentrations in the samples. (Slope = 1.041, R = 0.991).

Tissue plasminogen activator standards were run in each experiment, and the resulting standards curve (figure 1) was used in estimating the amount of t-PA left in each well. The instrument zero was set using a blank prepared the same way as in the t-PA activity assay. In each run, the endogenous inhibitory activity of the culture media was tested by substituting 5 ul of the culture media for the sample. The endogenous activity of the culture media was found to be zero in all samples tested.

The t-PA activity detected in each well was then subtracted out of 2 IU/ml (the original amount of t-PA added to the well in the first step of the assay). The final concentration is the amount of t-PA that was inhibited by PAI-1 in the sample. In other words, the amount of PAI-1 activity was inversely proportional to the amount of added t-PA remaining in the sample after incubation.

C. The PAI-1 antigen assay:

A double antibody sandwich ELISA technique using the Avidin-Biotin system was used to measure both PAI-1 and t-PA (130,131,132).

In this procedure, the sandwich is made up of the catcher (monoclonal antibody 1, MAB1) coating the plate, and the biotinilated chaser (monoclonal antibody 2, MAB2).

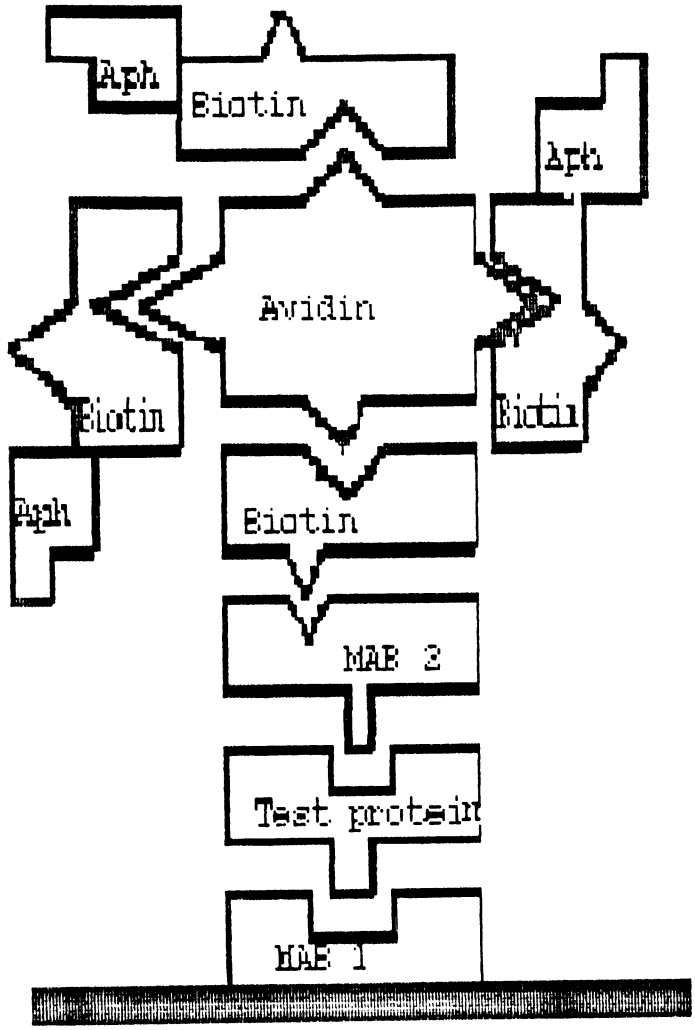

Figure 2: ***The sandwich built in the ELISA technique used in the determination of t-PA/PAI-1 Ag:*** MAB 1 and MAB 2 are monoclonal antibodies against t-PA when the assay was used for the t-PA determination, or against PAI-1 when the assay was used for the PAI-1 determination. MAB 2 is a previously biotinilated antibody. After this sandwich is built, p-Nitrophenyl phosphate solution (Alkaline phosphatase substrate) was added. After a certain period of time, the resulting color is proportional to the amount of Ag presented in the sample.

Between these two antibodies is the antigen. To this complex Avidin is added. One binding site of the avidin will bind to the biotinilated chaser. The other three binding sites are available for binding to the biotinilated enzyme, alkaline phosphatase, that is added after the avidin. The addition of alkaline phosphatase substrate, p-Nitrophenyl phosphate produces a color that is proportional to the amount of alkaline phosphatase bound to the avidin, biotin, MAB 2 complex. Since each avidin, biotin, MAB 2 complex can bind 3 alkaline phosphatase, the strength of the color signal will be amplified by a factor of three times.

1. Assay reagents:

- Two different monoclonal antibodies against two different sits of the PAI-1 molecules were a gift from Dr. L. Erikson (*Upjohn*).
- Biotin for the biotinilation of MAB2. (*Sigma*).
- Avidin (*Sigma*).
- Biotin-Alkaline phosphatase complex (*Sigma*).
- Alkaline phosphatase substrate (p-Nitrophenyl phosphate (PNPP) di tris salt) (*Calbiochem Cor.*).
- PAI-1 standards (*American Diagnostica*).

2. Biotinilating the monoclonal anti body:

The biotinilation technique of Erikson et al. (131) was slightly modified (132) and used in biotinilating our monoclonal antibodies. 1 ml of the 1 mg/ml anti PAI-1 (in 0.1 M $NaHCO_3$, ph 8.4) was mixed with 0.1 ml N-OH succinimidyl biotin (1.0 mg/ml in DMSO). The mixture was allowed to incubate for 2 hrs at room temperature. The reaction was stopped by adding 0.1 ml of 1 M glycine (in 0.1 M $NaHCO_3$, pH 8.4).

Free proteins were separated from the bound complexes by a membrane filtration chromatography technique using a membrane-tube by (Amicon 4208). The tube has a membrane that when centrifuged at 700 G, it allows low molecular weight molecules (free proteins) to pass through, while the high molecular weight are retained (biotin-MAB2 complex).

The protein mixture was placed in the membrane-tube, and the system was centrifuged at 700 X g. for 1 hr in a fixed angle rotor centrifuge. The fluid that remained on top of the membrane contains the Biotin-MAB2 complex was stored at -80°C until it is needed.

3. Assay procedure:

A flat bottomed immuno microtiter plate, was coated with anti PAI-1 by incubating the plate at 37^{0}C for 24-36 hrs, with 100 ul/well of MAB 1 in sodium bicarbonate buffer (0.05 M Na_2CO_3, pH 9.6, 0.2% sodium azide). The plate was then washed three times with washing buffer (0.01 M Na_2HPO_4, pH 7.4, 0.15 M NaCl, 0.01% tween 20), BSA buffer [washing buffer + 1% bovine serum albumin, (BSA)] was added in excess to each well, and incubated for 30 min to block the remaining free sites of the well and minimize the nonspecific binding of other proteins. After washing three times with the washing buffer, either 100 ul sample (CM), or standards, were added to the wells and incubated for 1 hr, at 37^{o}C. After washing three times, 100 ul of the biotinilated MAB 2 (in BSA buffer) were added to the wells and incubated for 1 hr, at 37^{o}C. Next, the plate was washed three times, avidin was added in excess to each well and incubated for 1 hr at 37^{o}C. After incubation and washing, Biotin-Alkaline phosphatase complex (in water) was added in excess and incubated at 37^{o}C, for 1 hr. Finally, the plate was washed three times and Alkaline Phosphatase substrate (PNPP) (in 100 mM NaHC03, 10 mM MgC12, pH 9.5 buffer) added in excess and incubated at 37^{o}C, for 100 min. The OD was measured at 410 nm using a Dynatech MR 300 microplate

spectrophotometer with instrument zero set on a well that had culture media added instead of samples in the second step. This will illuminate any endogenous interference from the culture media. OD's of the duplicate standards were averaged and plotted against their known concentrations. The resulted standards curve was linear within the range of the used standards (0 to 25 IU/ml) (figure 3). The amount of PAI-1 in the samples determined by comparing the OD of the sample to the standards curve.

D. The t-PA antigen assay:

Using two different MABs against t-PA (*a gift from Dr. L. Erikson*) the same ELISA technique used for the determination of PAI-1 Ag was used for the determinations of t-PA Ag in the samples. The procedure was basically the same except for using different MABs. An example of the resulting standards curve is shown in figure 4.

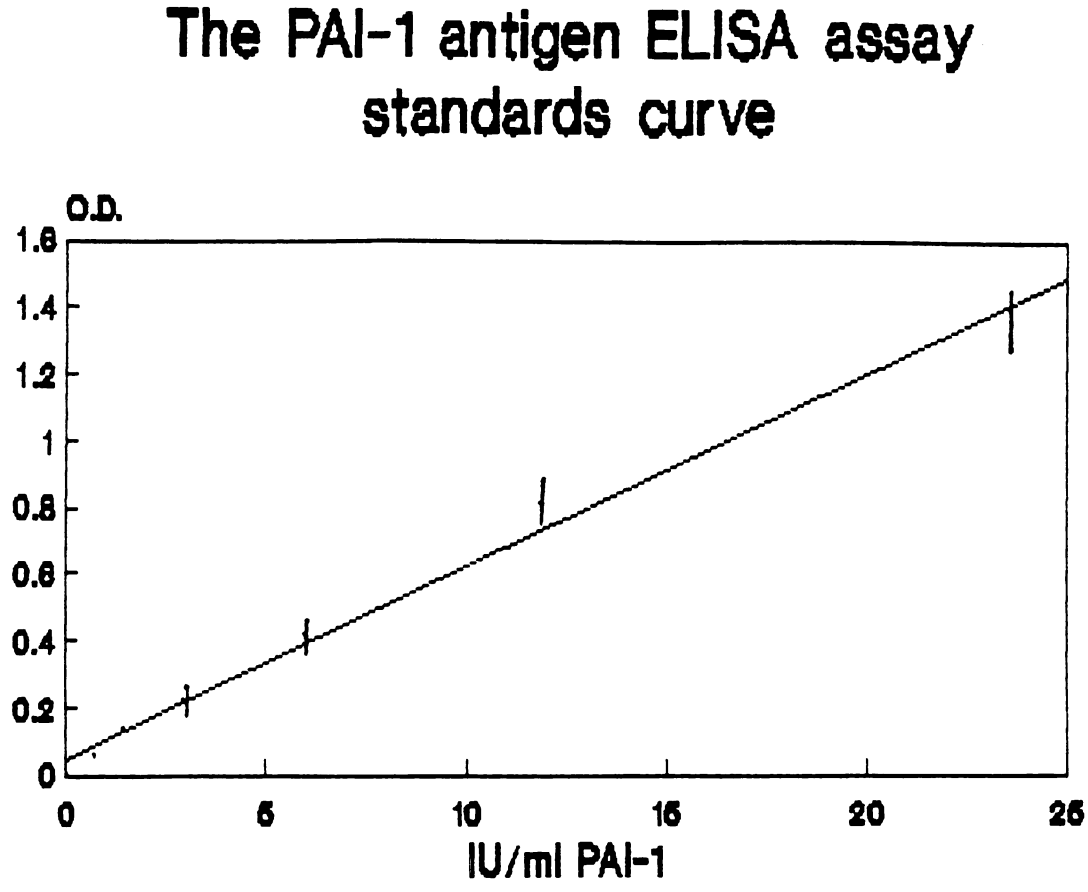

Figure 3: *The standards curve used in the ELISA technique for PAI-1:* PAI-1 standards were diluted prior to the assay, loaded to the wells of the microtiter plate, and were treated the same as the samples for the rest of the assay procedure. The resulting O.D.s were then plotted vs the concentration of each standard and the resulted curve was used to estimate the PAI-1 levels in the samples. (Slope = 17.13, R = 0.995).

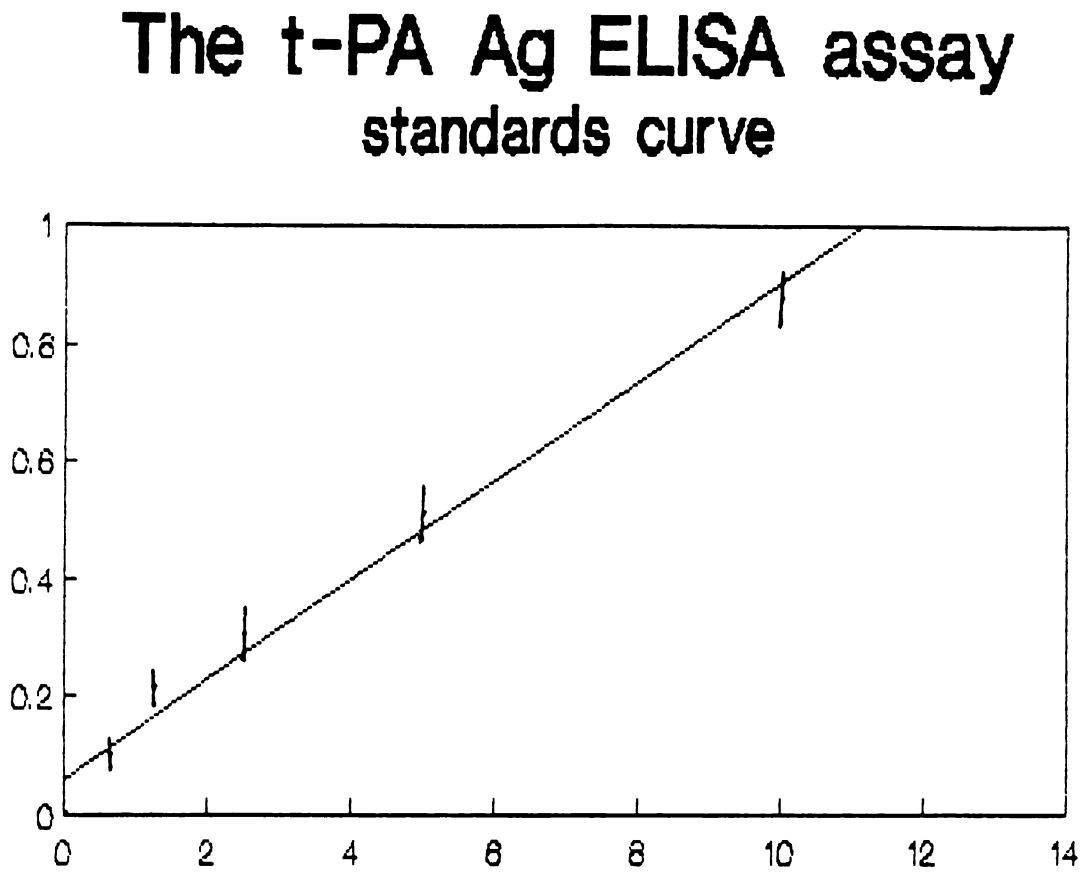

Figure 4: ***The standards curve used in the ELISA technique for t-PA:*** t-PA standards were diluted prior to the assay, loaded to the wells of the microtiter plate, and were treated the same as the samples for the rest of the assay procedure. The resulting O.D.s were then plotted vs the concentration of each standard and the resulted curve was used to estimate the t-PA levels in the samples. (Slope = 11.66, R = 0.992).

Results

I. Cell viability:

The number of the viable cells was determined after 1, 4, 17, and 24 hrs incubation at pH 6.0, 6.5, 7.5, 8.0, and 8.5. The total number of cells was about 300,000 cells/1 ml CM. The number of viable cells was found to be similar at all times at pHs between 6.0 and 8.0. This number was 290,000 cells/ml CM after 1 hr, 260,000 cells/ml after 4 hrs, 220,000 cells/ml after 17 hrs, and 210,000 cells/ml after 24 hrs. However at pH 8.5, 50% of the cells (150,000 cells/ml) were dead after 4 hrs incubation, and 90% (175,000 cells/ml) of the cells could not survive 17 hrs incubation at this pH.

II. t-PA activity assay:

None of the eighty different samples that have been tested for t-PA activity, have shown any fibrinolytic activity.

III. t-PA antigen test:

Table 1 shows the results of this test on the different samples that were tested. The changes in pH from pH 6.0 to 8.0 did not alter the t-PA rate of the time dependent increase in t-PA release into the culture media. In this pH range, no change in the level of t-PA Ag in the CM was measured until after four hours of incubation. After four hours, the level of t-PA in the CM increased with time reaching highest levels at 17 hours. The 24 hr level of t-PA Ag was not different from 17 hr. At pH 8.5 there was no change in the level of Ag over 24 hrs. After 24 hrs grater than 90% of the cells were dead as demonstrated by the trypan blue exclusion (figure 5).

Time of incubation / pH of CM	0.5 hr	1 hr	4 hr	12 hr	17 hr	24 hr
6.0	-	-	0.96 ± 0.77	2.0 ± 0.22	6.31 ± 1.5	6.40 ± 1.5
6.5	-	-	0.87 ± 0.60	2.40 ± 0.30	6.24 ± 1.3	6.53 ± 1.7
7.5	-	-	0.65 ± 0.60	2.87 ± 0.80	5.80 ± 0.35	6.37 ± 1.50
8.0	-	-	0.45 ± 0.40	2.17 ± 0.25	5.90 ± 1.40	5.68 ± 1.60
8.5	-	-	0.65 ± 0.50	0.27 ± 0.37	0.77 ± 0.77	2.20 ± 1.10

Table 1 : This table shows the results of running the ELISA technique for t-PA Ag on the samples. From this data, it appears that the pH of the culture media has no effect on the release of t-PA from the endothelial cells in the range of 6.0 to 8.0. The lower levels at pH 8.5 could be due to the lower number of the viable cells at this pH. t-PA secretion is time dependent and reaches its maximum value after 17 hrs.

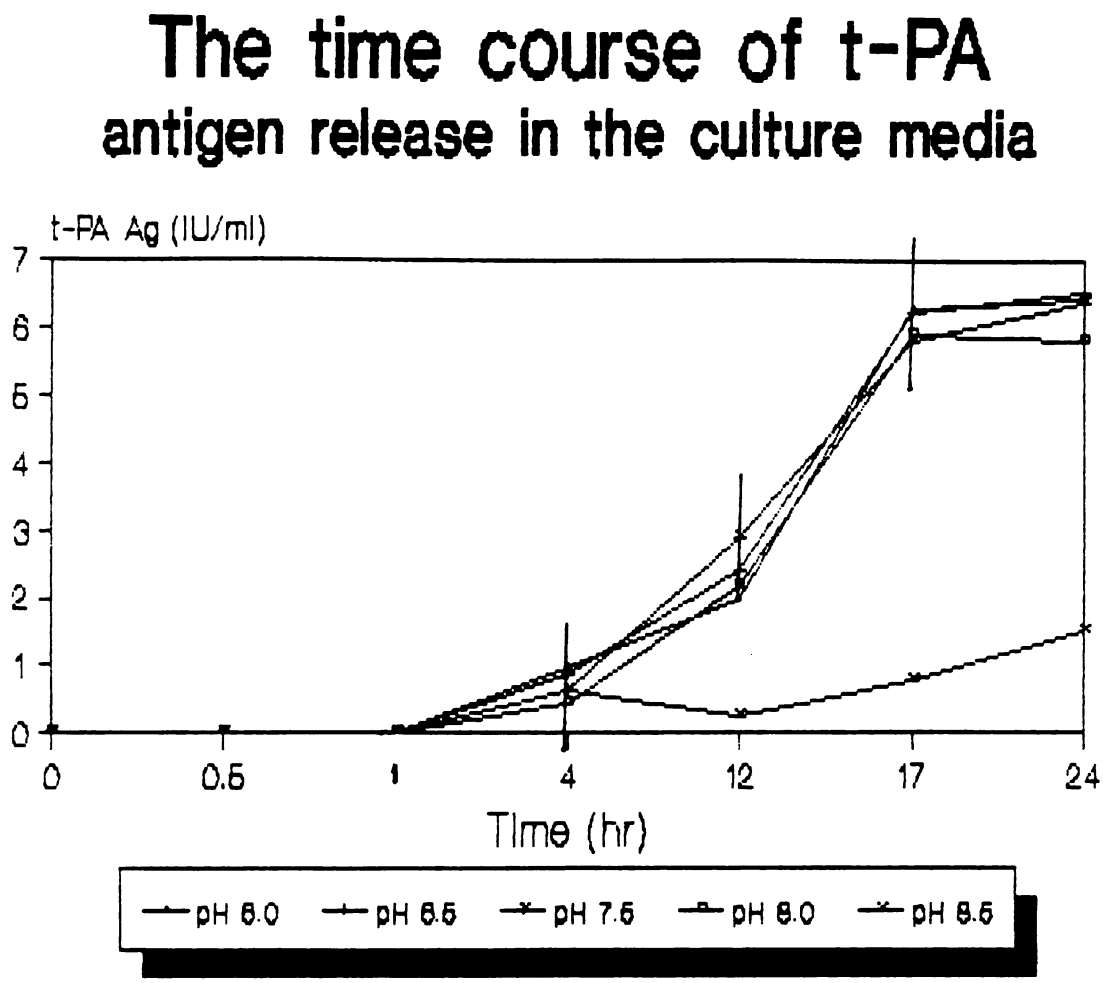

Figure 5: **the time course of the t-PA antigen release from the endothelial cells at different pHs:** Two different cell lines were tested for the effect of different culture media with different pHs (6, 6.5, 7, 7.5, 8, 8.5) on the release of t-PA Ag. Each cell line was tested twice. Samples were collected after different time periods (0.5, 1, 4, 12, 17, and 24 hrs). The t-PA Ag was measured in these samples using a double antibody ELISA technique. Except for pH 8.5, all samples collected at the same time point have shown similar results. The t-PA Ag level detected in the different experiments were averaged and plotted against time for the different pHs. This data suggests that the pH has no effect on the release of t-PA Ag from the endothelial cells. The low level detected at pH 8.5 could be due to the lower number of the viable cells at this pH.

IV. PAI-1 activity assay:

PAI-1 activity was measured on all samples collected at pH 6, 7.5, 8, and 8.5. Table 2, shows the PAI-1 level detected from each of these samples. Except for pH 8.5, the PAI-1 activity levels were the same for all samples collected at the same time point regardless of pHs. The lower activity was measured at pH 8.5, could be due to the lower number of the viable cells at this pH. The inhibitory activity at all pHs, seems to increase over time reaching highest level at 17 hrs. This level remained constant during the 17-24 hr period tested. Figure 6 shows the plot of the averaged PAI-1 activity levels detected in the different experiments verses time, at the different pHs. The figure indicates how this activity increases over time and remains constant after 17 hrs at all pHs.

Time of incubation / pH of CM	0.5 hr	1 hr	4 hr	12 hr	17 hr	24 hr
6.0	0.30 ± 0.10	0.33 ± 0.07	0.59 ± 0.14	1.02 ± 0.15	1.32 ± 0.31	1.33 ± 0.40
7.5	0.31 ± 0.08	0.39 ± 0.12	0.90 ± 0.16	1.28 ± 0.07	1.29 ± 0.26	1.28 ± 0.14
8.0	0.17 ± 0.40	0.32 ± 0.24	0.70 ± 0.36	1.05 ± 0.10	1.10 ± 0.14	1.17 ± 0.15
8.5	0.12 ± 0.40	0.26 ± 0.05	0.42 ± 0.49	0.12 ± 0.52	0.16 ± 0.16	0.29 ± 0.12

Table 2: This table shows the detected PAI-1 activity level using the titration assay by Moonen et al. This data indicates that the pH has no effect on the PAI-1 activity secreted from the endothelial cells. The lower PAI-1 activity level detected at pH 8.5 could be due to the lower number of the viable cells at this pH. PAI-1 activity is time dependent and reaches its maximum level after 17 hrs.

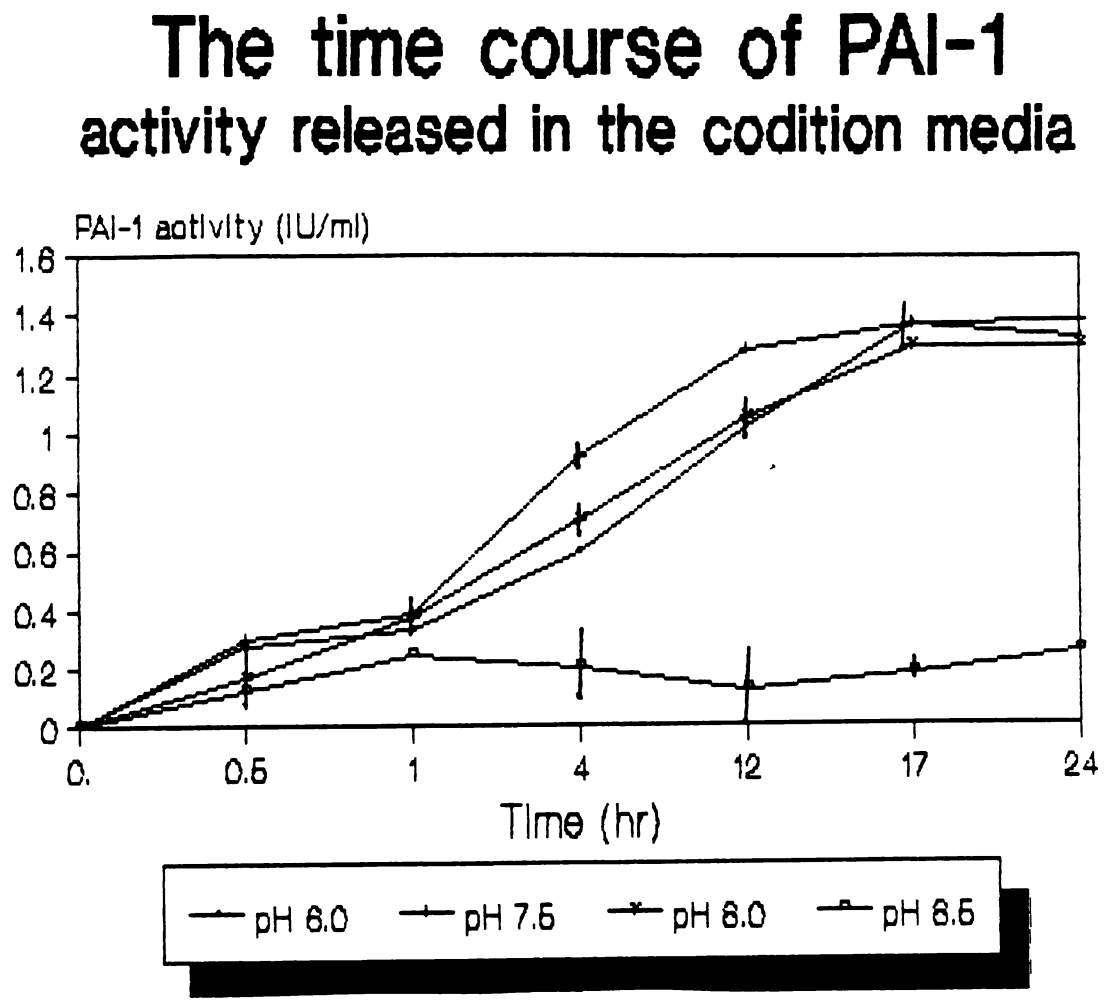

Figure 6: **the time course of the PAI-1 activity release from the endothelial cells at different pHs:** Two different cell lines were tested for the effect of different culture media with different pHs (6, 6.5, 7, 7.5, 8, 8.5) on the release of PAI-1 activity. Each cell line was tested twice. Samples were collected after different time periods (0.5, 1, 4, 12, 17, and 24 hrs). The PAI-1 activity was measured in these samples using the activity assay by Moonen et al. Except for pH 8.5, all samples collected at the same time point have shown similar results. The PAI-1 activity level detected in the different experiments were averaged and plotted against time for the different pHs. This data suggests that the pH has no effect on the release of PAI-1 activity from the endothelial cells. The low level detected at pH 8.5 could be due to the lower number of the viable cells at this pH.

V. The PAI-1 antigen assay:

The data presented in table 3 demonstrates that the pH of the CM in the range of 6.0 to 8.0 had no effect on the release of PAI-1 Ag from the endothelial cells. However the release of this Ag is time dependent reaching highest levels after 17 hrs. The 24 hrs level of PAI-1 Ag was not different from the 17 hr sample. Figure 7 shows that the relationship between time and PAI-1 Ag level in the CM is the same at the different pHs and in all cell lines.

Time of incubation / pH of CM	0.5 hr	1 hr	4 hr	12 hr	17 hr	24 hr
6.0	2.30 ± 2.10	2.37 ± 0.77	10.05 ± 5.5	19.57 ± 3.7	24.60 ± 1.2	25.82 ± 1.8
6.5	2.80 ± 1.06	4.22 ± 2.43	14.05 ± 2.9	22.22 ± 1.6	23.44 ± 1.0	23.95 ± 2.3
7.0	-	-	-	16.87 ± 1.7	21.74 ± 1.2	-
7.5	7.22 ± 1.60	2.85 ± 2.70	14.22 ± 3.8	22.62 ± 0.8	23.25 ± 1.3	23.47 ± 2.0
8.0	2.90 ± 2.30	5.60 ± 4.10	14.00 ± 2.2	21.63 ± 0.7	23.64 ± 0.3	23.87 ± 2.9
8.5	4.12 ± 0.67	5.17 ± 0.71	16.02 ± 5.9	21.05 ± 0.9	24.65 ± 1.7	22.05 ± 4.1

Table 3 : This table shows the results of running the ELISA technique for PAI-1 on the samples. From this data, it appears that the pH of the culture media had no effect on the release of PAI-1 from the endothelial cells. The lower levels at pH 8.5 could be due to the lower number of the viable cells at this pH. PAI-1 secretion is time dependent and reaches its highest value after 17 hrs.

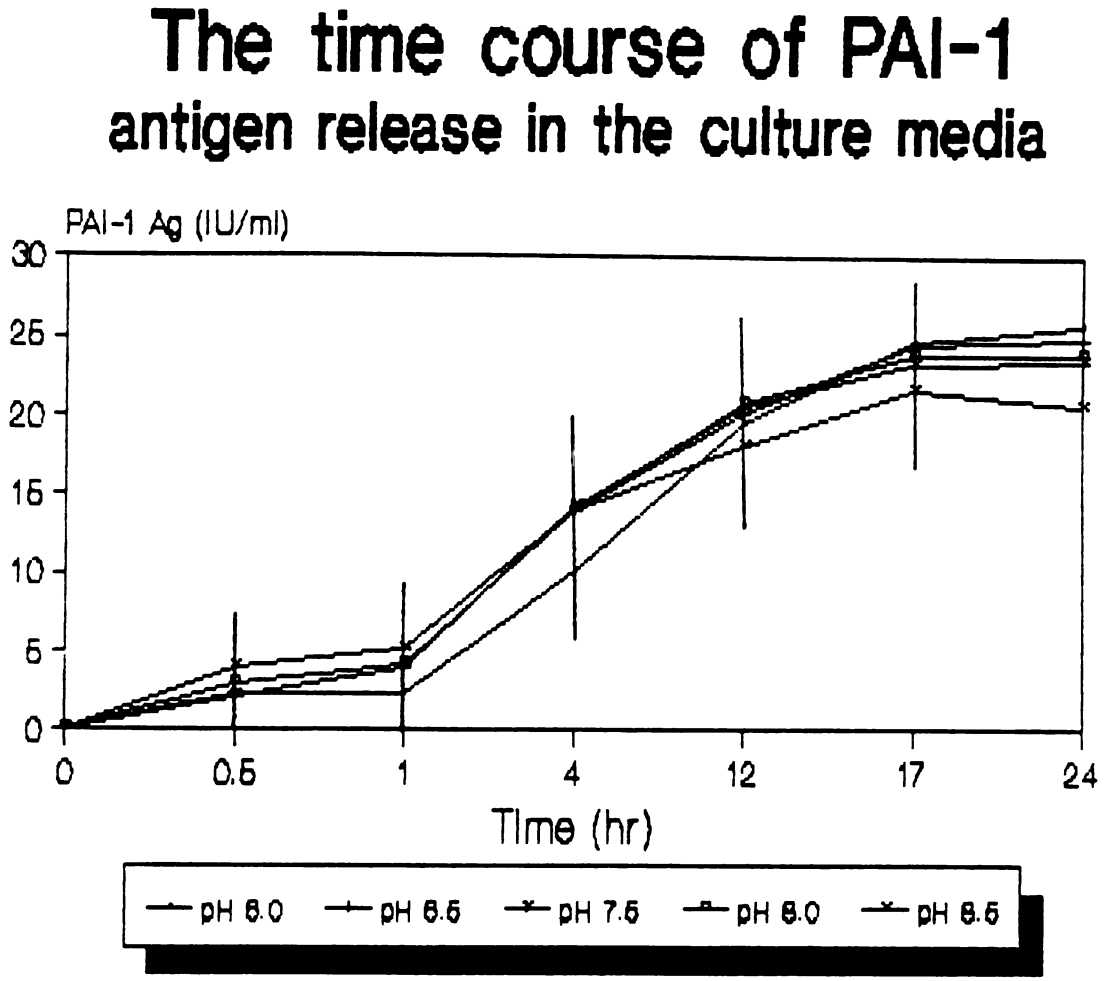

***Figure 7*: the time course of the PAI-1 antigen release from the endothelial cells at different pHs:** Two different cell lines were tested for the effect of different culture media with different pHs (6, 6.5, 7, 7.5, 8, 8.5) on the release of PAI-1 Ag. Each cell line was tested twice. Samples were collected after different time periods (0.5, 1, 4, 12, 17, and 24 hrs). The PAI-1 Ag was measured in these samples using a double antibody ELISA technique. Except for pH 8.5, all samples collected at the same time point have shown similar results. The PAI-1 Ag level detected in the different experiments were averaged and plotted against time for the different pHs. This data suggests that the pH has no effect on the release of PAI-1 Ag from the endothelial cells. The low level detected at pH 8.5 could be due to the lower number of the viable cells at this pH.

Discussion

The endothelium is the major site of control and regulation of both the coagulation and the fibrinolytic systems (133,134,135,136,137). In the regulation of the fibrinolytic system, the endothelial cells synthesize and release tissue plasminogen activator (t-PA) as well as its major inhibitor plasminogen activator inhibitor type 1 (PAI-1)(1). Many factors have been shown to alter the level of the synthesis and release of both proteins from cultured endothelial cells (28,33,57,93,94,95,96,97,98). Exercise, stress, venous occlusion, diabetes mellitus, and liver transplantation are all physiological conditions associated with altered levels of both t-PA and PAI-1 (75,76,78,79). These conditions are also accompanied by an altered plasma pH value (83,84,85,86,87,88,89,90,91).

In this study we have demonstrated that incubation of human endothelial cells in culture media with different pHs (6 to 8.5) has no effect on the release of t-PA and PAI-1. We have measured the t-PA activity and antigen as well as the PAI-1 activity and antigen after incubating confluent human endothelial cells in conditioned media at different pHs.

In controlled culture conditions, there is a slow increase in t-PA accumulation in the conditioned media (CM) over time. Peak levels were observed at 17 hrs. Levels at 24 hrs incubation were not different from 17 hrs values.

The rate of t-PA Ag released from endothelial cells incubated in culture media with pHs ranging from 6 to 8, was not different from control values. At pH 8.5, the amount released was less than the other pH values. Cell viability test indicated that the cell viability was not affected by the pH range from 6.0 to 8.0. At pH 8.5, less than 25% of the cells were viable at 17 hrs. This marked reduction in the number of live cells could account for the decrease levels of t-PA (antigen and activity) and PAI-1 (antigen and activity) in the 17 hrs CM. The difference in release of t-PA from the endothelial cells incubated at pH 8.5 was not different from the other pHs tested until after four hrs of incubation when the rate of t-PA release was reduced to zero.

Through out the time period tested, there was no change in t-PA activity even though there was a large increase in t-PA antigen. This finding was true for all pHs tested including the control. The failure to demonstrate an increase in t-PA activity can be accounted for by the immediate inhibition of t-PA activity by PAI-1 (63,138). The presence of up to 10 fold excess of inhibitor

relative to the activator has been reported by several investigators (17,104,120). It has also been reported that the interaction between t-PA and PAI-1 takes place on the surface of the endothelial cells (63). These findings suggests that as soon as the t-PA is released from the endothelial cells it will bind to PAI-1 on the surface of the cells, and is then released into the CM as a t-PA-PAI-1 complex.

The slow increase in the CM concentration of t-PA Ag during the first four hours of incubation might reflect an increase in the mRNA and protein synthesis (17,33). The highest level t-PA Ag (6-7 IU/ml) was reached at 17 hrs incubation. This finding supports the finding by Collen et al. and Laurence et al (17,57). Our values are similar to the post incubation levels of t-PA Ag reported by these investigator. The mechanism through which the level of t-PA Ag remains constant after reaching this particular concentration is still not clear. This mechanism might be due to either an autocatabolism of t-PA due to denaturation of the protein after a long incubation time, or it could reflect a concentration dependent inhibition of t-PA release.

The observation that human endothelial cells in culture do not secret free plasminogen activator activity into the surrounding medium questions the origin of the

fibrinolytic activity in circulating human plasma. Indeed, the occurrence of free t-PA in plasma in the presence of free PAI-1 remains a controversial issue. A few investigators have reported free t-PA along with the t-PA-inhibitor complex in the plasma (139). However, most investigators have been unable to detect any free t-PA in the plasma (140). A recent study has shown that in vivo thrombolysis by infusion of t-PA into animals with markedly elevated PAI-1 levels can occur in the absence of detectable free t-PA in the circulation (141). Thus indicates that the vascular endothelium plays a very important role in the regulation of in vivo fibrinolysis. These appear to be good agreement that free active PAI-1 is present in the plasma at low concentration (142).

PAI-1 antigen levels were not affected by changing the pH of the culture media. At pH 8.5, a lower concentration of PAI-1 was detected. This decrease in the release of the enzyme at this pH might be due to the low number of viable cells detected at this pH. The time course of the release of PAI-1 was time dependent and it follows the same pattern as the t-PA Ag time course release. Low levels of PAI-1 were detected during the first four hours of incubation, followed by a rapid increase in the concentration to a maximum value of 30 IU/ml that is reached after 17 hours of incubation. A similar level has been reported by Collen et al. and by

Laurence et al. (17,57). The PAI-1 activity was found also to follow the same time course pattern. The peak level of this activity was 1.5 to 2.0 IU/ml.

It appears that the amount of PAI-1 Ag released is greater than the amount of the measured activity. If the PAI-1 being released is inhibiting t-PA, this could explain in part this difference between activity and antigen. Another factor that would account for this difference is the previously reported observation that most of the PAI-1 is released in a latent inactive form (46).

Like t-PA, the mechanism via which a stable level of PAI-1 (Ag and activity) after 17 hours of incubation, is not known. As postulated for the t-PA Ag, this mechanism might be due to a feed back inhibition from the PAI-1 in the conditioned media on the cells. This inhibition could also be due to the possible effects of increased level of t-PA on PAI-1 release. The stability of the PAI-1 level after 17 hours of incubation could reflect increased protein denaturation after such a period of time. These suggestions for the mechanism that result in a stable level of t-PA and PAI-1 after 17 hrs of incubation, are in need for more investigation.

Factor VIII related antigen (*von Willebrand's factor*) was also measured. The release of this factor was found to

be independent of the pH of the culture media and followed the same time course as the PAI-1 and t-PA release. This observation supports our assumption that the cells are functioning properly at all pHs 6.0 to 8.0.

The conclusion that the altered pH has no affect on the release of either t-PA, PAI-1, or F VIII RAg from the endothelial cells, is very important in that it argues against the possibility that local alteration in pH affects the rate of secretion of these proteins. These observations suggests that it is highly unlikely that the altered fibrinolytic activity of the blood of a patient with severe pH changes can be explained by the effects of these changes on the endothelial cells. However, a pH value higher than 8.0 might have a damaging effect on the endothelial cells, whereas, these cells show good survival at pH values as low as 6.0.

The information from this study is important to future investigation using endothelial cells. The demonstration that the pH of the culture media can be altered between 6.0 and 8.0 with minimal to no effect on the release of t-PA, PAI-1, or factor VIII RAg, will allow more flexibility in studies using different agonist that might alter the pH of the culture media.

The effects of the alteration in pO2 and pCO2 on the rate of release and synthesis of t-PA and PAI-1 from the endothelial cells was not investigated. The pO2 and pCO2 are also important physiological variables that might affect the release of t-PA and PAI-1 from the endothelial cells, and might act synergistically to alter the rate of synthesis and release of these fibrinolytic proteins from the endothelial cells. More investigation into this area as well as on the mechanism of release and inhibition of release of t-PA and PAI-1, is needed.

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VITA

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The author was born in Damascus, Syria, on November 15, 1965. He graduated from AL-Thaqaffee High School, Damascus, Syria, in 1982.

In 1982 he enrolled in The University of Damascus, college of Pharmacy, and he was awarded a Bachelor of Science degree in Pharmacy in 1987. From September 1986 through June 1987, he interned at Teshreen Hospital and received a certification of laboratory training.

In 1988, he was accepted in the Medical Technology Program at Michigan State University, as a graduate student. During the period of the graduate school, he was also employed as teaching assistant in the undergraduate school of The Medical Technology Program.