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UTEROPLACENTAL FIBRINOLYSIS IN NORMAL PREGNANCY, PRE-ECLAMPSIA AND INTRAUTERINE GROWTH RETARDATION

By

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Doctor of Philosophy

University of Dublin

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Michaelmas Term 1999

DECLARATION

I hereby declare that this thesis is entirely my own work and that it has not been submitted previously for a degree at this or any other university.

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ABBREVIATIONS

α	Alpha
β	Beta
γ	Gamma
A	Absorbance
ADP	Adenosine diphosphate
Arg	Arginine
ATP	Adenosine triphosphate
BSA	Bovine Serum Albumin
DEAE	Diethylaminoethyl
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal Growth Factor
ELISA	Enzyme Linked Immunosorbent Assay
FDP	Fibrin(ogen) Degradation Products
FnDP	Fibrin Degradation Products
g	grammes
g/l	grammes per litre
Gly	Glycine
Glu	Glutamine
HBSS	Hank's Balanced Salt Solutions
His	Histidine
HMW	High Molecular Weight
Ile	Isoleucine
IU	International Units
IUGR	IntraUterine Growth Retardation
l	litre
Leu	Leucine
LMW	Low Molecular Weight
Lys	Lysine
m	milli
M	molar
M_r	Molecular Weight
mg	milligrammes
mls	millilitres
mm Hg	millimetres of mercury
mol/l	moles per litre
ng	nanogrammes (10^{-9})
nm	nanometres
OD	Optical Density
PAGE	polyacrylamide Gel Electrophoresis
PAI-1	Plasminogen Acivator Inhibitor - 1
PAI-2	Plasminogen Acivator Inhibitor - 2
PAP	Papanicolau
PBS	Phosphate Buffered Saline
PET	PBS-EDTA-Tween
PGI_2	Prostacyclin

Phe	Phenylalanine
pNA	para-Nitroaniline
rpm	revolutions per minute
SDS	Sodium Dodecyl Sulphate
SEM	standard error of the mean
t-PA	Tissue Plasminogen Activator
t-Paag	Tissue Plasminogen Activator antigen
t-Paact	Tissue Plasminogen Activator activity
TRIS	tris(hydroxymethyl) amino methane
TXA_2/PGH_2	Thromboxane A_2/Prostaglandin H_2
µg	micrognammes (10^{-6})
µl	microlitres
µM	micromolar
u-PA	Urokinase Plasminogen Activator
Val	Valine

SUMMARY

Normal pregnancy is accompanied by extensive changes in the coagulation and fibrinolytic systems. Within the uteroplacental vasculature decidual spiral arteries undergo extensive morphological changes to allow for increased blood flow to the placenta. These changes include fibrin deposition in the placenta and placental bed. Cytotrophoblasts have been found in proximity to these fibrin deposits. In pre-eclampsia (PET) and intrauterine growth retardation (IUGR) increased deposits of fibrin are found in the placenta and placental bed. Poor implantation into the placental bed by trophoblast cells is associated with PET and IUGR.

In this study the fibrinolytic ability of trophoblast cells from the placenta and placental bed were examined following cell culture. In addition the tissue levels of PAI-1, PAI-2, t-PA and u-PA were measured following organ culture. In addition the invasive properties of trophoblast cells was examined.

In placental and placental bed extracts PAI-1 was predominately secreted by the placenta. In pre-eclampsia and IUGR, PAI-1 levels are significantly increased while PAI-2 levels decreased. This suggests a decrease in placental expression of PAI-2 due to placental dysfunction. The raised levels of PAI-1 in extracts are supplemented by the secretion of PAI-1 from endothelial cells within the placenta. Cell culture identified that trophoblast cells produced and secreted PAI-1 and PAI-2. Increased PAI-1 levels are found in trophoblasts of pre-eclamptic and IUGR placenta and

placental bed. Invasion assays showed a decrease in invasive activity through fibrin and matrigel for trophoblast cells derived from the placenta of pre-eclamptic and IUGR pregnancies. The results suggest that PAI-1 may be involved in regulating trophoblast invasion. Furthermore, cell associated plasminogen activator activity is reduced in pre-eclamptic patients when compared to normal pregnancy. The results suggest that the fibrinolytic system is not only important in regulating fibrin deposition in the spiral arteries but in regulating trophoblast migration in the placental bed.

CHAPTER 1
INTRODUCTION

1.1 Haemostasis

Haemostasis is a complex process which depends upon the interaction of the vessel wall, platelets, blood coagulation and fibrinolysis. The primary role is to prevent blood loss and to restore the function of injured vessel walls.

1.2 Coagulation

Coagulation involves a complex interaction of soluble enzymes and substrates leading ultimately to the conversion of fibrinogen to fibrin. Fibrin acts locally at the site of vessel injury to stabilise the platelet clot. The clot so formed will be dissolved by the process of fibrinolysis. The haemostatic process by which excessive blood loss is prevented involves a dynamic interaction between coagulation, platelets, the vessel wall and fibrinolysis. The coagulation system is organised into two pathways; the intrinsic pathway in which all the components are present within the blood and the extrinsic pathway so called because some of the key factors lie outside the blood.

Intrinsic Blood Coagulation

Activation of the intrinsic pathway *in vivo* is probably the result of blood, at the site of a damaged vessel, coming in contact with collagen elastic fibres and other tissues (Walsh, 1972). The coagulation system is organised so that the product of each reaction acts as the enzyme in a subsequent reaction. This is known as the "cascade" or "waterfall" system (Davie and Ratnoff,

1964; MacFarlane, 1964). The key contact factor is factor XII (Hageman factor) which interacts with prekallikrein and high molecular weight kininogen to form an active serine protease (factor XIIa) which in turn activates factor XI to XIa. Subsequently activated factor XI (XIa) clears the factor IX molecules by a two-step reaction to give an active molecule which then complexes with factor VIII and calcium ions on phospholipid micelles which have been produced from the aggregated blood platelets. The complex formed by the adsorption of VIII-IXa-Ca ions on the phospholipid then acts on factor X in the final common pathway.

Extrinsic Coagulation

The extrinsic pathway is initiated by the release of tissue factor (thromboplastin) from injured tissue. Tissue factor consists of a specific protein (apoprotein III) and a mixture of phospholipids (Nemerson and Pitlick, 1970). Tissue factor activates plasma factor VII in the presence of calcium ions and in turn activates factor X in small amounts (Jesty and Nemerson, 1977). This response is amplified by a feedback mechanism as factor Xa further activates VIIa to a more active enzyme form α - VIIa which accelerates the conversion of prothrombin to thrombin (Blomback *et al.*, 1978; Jackson *et al.*, 1984; Bajal *et al.*, 1981).

The Final Common Pathway

A complex of activated factor X, calcium, factor V and phospholipid activates

prothrombin by two proteolytic cleavages to the active enzyme thrombin (Milstone, 1964). This complex provides a powerful feedback mechanism by altering the molecular structure of factor VIII and enhancing its proteolytic action (Marler *et al.*, 1982). Thrombin's proteolytic action cleaves inactive fibrinogen to fibrin in a two-step reaction. The triggering event for the transformation of fibrinogen into fibrin is the thrombin-catalysed release of the fibrinopeptides A and B. As a result of the removal of these relatively small, polar peptides, the parent molecules spontaneously polymerise into the fibrin network. Cross-linking by FXIIIa makes fibrin less susceptible to lysis by fibrinolytic enzymes (Gormsen *et al.*, 1967).

Although the intrinsic and extrinsic systems have been described separately there are cross over activities which are of clinical relevance. These include the fact that factor XIIa can activate factor VII; factor VIIa can activate factor IX; factor VIII is activated by IIa and Xa and factor IXa can activate factor VII.

Inhibitors of Coagulation

A system of naturally occurring coagulation inhibitors prevent inappropriate activation of coagulation and maintain homeostatic balance.

Antithrombin III (ATIII) is a glycoprotein of molecular weight 58kDa which is produced in the liver. It is the principal inhibitor of blood coagulation. It is present in a concentration of 3μmol/L and it is a major inhibitor of thrombin and activated factor X (Biggs, 1970). The formation of thrombin -

antithrombin III complex involves initial cleavage of a peptide bond in ATIII between Arg-385 and Ser-386, near the carboxy-terminal (Bjork and Lindahl, 1982). Upon dissociation of the complex, active thrombin is liberated (Seegers, 1980) but the antithrombin III is in a proteolytically modified form which has lost the ability to neutralise thrombin (Danielsson and Bjork, 1980). Critical to the function of ATIII is the presence of minute amounts of heparin which will enhance its actions (Rosenberg and Damus, 1973). Antithrombin III also inhibits factors IXa, XIa and XIIa (Barrowcliffe and Thomas, 1987).

Intravascular activation of coagulation results in the formation of thrombin which, in turn, is inactivated by a complex formed with its major inhibitor, antithrombin III. This process leads to the appearance of thrombin-antithrombin III (TAT) complexes in peripheral blood (Kobayashi and Terao, 1987). Consequently measurement of plasma TAT concentrations allows the detection of an activation of the coagulation cascade.

Protein C is a serine protease, a glycoprotein of molecular weight 62kDa, which is produced by the liver. It circulates in the plasma in a concentration of approximately 0.08μmol/L. Protein C was found to be an anticoagulant (Kisiel, 1976) and to be the anticoagulant previously identified as autoprothrombin IIa (Seegers *et al.*, 1976). Cleavage of an N-terminal tetradecpeptide from the heavy chain of protein C converts the zymogen into the fully active enzyme. The activating reaction is regulated in the manner of the family of Vitamin K dependent zymogens: the activating proteinase

(thrombin) associates with a cell surface cofactor to attain the catalytic efficiency required to activate Protein C under physiological conditions. The rate of conversion is enhanced many thousand fold by the presence of thrombomodulin (Esmon, 1982). Activated protein c is a powerful inhibitor of both activated factors VIII and V and is one of the prime methods of control of coagulation activation (Kisiel, 1977; Vehar and Davie, 1980).

Protein S acts synergistically with activated protein C. it is a glycoprotein of molecular weight 69kDa and it is present in plasma at a concentration of 0.3μmol/L (Forbes and Greer, 1992). It was reported in 1980 that protein S enhances both the binding of activated protein C to phospholipids and the degradation of factor Va (Walker, 1980). Protein s exhibits anticoagulant activity which is independent of the presence of activated protein C and is mediated by competitive inhibition of factor Xa (Mitchell, 1988). Protein S has also been shown to accelerate the inactivation of purified factor VIII by activated protein C (Gardiner *et al.*, 1984). Thrombin degrades protein S to its inactive form. As with protein C, protein S deficiency has been described and is associated with venous thrombosis (Comp, 1984; Schwarbz, 1984).

CI inactivator has inhibitory activity on the initial phases of the intrinsic pathway, inhibiting factors XIIa, XIa and kallikrein. It is a neurominoglycoprotein with a molecular weight of 104kDa and is a single chain structure (Harpel *et al.*, 1976). It has a broad spectrum of activity but was first described as an inhibitor of the first step of complement (Harpel *et al.*, 1976). It has also been described as an inhibitor of the fibrinolytic and

esterolytic activities of plasmin (Schreiber, 1973).

Other thrombin inhibitors include α-2-macroglobulin (Jesty, 1986), α-1-antitrypsin (Learned, 1976) and heparin co-factor (Tollesfon, 1982). α-2-macroglobulin is capable of inactivating a large number of serine proteases such as plasmin, thrombin, kallikrein and factor XIIa (Harpel, 1970; 1976; Jesty, 1976).), α-1-antitrypsin inhibits a number of proteases in irreversible reactions. The main coagulation components inhibited are kallikrein, factor XIa and thrombin (Learned, 1976; Scott, 1982). Heparin co-factor has relatively little action on activated factor X (Tollefson, 1982).

1.3 Fibrinolysis

Fibrinolysis is a Latin-Greek hybrid and means "fiber dissolution". It is best summarised as the enzymatic breakdown of fibrinogen and fibrin by plasmin. The fibrinolytic system acts as the major defence against fibrin deposition on the vessel wall and is thus an important protection against thrombosis. It plays a role not only in the removal of fibrin from the vascular bed (Astrup, 1956; Fearnley, 1973; Astrup, 1978; Collen, 1980) but also in many other biological processes including macrophage migration, ovulation, blastocyst implantation, tissue remodelling and malignant cell invasion (Gaffney, 1994).

The basic components of the fibrinolytic system include plasminogen and plasmin, plasminogen activators, inhibitors of fibrinolysis and fibrin degradative products. The proenzyme, plasminogen can be converted into an

active form, plasmin, by plasminogen activators (Castellino, 1979). The active enzyme then attacks and breaks down the insoluble polypeptide into a number of small soluble peptides called fibrin degradative products. These soluble products are then carried by the bloodstream to the kidneys and excreted.

1.4 Historical Background

In 1893, Dastre coined the term "fibrinolysis", which he proposed after his experiments on the disappearance of fibrin from blood of dogs stressed by repeated haemorrhagic shocks (for review, see Marsh, 1981).

At the beginning of the twentieth century Morawitz (1906) showed that post-mortem fluidity of blood was due to an absence of fibrinogen and conjectured that this resulted from enzyme digestion. In 1908, Nolf proposed the existence of a dynamic equilibrium, where fibrin formed during coagulation was removed by an active lytic substance released from the vessel wall.

In 1933, Tillet and Garner observed that culture filtrates from ß-haemolytic streptococci could dissolve fibrin. They suggested that the streptococcus produces a fibrinolytic enzyme which they termed "streptococcal fibrinolysin". It was later shown that, apart from the streptococcal factor, lysis was also dependent on some "lytic factor" present in plasma globulins (Milstone, 1941). Christensen and MacLeod (1945), believing that the active factor produced by streptococci was an enzyme, named it streptokinase. Furthermore, they showed that the "lytic factor" described by Milstone was

plasminogen and that plasminogen could be converted into its active form, plasmin, by streptokinase (Christensen and MacLeod, 1945).

In 1947, Astrup and Permin demonstrated that a tissue activator of plasminogen was responsible for cellular fibrinolysis. Todd (1959) showed that the tissue plasminogen activator is localised to the endothelial cells of the vasculature.

Fibrinolytic activity in urine was first observed by MacFarlane and Pilling (1947). Williams (1951) recognised that this fibrinolysin in urine was a plasminogen activator and Sobel and his coworkers coined the term "urokinase" in 1952.

In 1963, Brakman and Astrup noted that plasma from pregnant women inhibited urokinase-induced fibrinolysis. Further studies demonstrated that plasminogen activator inhibitor activity was present in placenta (Kawano *et al.*, 1968, 1970; Kawano & Uemura, 1971) and produced by placental tissue explants *in vitro* (Bernik & Kwaan, 1971; Astedt *et al.*, 1972). However, in these early studies inhibition of plasmin was not excluded, as inhibition of plasminogen activator activity was measured via plasmin-mediate degradation of fibrin. In 1978, Holmberg *et al.* purified an inhibitor from placental homogenates that rapidly formed complexes with urokinase.

Luskutoff and Edington (1977) reported that endothelial cells secreted a molecule with plasminogen activator inhibiting activity. Some years later several groups demonstrated that human plasma did contain a specific plasminogen activator inhibitor which rapidly inactivated physiological

concentrations of t-PA (Kruithof *et al.*, 1983; Chmielewska *et al.*, 1983; Verheijen *et al.*, 1984; Wiman *et al.*, 1984a).

1.5 Plasminogen-Plasmin

Plasminogen is the inactive precursor of plasmin and is produced in the liver (Raum *et al.*, 1980, Saito *et al.*, 1980) and is found in small quantities in most tissues where it is involved in physiological cell migration and repair processes. It is a single-chain glycoprotein of 92kDa, containing 791 amino acids and 2% carbohydrate. The native form of plasminogen has glutamic acid at the N-terminal and is called Glu-plasminogen. It has a clearance rate of 2.2 days. A slightly degraded form, Lys-plasminogen is cleared more rapidly with a half-life of 0.8 days (Collen & de Maeyer, 1975).

The domains that can be recognised in plasminogen are the preactivation peptide, five kringles and the protease domain. The preactivation peptide is located at the N-terminal sequence. Plasmin cleaves the plasminogen molecule at several peptide bonds within this sequence giving rise to a shorter molecule with Met, Lys or Val at the new N-terminal. All these forms are commonly called Lys-plasminogen. The peptide bonds which undergo proteolytic cleavage by plasmin are the Arg 68 - Met 69, Lys 77 - Lys 78 or Lys 78 - Val 79 (Wallen & Wiman, 1970, 1972). This cleavage results in profound conformational changes that render Lys – plasminogen more easily activatable by plasminogen activators. In addition, this step plays an important role in regulating fibrinolysis because Lys – plasminogen has a

higher affinity for fibrin than Glu – plasminogen. Kringles 1 and 5 have been shown to have high affinity for native fibrin (Petersen and Suenseon, 1990; Wu *et al.*, 1990). In the presence of partially digested fibrin, the affinity of Glu – plasminogen approaches that of the Lys – form. This is due to a conformational change in the structure of Glu – plasminogen (Tran – Thang *et al.*, 1986; Fleury and Angles – Cano, 1991).

The five kringles confer to plasminogen the ability to bind α_2-antiplasmin (Wiman *et al.*, 1979), histidine-rich glycoprotein (Lijnen *et al.*, 1980), thrombospondin (Silverstein *et al.*, 1984), tetranectin (Clemmensen *et al.*, 1986), extracellular matrix (Knudsen *et al.*, 1986).

The protease domain is located at the C-terminal end of the molecule. It contains 230 amino acids and resembles that of other serine proteases. The catalytic site contains regions of high homology surrounding the three amino acids His 603, Asp 646 and Ser 741.

Two distinct glycoforms are found in plasma. Variant I plasminogen is glycosylated at Asn 289 and Thr 346. Variant II plasminogen is glycosylated only at the threonine site (Castellino, 1984).

Conversion of plasminogen to plasmin is accomplished by proteolytic cleavage of a single bond, Arg 560 – Val 561, which transforms the molecule to plasmin, a two chain serine protease linked by internal disulphide bonds. The larger A chain (60kDa) and the smaller B chain (25kDa) are connected by disulphide bridges at Cys 547 – Cys 665 and Cys 557 – Cys 565. The first bond is homologous to the interchain disulphide bonds of thrombin and

factor X, while the activator – sensitive Arg 560 – Val 561 is located within the Cys 557 – Cys 565 disulphide bridge; this arrangement is unique to plasmin and appears to facilitate interaction between activators and the cleavage site (Summaria *et al.*, 1972; Sottrup – Jensen *et al.*, 1978). The serine active site of plasmin is located on the B chain. The active centre is formed by His 602, Asp 645 and Ser 740.

Plasmin functions as an endopeptidase acting specifically as a serine protease. It cleaves only peptide bonds having either Lys or Arg on the carboxyl side of the molecule.

1.6 Plasminogen Activation

The conversion of plasminogen to the active protease plasmin is central to fibrinolysis. Plasminogen activation can occur by three different mechanisms; the intrinsic pathway, the extrinsic pathway and exogenous activation.

The intrinsic pathway has a common link with blood coagulation as all the factors involved in initiating fibrinolysis are present in the blood (factor XII, high molecular weight kinnogen and pre-kallikrein) (Niewiarowski and Prou-Wartelle, 1959; Colman, 1969). While the extrinsic system is activated by fibrin, the physiological trigger of the intrinsic system remains unknown.

In the extrinsic pathway of plasminogen activation, the tissue plasminogen activator (tPA) is released from the tissues or endothelium in response to various stimuli. The exogenous pathway is activated by urokinase (uPA),

streptokinase, APSAC (anisoylated plasminogen streptokinase activator) and staphylokinase.

1.7 Physiological (Endogenous) Plasminogen Activators

Tissue Plasminogen Activator

The principal site of synthesis of tPA is the endothelial cell (Levin & Loskutoff, 1982; Kristensen *et al.*, 1984). Further studies have shown that mesothelial cells, megakaryocytes and monocytes are also capable of synthesising tPA (Brisson-Jeanneau *et al.*, 1990a, 1990b; Hart *et al. 1989;* Van Hindsbergh *et al.*, 1990; Latron *et al.*, 1991).

tPA, like other serine proteases, is synthesised and released from cells in a single chain form. It is a glycoprotein with a molecular weight of approximately 69kDa. The native molecule consists of a single polypeptide chain of 527 or 530 amino acids and contains between 7 and 13% carbohydrate. The A-chain of tPA contains four distinct domains. At the N-terminus is the finger domain which extends from residues 6 to 43 and exhibits homology with the first finger of bovine fibronectin (Petersen *et al.*, 1983). The epidermal growth factor (EGF) domain is homologous to the murine and human EGF (Savage *et al.*, 1973) and extends from residues 44 to 92. This region probably is responsible for the recognition of clearance receptors on cell surfaces. There are two kringle domains. Most investigators have not discovered a particular function for kringle 1 (van Zonneveld *et al.*,

1986a; Verheijen *et al.*, 1986a; de Munk *et al.*, 1989), except one group who claim that kringle 1 has a lysine-binding site and thus binds to fibrin (Gething *et al.*, 1988) and another which reported that a tPA mutant which lacked the finger- and the kringle 2 domain stimulated plasminogen activation in the presence of fibrin (Stern & Weidle, 1990). Kringle 2 has an affinity for fibrin, and for lysine and ∞-amino carboxylic acids, such as ε-amino caproic acid (EACA) (van Zonneveld *et al.*, 1986a, 1986b; Verheijen *et al.*, 1986a). The protease domain, also called B-chain, shows homology with the B-chains from other serine proteases. It spans 230 amino acids and contains the active site made up of three amino acids: His 322, Asp 371 and Ser 478 (Pennica *et al.*, 1983). The histidine residue is methylated when tPA is irreversibly inhibited by the inhibitor D-Phe-Pro-Arg chloromethyl ketone (PPACK). The serine residue is blocked when tPA is irreversibly inhibited by diisopropylflourophosphate (DFP) or phenyl methyl sulphonyl flouride (PMSF). Cleavage at Arg 275 – Ile 276 converts the single chain to the two-chain form, which is held together by a single disulphide bond. The B-chain is involved in the activation of plasminogen while the A-chain is required for the stimulation of tPA by fibrin (Rijken & Groeneveld, 1986).

The activity of tPA is fibrin specific. In the absence of fibrin, tPA is a very poor plasminogen activator. In the presence of fibrin, the catalytic activity of tPA is up to 1000-fold higher and may be attributed to the formation of a ternary complex between tPA, plasminogen and fibrin (Ranby, 1982; Hoylaerts *et al.*, 1982).

Single-chain tPA (sctPA) has a slightly lower catalytic activity in activating plasminogen in the absence of fibrin than tctPA (Ranby, 1982; Rijken *et al.*, 1982). However, these differences disappear in the presence of fibrin, probably because sctPA bound to fibrin takes on a configuration that is similar to tctPA.

Urokinase Plasminogen Activator

In human plasma, uPA antigen concentrations range from 2 to 7 ng/ml (Kirchheimer & Binder, 1984; Darras *et al.*, 1986). There is no sex difference, but values tend to be higher in older subjects and higher concentrations are found in patients with liver cirrhosis, and hepatoma (Kirchheimer *et al.*, 1985). uPA is synthesised widely by fibroblasts, epithelial cells and macrophages (Bachmann, 1994; Grav and Moroz, 1989).

Unlike tPA, uPA does not form complexes with PAI-1 in normal human plasma. Single- (scuPA) and two-chain (tcuPA) are rapidly cleared from the circulation with a half-life of approximately 5 to 10 minutes (Stump *et al.*, 1987; Kohler *et al.*, 1991).

The mature uPA is a single chain glycoprotein (pro-uPA or scuPA) with a molecular weight of 55kDa, consisting of 411 amino acids and containing about 7% carbohydrates (Gunzler *et al.*, 1982a, 1982b; Steffens *et al.*, 1982; Kasai *et al.*, 1985). A number of proteases are able to convert scuPA to tcuPA (also called HMW - uPA) by cleavage of the Lys 158 - Ile 159 bond. In human plasma these are plasmin, kallikrein, factor XIIa (Ichinose *et al.*, 1986;

Hauert *et al.*, 1989) and in tissues cathepsin B and L (Kobayashi *et al.*, 1991; Goretzki *et al.*, 1992).

tcuPA is a multidomain protein in which the N-terminal A chain (20kDa) and the C-terminal catalytic, serine protease B chain (34kDa) are linked by a single disulphide bond (Gunzler *et al.*, 1982a, 1982b). A low molecular weight uPA (LMW - uPA), which is endowed with catalytic activity and inhibitor - binding capacity but is missing the amino-terminal 135 residues, can be isolated after cleavage of the bond between Lys 135 and Lys 136 by plasmin.

The A chain contains the receptor binding region of uPA, corresponding to amino acid residues 20 - 32, in the so- called Growth Factor Domain (GFD), and a kringle domain (Appella *et al.*, 1987). Pro-uPA, active uPA and the amino - terminal 135 residues long fragment of uPA (ATF) bind to cell surface urokinase receptor (uPAR) with high affinity unlike LMW uPA, which does not bind uPAR. The kringle domain of scuPA lacks high affinity for fibrin and its analogs. scuPA does not circulate complexed to PAI-1. However, the free molecule has comparatively low intrinsic catalytic activity (Petersen *et al.*, 1988; Kasai *et al.*, 1985; Hussain, 1991).

The B chain contains the full proteolytic activity (Sumi & Robbins, 1983). In analogy to other serine proteases, the amino acids His 204, Asp 255 and Ser 356 form the catalytic site.

Binding to uPAR regulates uPA activity by mechanisms that either accelerate its conversion from pro-uPA to its active form or inhibit the active

enzyme through specific inhibitors (Cubellis *et al.*, 1989; Ellis *et al.*, 1989, 1990,1991; Stephens *et al.*, 1989; Berkenpas & Quigley, 1991; Manchanda & Schwartz, 1991).

1.8 Exogenous Plasminogen Activators

Streptokinase

Streptokinase (SK) is a non-enzyme protein which requires the formation of a complex with plasminogen to act upon other plasminogen molecules. It has a molecular weight of 47 kDa and contains 414 amino acids. SK forms a 1: 1 stoichiometric complex with human plasminogen (Wohl *et al.*, 1978, Castellino, 1979) which results in a conformational change of the plasminogen molecule exposing the active site. The SK-plasminogen complex is then converted into a SK-plasmin complex in which SK undergoes proteolytic degradation.

Both the SK-plasminogen and the modified SK-plasmin complex are efficient activators of plasminogen. Plasminogen and plasmin have some affinity for fibrin which is not lost upon complex formation with SK (Cederholm-Williams, 1980). α_2-antiplasmin rapidly inactivates plasmin which is not bound to fibrin but the SK-plasmin(ogen) complexes are protected to a certain extent from this deactivation (Cederholm-Williams *et al.*, 1979). Streptokinase has a half-life of 20 to 30 minutes in humans (Pfeifer *et al.*, 1969; Gonias *et al.*, 1982) and has been widely used for the

thrombolytic treatment of venous and arterial thrombosis.

APSAC

The Anisoylated Plasminogen –SK Activator (APSAC) is a man-made astute modification of the SK-plasminogen complex, which is based on the idea of utilising the preserved fibrin binding capacity of the SK-plasminogen complex, but rendering the active site centre temporarily inactive until the complex has bound to fibrin. This was achieved by coupling of *p* – anisoyl to the active site serine of plasminogen (Smith *et al.*, 1981).

Staphylokinase

The thrombolytic properties of staphylokinase have been known for more than 40 years (Lewis and Ferguson, 1951). It is a protein produced by *Staphylococcus aureus* with a molecular weight of 15 kDa (Lack, 1948). As with SK, staphylokinase is not an enzyme, but form 1: 1 stoichiometric complexes with plasminogen which act as plasminogen activator. In the absence of fibrin, the activator complex is rapidly inhibited by α_2-antiplasmin (Lijnen *et al.*, 1991,1992). The fibrin affinity of plasminogen is preserved in the activator complex and fibrin-bound complex is somewhat protected from inactivation by α_2-antiplasmin. *In vitro*, staphylokinase appears to be more effective and fibrin specific than SK (Matsuo *et al.*, 1990; Lijnen *et al.*, 1991, 1992).

1.9 Inhibitors of Fibrinolysis

Inhibitors of the fibrinolytic system constitute two main types: antiplasmins which inhibit plasmin and anti-activators, the inhibitors of plasminogen activation.

The majority of fibrinolytic inhibitors are members of the serine protease inhibitor (SERPIN) family. Serpins are highly homologous proteins that are derived from a common ancestral gene some 500 million years ago (Travis and Salvesen, 1983; Carrell and Boswell, 1986; Huber and Carrell, 1989). Inhibition of serine proteases by serpins occurs through interaction of the catalytic centre of the protease with the exposed loop of the inhibitor. The interaction of a serpin with its target protease may follow either one of two pathways, denoted the 'substrate' and 'suicide' pathway, respectively.

Four immunologically distinct molecules with plasminogen activator inhibitor (PAI) activity have been identified. These include PAI-1, PAI-2, PAI-3 and protease nexin.

Plasminogen Activator Inhibitor 1

Plasminogen activator inhibitor 1 (PAI-1) is a 50kDa glycoprotein that rapidly forms inactive, 1:1 equimolar complexes with tPA and uPA with second-order association rate constants k_1 of 1-2 x 10^7 M^{-1} s^{-1} (Kruithof *et al.*, 1984; Thorsen *et al.*, 1988).

PAI-1 was first identified in conditioned media of human endothelial cells

(Loskutoff *et al.*, 1977; 1983) and rat hepatoma cells (Seifert & Gelehrter, 1978; Colman, *et al.*, 1982). It is found in a variety of other sources such as human liver and spleen (Simpson *et al.*, 1989). In 1986, Philips *et al.* suggested a placental source for PAI-1. This was confirmed when Feinberg *et al.* (1989) demonstrated the presence of PAI-1 mRNA in cultured trophoblast from term placenta.

Blood contains two pools of PAI-1 (Erickson *et al.*, 1984; Sprengers *et al.*, 1984). Platelets constitute the main reservoir (100-200 ng/ml blood), while a minor pool is present in plasma (10 ng/ml) (Kruithof *et al.*, 1984). Studies have indicated that PAI-1 is stored in an active configuration within the α-granules of platelets (Sprengers *et al.*, 1984).

In 1986, four groups independently isolated full-length human PAI-1 cDNAs (Andreasen *et al.*, 1986; Ginsburg *et al.*, 1986; Ny *et al.*, 1986; Pannekoek *et al.*, 1986). The cDNA encodes a precursor protein of 402 amino acids, consisting of a single peptide (23 residues) and a mature protein of 379 amino acids. The reactive centre is located 33 residues from the carboxyl-terminus and comprises the amino acids Arg-346 and Met-347 (Andreasen *et al.*, 1986). The mature protein is devoid of cysteine residues and thus contains no disulphide bonds. PAI-1 has three asparagine-linked glycosylation sites which do not affect its biological function. Sequence comparisons reveal that PAI-1 is approximately 30% homologous with α_1 - antitrypsin and antithrombin III.

PAI-1 exists in at least four different structural forms: the active form,

the latent form, a non-inhibitory substrate form and the inactive cleaved form. Pai-1 is synthesised as an active inhibitor. It is not stable, but is spontaneously converted into a latent, non-inhibitory form. The half-life is 2 and 4 hours in a purified system and in plasma, respectively. The longer half-life in plasma is ascribed to the binding of PAI-1 to vitronectin. The stability can be dramatically improved by lowering the pH from 7.4 to 5.5.

Latent PAI-1 can be partially reactivated *in vitro* by treatment by denaturing agents such as sodium dodecyl sulphate, guanidinium chloride and urea, followed by renaturation of the unfolded protein. *In vitro* experiments suggest that some reactivation of latent PAI-1 occurs in the circulation possibly on negatively charged phospholipids of vascular endothelial cell membranes (Vaughan *et al.*, 1990).

A non-inhibitory substrate form of PAI-1 has been isolated which, in contrast to active PAI-1, does not form stable complexes with tPA but is cleaved at the P_1-P_1' bond (Declerck *et al.*, 1992).

PAI-1 is the primary inhibitor of both tPA and uPA. The second-order rate constant for the inhibition of sctPA by PAI-1 is 5.5×10^6 M^{-1} s^{-1}, which is about three times lower than that of tctPA and tcuPA (1.8×10^7 M^{-1} s^{-1}) (Thorsen *et al.*, 1988).

The absence of the growth factor-like domain and the kringle domain in LMW tcuPA does not affect the inhibition of PAI-1, suggesting that only the protease domain is responsible for the interaction (Thorsen *et al.*,

1988). Furthermore, Bjorquist *et al.* (1994) reported that no important interaction sites for PAI-1 are present or exposed in the nonenzymatic part of tPA.

Within the protease domain of uPA and tPA two sites are essential for the rapid interaction with PAI-1. The first is the active centre, which recognises PAI-1 as a pseudo-substrate. The second site is located on a positively charged surface loop adjacent to the active centre of the enzymes. This site is comprised of residues 296 – 302 in tPA (Madison *et al.*, 1990) and residues 179 – 184 in uPA (Adams *et al.*, 1991). Deletions of or mutations in the surface loop result in PAI-1 resistant activators (Li *et al.*, 1992; Keyt *et al.*, 1994).

A well-established cofactor of PAI-1 is vitronectin which binds active PAI-1 but not latent PAI-1 (Seiffert and Loskutoff, 1991). Complexes of vitronectin and PAI-1 have been demonstrated in plasma as well as the matrix of cultured endothelial cells. Vitronectin may regulate the pericellular distribution of PAI-1. In addition, it may protect PAI-1 against inactivation by oxidants (Reilly *et al.*, 1994).

Vitronectin has only a modest effect on the interaction between PAI-1 and tPA or uPA (Edelberg *et al.*, 1991; Keijer *et al.*, 1991). However, binding results in an altered specificity, as the vitronectin – PAI-1 complex becomes an efficient inhibitor of thrombin (Ehrlich *et al.*, 1990).

A second potential cofactor of PAI-1 is heparin, which specifically binds the inhibitor. Although heparin does not increase the reaction rate

between PAI-1 and tPA or uPA, heparin, like vitronectin, strongly accelerates the inhibition of thrombin by PAI-1 (Keijer *et al.*, 1991). In contrast to vitronectin, heparin does not stabilise PAI-1 activity.

PAI-1 has an affinity for fibrin which may result in the accumulation of the inhibitor in thrombi (Wagner *et al.*, 1989). Two binding sites have been identified on fibrin: a small number of high affinity sites and a large number of low affinity sites (Reilly and Hutzelmann, 1992). Occupation of these sites with PAI-1 protects fibrin from proteolytic degradation.

Plasminogen Activator Inhibitor 2

Plasminogen activator inhibitor 2 (PAI-2) is the second well-characterised, rapid and specific PA inhibitor relevant to the *in vivo* regulation of PA activity. PAI-2 was initially named placental-type plasminogen activator inhibitor as a result of its discovery as a PA inhibitory activity in placenta (Kawano *et al.*, 1968). Further studies indicated that PAI-2 is an alpha globulin molecule, which is stable at pH 5.0 - 7.5 (Kawano *et al.*, 1970). By immunohistochemical staining, PAI-2 has been localised to the trophoblastic epithelium (Astedt *et al.*, 1986). This was in accordance with an early observation of absence of fibrinolytic activity in syncytiotrophoblastic cells (Sheppard & Bonnar, 1978). Further studies have shown that PAI-2 is identical to the inhibitor isolated from monocytes, macrophages, granulocytes and human U937 monocyte-like cells (Kopitar *et al.*, 1985; Chapman & Stone, 1985; Kruithof *et al.*, 1986).

Two forms of PAI-2 exist, both of which have identical inhibition characteristics (Mikus *et al.*, 1993); a nonglycosylated form with a molecular weight of 47kDa (Kruithof *et al.*, 1986) and a 60kDa, glycoslyated form (Kruithof *et al.*, 1986; Genton *et al.*, 1987; Wohlwend *et al.*, 1987). A distinct difference in cellular localisation has been noted: 47kDa PAI-2 being intracellular and 60kDa PAI-2 being secreted (Genton *et al.*, 1987; Wohlwend *et al.*, 1987a; Wohlwend *et al.*, 1987b).

Partial amino acid and cDNA sequencing has demonstrated that PAI-2 is a member of the serpin superfamily of proteins (Kruithof *et al.*, 1986; Kiso *et al.*, 1988). PAI-2 is a single chain protein of 415 amino acids. The reactive centre (Arg-380 and Thr-381) corresponds to the Arg-Val bond in the plasminogen molecule, which is cleaved by plasminogen activators. PAI-2 has three potential N-linked glycosylation sites (asn-75, asn-115 and asn-339), which all appear to be occupied in glycosylated PAI-2 (Ye *et al.*, 1988). Two common variants of PAI-2 have been described differing at three positions: one having Asp-120, Asn-404, Ser-413 (type A), the other having Asp-120, Lys-404, Cys-413 (type B), both of which have been identified in U-937 cells (Ye *et al.*, 1987; Webb *et al.*, 1987; Schleuning *et al.*, 1987; Antalis *et al.*, Ny *et al.*, 1989; Ye *et al.*, 1989). The presence of transglutamination sites (gln-83, gln-84 and gln-86) provides a mechanism for the localisation or concentration of both types of PAI-2 to cellular or extracellular structures such as trophoblast membranes (Jensen *et al.*, 1989; Jensen *et al.*, 1993; Jensen *et al.*, 1994).

PAI-2 is an efficient inhibitor of uPA (rate constant 10^6 M^{-1} s^{-1}) and two chain tPA (2 x 10^5 M^{-1} s^1), albeit slower than PAI-1, by a factor of 10 and 50 times, respectively (Kruithof *et al.*, 1986; Mikus *et al.*, 1993; Thorsen *et al.*, 1988; Kruithof, 1988). In comparison with PAI-1, PAI-2 is a poor inhibitor of single chain tPA (10^3 M^{-1} s^{-1} *v* 10^7 M^{-1} s^{-1}), which makes it unlikely that PAI-2 contributes to the inhibition of single chain tPA *in vivo* (Kruithof *et al.*, 1986; Astedt *et al.*, 1985). Furthermore, since fibrin-bound tPA appears to be protected from inhibition by PAI-2 (Leung *et al.*, 1987), it is generally accepted that PAI-2 functions primarily to inhibit uPA in the extravascular space and plays only a minor role in controlling tPA-mediated fibrinolysis. PAI-2 binds irreversibly to its target protease, is cleaved in the process and rendered completely inactive; behaving therefore, as a suicide substrate, a feature common to SERPINS in general (Potempa *et al.*, 1983; Remold-O'Donnell, 1983).

Plasminogen Activator Inhibitor 3

Plasminogen activator inhibitor 3 (PAI-3) was purified from human urine by Stump *et al.* (1986) and has a molecular weight of 50kDa (Geiger, 1988). PAI-3 inhibits uPA and thrombin in reactions stimulated by heparin while tPA is weakly inhibited by PAI-3. Its primary role may in fact be as an inhibitor of activated protein C rather than plasminogen activators.

α_2-Antiplasmin

α_2-antiplasmin (α_2-AP) was discovered independently by three groups (Collen, 1976; Mullertz and Clemmensen, 1976; Moroi and Aoki, 1976). It is the major fast-acting plasma inhibitor of plasmin, for which it has a high affinity. It is a glycoprotein with an apparent molecular mass of 70kDa; the actual mass based on amino acid content is 50.5kDa (Holmes *et al.*, 1987). It is present in plasma at a concentration of 1μM. Therefore, it can neutralise the plasmin produced, even if a relatively high proportion of the plasma plasminogen is activated.

Two forms of α_2-AP are detectable in normal human plasma (Clemmensen *et al.*, 1981). One form binds plasminogen and this is the native form of α_2-AP. The second form retains its inhibitory capacity but it cannot bind plasminogen because of its lack of a C-terminal peptide (Sasaki *et al.*, 1986). The C-terminal end of the molecule contains a second site of interaction between plasmin(ogen) and α_2-AP, in addition to the reaction at the plasmin active site. This second site is of great biological significance, in that it reinforces the fibrin specificity of physiological plasminogen activation. Since both tPA and plasminogen bind to fibrin, plasmin can be formed on the fibrin surface. In that situation, both the active site for plasmin and the other site for interacting with α_2-AP are occupied by fibrin. Therefore, the fibrin-bound plasmin is relatively protected from the action of α_2-AP (Collen, 1980).

α_2-AP has three functional roles. The inhibitor's main target is plasmin with

which it forms a 1:1 stoichiometric complex - the inhibition process is dependant upon the presence of free lysine binding sites and an available binding site on the plasmin molecule. Earlier studies described a two stage binding mechanism whereby, a reversible step is followed by an irreversible transition to the final inhibited complex (Christensen and Clemmensen, 1977; Wiman and Collen, 1978). However, a mechanism of reaction in which both steps are reversible has been described (Longstaff and Gaffney, 1991, 1992).

Apart from rapidly inactivating plasmin, α_2-AP also interferes with plasminogen adsorption to fibrin. Furthermore, the inhibitor cross-links with the α-chains of forming fibrin within which it is thought to neutralise the active plasmin (Sakata and Aoki, 1980; Ichinose *et al.*, 1983). α_2-AP is crosslinked to fibrin by the action of factor XIII. The linkage occurs between Gln-2 of α_2-AP and a lysine residue in the fibrinogen Aα chain (Kimura and Aoki, 1986). This process delays the premature dissolution of the clot.

α_2-Macroglobulin

α_2-macroglobulin is a large glycoprotein and is made up of four identical subunits with a total molecular weight of 740kDa (Barrett, 1981). It inhibits plasmin in a slow non-specific manner. It appears to inhibit plasmin formed in excess of the inhibitory capacity of α_2-antiplasmin (Collen, 19760. It is a proteinase inhibitor of wide specificity that inhibits tPA and uPA (Aoki and Harpel, 1984).

α_1-Antitrypsin

α_1-antitrypsin is a glycoprotein of the serpin family with a molecular weight of 55kDa. It inhibits plasmin in a time dependent reaction. Although the exact mechanism of this inhibition is unknown, it is believed to involve enzymatic clearance (Rimon *et al.*, 1966).

C1-Inhibitor

C1-inhibitor is a neuroamino-glycoprotein with a molecular weight of 105kDa (Ratnoff and Lepow, 1957). It is a member of the serpin family but it is unusual in having a large N-terminal domain. It is heavily glycosylated. It is not a significant inhibitor of plasmin or uPA but can form complexes with tPA during exercise and in various pathological states (Booth *et al.*, 1984, 1987).

Antithrombin III

Antithrombin III inhibits plasmin in a time – dependent manner, the inhibitory reaction is accelerated greatly in the presence of heparin (Highsmith and Rosenberg, 1974; Crawford and Ogston, 1975; Machovich *et al.*, 1981). The inhibitor is a glycoprotein of molecular weight 65kDa and is composed of a single polypeptide chain with three disulphide bonds and four oligosaccharides (Petersen *et al.*, 1978). Antithrombin III is synthesised in the liver and has a half-life in the circulation of about three days (Collen *et al.*, 1977).

1.10 Fibrin(ogen)

Fibrinogen is defined as that soluble protein in blood and tissue extract, which, in presence of thrombin, is transformed into the insoluble fibrin network structure.

Fibrinogen is estimated to have a molecular weight of 340kDa (Caspary and Kekwick, 1957). Recently, a new form of fibrinogen has been described which has a molecular weight of 420kDa (Fu *et al.*, 1992; Fu and Grieninger, 1994). This variant occurs in homodimeric form in plasma and constitutes 1 - 2% of the total fibrinogen pool.

Fibrinogen is a dimeric molecule. The two halves consist of three polypeptide chains with a molecular formula of (Aα, bß, γ)2. The half molecules are covalently linked through symmetrical disulphide bonds at the N-terminal ends. Structurally, fibrinogen contains a central nodule and two larger outer nodules (Fowler and Erickson, 1979). The central nodule contains the N-terminal ends of all six polypeptides and is called the E-domain or N-DSK. The outer nodules are known as the D-domains and contain the middle and C-terminal segments of the six chains (Doolittle, 1987).

Fibrin formation is initiated by the thrombin - mediated release of small polar peptides known as fibrinopeptides A and B (FPA and FPB) from the N-terminal portion of the Aα and Bß chains, respectively (Blomback, 1978). FPA is always released at the fastest rate and appears to be required for the activated molecule to be incorporated into the growing polymer (Blomback and Vestermark, 1958; Blomback *et al.*, 1978; 1984).

At the time of gel formation, only small amounts of FPB have been released. Subsequent to gel formation, FPB is release is greatly accelerated and this acceleration may be associated with conformational changes occurring as a result of polymer organisation.

The current view is that polymerisation of activated fibrinogen molecules involves complementary intermolecular interactions of domains in fibrinogen following release of fibrinopeptides. The functional sites uncovered following the release of FPA and FPB are denoted *A* and *B*, respectively, and are located in the N - DSK. The dimeric fibrinogen molecule has two *A* and two *B* sites. Complementary site, *a*, is located in the fragment D domain. The *b* site may be in the fragment D or αC domain. In the dimeric molecule there are two *a* and two *b* sites. In the early phase of polymerisation the *A* site of one molecule interact with the *a* site of another in a half - staggered fashion as was originally suggested by Ferry (1952).

The early fibrin polymers serve as substrate for thrombin in release of FPB and the following interaction of *B*:b sites. The release of FPB is accompanied by increased calcium ion binding and pH shift (Mihalyi, 1988a, 1988b; Mihalyi *et al.*, 1988). This leads to a lateral aggregation of polymers or branching or both (Laurent and Blomback, 1958). The mature fibrin products where *A*:a sites are involves is called fibrin I (or des AA fibrin) and the one when *A*:*a* as well as *B*:*b* sites are operative is names fibrin II (or des AABB fibrin). The domains are functional in fibrinogen as it circulates in blood, but prior to activation of the *A* or *B* domains no polymerisation and

clot formation occurs (Blomback, 1996).

The fibrin polymer that is formed after activation with thrombin undergoes secondary transformation through factor XIII catalysed formation of covalent crosslinks between glutamine and lysine residues in fibrin (Loewy, 1972; Lorand, 1972; Doolittle *et al.*, 1972; McKee *et al.*, 1972). Crosslinking of fibrin involves both γ and α chains in fibrin protomers. ε(γ-glutamyl) lysine bonds are formed between a glutamine residue in one chain and a lysine residue in another chain. γ chains of juxtapositioned fragment D domains are crosslinked in an antiparallel fashion, thus forming fibrin dimers (Doolittle *et al.*, 1972). These crosslinks are by some investigators believed to be longitudinally arranged between contacting ends of two fibrin D domains in one strand of the protofiber (Weisel *et al.*, 1993), whereas others find evidence for the crosslinks to be between fragment D domains of opposing strands in the protofiber (Siebenlist *et al.*, 1995).

1.11 Fibrin Degradation Products

The molecular fragmentation of fibrinogen was first investigated in 1945 (Seegars *et al.*, 1945). Five major fragments, A, B, C, D and E were obtained from the interaction of fibrinogen with plasma and were identified in 1961 (Nussenzweig, 1961). It was subsequently shown that fragments D and E were synonymous with the α and β fragments demonstrated earlier by Seegars *et al.* (1945). Intermediate fibrinogen degradation products, named X and Y fragments, were also reported (Nussenzweig, 1961).

The first major fragment to appear when plasmin digests fibrinogen is termed fragment X and its formation coincides with the appearance of low molecular weight peptides (fragments A, B and C). Fragment X is split further to form two fragments called Y and D. Fragment Y is finally cleaved by plasmin to provide an additional fragment D and fragment E. Fragment D and fragment E are the plasmin resistant core of products of fibrinogen degradation (Marder, 1969; Gaffney, 1987).

While the natural substrate for plasmin is fibrin, much of the experimental data on fibrin dissolution has been obtained from studies on fibrinogen. The conversion of fibrinogen to fibrin which has been described earlier results in the formation of at least three different types of fibrin: non-cross linked, partly cross linked (being joined by γ chains only), or fully cross linked (having both γ - γ and α - α cross links). Since non-cross linked fibrin resembles fibrinogen, digestion by plasmin yields fragments similar to those observed for fibrinogen (Gaffney, 1973; Slade *et al.*, 1976). The spectrum of fragments obtained from partly or fully cross-linked fibrin are similar (Gaffney *et al.*, 1975). Cross-linked fibrin yields a large plasmin resistant fragment termed D dimer (Gaffney, 1973). In addition to D dimer, complexes of D dimer with fragment E have been found in plasmin digests (Hudry-Clergeon *et al.*, 1974). Using chromatography a number of soluble cross-linked fibrin fragments have been obtained after digestion by plasmin (Gaffney, 1980), and have been classified as cross-linked X-oligomers. It has now been established that X-oligomers are the earliest fragments produced

from cross-linked fibrin (Gaffney and Philo, 1981).

1.12 Cellular Receptors for Plasmin Generation

Plasmin generation is fundamental to the solubilisation of fibrin containing thrombi as well as many other biological processes (Collen and Lijnen, 1994; Hajjer and Nachman, 1994). There has been increasing realisation over the past number of years that binding of components of fibrinolysis to surfaces other than fibrin may play a vital role in the regulation of plasmin generation. New studies have investigated the plasmin generating properties of endothelial cells, blood cells, neuronal cells and tumour cells. Of particular interest are cellular receptors, which regulate cell surface plasmin generation.

1.13 Plasminogen Receptors

The plasminogen receptors represent a widespread group of structurally diverse molecules that serve to localise plasminogen on cell surfaces (Hajjer and Nachman, 1994; Plow *et al.*, 1991; Miles and Plow, 1988). They have been reported, for example, on human platelets, monocytes, granulocytes and lymphocytes (Miles and Plow, 1988) and on endothelial cells from several species (Hajjer and Nachman, 1994). The binding of plasminogen to these receptors is rapid, reversible and of physiologically significant affinity and capacity (Miles and Plow, 1988; Hajjar *et al.*, 1988). Glu – plasminogen interaction with the cell surface shows an order

of magnitude increase in the catalytic efficiency of plasmin generation by tPA (Hajjar *et al.*, 1988; Schafer *et al.*, 1989). Lys–plasminogen does not appear to represent a physiologic intermediate in the fibrin–free fluid phase activation of plasminogen (Collen and Lijnen, 1994). However, it may represent a significant intermediate in the cell surface activation of plasminogen (Hajjar and Nachman, 1988).

1.14 Control and Regulation of Fibrinolysis

In the late 1950s theories were published regarding the roles of plasminogen, plasminogen activator and α_2-antiplasmin in the fibrinolytic process. The localisation of fibrinolytic activity, upon the fibrin surface, is common to each of the theories presented (Astrup, 1956; Alkjaersig *et al.*, 1959; Ambrus and Markus, 1960).

In 1980, Collen proposed a model for the regulation and control of fibrinolysis upon the fibrin surface. In the absence of fibrin, tPA interacts quite weakly with plasminogen. However, during fibrin formation, tPA and plasminogen absorb onto the clot and the activation of plasminogen is enhanced greatly. This can be explained by an increase in the affinity of fibrin - bound tPA for plasminogen. The resulting plasmin proceeds to proteolytically degrade the clot. Any free plasmin is inactivated by α_2-antiplasmin in order to avoid unwanted fibrinolytic digestion in the general circulation (Collen, 1980).

The role of uPA in the fibrinolytic process is still under investigation. At the

present time, uPA is thought to be involved in localised fibrinolytic activity within tissue. In tPA-mediated fibrinolysis in the general circulation, uPA appears to play a secondary role. Following activation by tPA, high affinity sites become available in the fibrin-bound plasminogen which induce conformational changes in the molecule. scuPA binds to these sites and assist tPA in breaking down the fibrin structure.

tPA circulates in plasma complexed to PAI-1. Gaffney (1987) suggested that this tPA/PAI-1 complex dissociates in the presence of forming fibrin and activates plasminogen but that it is relatively inactive in the presence of preformed clots. Subsequently, it was reported that tPA is inhibited by fibrin -bound PAI-1 and that free tPA competes with tPA/PAI-1 complexes for common binding sites (Wagner *et al.*, 1989). This study suggested that PAI-1 activity is controlled by and confined to the fibrin surface. In 1990, Paramo suggested that α_2-antiplasmin is the major inhibitor in the fluid phase of blood. In recent years, many studies have to examine the role of cell receptors in cell surface fibrinolysis. Receptors for both plasminogen (Miles and Plow, 1988) and plasminogen activators have been isolated from endothelial cells.

It has been suggested that these cell surface receptors may play a significant role in the regulation of plasmin generation (Hajjar, 1995). Although structurally diverse, these receptors can be classified on a functional basis into two groups. Activation receptors for plasminogen and plasminogen activators serve to localise, and in some cases, potentiate

plasminogen activation and are expressed on endothelial cells, blood cells, neuronal cells and tumour cells. Clearance receptors, on the other hand, serve to eliminate plasmin and plasminogen activators from blood focal microenvironments. They are found mainly on parenchymal hepatocytes and tissue macrophages. The integrated actions of both classes of receptors are essential to the homeostatic control of plasmin activity.

1.15 Normal Pregnancy

The placenta, essentially a foetally derived organ, has evolved as an indispensable element of pregnancy in mammals. It is a structure that allows maternal and foetal blood to come into close proximity to each other for an efficient and proper exchange of molecules. In humans, the establishment of the haemochorial placenta requires that the trophoblast invade the endometrium penetrating into its deep layers until it locates an abundant supply of maternal blood. The human placenta receives its blood supply from more than 100 uteroplacental arteries (Brosens and Dixon, 1966; Boyd and Hamilton, 1970) which are elaborated by the action of migratory interstitial and endovascular trophoblast on the spiral arteries in the placental bed (Harris and Ramsey, 1966; Brosens *et al.*, 1967; Boyd and Hamilton, 1970). To achieve this, trophoblast cells breach the uterine epithelium inclusive of its basement membrane, as well as the subepithelial connective tissue, and eventually invade maternal vessels to come into contact with maternal blood. The mechanisms by which trophoblast cells

invade the uterus, as well as those which control this invasion, have been under investigation for many years. The concept of the trophoblast as an invasive cell was proposed by Graf von Spee at the turn of the century (for review, see Denker, 1977). From histological analysis of implantation sites in the guinea pig, he suggested that trophoblast cells secrete degradative enzymes since the uterine tissue surrounding the trophoblast cells showed signs of degeneration. Kirby (1960, 1963*a*, 1963*b*, 1965) demonstrated that, at least in the mouse, the decidual tissue of the pregnant uterus is likely to prevent trophoblast cells from excessive invasiveness.

Although chemical, physical and immunological mechanisms were proposed for the putative anti-invasive role of the decidual tissue (for review, see Billington, 1971), the precise mechanisms remained to be identified.

1.16 Placentation

The formation of the human placenta begins when the blastocyst penetrates the maternal endometrium to become rapidly embedded in the uterine stroma. As the morula is converted into a blastocyst, trophoectoderm cells form a layer encircling the blastocyst with an inner cell mass at one pole. The human blastocyst usually attaches via its embryonic pole to the posterior wall of the uterus on the sixth day after fertilisation (for review, see Moore, 1988). By the eight day, the blastocyst has differentiated into a peripheral layer of syncytiotrophoblast and an inner layer of cytotrophoblast. The syncytial mass penetrates between epithelial cells and rapidly expands

into the underlying stroma. Cells from the inner cytotrophoblast cell mass contribute to the syncytial mass by proliferation and fusion. By the ninth day, vacuoles or lacunae appear in the syncytium and these rapidly enlarge and fuse. The establishment of a potential uteroplacental circulation occurs when maternal venous capillaries are eroded by the syncytium thus allowing blood to seep into the lacunae system. The lacunae will eventually evolve into the intervillous space of the definitive placenta. By 12 - 13 days after fertilisation, the blastocyst is completely embedded in the decidual stroma.

After this initial phase of implantation, trophoblast differentiation may be considered to occur along two main pathways - villous and extravillous. Villous trophoblast ultimately covers all the chorionic villi of the definitive placenta and is concerned with oxygen and nutrient transport from mother to child. The villi are bathed with blood and hence contact is only made with maternal cells present in the circulation. In contrast, extravillous trophoblast migrates deep into the uterine mucosa as far as the myometrium and encounters many maternal cell types as it invades through uterine tissues.

1.17 Villous Trophoblast

The main function of the complex villous tree, which eventually forms the definitive placenta, is the transfer of nutrients and oxygen from the maternal to the foetal circulation. During the second week of gestation buds of cytotrophoblast start to protrude into the primitive syncytium. These

primary villi are converted into secondary villi by the penetration of the solid cytotrophoblast buds by mesenchymal cells and then into tertiary villi when the mesenchymal core are supplied by embryonic blood vessels. These embryonic vessels link with vessels in the mesoderm of the chorionic plate and ultimately with vessels in the foetus so that by the fourth week the fetoplacental circulation is established.

During the second and third months of pregnancy, rapid extension and branching of the villi result in the villous tree characteristic of the mature placenta. Two layers of trophoblast cover the villi. The inner cytotrophoblast cells sit on a basement membrane covered by the outer syncytiotrophoblast layer. Consequently, it is this syncytiotrophoblast, which lines the entire intervillous space, which comes into contact with maternal blood in a manner similar to endothelial cells. The villous syncytiotrophoblast is also the major source of placental proteins produced during pregnancy.

1.18 Extravillous Trophoblast

Interstitial trophoblast

During the second week of gestation, cytotrophoblast cells rupture the primitive syncytium. At the tips of these early villi, cytotrophoblast cells form a solid core known as the cytotrophoblast cell columns, which fix the villi to the decidua. These early villi are termed anchoring villi. The cytotrophoblast cell columns spread laterally and fuse with neighbouring

columns to form a cytotrophoblast shell. Once the shell has come into contact with the decidua, invading trophoblast cells known as interstitial trophoblast cells enter the uterine mucosa. These cells apparently move towards the decidual spiral arteries, as trophoblast is always concentrated around the arteries with a much sparser number of invading cells in the intervening areas.

Spiral arteries in the decidua basalis that are surrounded by trophoblast show endothelial swelling and a characteristic destruction of the muscular media, which is replaced with fibrinoid material. By 8 weeks of pregnancy interstitial trophoblast has invaded the full thickness of the decidua. As the cells move deeper into the decidua, they become multinucleated and more rounded. These trophoblast cells are known as placental bed giant cells, and are regarded as the terminally differentiated sessile end-point of the extravillous pathway.

During the second trimester a second wave of trophoblast invasion occurs into the myometrium. Destruction of the musculo-elastic tissue of the intramyometrial segments of the spiral arteries occurs with similar fibrinoid deposition ensuing. However, the infiltration in the myometrium is diffuse and the trophoblast cells have the morphology of placental bed giant cells.

Endovascular trophoblast

The cytotrophoblast shell formed from the cell columns of the anchoring villi provides almost a continuous and complete rim around the developing

villous tree during the first trimester. In addition to the interstitial trophoblast, this shell is the origin of endovascular trophoblast.

Where the cytotrophoblast comes into proximity to maternal spiral arteries, the trophoblast cells form a plug seemingly occluding the lumen. From these plug-like areas of the shell, trophoblast move in a retrograde manner down the spiral arteries "like wax dripping down a candle" (Ramsey and Donner, 1980). Endovascular trophoblast invasion continues into the spiral arteries throughout the first trimester. However, this invasion is incomplete and sections of the lumen furthest from the intervillous space may retain residual endothelial cells.

In the second trimester intramyometrial arterial segments also come to be lined partly by endothelial cells overlying the fibrinoid material and trophoblast. Endovascular trophoblasts are only seen in vessels surrounded by interstitial trophoblast and undergone medial disruption. The end result of endovascular trophoblast invasion is the establishment of the so-called physiological changes within the spiral arteries (Brosens *et al.*, 1967; Sheppard and Bonnar, 1974). These physiological changes convert the vessels from muscular end arteries to wide – mouthed sinusoids, so transforming the vascular supply from a high pressure – low flow system to a low pressure – high flow system (Moll *et al.*, 1975). Furthermore, loss of the endothelium and muscular layers render the vessels unable to respond to vasomotor stimuli.

Fibrin deposition occurs in uterine spiral arteries of the placental bed as the

vessels undergo physiological adaptations, required to facilitate increased blood flow to the fetoplacental unit, following the invasion of endovascular trophoblast in early pregnancy (Brosens *et al.*, 1967; Sheppard and Bonnar, 1974). In normal pregnancy, these trophoblast-induced vascular adaptations extend back from their openings into the intervillous space to where the spiral arteries originate from their parent radial arteries in the myometrium. It is thought that these physiological changes are effected in two stages, the conversion of the decidual segments of the spiral arteries by a wave of endovascular trophoblast migration in the first trimester and the myometrial segments by a subsequent wave in the second trimester (Robertson *et al.*, 1975; Pijnenborg *et al.*, 1983). The physiological fibrin deposition within the spiral arteries in normal pregnancy may be due either to an accelerated fibrin formation or an inhibition of fibrinolytic activity. Degenerative changes of placental villi have been shown to be related to the degree of luminal occlusion of decidual spiral arteries (Sheppard and Bonnar, 1980). These changes may cause alterations in fibrinolytic activity within the placenta resulting in greater intervillous and perivillous fibrin deposition.

In preeclampsia trophoblast invasion is somewhat impaired and only about half to two – thirds of the decidual spiral arteries undergo physiological changes (Khong *et al.*, 1986). Furthermore, the conversion of myometrial components of the spiral arteries fails to occur even in vessels where the decidual segments have undergone physiological change (Brosens *et al.*,

1972; Sheppard and Bonnar, 1981; Gerretsen *et al.*, 1981). This suggests that the primary invasion of trophoblast is partially impaired, and the second wave fails to occur or is inhibited. Thus, a qualitative and quantitative restriction in the physiological process of trophoblast invasion results in reduced placental blood flow (Robertson and Khong, 1987). This will become more threatening as the pregnancy advances and the demands of the conceptus increases. Additionally, as the vessels maintain muscular coats, they remain sensitive to vasomotor stimuli.

Preeclampsia is also associated with a typical though non-specific muscular legion termed 'acute atherosis" (Zeek and Assali, 1950; Sexton *et al.*, 1950; Labarrere, 1988) which can be seen in the intramyometrial segments of the spiral arteries in the placental bed, the basal arteries and the decidual parietalis but not in the intradecidual vessels of the placental bed which have undergone physiological change. This was first noted by Hertig in 1945 and was subsequently termed acute atherosis because of the presence of foam cells in the damaged vessel wall (Hertig, 1945).

Acute atherosis is a necrotizing arteriopathy characterised by fibrinoid necrosis, accumulation of lipid-laden macrophages and damaged cells, fibroblast proliferation and a mononuclear cell perivascular infiltrate (Labarrere, 1988). In the early stages, this lesion is characterised by endothelial damage, insudation of the plasma constituents into the vessel wall, cell proliferation and medial necrosis of the lipid containing muscle cells (de Wolf *et al.*, 1975). The lipid released is subsequently taken up by

macrophages which accumulate in the vessel wall. More recently, Shanklin and Sibai (1989) have shown that endothelial damage can be seen ultrastructurally in the decidua at sites outwith the placental bed throughout the maternal – foetal boundary.

Failure of trophoblast invasion and acute atherosis are not specific to preeclampsia as these lesions can be seen in normotensive intrauterine growth retardation (Sheppard and Bonnar, 1976, 1981; de Wolf *et al.*, 1980), chronic and nonproteinuric gestational hypertension (Pijnenborg *et al.*, 1991). Furthermore, this is not an all or none phenomenon as there is a great deal of variation in the extent of these pathological findings in the hypertensive disorders of pregnancy (Pijnenborg *et al.*, 1991).

These abnormalities in the maternal placenta may produce ischaemia of the foetal placenta. Pathological examination of the foetal placenta in preeclampsia reveals infarcts, patchy necrosis and intracellular damage of the syncytiotrophoblast, and an increase in villous cytotrophoblastic cells (Fox, 1988).

1.19 Matrix-Degrading Proteinases

Enzymes that can digest matrix proteins are believed to play an important role in the invasion by trophoblasts of the placental bed. There are two main families of enzymes that have been implicated in the degradation of the protein components of the extracellular matrix (ECM). The two families of enzymes are interactive, initiating a cascade of proteolytic events that result

in matrix degradation.

The first of these families include the plasminogen activators (t-PA, u-PA) and their inhibitors (PAI-1, PAI-2 and PAI-3). The second important family of enzymes are the metalloproteinases. These are usually secreted as latent proenzymes which need to be activated and u-PA has been implicated as an important physiological activator. Metalloproteinases are usually described under three subclasses - collagenases, gelatinases and stromelysins - with distinctive but overlapping substrate specificities.

The collagenase cubclass (MMP-1, MMP-5) act on fibrillar collagens. The substrates for the gelatinases (MMP-2, MMP-9) is gelatin (denatured collagen) as well as intact type IV basement membrane collagen and type V collagen. The stromolysins (MMP-3, MMP-10, MMP-7) have broad substrate specificity, which include fibronectin and laminin. Like the plasminogen activators, the activities of metalloproteinases are subject to tight control by a special class of tissue inhibitors of metalloproteinases (TIMP) which complex with the enzymes and thus inhibit their proteolytic action.

1.20 Trophoblast Degradation of ECM Proteins

It has been recognised that trophoblast cells have proteolytic activity for over 50 years. Subsequent studies have shown that explant cultures of chorionic villi would adhere to and then degrade the surrounding ECM proteins on which they were seeded (Fisher *et al.*, 1985). The same research later confirmed that it is the cytotrophoblast cells migrating out of the chorionic

villi which are responsible for the matrix degradation (Fisher *et al.*, 1989). Available evidence indicates that trophoblast degradation of matrix proteins is by the production of appropriate proteins. Immunohistology has demonstrated the presence of collagenase in extravillous cytotrophoblast cells (Moll and Lane, 1990; Fernandez *et al.*, 1992). Fisher *et al.* (1989) have shown that first trimester trophoblast express a wide range of cell-associated gelatin-degrading proteinases ranging from 68 to >200kDa in size.

Bischof *et al.* (1991) demonstrated that first trimester culture supernatants exhibit an array of seven bands of digestion ranging from 59 to 230 kDa, with three of these (230, 94, 64kDa) being particularly intense. Only four of these seven bands (230, 128, 94, 65 kDa) were seen in cell lysates. There are, therefore, more proteinases secreted by trophoblast than associated with the cell membrane. Furthermore, Bischof *et al.* (1991) identified four secreted gelatinases of 197, 94, 64 and 59 kDa which were considered to be trophoblast -specific products.

1.21 Trophoblast Plasminogen Activators and their Inhibitors

Cultured human trophoblast cells have also been demonstrated to secrete u-PA with proteolytic activity (Martin and Arias, 1982; Queenan *et al.*, 1987). Using specific rabbit antisera, Feinberg *et al.* (1989) localised both PAI-1 and PAI-2 in cultured trophoblast, with PAI-1 being present in the cytoplasm and cell surface membrane while PAI-2 occurs only in the cytoplasm. Immunohistological staining of placental sections localised PAI-2 only to

villous syncytiotrophoblast, suggesting a possible role of this inhibitor in regulating fibrinolysis within the intervillous space. In contrast, PAI-1 is found mainly among the invading population of extravillous cytotrophoblast, which could reflect a role in trophoblast migration. More recently, an immunological study claimed to detect u-PA as well as both PAI-1 and PAI-2 in all trophoblast populations (Hofmann *et al.*, 1994), although the tissues used were from ectopic rather than uterine pregnancies.

1.22 Trophoblast Invasion *In Vitro*

Yagel *et al.* (1988) demonstrated trophoblast invasion through human amnion. This invasion was prevented by a collagenase inhibitor (HCI), a metalloproteinase inhibitor (1-10 phenanthrolin), a serine proteinase inhibitor (trasylol) and an antibody directed against u-PA. In contrast, mersalyl, a mercurial compound known to activate procollagenase, enhanced trophoblast invasion. These observations are in support of the role of proteases in trophoblast migration. A further study demonstrated that conditioned media from first trimester decidual cells could suppress trophoblast invasion (Graham and Lala, 1991). This study concluded that the cytokine, TGF-β and the metalloproteinase inhibitor, TIMP, were the main components in decidual cell supernatants which inhibited trophoblast invasion. Both TGF-β and TIMP are produced by trophoblast cells (Yagel *et al.*, 1988) and this suggests trophoblast cells may be able to control their ability to invade through ECM. Librach *et al.* (1991) demonstrated

trophoblast invasion through an artificial ECM (Matrigel). Both TIMP and TIMP-2 effectively blocked trophoblast invasion in this assay. Furthermore, they ascertained that it is the 92 kDa type IV collagenase which is an important mediator of trophoblast invasion. Specific antibodies to u-PA and exogenous PAI-1 and Pai-2 only resulted in partial inhibition. They concluded that the metalloproteinase rather than the serine proteinase enzyme system plays the major role in the final pathway of first trimester trophoblast invasion (Librach *et al.* 1991).

1.23 Fibrinolytic Changes during Normal Pregnancy

The central role of the fibrinolytic system during pregnancy is believed to be the control of physiological fibrin deposition in the uteroplacental circulation and the prevention of fibrin deposition in the vascular bed. As will be discussed in greater detail in the following chapters, profound changes occur in coagulation and fibrinolysis in normal pregnancies and pregnancies complicated by pre-eclampsia and intrauterine growth retardation (IUGR). During normal pregnancy, the fibrinolytic system appears to be actively diminished. Plasminogen activator levels are believed to reflect fibrinolytic potential. Studies have shown high levels of circulating immunologic t-PA and u-PA during normal pregnancy. Accumulated evidence suggests the placenta to be a producer of uPA (Shimada *et al.*, 1989). The decrease in fibrinolytic activity may be due to increased levels of PAI-1 and PAI-2 which circulate during pregnancy. Several studies have reported an increase in

PAI-1 activity as pregnancy progresses (Estelles *et al.*, 1989; Bonnar *et al.*, 1990; Halligan *et al.*, 1994). PAI-2 appears in increasing amounts during pregnancy (Kruithof *et al.*, 1987; Lecander and Astedt, 1987; Wright *et al.*, 1988; Booth *et al.*, 1988; Estelles and Gilabert, 1989; Estelles and Gilabert, 1990; Halligan *et al.*, 1994; He *et al.*, 1996). The placenta has been cited the source responsible for increased PAI-1 and PAI-2 during pregnancy (Phillips *et al.*, 1986; Astedt *et al.* 1986; Feinberg *et al.*, 1989).

The placenta plays a central role in pre-eclampsia. This is supported by the observation that the condition resolves itself when the placenta is removed and that pre-eclampsia can occur without the presence of the foetus, as in cases of hydatidiform mole (Newman and Eddy, 1988). A haemostatic imbalance between fibrin deposition and fibrinolysis is now known to occur in pre-eclampsia (Hathaway and Bonnar, 1987). The origin of these haemostatic changes remains unknown. As yet, the possible role of the fibrinolytic system in the pathogenesis of pre-eclampsia is not fully understood. It can be stated that the consistently higher levels of tPA antigen observed suggests higher fibrinolytic potential in pre-eclampsia compared with normal pregnancy.

Several studies have shown that uteroplacental blood flow is impaired in pre-eclampsia (Browne and Veall, 1953; Lunnell *et al.*, 1982; Trudinger *et al.*, 1985; Leiberman *et al.*, 1988). A failure of physiological changes to extend into the spiral arteries of the placental bed has been well documented in pregnancies complicated by pre-eclampsia (Brosens *et al.*, 1972; Sheppard

and Bonnar, 1976, 1981, 1988; Gerretsen *et al.*, 1981; Robertson and Khong, 1987). The absence of these physiological changes is a prerequisite for the development of pathological lesions termed "acute atherosis" (Zeek and Assali, 1950). The lesion is characterised by fibrinoid necrosis, accumulation of lipophages in the damaged arterial walls and plaques on the intimal surface of the vessels containing large amount of lipids, smooth muscle cells and fibrin (Brosens *et al.*, 1972; Sheppard and Bonnar, 1976; Sheppard and Bonnar, 1988). Vascular occlusion ultimately occurs and the blood flow to the placenta, and the supply of essential nutrients to the developing baby, is compromised.

In pre-eclampsia, the defective physiological changes of the spiral arteries are due to a failure of complete trophoblastic invasion (Robertson *et al.*, 1975; Khong *et al.*, 1986).

Higher levels of tPA antigen are observed during pregnancy which suggest higher fibrinolytic potential in pre-eclampsia compared with normal pregnancy. A decrease in plasma fibrinolytic activity caused by increased PAI-1 levels has been reported in pre-eclamptic patients (Estelles *et al.*, 1989; Estelles *et al.*, 1991; Estelles *et al.*, 1994; Balleger *et al.*, 1991; Caron *et al.*, 1991; Reith *et al.*, 1993; Sheppard *et al.*, 1990; Halligan *et al.*, 1994; Friedman *et al.*, 1995).). In 1976, Elder suggested that the altered placenta may be the source of increased PAI-1 (Elder, 1976). Similarly, lower levels of both antigenic and functional PAI-2 in severe pre-eclampsia compared with a normal pregnant group have been reported (Estelles *et al.*, 1989; Estelles *et*

al., 1991; Reith *et al.*, 1993; Halligan *et al.*, 1994; He *et al.*, 1996). In pre-eclampsia, the defective physiological changes of the spiral arteries are due to a failure of complete trophoblastic invasion (Robertson *et al.*, 1975; Khong *et al.*, 1986). Trophoblast cells lining decidual spiral arteries are known to have a reduced capacity for fibrinolysis in normal pregnancy (Sheppard and Bonnar, 1978). In a recent study, the *in vitro* degradative properties of trophoblast cells isolated from placentas of pre-eclamptic pregnancies were compared with trophoblast cells isolated from normal placentas. This study revealed that the trophoblast cells from normal and preeclamptic placentas secreted similar amounts of uPA and PAI-1. However, the pre-eclamptic trophoblast cells expressed significantly less cell surface plasminogen activator activity (Graham and McCrae, 1996).

Several studies have examined plasminogen activator activity and plasminogen activator inhibitor activity in placental extracts of pre-eclamptic pregnancies. A significant increase in PAI-1 antigen levels and a significant decrease in PAI-2 antigen levels in placental extracts from pre-eclamptic pregnancies complicated with IUGR have been reported (Estelles *et al. 1994, 1998).* In contrast, Kanfer *et al.* (1996) could find no differences in PAI-1 antigen levels while the PAI-2 content was significantly increased.

Placental abnormalities such as a reduction in mass and area, altered function and abnormal placental location are known causes of growth retardation (Baur, 1977; Hoogland *et al.*, 1980). Maternal vascular disease whether it is chronic hypertension, pre-eclampsia or diabetes has been

associated with impaired foetal growth (Long *et al.*, 1980; Katz *et al.*, 1980). Idiopathic IUGR is associated with reduced blood placental perfusion. At least 35% of cases of IUGR are attributable to a disturbance in the uteroplacental circulation (Dawes, 1974). The pathological role of fibrin, or the factor(s) that stimulates its excessive deposition within the uteroplacental vasculature of these complicated pregnancies, is as yet unknown. Several studies have reported only slight changes in the levels of components of the haemostatic mechanism in normotensive patients with intrauterine growth retardation when compared with normal pregnancy (Frigoletto *et al.*, 1971; Howie *et al.*, 1971; Elder and Myatt, 1976; Persson *et al.*, 1982). It is generally believed in IUGR, disordered activity of platelets and the coagulation system is confined to the uteroplacental circulation (Howie *et al.*, 1971; Howie, 1977; McKillop *et al.*, 1978; Wallenburg and van Kessel, 1979; Persson *et al.*, 1982).

Boyle (1992) found similar levels of tPA antigen and activity in normotensive IUGR compared with normal pregnancy. Estelles *et al.* (1991) suggested that uPA might correlate with placental function. Later, Lindoff and Astedt (1994) reported a significant decrease in uPA antigen in plasma from IUGR pregnancies in comparison to normal pregnancy. Furthermore, they reported that uPA antigen were significantly correlated with placental and foetal growth and suggested that uPA plasma levels appear to reflect placental remodelling and function.

Many studies have investigated PAI-1 and PAI-2 levels in pregnancies

complicated by IUGR. PAI activity and PAI-1 antigen were found to be significantly higher in pre-eclamptic patients with IUGR (Estelles *et al.*, 1991). In a recent study, the authors confirmed that plasma levels of PAI-2 antigen were also decreased in both hypertensive and normotensive IUGR groups (Estelles *et al.*, 1991). The reduced circulating levels of this pregnancy-specific inhibitor were proposed as an adequate marker of impaired placental function.

Endothelial cells line vessels in every organ system and regulate the flow of nutrient substances, diverse biologically active molecules and the blood cells themselves. Studies with endothelial cells cultured from various tissues have lead to the inference that t-PA production and secretion is a property of all endothelial cells (Hirsh *et al.*, 1994). U-PA is expressed by endothelial cells involved in wound repair or angiogenesis (Bacharach *et al.*, 1992), consistent with the hypothesised importance of u-PA in cell migration and tissue remodelling. Endothelial cells in culture produce abundant PAI-1 that is associated primarily with its extracellular matrix. PAI-2 is found normally in plasma only during pregnancy (Astedt *et al.*, 1986) and is not synthesised by endothelial cells to an appreciable extent. Alterations in endothelial cells morphology occur within the placenta (Khong *et al.*, 1992) and in glomerular capillaries (glomerular endotheliosis), the latter being characterised by endothelial cell swelling and lipid accumulation (see Ferris (1995) for review). Fibrin deposition in microvasculature is common. Alterations in the extent of plasminogen

activator and inhibitor levels in endothelial cells may account for the increased fibrin deposition seen in the placenta and the placental bed.

The decidua at term is made up of a number of cell types, stromal cells, epithelial cells, endothelial cells, vascular smooth muscle cells and leukocytes. Sheppard *et al.* (1990) studied the levels of PAI-1 and PAI-2 in the placental bed decidua. They showed that the decidual extracts contained greater amounts of PAI-1 than PAI-2. In both pre-eclampsia and IUGR the levels of both PAI-1 and PAI-2 increase. However, these increases are not significant. A decreased ability of trophoblast cells to generate plasmin due to increased levels of PAI-1 and PAI-2 may result in decreased basement membrane degradation and thus result in impaired invasion into the placental bed. The presence of plasminogen activator inhibitors within the placental bed could yield the same result.

1.24 Objectives of the Study

As discussed above profound changes occur to the fibrinolytic system during pregnancy. It is marked with increased levels of both plasminogen activators and their inhibitors, which leads to an overall reduction in fibrinolytic activity. These changes are seen not only in the systemic system but also in the uteroplacental circulation. Many studies have suggested that the placenta is a major source for PAI-1 and PAI-2. These inhibitors have been immunologically associated with the placenta and have been shown to be present in placental extracts. However, no study has measured the secretory

levels of the inhibitors by the placenta and placental bed. Therefore, the contribution of these inhibitors to the local uteroplacental circulation has not been measured. In pre-eclampsia and intrauterine growth retardation there is an increase in fibrin deposition in the placenta and placental bed. This may be the result of increased PAI activity in these tissues.

The placenta and placental bed are made up of many different cell types including trophoblast cells, stromal cells and endothelial cells. The contribution of these cells to the levels of PAs and PAIs in the local tissue has not been measured. Significant changes of PAIs, in some but not all of these types may contribute to the changes in fibrin deposition seen in pregnancies associated with pre-eclampsia and intrauterine growth retardation. One aim of this study was to try and identify any changes in the levels of secretion.

Over the last decade, cultured trophoblast cells have been used to try and examine the invasive behaviour of trophoblast cells seen *in vivo*. Plasminogen activators and their inhibitors are believed to play a crucial role in the invasive ability of trophoblast cells. As stated earlier, pre-eclampsia and intrauterine growth retardation are associated with reduced trophoblast invasion. This decrease in invasive ability by trophoblast may be due to changes in secretory levels of plasminogen activators and their inhibitors by trophoblast cells. Furthermore, within the placental bed, it may be due to changes in secretory levels of PAs and PAIs by endothelial and decidual cells. In this study, changes in overall PA and PAI activity within

the placental bed were to be examined. Furthermore, the secretory levels of PAs and PAIs by trophoblast cells, decidual cells and endothelial cells were examined.

The first aim of this project was to determine the contribution of cells from the placenta and placental bed to the uteroplacental circulation in normal pregnancy. This was achieved by maintaining placenta and placental bed biopsies from normal pregnancies in organ culture and measuring the *in situ* secretion and fibrinolytic enzymes by ELISA.

Secondly, fibrinolytic enzyme levels within homogenised uteroplacental tissue were measured by ELISA in order to determine their contribution to localised fibrin deposition.

Thirdly, trophoblast cells were isolated from the placenta and placental bed, cultured and their fibrinolytic activity measured so as to determine their contribution to the overall fibrinolytic activity seen in the uteroplacental compartment.

Furthermore, cultured term villous trophoblasts have been suggested to display characteristics to extravillous trophoblast, which invade the placental bed. As discussed earlier, plasminogen activators and their inhibitors may play a role in trophoblast invasion. Therefore, the invasive properties of term trophoblasts were investigated by culture on fibrin and extracellular matrix.

Further studies assessed the levels of production of fibrinolytic activators and inhibitors by cultured endothelial cells isolated from placentas and

placental beds from healthy pregnant women and those with PET and IUGR.

In addition, the levels of production of fibrinolytic activators and inhibitors by decidual cells isolated from placental beds from healthy pregnant women and those with PET and IUGR were analysed.

CHAPTER 2
PATIENTS AND METHODS

2.1 Patients

2.1.1 Caesarean Section (Group 1)

Nineteen women who were delivered by elective Caesarean section were recruited to the study. Reasons for delivery of these women by Caesarean section included breech or complicated presentations, cephalopelvic disproportion and a history of previous Caesarean section. Four patients smoked between 5 and 20 cigarettes a day while the rest were non-smokers. Patients sectioned for placenta praevia, diabetes mellitus or any cases where pathology of the uteroplacental bed and/or placenta was suspected, were excluded from this group. Having given full and informed consent to take part in the study, these patients had the required tissue samples of placenta and placental bed taken following Caesarean section. Full patient details are given in Table 2.1.1.

2.1.2 Normal Pregnancy (Group 2)

Seventeen healthy primigravidae aged between 16 and 33 were recruited as a control group. All of the women had normal deliveries with a mean gestational age of 35 weeks (range 32 – 37). Five of the women smoked 6- 22 cigarettes per day; the remaining twelve were non-smokers. None of the women had raised blood pressure during their pregnancy or had any history of thromboembolic disorders. All patients were delivered normally. Full patient details are given in Table 2.1.2.

2.1.3 Pre-eclampsia (Group 3)

Thirty-four women aged between 22 and 33 years with diagnosed pre-eclampsia, having given full and informed consent were recruited for the study.

Pre-eclampsia was defined according to the classification approved by the International Society for the Study of Hypertension in Pregnancy (Davey and MacGillivray, 1986).

Under this classification hypertension is defined as one diastolic blood pressure reading of at least 110mmHg or two consecutive diastolic blood pressure readings of at least 90 mmHg no less than four hours apart. Significant proteinuria is defined as greater than or equal to 300mg of total protein in a twenty – four hour urine collection or if this is not available 1+ dipstick proteinuria on two consecutive occasions four or more hours apart.

Using this definition, pre-eclampsia is defined as hypertension in combination with proteinuria developing after twenty weeks gestation in a previously normotensive non-proteinuric woman. The pre-eclamptic group was subdivided according to the birth weight of the baby:

A. Seventeen women with diagnosed pre-eclampsia whose babies' weights were above the 10th centile. All were delivered by Caesarean section. This group included a 35-year old para 3, who delivered a well grown baby at 39 weeks' gestation. In older women there may be a possibility of a co-existing medical condition. This patient was initially

removed from the patient group and the statistics of the study examined. However, no significant differences could be seen having removed the patient from the group. In the light of this result, the patient was included in the study.

B. Seventeen women with pre-eclampsia who were also diagnosed as having intrauterine growth retardation (IUGR). Antenatal diagnosis was made using ultrasound techniques. At delivery, IUGR was confirmed if the baby birth weight was less than the 10th centile. Of these patients six smoked an average of 5-20 cigarettes per day and the remaining ten were non-smokers. Having given full and informed consent to take part in the study, these patients had the required tissue samples of placenta and placental bed taken following Caesarean section. Patient details are given in Tables 2.1.3 and 2.1.4.

2.1.4 Intrauterine Growth Retardation (Group 4)

Seventeen normotensive women aged between 23 and 37 who were diagnosed as having IUGR (baby birth weight less than the 10th centile) were recruited for the study. Of these patients twelve smoked an average of 5-25 cigarettes per day. Diagnosis was made using ultrasound techniques and patients who were diagnosed before delivery as having IUGR but whose baby birth weights on delivery were greater than the 10th centile were excluded from the study. Having given full and informed consent to take part in the study, these patients had the

required tissue samples of placenta and placental bed taken following Caesarean section. Patient details are given in Table 2.1.5.

No.	Age (y)	Parity	Gestation (weeks)	Baby (g)	Sex	Placenta (g)	Del. Gest. (weeks)	Cigs/day
1	29	1	37	3420	M	585	37	-
2	38	2	42	3760	M	790	42	-
3	27	0	38	3585	F	630	38	-
4	34	2	38	3675	M	620	38	5
5	23	0	36	2860	F	590	36	-
6	32	1	40	3820	F	760	40	-
7	35	1	42	3905	M	860	42	10
8	28	0	39	3720	F	720	39	-
9	31	2	38	3360	F	680	38	-
10	24	0	36	3480	M	545	36	-
11	33	1	39	3580	M	640	39	20
12	34	2	38	2950	F	570	38	-
13	30	0	36	2885	M	510	36	-
14	28	1	40	3515	F	660	40	-
15	29	2	42	3940	F	850	42	-
16	32	0	41	3865	F	740	41	5
17	35	1	38	3390	M	510	38	-
18	26	0	40	3410	F	620	40	-
19	28	0	42	3890	M	820	42	-

Mean gestation at delivery = 39.0 (S.D. = 2.1)

Mean baby weight = 3527 (S.D. = 336)

Mean placental weight = 668 (S.D. = 110)

Mean maternal age = 30y (S.D. = 4)

Table 2.1.1: Control Caesarean Section (Group 1) – Patient Details

No.	Age (y)	Cigs/day	Gestation (weeks)	Baby (g)	Sex	Placenta (g)
1	32	-	34	3240	F	630
2	28	10	37	4100	F	790
3	24	-	35	3935	F	660
4	3	-	37	3745	M	640
5	25	15	33	3150	M	570
6	28	-	35	3230	M	620
7	32	10	36	3710	F	660
8	20	-	35	3690	M	690
9	25	6	36	3440	M	520
10	31	-	34	3180	F	580
11	30	-	34	2770	M	455
12	16	5	35	3640	M	720
13	28	-	32	3280	F	615
14	24	-	37	3975	M	560
15	26	-	33	3120	M	515
16	31	22	36	3025	F	685
17	30	-	36	3270	F	595

Mean gestation at delivery = 35.0 weeks (S.D. = 1.5)

Mean baby weight = 3441g (S.D. = 375)

Mean placental weight = 617g (S.D. = 82)

Table 2.1.2: Normal Pregnancy Section (Group 2) – Patient Details

No.	Age (y)	Parity	Gestation (weeks)	Baby (g)	Sex	Placenta (g)	D.B.P. (mmHg)	Protein (g/L)	Uric Acid (μM)
1	36	1	34	3160	M	595	105	2.0	310
2	32	0	34	2750	M	530	100	10.0	345
3	33	1	35	1590	F	555	110	10.0	413
4	28	0	30	2340	M	480	105	5.0	427
5	29	1	34	2860	F	625	95	10.0	382
6	25	2	37	3665	M	740	110	3.0	441
7	35	3	39	3790	M	780	105	1.0	354
8	31	0	36	3384	M	665	100	3.1	458
9	33	0	34	3420	F	510	110	1.0	479
10	22	0	37	2996	F	590	105	3.2	296
11	23	0	35	2180	M	540	110	3.0	267
12	28	0	36	2340	M	550	110	4.3	385
13	35	2	32	1780	F	380	105	10.0	367
14	29	0	31	1540	M	340	110	5.1	239
15	28	0	33	2160	F	465	95	1.0	359
16	30	1	36	3125	M	660	100	10.0	395
17	33	1	36	3255	F	595	100	5.5	390

Mean gestation at delivery = 34.7 weeks (S.D. = 2.3)

Mean baby weight = 2725g (S.D. = 714)

Mean placental weight = 565g (S.D. = 114)

Mean Blood Pressure = 104mm hg (S.D. = 5.3)

Proteinuria = 5.1g/L (S.D. = 3.5)

Uric acid = 371μM (S.D. = 66)

Table 2.1.3 Pre-eclampsia (without IUGR) (Group 3A) – Patient Details

No.	Age Centile (y)	Parity	Gestation (weeks)	Baby (g)	Sex	Placenta (g)	D.B.P. (mmHg)	Protein (g/L)	Uric Acid (μM)	Cigs/day	
1	25	0	39	2480	F	640	110	10.0	358	-	<5
2	33	0	34	1350	M	380	90	8.4	505	5	<10
3	36	0	36	1945	M	450	130	10.0	425	-	<5
4	31	0	35	2565	M	560	125	5.8	631	-	<10
5	26	0	33	1770	F	600	100	2.9	547	-	<10
6	33	0	38	2315	F	530	110	10.0	440	10	<5
7	36	1	31	1190	M	240	130	1.0	335	-	<10
8	28	0	34	1860	F	490	120	10.0	601	-	<5
9	30	0	36	1585	M	390	120	3.2	580	20	<10
10	34	0	33	1790	M	420	110	9.0	624	-	<5
11	31	0	38	2605	M	620	130	5.0	597	-	<5
12	27	0	37	2035	M	550	90	10.0	316	-	<10
13	35	1	34	1930	F	370	100	1.0	495	20	<10
14	33	0	38	2160	F	430	110	3.8	578	-	<5
15	33	0	35	2380	M	570	125	10.0	465	-	<5
16	34	0	34	1855	F	440	95	5.3	411	10	<5
17	32	0	34	2080	F	510	105	3.0	378	5	<10

Mean gestation at delivery = 35.0 weeks (S.D. = 2.2)

Mean baby weight = 1994g (S.D. = 402)

Mean placental weight = 482g (S.D. = 105)

Mean Blood Pressure = 112mm hg (S.D. = 4)

Proteinuria = 6.4g/L (S.D. = 3.5)

Uric acid = 487μM (S.D. = 106)

Table 2.1.4: Pre-eclampsia with IUGR (Group 3B) - Patient Details

No.	Age (y)	Parity	Gestation (weeks)	Baby (g)	Sex	Placenta (g)	Centile	Cigs/day
1	32	0	32	1170	M	220	<5	10
2	36	1	37	2140	F	420	<10	-
3	23	0	39	2570	F	610	<10	25
4	35	2	35	1745	F	350	<5	5
5	36	2	36	2080	M	540	<5	5
6	33	0	33	1330	F	270	<5	20
7	37	1	38	2270	M	495	<5	-
8	33	2	34	1620	M	330	<5	20
9	31	0	40	2890	M	705	<10	-
10	36	1	35	1530	M	310	<5	15
11	36	2	36	1865	F	410	<10	20
12	34	2	34	1326	M	250	<5	-
13	24	0	39	2455	F	530	<10	10
14	38	1	38	2285	F	460	<5	10
15	36	3	36	2010	F	490	<10	4
16	32	0	35	2185	M	505	<10	-
17	33	1	36	2070	M	447	<10	15

Mean gestation at delivery = 36.0 weeks (S.D. = 2.2)

Mean baby weight = 1973g (S.D. = 472)

Mean placental weight = 432g (S.D. = 132)

Table 2.1.5: Intrauterine Growth Retardation (Group 4) – Patient Details

2.2 Methods

2.2.1 Preparation of Placental Cells

Human cytotrophoblasts were purified from placentas following Caesarean section using the method of Morrish *et al.* (1986). Placental tissue was gently separated by sterile dissection from different cotyledons, excluding chorionic and basal plates, minced with scalpel blades, and washed repeatedly with 0.9% NaCl to remove blood from the intervillous space. This process was continued until approximately 50g of placental tissue was collected. The tissue was then minced into smaller pieces of about 0.2 cm across. 150ml of warmed CMF Hank's was added to enzyme flask 1 which contained the Trypsin/DNAase (0.18% trypsin and 500 Kunitz units of DNAase 1) and mixed. 1ml of the PS solution was added along with the $CaCl_2$ and $MgSO_4$ solutions to this flask. Finally, the placental tissue was added to the flask, mixed well and placed in the water bath at 37°C. The digestion flask was removed from the water bath every five minutes and swirled vigorously in order to resuspend the tissue. After 30 minutes, the flask was removed from the water bath and allowed to stand at a 45-degree angle for 1 minute. Approximately 80 ml of the supernatant was removed using a sterile pipette and this supernatant was discarded. 100 ml of CMF Hank's was added to enzyme flask 2 and mixed well. After addition of the $CaCl_2$ and $MgSO_4$ solutions, the solution was added to the digestion flask containing the tissue fragments, returned to the water bath for a further 30 minutes. As before,

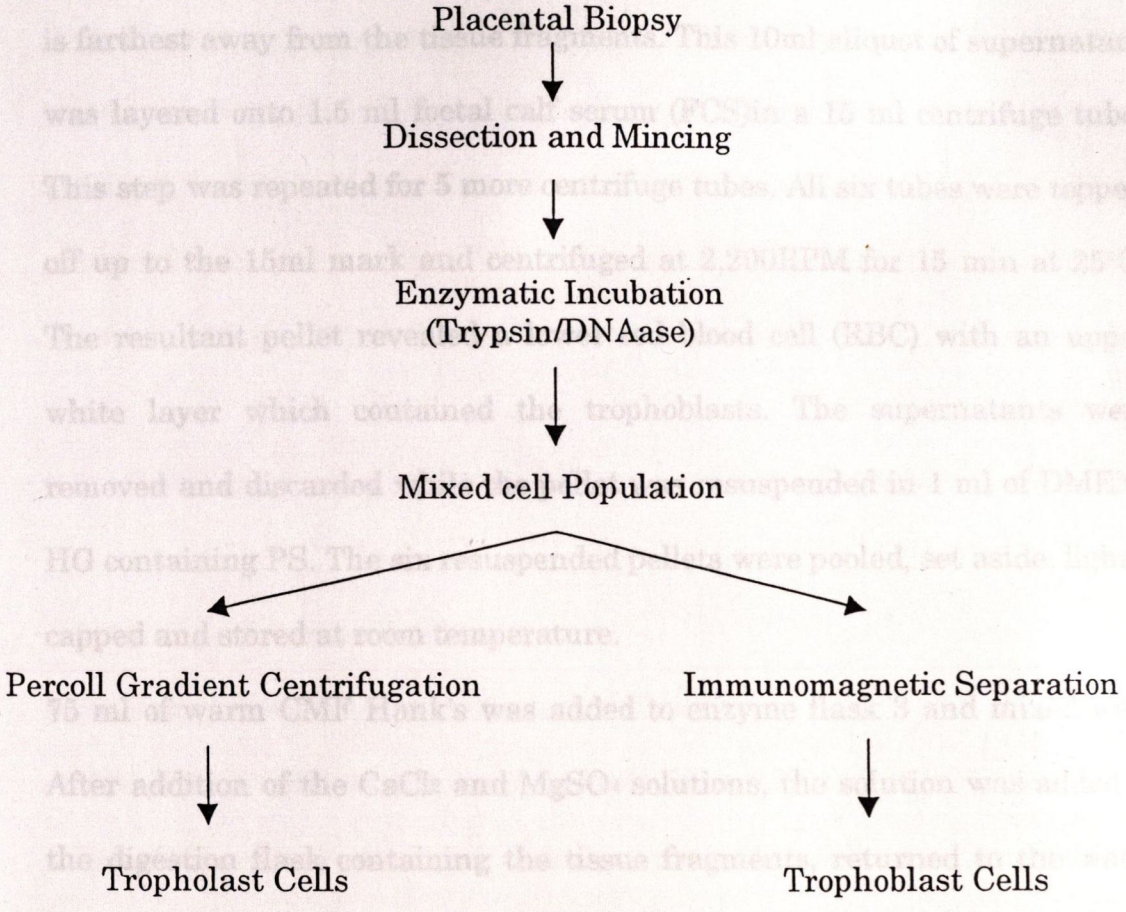


Table 2.2.1 Flowchart for isolation of trophoblast cells

the solution was vigorously shaken every 5 minutes.

After 30 minutes, the flask was removed, placed at a 45-degree angle and the tissue allowed to settle for 1 minute. With a clean 10 ml pipette the supernatant was slowly removed from just below the surface at a point which is farthest away from the tissue fragments. This 10ml aliquot of supernatant was layered onto 1.5 ml foetal calf serum (FCS)in a 15 ml centrifuge tube. This step was repeated for 5 more centrifuge tubes. All six tubes were topped off up to the 15ml mark and centrifuged at 2,200RPM for 15 min at 25°C. The resultant pellet revealed a lower red blood cell (RBC) with an upper white layer which contained the trophoblasts. The supernatants were removed and discarded while the pellet was resuspended in 1 ml of DMEM-HG containing PS. The six resuspended pellets were pooled, set aside, lightly capped and stored at room temperature.

75 ml of warm CMF Hank's was added to enzyme flask 3 and mixed well. After addition of the $CaCl_2$ and $MgSO_4$ solutions, the solution was added to the digestion flask containing the tissue fragments, returned to the water bath for a further 30 minutes. As before, the solution was vigorously shaken every 5 minutes.

Once again, the supernatant was removed, layered over FCS, centrifuged and the pellets resuspended and pooled.

A final 75ml of CMF Hank's was added to enzyme flask 4 and the digestion step repeated for 30 minutes. At the end of this time, as much of the supernatant was removed from the tissue solution and the trophoblasts

pelleted and resuspended as before. This last digestion was pooled into two sets. At the end of the digestion process, there were 4 tubes containing pooled calf serum pellets which were then centrifuged for 10 minutes at 2,200 RPM in a bench centrifuge. The supernatants were discarded while the pellets were resuspended with 1 ml DMEM-HG containing PS. Finally, the 4 tubes were pooled. On average, between 80 and 170 million cells were obtained from 30g of starting placental tissue. Further purification of the placental cells was performed by either affinity chromatography or Percoll gradient centrifugation/immunomagnetic separation.

2.2.2 Cell Separation by Percoll Gradient Centrifugation

Preparation of Percoll Gradient

Each Percoll gradient was made fresh as follows:

A 90% Percoll solution was prepared by adding 2ml 10X Hank's solution to 18ml of Percoll. This Percoll solution was diluted to prepare different strength Percoll solutions.

%Percoll	ml 90% Percoll	ml Hank's
70	2.33	0.67
65	2.17	0.83
60	2.00	1.00
55	1.83	1.17
50	1.67	1.33
45	1.50	1.50
40	1.33	1.67
35	1.17	1.83
30	1.00	2.00
25	0.83	2.17
20	0.67	2.33
15	0.50	1.50
10	0.33	2.67
5	0.17	2.83

Each solution was mixed and beginning with the 70% Percoll, the Percoll solutions were layered into a 50ml polystyrene conical centrifuge tube using a clean Pasteur pipette.

The pooled digests were very carefully layered onto the Percoll gradient and centrifuged at 1,200 X g for 20 minutes without the brake.
The tube was removed carefully from the centrifuge and placed in a support in a completely upright position. The top fluffy layer was removed using a

Pasteur pipette and discarded. The next two layers (which contain the trophoblast cells) were carefully removed using a 10ml pipette and placed into a clean 50ml polystyrene centrifuge tube.

The collected bands were diluted to the 50ml mark with DMEM-HG containing PS and centrifuged at 2,200 RPM for 10 minutes. The pellet was finally resuspended in 10 ml of serum-free DMEM-HG-PS. A cell count was then performed using a haemocytometer. The suspension was finally plated to the desired plating concentration with DMEM-HG-PS and maintained in a humidified incubator at 37° C in a 95% air, 5% carbon dioxide atmosphere.

2.2.3 Immunomagnetic Separation of Trophoblast Cells

Cells (either freshly isolated or thawed) were suspended in ice-cold medium containing 17% FCS to give 4 x 10^6 cells/ml. All subsequent steps were performed in the cold. For simplicity, the following description is based on processing 1ml of cell suspension.

To the suspension (1ml) was added 20ml of a monoclonal anti-HLA-ABC antibody and 40ml of monoclonal anti-HLA-DR antibody. The mixture was incubated on ice for 45 minutes with occasional gentle shaking and then centrifuged at 350 x g for 5 minutes. The supernatant was removed and the cell pellet was resuspended in HBSS (without Ca^{2+} and Mg^{2+}) supplemented with 1% FCS. After a further centrifugation step, the pellet was resuspended in medium at a ratio of 1ml medium:4 x 10^6 cells. To this was added 40ml of a suspension of magnetic beads coated with goat anti-mouse IgG. The

mixture was placed on a rotating platform at 3RPM for 30 minutes at 4C. Cells were collected by applying a magnet to the side of the tube, followed by withdrawal of the bead-free supernatant. This process was repeated three times, interspersed by 1 minute washes with cold medium. The supernatant was pooled and centrifuged at 350 x g for 10 minutes. The pelleted cells were resuspended in culture medium and either plated or cryopreserved. Identification of the cells as trophoblast was determined by immunocytochemical staining for cytokeratin and their ability to produce hCG and hPL.

2.2.4 Isolation of Placental Bed Cell Population

Placental bed tissue was routinely removed from the centre of the placental bed site following removal of the placenta. The biopsies were wedged shaped, normally two inches long, and one inch in thickness. There were examined microscopically prior to use.

Placental bed tissue was washed, teased apart and incubated for 15 minutes at 37C in DMEM containing 0.02% protease type XIV, 1nM L-glutamine, 100mg/ml streptomycin and 100 IU/ml penicillin. Tissue fragments were washed in PBS/0.1% BSA and resuspended in DMEM containing 10% FCS, 0.2% hyaluronidase (Type 1-S) and 0.25% collagenase and stored overnight at 4C. The following day, the suspension was incubated for 15 minutes at 37C with DMEM containing 0.18% Trypsin and 500 IU DNAase 1.

The obtained solution was filtered initially through a 100mm nylon sieve to

remove undigested tissue fragments and then through a 40mm sieve to retain whole glands and cell aggregates. Cells were extensively washed in PBS/0.1% BSA and left overnight in DMEM/10% FCS at 4C.

To separate viable cells from debris and dead cells, cells were resuspended in 25% Percoll and layered over 60% Percoll and under PBS. After centrifugation at 670 x g for 30 minutes, viable cells were collected at the 25%/60% Percoll interface, washed in PBS and counted in a haemocytometer (Improved Neubauer). Cell viability was assessed by Trypan Blue exclusion. The cells were verified as been of trophoblastic origin by cytokeratin and W6/32 immunostaining and their ability to produce hCG and hPL.

2.2.5 Purification of Placental Cells via Negative Selection

Plastic columns were filled with 1ml of goat anti-mouse immunoglobulin (Ig)-conjugated glass beads prepared according to the manufacturer's recommended procedure (Biotex Lab.). Meanwhile, placental cells were incubated in PBS/20% normal goat serum for 20 minutes on ice to reduce non-specific binding. The cells were then washed, resuspended in PBS/2% FCS and incubated with mouse-anti-human CD9 antibody at a ratio of 1μg antibody to 2 million cells. After a forty minute incubation on ice, the cells were washed twice with cold PBS/2% FCS. This preparation was then loaded onto the columns which had been washed with PBS and the flow rate adjusted to one drop every 8 seconds. A maximum of 40 million cells can be loaded onto each column without impeding the flow rate. Directly after

Table 2.2.2 Cell Identification and Viability

Placental trophoblast:	92 ± 4% Cytokeratin +ve
	90 ± 8% viability
Placental bed trophoblast:	85 ± 7% Cytokeratin +ve
	81 ± 9% W6/32 +ve
	92 ± 5% viability

Values are mean ± range (n = 40)

Cytokeratin is a marker for trophoblast cells

W6/32 is expressed by extravillous cells but not by villous cells.

loading, non-adhering cells were eluted with 13ml of PBS/2% FCS. These placental cells were centrifuged, resuspended in FCS at 10 x 10^6 cells/ml and either plated or cryopreserved in 10& (v/v) DMSO in FCS under liquid nitrogen.

2.2.6 Invasion Assays

Cytotrophoblast cells (5 x 10^6 cells per filter) were cultured on coated Transwell inserts (6.5mm; Costar Corp.) containing polycarbonate filters with 8-μm pores. The inserts were coated with matrigel or fibrin. Every 24 hours the cultures were rinsed in PBS, fixed and stained with anticytokeratin antibodies.

To assay invasion, the filters were dissected from the inserts with a scalpel blade and mounted on poly -L- lysine-coated slides in a drop of mounting medium such that the underside of the filters faced upwards. Serial optical sections were obtained by using confocal microscopy. The number of cytokeratin-positive cell processes that penetrated the matrigel or fibrin were counted. Six replicate filters were analysed for each time point for each isolation procedure.

2.2.7 Organ Culture of Placenta and Placental Bed Biopsies

All biopsies were washed and dissected as previously described. Biopsies were chopped into small pieces (about 1mm diameter) and placed on Transwell inserts containing 8mm pore polycarbonate filters. Exactly 1.6ml

of medium (DMEM containing 10% FCS) was added to each well. This volume was determined to be the minimum needed to cover each biopsy with medium. Biopsies were incubated at 37° C and the medium changed every 24 hours. The removed medium was snap frozen and stored for analysis later. Each day sample biopsies were removed, frozen in liquid nitrogen, pulverised and stored for analysis. Viability was determined by hCG measurement, Trypan blue exclusion assays and MTT analysis.

2.2.8 Measurement of PA Activity in Trophoblast Conditioned Medium

u-PA activity in trophoblast conditioned medium was measured in microtiter wells by incubating indicator-free conditioned medium with Glu-plasminogen (final concentration 0.1mg/ml) and the chromogenic plasmin substrate S-2251 (final concentration 60mM) at 37°C. The optical density was then measured at 405nm in a microplate reader (Titertek). Trophoblast conditioned medium was collected at 24 hour intervals over a period of five days.

2.2.9 Measurement of PA Activity Expressed by Cultured Trophoblast Cells

To measure cell-associated u-PA activity, trophoblast cells were cultured in serum-free DMEM-HG containing 2% Ultroser G for 12 or 24 hours. The conditioned medium was then removed and the cells washed twice with

indicator-free DMEM-HG containing 0.5% BSA. The cells were then incubated in indicator-free medium containing 25mg/ml Glu-plasminogen and 60mM S-2251 at 37°C. At various time points over a twelve hour period, the optical density was then measured at 405nm in a microplate reader (Titertek).

2.2.10 Endothelial Cell Isolation

Goat anti-mouse IgG-coated Dynabeads M-450 (Dynal, Wirral, UK) were coated with purified QB-End/40 monoclonal antibody to thrombomodulin (Serotec, UK) according to the manufacturer's instruction. The beads were washed in PBS/1% NCS prior to use. The beads were added to the dissociated placental or placental cells at a bead/cell ratio of 3:1 and incubated at slow rotation for 30 minutes at 4C. Rosetted cells were collected by applying a magnet to the side of the tube, followed by withdrawal of the bead-free supernatant. This process was repeated three times, interspersed by 1 minute washes with cold RPMI/1% NCA. Purified cell clusters were resuspended in Medium 199 plus 40% FCS, glutamine and antibiotics.

2.2.11 Culture of Purified Endothelial Cells

Rosetted cells were seeded into 35mm^2 plastic culture dishes pre-coated with 1% gelatin. The culture medium was supplemented with 0.1% of both endothelial cell growth supplement and heparin (170 IU/mg; Sigma). The medium was replaced every 2 days. The cells reached confluence by 6 days

and were subcultured every 10 days by trypsinisation and reseeded at a split ratio of 1:3. Endothelial cells were characterised by staining with von Willebrand factor and QB-End/40.

2.2.12 Isolation and Culture of Decidual Cells and Decidual Endothelial Cells

Each placental bed biopsy was cut into 5 mm cubes and incubated at 37°C for 8 minutes in trypsin 1:2500 and Pronase (80μg/ml). The resultant fragments were washed five times with Hank's balanced salt solution (HBSS), capillary segments were squeezed from the cut edges by downward pressure with the flat edge of a scalpel blade and the resultant mixed cell population was collected, centrifuged (1000 X *g*, 5 minutes) and resuspended in 80μl of HBSS plus 5% foetal calf serum. This cell suspension was incubated at 4°C for 10 minutes with 20μl of the lectin ulex europaeus (UEA-1) - coated dynabeads (4 x 10^8 beads/ml) and washed five times with 2ml HBSS plus 5% foetal calf serum, separation after each wash being achieved in a magnetic particle concentrator. The cell-bead complex was dissociated by competitive binding of L-fucose. The cells were resuspended in growth medium and plated down on a gelatin - coated 35mm tissue culture dish. Cells were routinely maintained in a humidified incubator at 37° C in a 95%air, 5% carbon dioxide atmosphere. The growth medium used was McCoy's medium containing growth factors 0.1 mg/ml endothelial cell growth supplement, 0.1 mg/ml heparin, 2.5 μg/ml fungizone, 100mU/ml

penicillin and 100 μg/ml streptomycin, with 40% FCS. Cells were examined by phase contrast microscopy, refed at 2 day intervals, and passaged at confluence with the use of trypsin 1:250, 0.05%/0.02% (w/v) EDTA in PBS solution. Endothelial cells were identified by their expression of von Willebrand factor antigen with immunocytochemistry.

2.2.13 Immunocytochemistry of Cultured Cells

Primary Antibodies

Cytokeratin (Dako M821)

uPA (A.D. 394)

tPA (A.D. 373)

PAI-1 (A.D. 3785)

PAI-2 (A.D. 3750)

vWF (Dako)

QBEND/40 (Dako)

w6/32 (SeraLab)

hCG (Dako)

hPL (Dako)

vimentin (Dako)

Reagent Preparation

Tris Buffer (TB): 1.39g/L Trizma Base, 6.06g/L Trizma Hydrochloride

Tris Buffered Saline (TBS):9 parts Sodium Chloride %.84g/L, 1 part Tris

Buffer

Biotinylated Rabbit anti Mouse IgG (1:100 (TBS)

StreptAB Complex (22.5ml A, 22.5ml B and 2.5ml TB)

Procedure

1. The cells grown on coverslips in 16-well plates were gently washed with PBS at room temperature.

2. Cold methanol (-20C) was added to the wells and transferred to a fridge at 4° C for 5 minutes.

3. The coverslips were then transferred into cold (-20° C) acetone in an acetone resistant dish for 2 minutes.

4. The coverslips were subsequently washed twice with TBS.

5. The coverslips were then incubated with the diluted primary antibody for 30 minutes at room temperature.

6. Coverslips washed with TBS.

7. The coverslips were incubated with the diluted secondary antibody against mouse IgG for 30 minutes at room temperature.

8. Coverslips washed with TB.

9. StreptAB complex added and incubated 30 minutes at room temperature.

10. The coverslips were washed in TB and developed with DAB chromogen which was followed by washing with distilled water.

11. Finally the coverslips were counterstained with haemotoxylin and mounted in Glycerol gel.

2.2.14 Tissue Plasminogen Activator Activity

(Biopool Spectrolyse™/fibrin, Sweden)

Assay Principle

This assay is based on the functional parabolic rate assay described by Ranby et al. (1982) and Wiman et al. (1983). The t-PA activity is measured by adding Glu-plasminogen, chromogenic plasmin substrate and fibrin and neutral pH. In the presence of fibrin, t-PA converts plasminogen to plasmin, which subsequently cleaves the chromogenic substrate. The amount of colour development in a given time is proportional to the amount of t-PA activity in the sample.

The assay is insensitive to plasminogen and fibrinogen. The assay measured t-PA in samples in the range of 0.01 IU/ml to 30IU/ml. The t-PA standard was calibrated against the World Health Organisation (WHO) First International Standard for t-PA coded 86/670 from the National Institute for Biological Standards and Control, England.

Reagents

PAR: 0.5mg human Glu-plasminogen colyophilised with D-But-Lys-pNA (6μmol)

DESAFIB-X™: stimulator for t-PA, 2mg solubilised desAA fibrinogen (fibrin 1)

t-PA standard: 10μg human melanoma cell line derived single chain

Buffer: Tris HCl buffer, 50mmol/L containing 0.1g/L Tween 80, pH8.7

Acetate: 1M Sodium Acetate buffer, pH3.9

Stop/fibrin: 25g/L Sodium Dodecyl sulphate in 50g/L acetic acid

deionised water

Citrate buffer, pH4.5; 1M citric acid and 1M trisodium citrate mixed to achieve the correct pH

Preparation of Reagents

Tris buffer was diluted 10X with water.

PAR was reconstituted with 12.5ml of Tris buffer. This was stable for 24 hours at 6°C or for 4 weeks at -20°C.

DESAFIB-X™ was reconstituted with 750µl Tris buffer.

Preparation of Samples

Tissue plasminogen activator activity in samples is unstable. The half-life ranges from 1 minute to 4 hours depending on PAI activity. Nine volumes was added into 1M citrate buffer pH 4.5, thus lowering the pH of the sample. This stabilises the t-PA activity by blocking inhibition by PAI.

Procedure

Preparation of standards

1ml of acetate was added to the t-PA standard yielding a 6000IU/ml solution. 50µl was added to 7.5ml Tris buffer to yield a 49IU/ml solution. Working standards were prepared as follows:

0IU/ml t-PA standard:100µl of PAI-1 free sample was mixed with 400µl acetate buffer, incubated at room temperature for 20 minutes and then

diluted with 8ml of water.

40IU/ml t-PA standard:100µl of PAI-1 free sample was mixed with 400µl acetate buffer, incubated at room temperature for 20 minutes. 100µl of 40IU/ml t-PA standard was added and then diluted with 7.9ml of water.

4IU/ml t-PA standard:150µl 40IU/ml standard mixed with 1350µl 0IU/ml t-PA standard.

0.4IU/ml t-PA standard: 50µl 4IU/ml t-PA standard mixed with 450µl 0IU/ml t-PA standard. Additional t-PA standards were prepared:

Standards (IU/ml)	Diluted t-PA standard	0 IU/ml Standard
3.0	300µl (4IU/ml)	100µl
2.0	200µl (")	200µl
1.5	150µl (")	250µl
2.0	100µl (")	300µl
0.5	50µl (")	350µl
0.3	300µl (0.4 IU/ml)	100µl
0.1	100µl (0.4 IU/ml)	300µl
0	-	400µl

t-PA assay in microtest plate

20µl of diluted sample or diluted standard was added in duplicate to microtest plate wells. 200µl PAR was added to the wells using an eight-

channel pipette and the plate placed in the refrigerator for 15 minutes. 10µl of DESAFIB-X™ was added to each well with an eight-channel pipette within two minutes and the plate agitated for 10 minutes. The plate was then incubated for 19 hours at 37°C. After this time elapsed, 25µl STOP/fibrin was added to each well and the plate agitated for 15 minutes.

Measurement

The absorbance was measured at 405nm and 492nm and the difference calculated. Paranitroanilide absorbs light at 405nm but not at 492nm whereas the absorbance due to turbidity is approximately equal at 492nm. Therefore, absorbance at A_{492} was measured and subtracted to correct for background due to turbidity.

Results

A standard curve was obtained by plotting standard A_{405}-A_{492} values against their corresponding t-PA activity levels. Sample t-PA activity levels were then determined from the curve.

2.2.15 Tissue Plasminogen Activator (t-PA) Antigen

(Biopool Immunolyse™, Sweden)

Assay Principle

An enzyme immunoassay (ELISA), utilising the double antibody principle,

was used for the quantitative determination of t-PA antigen in human plasma and tissue extracts (Bergsdorf et al., 1983; Ranby et al. 1986; Ranby et al., 1989). The ELISA measures free, single and two chain forms of t-PA and also the bound forms (t-PA complexed to α_2-antiplasmin, PAI-1 and PAI-2).

Samples were added to wells pre-coated with goat anti-human uterine t-PA antibodies. Following incubation, goat anti-human t-PA antibodies labelled with horseradish peroxidase (conjugate) was added to the wells. Unbound conjugate was then washed away. The amount of peroxidase remaining in the wells was then proportional to the amount of t-PA antigen. A peroxidase enzyme substrate was added and the resulting coloured development measured.

Assay Characteristics

Biopool Imulyse™ t-PA in the range 1.5-30 ng/ml. Maximum sensitivity was 1.5ng t-PA/ml of sample. No cross reaction with urokinase has been observed

Reagents

Flat bottom 96-well microtest plates

PET-buffer: PBS-EDTA-Tween buffer (0.1M NaCl, 5mM EDTA, 20mM Sodium Phosphate buffer, 0.1% Tween 20), pH 7.3

Coat/Pre-fill A: 3mg goat anti-human t-PA antibody

Pre-fill N: 1mg normal goat antibody

Coating Solvent: 0.1M sodium hydrogen carbonate ($NaHCO_3$)

t-PA standard: 500ng single chain t-PA

Conjugate: horseradish peroxidase labelled goat anti-human t-PA antibody

Substrate: orthophenylenediamine di-HCl (OPD)

1M potassium hydrogen carbonate ($KHCO_3$)

4.5M sulphuric acid (H_2SO_4)

30% hydrogen peroxide(H_2O_2)

Glycerol (concentrated)

deionised water

Human control plasma (Reference plasma 100%; Immuno AG Gmbh)

Preparation and Storage of Bioreagents

The bioreagents were prepare and stored as follows:

Coating/prefill A: 3ml of water was added to the coating vial, agitated for 10 minutes and stored as 5 x 350 µl aliquots (labelled coat) and 5 x 200µl aliquots (labelled prefill A).

Prefill N: 1ml of water was added to the pre-fill N vial, gently agitated for 10 minutes and stored as 5 x 190µl aliquots.

t-PA Standard: t-PA standard was dissolved with 500µl 1M $KHCO_3$ to yield 1µg t-PA/ml and stored as 5 x 95µl aliquots.

Conjugate: 125µl of water was added to the conjugate vial and following dissolution, 125µl concentrate glycerol was added and mixed well.

All aliquots were stored in small tightly capped plastic tubes at -70°C. The conjugate aliquots were stored in tin-foil covered tubes.

Procedure

DAY 1

Coating of plate

Microtest plates were coated with anti-human t-PA antibodies. 250µl of the coating antibody was diluted to 25ml with coating solvent. Using an eight-channel pipette (Titertek), 200µl of the coating solution was added to each well of a 96-well microtest plate. The plate was then incubated for 16-18 hours in a moisture chamber at 25°C.

DAY 2

Washing

The coating solution was discarded and the plate washed four times with PET buffer using an automatic microplate washer (Titertek).

Prefilling the plate

150µl of normal goat antibodies (prefill N) was diluted to 12ml with PET-

buffer. 150µl of this solution was added to each odd-numbered (N) well. 150µl of anti-human t-PA antibodies (prefill A) was dilute to 12ml with PET-buffer. 150µl of this solution was added to each even-numbered (A) well. Extreme care was taken not to contaminate prefill N wells with prefill A wells.

Preparing the standard curve

15µl of the reconstituted t-PA standard was added to 485µl of control human plasma and mixed, yielding a t-PA concentration of 30+X ng t-PA/ml of plasma. X was the original t-PA content in the control plasma (3-6ng/ml). The standard dilutions were prepared as follows:

t-PA (ng/ml)	t-PA enriched plasma	Control plasma
30.0 + X	100µl	0µl
24.0 + X	80µl	20µl
18.0 + X	60µl	40µl
12.0 + X	40µl	60µl
6.0 + X	20µl	80µl
3.0 + X	10µl	90µl
1.5 + X	0µl	100µl

Addition of standards and samples

10µl of each standard solution was added to both an N-well and an adjacent A-well in consecutive columns 1 and 2 of the plate. Columns 3-12 were used for the samples. Tissue extracts and supernatants were thawed and added

undiluted to the plate. 10µl of each sample was added to adjacent N- and A-wells. The plate was incubated for three hours at 25°C with gentle agitation on a microtest plate shaker (400-500RPM).

Conjugate

7.96ml of PET-buffer was added directly to 40µl of the conjugate and agitated for 3 minutes. 50µl of conjugate was added to all wells using an eight channel pipette.

Incubation

The microtest plate was incubated, with cover, for two hours at 25°C and agitated on a microtest plate shaker (400-500 RPM).

Washing

The plate was emptied and washed four times with PET-buffer using a microtest plate washing machine (Titertek).

Substrate addition

The substrate (OPD) was dissolved within 10 minutes of use. The lyophylised substrate was reconstituted with 22ml of water and gently agitated for 5 minutes. 1.5ml of 0.15% H_2O_2 was added immediately before use and mixed. 200µl of this substrate solution was then added to each well and the plate was incubated for 30 minutes in the dark at room temperature.

Stopping the reaction

50µl of stop solution (4.5M H_2SO_4) was added to each well with the same

speed and order as the addition of the substrate. Ten minutes was allowed for colour stabilisation.

Measurement

The absorbance was read at 492nm in a microtest plate spectrophotometer (Titertek Multiscan).

Calculations

The difference between the N-value and the corresponding A-value was calculated for both standards and test samples and was denoted A. To obtain the standard curve, A standard values were plotted against the amount of t-PA added to the control plasma (0, 1.5, 3, 6, 12, 18, 24 and 30ng/ml). The standard curve passed through the origin. Sample A values were read from the curve to determine sample t-PA concentrations.

2.2.16 Urokinase Plasminogen Activator (u-PA) Antigen

(Biopool TintElize® u-PA, Sweden)

Assay Principle

This enzyme immunoassay (ELISA) was developed with u-PA monoclonal catch antibody and polyclonal tag antibodies used in a radioimmunoassay described by Astedt et al. (1989). The assay was used to determine quantitatively, urokinase (u-PA) antigen in blood plasma and tissue extract

samples. The assay measured single chain (scu-PA) and high molecular weight (HMW-u-PA) forms of urokinase. The detection limit was 0.1ng/ml. Samples were added to micro-test wells. A-wells were pre-coated with anti u-PA antibodies. N-wells were pre-coated with non-immune antibody. u-PA in the samples bound to the coat antibodies in the n-wells but was prevented from doing so in the A-wells because of the presence of soluble antibodies which bound rapidly to u-PA, thereby blocking the sites necessary to bind u-PA to the wells. Anti-u-PA peroxidase-conjugate goat polyclonal antibody was added to the wells. This bound to antigenic determinants on the bound u-PA molecule forming a sandwich: coat antibody-u-PA-conjugate. The wells were washed to remove unbound conjugate. The substrate (OPD) was added to produce a coloured product at a rate proportional to the amount of coated antibody-u-PA-conjugate in the wells. The conjugate was only bound by non-specific mechanisms in the A-wells. The difference in response between the N- and A-wells was the specific u-PA response.

Reagents

Ready to use microtest plate pre-coated with mouse anti-urokinase monoclonal antibodies and pre-filled with control antibodies

PET-buffer: PBS-EDTA-Tween buffer (0.1M NaCl, 5mM EDTA, 20mM Sodium phosphate buffer, 0.1% Tween 20, pH 7.3)

Standard: highly purified single-chain urokinase

Conjugate:horseradish peroxidase labelled goat anti-human u-PA antibody

u-PA depleted plasma

Substrate: orthophenylenediamine (OPD)

0.15% hydrogen peroxide (H_2O_2)

Stop solution: 3M sulphuric acid (H_2SO_4)

Deionised water

Procedure

Reconstitution of microtest plate

25µl of PET-buffer was added to wells in column 1 and 2. 75µl of PET-buffer was added to the wells of the remaining columns 3-12. The plate was gently agitated for 1 minute. Care was taken not to contaminate between A- and N-wells.

Preparation of standards

u-PA standard was dissolved in 1ml of PET buffer to yield a final concentration of 100ng u-PA/ml. A 4ng/ml solution was prepared by transferring 15µl to 360µl u-PA depleted plasma (PET-buffer was used when assaying tissue extracts).

Standard dilutions were prepared as follows:

scu-PA ng/ml	4ng/ml	u-PA depleted plasma (or PET)
4.00	120µl	0µl
2.00	60µl	60µl
1.00	30µl	90µl
0.50	15µl	105µl
0.16	5µl	120µl
0.00	0µl	120µl

Addition of samples and plasma samples

50µl of u-PA depleted plasma was added to wells in columns 1 and 2 (this made standard and sample wells equal in plasma content). 50µl of each standard was added to N- and corresponding A-wells in column 1 and 2. 50µl of each sample was added to adjacent A- and N-wells.

Incubation

The microtest plate was incubated, with cover, for two hours at 25°C and agitated on a microtest plate shaker (400-500 RPM).

Addition of conjugate

6ml of PET-buffer was added directly to the conjugate and agitated for 3 minutes. 50µl of conjugate was added to all wells using an eight channel

pipette. The plate was incubated for a further one hour at 25°C.

Washing

The plate was emptied and washed four times with PET-buffer using a microtest plate washing machine (Titertek).

Substrate addition

The substrate (OPD) was dissolved within 10 minutes of use. The lyophylised substrate was reconstituted with 22ml of water and gently agitated for 5 minutes. 2ml of 0.15% H_2O_2 was added immediately before use and mixed. 200µl of this substrate solution was then added to each well and the plate was incubated for 15 minutes in the dark at room temperature.

Stopping the reaction

50µl of stop solution (3M H_2SO_4) was added to each well with the same speed and order as the addition of the substrate. Ten minutes was allowed for colour stabilisation.

Measurement

The absorbance was read at 492nm in a microtest plate spectrophotometer (Titertek Multiscan).

Calculations

The difference between the N-value and the corresponding A-value for all standards and samples was calculated, **A**. To obtain the standard curve, **A** standard values were plotted against the standard u-PA concentrations (ng/ml). Sample **A** values were read from the curve to determine sample u-PA concentrations.

2.2.17 Plasminogen Activator Inhibitor-1 (PAI-1) Antigen

(Biopool TintElize^{R} PAI-1, Sweden)

Assay Principle

This enzyme immunoassay is based on the ELISA described by *Declerck et al.* (1988). It was used for the quantitative determination of human PAI-1 (plasminogen activator inhibitor 1, endothelial type) in plasma and tissue extracts. Samples were added to micro-test wells coated with anti-human PAI-1 mouse monoclonal antibodies, which bound the PAI-1 in the sample. A second goat polyclonal anti-PAI-1 antibody, conjugated to peroxidase, was also added. The conjugated antibody bound to the free antigenic determinants on the immobilised PAI-1 forming a coat antibody-PAI-1 antigen-conjugated antibody "sandwich". Unbound conjugated antibody and other unbound materials were washed away and a peroxidase substrate added. The substrate coloured through catalytic oxidation by peroxidase-conjugated antibody and hydrogen peroxide. The colour density was directly

proportional to the amount of PAI-1 in the sample.

The Biopool TintElize^{R} PAI-1 quantified human plasminogen activator inhibitor type 1 (endothelial type). The PAI-1 antigen content of the standard was based on amino acid quantification of PAI-1 using the known composition and the molecular weight (Pannekoek et al., 1986). The assay detected active and latent (inactive) forms of PAI-1. Complexes of PAI-1 (tPA/PAI-1 and uPA/PAI-1) were recovered with about the same efficiency as free PAI-1. The lower detection limit for the assay was about 2 ng PAI-1/ml of fluid.

Reagents

Microtest plate: A-wells pre-coated with anti-PAI-1 mouse monoclonal antibodies and filled with control antibodies. N-wells also contain soluble antibodies directed against non-related structures

PET-buffer: PBS-EDTA-Tween buffer (0.1M NaCl, 5mM EDTA, 20mM Sodium phosphate buffer, 0.1% Tween. pH 7.3)

Conjugated antibody:anti-PAI-1 goat antibody conjugated to peroxidase

PAI-1 depleted plasma

PAI-1 antigen standard plasma

Substrate: orthophenylenediamine (OPD)

0.15% hydrogen peroxide (H_2O_2)

Stop solution: 3M sulphuric acid (H_2SO_4)

Deionised water

Procedure

Reconstitution of microwells

100µl of PET-buffer was added to each well.

Preparation of standards

0.5ml of water was added to both the PAI-1 depleted plasma and the PAI-1 antigen standard (40ng/ml) and were then mixed in small test-tubes, in the following proportions:

PAI-1 Standards (ng/ml)	40	20	10	0
PAI-1 Antigen Plasma (µl)	50	25	12.5	0
PAI-1 depleted Plasma (µl)	0	25	37.5	50

20µl of each standard was added to both N- and A-wells.

Samples

20µl of each sample was added to both an N-well and an adjacent A-well.

Conjugate

7ml of PET-buffer was added directly to the conjugate and agitated for 3 minutes. 50µl of conjugate was added to all wells using an eight channel pipette.

Incubation

The microtest plate was incubated, with cover, for two hours at 25°C and agitated on a microtest plate shaker (400-500 RPM).

Washing

The plate was emptied and washed four times with PET-buffer using a microtest plate washing machine (Titertek).

Substrate addition

The substrate (OPD) was dissolved within 10 minutes of use. The lyophylised substrate was reconstituted with 22ml of water and gently agitated for 5 minutes. 1.5ml of 0.15% H_2O_2 was added immediately before use and mixed. 200µl of this substrate solution was then added to each well and the plate was incubated for 15 minutes in the dark at room temperature.

Stopping the reaction

50µl of stop solution (3M H_2SO_4) was added to each well with the same speed and order as the addition of the substrate. Ten minutes was allowed for colour stabilisation.

Measurement

The absorbance was read at 492nm in a microtest plate spectrophotometer (Titertek Multiscan).

Calculations

The difference between the N-value and the corresponding A-value for both standard and test samples represented the PAI-1 specific response and was denoted **A**. To obtain the standard curve, **A** standard values were plotted against the standard PAI-1 concentrations (40, 20, 10 and 0ng/ml PAI-1). The standard curve passed through the origin. Sample **A** values were read from the curve to determine sample PAI-1 concentrations.

2.2.18 Plasminogen Activator Inhibitor - 2 (PAI-2) Antigen

(Biopool TintElize® u-PA, Sweden)

This assay is similar to the assay described for PAI-1 determination. It is an enzyme immunoassay (ELISA), which detects both the low molecular weight (46.6kDa) and the glycosylated high molecular weight (60kDa) forms of PAI-2. The assay is based on the ELISA described by Lecander and Astedt (1986), using a monoclonal catch (immobilised) antibody and a polyclonal HRP-conjugated antibody.

The only reagent that differs is the PAI-2 standard plasma. All stages of the procedure are the same as that described for PAI-1, except that the samples

were diluted 1/5 with PET-buffer so that the sample absorbance values fell within the range of the standard curve. The PAI-2 standards were prepared as follows:

Concentration (ng/ml)	PAI-2 Standard Plasma (µl)	PAI-2 Depleted Plasma (µl)
100	100	0
75	75	0
50	50	50
25	25	75
0	0	100

2.2.19 Plasminogen Activator Inhibitor-2 (PAI-2) Antigen

(Biopool Imubind^{R} PAI-1, Sweden)

Assay Principle

The IMUBIND PAI-2 ELISA employed a polyclonal antibody against human PAI-2 as the capture antibody. Samples were incubated in precoated microtest wells and were detected with a secondary biotinylated antibody which recognised the bound PAI-2 molecules. The addition of streptavidin conjugated horseradish peroxidase (HRP) completed the formation of the antibody-enzyme detection complex.

The addition of perborate 3, 3', 5, 5' tetramethylbenzidine (TMB) substrate results in a blue coloured solution due to the reaction between TMB and

HRP. Addition of sulphuric acid stop solution yielded a yellow colour. PAI-2 levels were quantified by measuring the solution absorbances at 450nm and comparing them to a standard curve.

The lower detection limit of the assay was 50pg/ of sample. The assay detected the low molecular weight (48 kDa) form found in placental tissue and the high molecular weight (60 kDa) form found in maternal blood and ascitic fluid. free PAI-2 and PAI-2/uPA complexes are recognised with equal sensitivity. The assay was insensitive to PAI-1.

Reagents

6 x 16 well precoated microtest strips with holder and lid

6 vials PAI-2 Standards, 0-10ng/ml (lyophilised)

2 vials Detection Antibody, biotinylated anti-human PAI-2 (lyophilised)

1 vial Enzyme Conjugate, Streptavidin-horseradish peroxidase (100µl)

1 vial Enzyme Conjugate Diluent (11ml)

1 vial Substrate, TMB (11ml)

1 vial Detergent, 25% Triton X-100 (10ml)

2 packets PBS Buffer, pH 7.4

Deionised water

Reagent Preparation

Standards

1ml of water was added to the 0.5, 1.0, 2.5, 5.0 and 10.0ng/ml standard

vials. 2ml of water were added to the 0.0ng/ml standard vial. All vials were then gently agitated for 3 minutes.

Detection Antibody

5.5ml of water was added to each of the two vials and the vials were agitated gently for 3 minutes.

Enzyme Conjugate Diluent

The contents of the enzyme conjugate diluent vial (11ml) were added to 55ml of water and mixed.

Wash Buffer

The contents of one PBS packet was dissolved in 900ml of water. 4ml of 25% Triton X-100 was added and the volume made up to 1 litre.

Sample Buffer

100ml of sample buffer was prepared by adding BSA to wash buffer to a final concentration of 1% w/v (1g BSA/ 100ml wash buffer).

Sample Preparation

All samples were diluted with sample buffer to give a protein content in the range 100μg/ml to 150μg/ml.

Assay Procedure

100µl of PAI-2 standard and samples were added to the microtest wells in duplicate. temperature. The microtest plate was incubated, with cover, for two hours at 25°C. The plate was emptied and washed four times with wash buffer using a microtest plate washing machine (Titertek). 100µl of biotinylated detection antibody was added to each well and the plate incubated for 1 hour at room temperature. The plate was washed 4 times with wash buffer. 10µl of enzyme conjugate was added to 10ml of enzyme conjugate diluent and 100µl of this diluted enzyme conjugate was added to each well. The plate was incubated for 1 hour at room temperature after which it was washed with wash buffer.

100µl of substrate solution was added to each well and allowed to incubate for 20 minutes at room temperature in order to allow the blue colour to develop. The enzymatic reaction was stopped by adding 50µl 0.5M H_2SO_4. The absorbance was read at 450nm within 30 minutes in a microtest plate spectrophotometer (Titertek Multiscan).

Sample PAI-2 concentrations were obtained by comparing the mean absorbance to the standard curve.

2.2.20 Extraction of Fibrinolytic Activators and Inhibitors

Placental and placental bed tissue samples were extracted for estimation of fibrinolytic activators and inhibitors in tissue.

Tissue samples were homogenised in 2M sodium thiocyanate, centrifuged and the supernatant dialysed against PBS. This technique used was based on the method described by Astrup and Stage (1952) and Rybo (1966).

Reagents

Phosphate buffered saline (PBS) 0.14M, pH 7.4

7.2 g Sodium Hydrogen Phosphate ($Na_2HPO_4.2H_2O$)

1g Potassium Hydrogen Phosphate (KH_2PO_4)

1g Potassium Chloride (KCl)

40g Sodium Chloride (NaCl)

The above chemicals were dissolved in 5L of distilled water. The pH was checked and adjusted if necessary.

2M Sodium Thiocyanate (NaSCN)

234.2g Sodium Thiocyanate per litre of water

The pH was adjusted to 7.75 with solid sodium bicarbonate ($NaHCO_3$)

Equipment

TAAB Rotator (type N, 2RPM) for gentle agitation of extracts

Bench- top centrifuge

Polytron tissue pulveriser

Extraction Method

Each frozen sample was weighed and placed in a plastic tube. 2M thiocyanate was added in a ratio of 30µl/mg tissue. The tissue was homogenised for 1 minute in ten second bursts using the Polytron tissue pulveriser. The homogenate and the tip of the pulveriser were kept on ice in between bursts.

The tissue homogenates were incubated at room temperature for one hour and gently agitated.

The homogenates were centrifuged for 10 minutes at 4,500RPM. The supernatants were recovered and the tissue debris pellets discarded. Each supernatant was poured into Visking tubing and dialysed overnight against PBS. Three changes of the PBS were performed.

Following dialysis, the supernatants were stored as 100µl aliquots in pre-labelled tightly capped plastic tubes, snap frozen and stored at -70°C until analysed.

2.2.21 Protein Extraction in Tissue Extracts

The method used to estimate the protein content in the tissue extracts is that described by Lowry et al. (1951). The principle of the assay is protein complex formation with copper and reduction of phosphomolybdic-phosphotungstic (Folin-Ciolteau) reagent by the hydroxyl and sulphydryl groups in the protein results in a colour reaction which is proportional to the protein concentration.

Reagents

Solution A : 2% Potassium Sodium Tartrate, 0.1ml

1% copper sulphate, 0.1ml

2% Sodium Carbonate in 0.1M Sodium Hydroxide, 10ml

The individual solutions were made up and stored separately at 4°C for up to several months. Solution A was prepared immediately prior to use.

Folins Reagent

Bovine Serum Albumin (BSA)

Method

Preparation of Standard Curve

BSA solution was made up immediately before use in a concentration of 0.01g/10ml of distilled water. Dilutions were prepared from the stock BSA to obtain the following standard solutions:

Standard (μg/ml)	BSA (μl)	dH_2O (μl)
0	0	1000
5	5	995
10	10	990
15	15	985
20	20	980
25	25	975
30	30	970
35	35	965
40	40	960
50	50	950
60	60	940

Dilution of samples

Samples were diluted to ensure that the protein content fitted the standard range. 12.5μl of extract was diluted to 1ml with distilled water (1:80 dilution). To obtain a 1:160 dilution, two extract aliquots of 500ml were further diluted to 1ml with water. Standard and sample volumes were equal.

Protein Estimation

2ml of solution A was added to each standard and sample. The test-tubes were vortexed and incubated at room temperature for 10 minutes. 120μl of

Folins Reagent was added and the tubes immediately mixed. The tubes were left standing at room temperature for 30 minutes to allow colour to develop. Absorbances were then read at 650nm on an LKB Ultraspec K spectrometer.

2.2.22 Statistical Analysis

All data was analysed using a statistical graphics package system. A normal probability plot was carried out on each data set to identify parametric and non-parametric data. The following statistical tests were used:

Analysis of Variance (ANOVA)

ANOVA estimates the effects of one or more independent factors on a dependent or response variable in a set of parametric data. In cases where the effects of two factors are measured, the interaction between these two factors may also be tested. The probability that the effect of a given factor is due to chance is measured as the f value. F values less than 0.05 were said to be significant.

Students' t-test

Students' t-test compares the means and variances of two samples where the data is continuous and parametric. Unpaired Students' t-test compared data from two different groups of subjects, paired Students' t-test compared data from the same group of individuals at different stages.

Mann-Whitney-Wilcoxan Test

This test is used for unpaired non-parametric data. The procedure combines and ranks the data from the two samples, the ranks are then summed over all the observations contained in each sample and a statistic is calculated to compare these rank sums, a large statistic represents a low probability that there is no difference between the two samples.

For the above tests, alpha was set at 0.05; therefore all p values obtained which were less than 0.05 were taken as significant. When serial data was analysed, alpha was set at 0.01 to allow for the repeated use of t tests.

Simple Regression

The simple regression procedure fits a model relating one dependent set of data to an independent set of data by minimising the sum of squares of the residuals for the fitted line. Both data sets must be parametric. This model may be linear, multiplicative, exponential or reciprocal. The correlation coefficient (r) provides an estimate of how closely the values adhere to the fitted line. A perfect correlation would give an r value of 1. The p value estimates the probability of the fitted line being horizontal i.e. no relationship between the two sets of data. However, the p value does not give any information on the adherence of the data to the fitted line.

Spearman Rank Correlation Coefficient

This procedure is used to estimate the relationship between the independent and dependent set of non-parametric data. Unlike simple regression, it uses ranks of data rather than the actual values themselves. The r value estimates the correlation between the ranks.

CHAPTER 3
FIBRINOLYSIS IN NORMAL PREGNANCY

3.1 INTRODUCTION

Normal pregnancy is accompanied by dramatic changes in the haemostatic system involving both the coagulation and fibrinolytic system. The physiological changes appear first to maintain the integrity of expanding maternal and foetal circulations at the interface of the placenta and, secondly, to ensure the rapid and effective control of bleeding from the placental site during and after placental separation. Haemostasis during pregnancy and delivery is maintained by an increase in the concentration of several coagulation factors and, most importantly, by a decreased fibrinolytic system. The central role of the fibrinolytic system in normal pregnancy is believed to be control of physiological fibrin deposition in the uteroplacental circulation and the prevention of fibrin deposition in the vascular bed.

3.1.1 Fibrinolysis in the Systemic Circulation during Pregnancy

Fibrinolytic Activity

Biezenski and Moore (1958) were among the first to report a gradual decrease in fibrinolysis during pregnancy with lowest values in the third trimester and during labour, followed by a rapid increase in fibrinolytic activity in the early puerperium. Prior to this, Macfarlane and Biggs (1946) had found no evidence of plasma proteolytic activity in healthy pregnant

women when compared to nonpregnant women. Marguilis *et al.* (1954) reported a higher incidence of fibrinolytic activity in blood during the puerperium than during the antenatal period. Employing similar clot lysis methods, several studies have confirmed a pregnancy-induced reduction in fibrinolytic activity (Shaper *et al.*, 1965; Nilsson and Kullander *et al.*, 1967; Bonnar *et al.*, 1969; Condie and Ogston, 1976; Stirling *et al.*, 1984; Wright *et al.*, 1988). Some studies, however, have questioned the validity and sensitivity of the clot lysis techniques used in many of these reports (Kruithof *et al.*, 1987; Woodhams *et al.*, 1989; Gerbasi *et al.*, 1990). High levels of fibrin degradation products are known to circulate during pregnancy (Balleger *et al.*, 1987; Gaffney *et al.*, 1988; Wright *et al.*, 1988). This suggests an increased rather than a diminished fibrinolytic system associated with pregnancy. Fletcher *et al.* (1979), using fibrinogen chromatography, reported a substantial increase in fibrin deposition from the second gestational month, accompanied by a compensatory increase in fibrinolytic activity. Arias *et al.* (1979), using a whole blood technique, concluded that fibrinolysis was extremely active in term pregnancies. This disputed the interpretation of previous reports where plasma was used, emphasising the existence and importance of cell-mediated fibrinolysis. In 1987, Kruithof *et al.* found that the overall fibrinolytic activity as measured by a radioiodinated fibrin plate assay did not alter significantly during pregnancy.

The precise mechanism of how the fibrinolytic activity is depressed during

pregnancy has been the subject of much discussion and remains uncertain. The complex inter-relationship of fibrinolytic activators and inhibitors during pregnancy has been comprehensively investigated.

Tissue Plasminogen Activator

Plasminogen activator levels are believed to reflect fibrinolytic potential. Early authors concluded that the fall in fibrinolytic capacity during pregnancy was due to decreased levels of plasminogen activator (Brakman, 1966; Nilsson and Kullander, 1967; Bonnar *et al.*, 1969; Astedt *et al.*, 1970; Astedt, 1972). In early studies, Astedt (1970) concluded that when compared with the low fibrinolytic activity in the blood, the activity in the vessel wall is relatively high, so that the release of activators into the blood stream was probably inhibited.

Fletcher *et al.* (1979) proposed a consumptive process whereby absorption to fibrin deposits resulted in low circulating levels of the plasminogen activator. Gore *et al.* (1987), using a chromogenic substrate assay, reported a profound suppression in the release of vascular tPA throughout pregnancy. Wright *et al.* (1988) likewise confirmed a marked reduction in tPA activity levels in the second and third trimester, with a rapid return to nonpregnant levels postpartum.

The introduction of highly specific immunoassays has enabled investigators to measure the antigenic levels of fibrinolytic components. There are conflicting reports on the behaviour of antigenic tPA in pregnancy. Balleger

et al. (1987) concluded that induced tPA release is significantly reduced during pregnancy. However, in contrast, other studies report increases or no variation of tPA antigen during pregnancy (Balleger *et al.*, 1987; Kruithof *et al.*, 1987; Shimada *et al.*, 1989; Daly, 1989; Aznar *et al.*, 1986). Halligan *et al.* (1994) reported that tPA antigen levels increase significantly as pregnancy progresses and remain elevated at six weeks postpartum. Furthermore, Bellart *et al.* (1998) showed a significant increase in tPA levels, which returned to the normal nonpregnant range three days postdelivery.

Urokinase Plasminogen Activator

Over the last ten years attention has been drawn to the urinary plasminogen activator, urokinase (uPA) during pregnancy. Kruithof *et al.* (1987) using a radioimmunoassay measured antigenic levels of pro-urokinase (scu-PA) and reported a 200% increase in levels of scuPA antigen throughout the antenatal period. In contrast, Shimada *et al.* (1989) observed a significant increase in uPA levels confined to the third trimester. Recently, increased levels of combined activable single chain urokinase (scu-PA) and active uPA were recorded during pregnancy (Estelles *et al.*, 1990). Further studies have demonstrated similar results (Koh *et al.*, 1992; Lindoff *et al.*, 1993). Accumulated evidence suggests the placenta to be a producer of uPA. Shimada *et al.* (1989) found the concentration of uPA in uterine vessel blood to be higher than that in peripheral blood, but to decrease after

placental separation, and hypothesised that the trophoblasts are the main source of production.

In 1987, Queenan *et al.* demonstrated the synthesis and secretion of uPA by cultured cytotrophoblasts from term placentas. In a recent study, Graham and McCrae (1996) showed that uPA is the predominant plasminogen activator secreted by trophoblasts isolated from the placenta of normal patients. A high level of circulating immunologic tPA and uPA appears to contradict the depressed fibrinolytic system normally associated with pregnancy.

Plasminogen Activator Inhibition

Brakman and Astrup (1963) were among the first to suggest a fibrinolytic inhibitory mechanism at work during pregnancy. Kawano *et al.* (1968) extracted a selective inhibitor of urokinase from placental homogenate. Subsequent studies confirmed the existence of the placental urokinase inhibitor (Astedt *et al.*, 1970b; Uszynski and Abildgaard, 1971; Holmberg *et al.*, 1978). Opinions differed on the inhibitory effect of blood on urokinase during pregnancy, some authors finding it to be increased (Brakman and Astrup, 1963; Shaper, 1968; Walker *et al.*, 1983; Lottenberg and Willis, 1986) others, normal or even decreased (Nilsson and Kullander, 1967; Bonnar *et al.*, 1969; Hedner and Astedt, 1971). Astedt (1972) investigated the significance of the placenta in the depression of fibrinolytic activity during pregnancy. He disputed a placental urokinase inhibitory effect and

instead, attributed to the placenta a hormonal influence responsible for the decrease in blood fibrinolytic activity during pregnancy. However, the prevailing opinion within the field continues to hold increased plasma inhibition responsible for the decreased fibrinolytic activity observed throughout pregnancy. Walker *et al.* (1983) suggested the possibility of two plasminogen activator inhibitors contributing to increased fibrinolytic inhibition when they discovered that the activity of both urokinase and tissue activator on fibrin plates was inhibited by third trimester plasma to a greater extent than non-pregnant plasma. By incubating increased amounts of t-PA with plasma and then assaying the residual plasminogen activator, Wiman *et al.* (1984) demonstrated the presence of a "novel fast-acting" plasminogen activator inhibitor in increasing amounts during pregnancy. In a subsequent study, Astedt *et al.* (1985) confirmed the presence of this inhibitor in pregnancy plasma. They refuted earlier findings that low fibrinolytic activity during pregnancy was due to impaired release of t-PA from vessel walls but instead, due to inactivation of t-PA by this inhibitor. Subsequent studies reported similar findings (Aznar *et al.*, 1986; Gore *et al.*, 1987). During this period of time a certain amount of confusion existed as to the source of this "novel" inhibitor. Astedt *et al.* (1985a) suggested a similarity between the placental inhibitor of Kawano *et al.* (1958) and the plasminogen activator inhibitor described in their own study. Consequently, Lecander and Astedt (1986) isolated from pregnancy plasma a specific plasminogen activator inhibitor with a

placental source and ascribed this inhibitor responsibility for depressed fibrinolytic activity in pregnancy. Nillson *et al.* (1986) proceeded to demonstrate the existence of two unidentical forms of plasminogen activator inhibitors, endothelial type (PAI-1) (van Mourik *et al.*, 1984) and placental type (PAI-2) (Astedt *et al.*, 1985b), in pregnancy plasma.

Plasminogen Activator Inhibitor 1

Kruithof *et al.* (1987) reported PAI-1 levels ten times higher in pregnant women at term than in non-pregnant women. Similarly, Jorgensen *et al.* (1987) measured a 4 to 8 fold increase in PAI-1 levels reaching peak levels at term. Estelles *et al.* (1989) reported an increase in PAI-1 activity from the first to the third trimester with a corresponding three-fold increase in PAI-1 antigen also determined during pregnancy. Further studies have confirmed these results (Bonnar *et al.*, 1990; Halligan *et al.*, 1994).

Plasminogen Activator Inhibitor 2

In recent years a considerable number of studies have investigated the behaviour of PAI-2, the pregnancy-specific placental inhibitor. Authors have consistently agreed that PAI-2, undetectable in non-pregnant plasma, appears in increasing amounts during pregnancy. This gradual increase in antigenic PAI-2 has found to be almost linear with gestational age and at term, and is from below detection limit (<5ng/ml) to a maximum of 250ng/ml (Kruithof *et al.*, 1987; Lecander and Astedt, 1987; Wright *et al.*,

1988; Booth *et al.*, 1988; Estelles and Gilabert, 1989; Estelles and Gilabert, 1990; Halligan *et al.*, 1994; He *et al.*, 1996). Estelles *et al.* (1989) reported both PAI-2 antigen and functional PAI-2 to increase eight-fold as early as the first trimester, which thereafter increased consistently during each trimester. It has been the objective of several studies to focus on both inhibitors, PAI-1 and PAI-2, in an attempt to establish their relative contribution to the inhibitory capacity of pregnancy plasma. Using quenching antibodies, Jorgensen *et al.* (1987) concluded that PAI-1 was the primary inhibitor of both single-chain and two-chain t-PA and that PAI-2 was the secondary inhibitor of two-chain t-PA in pregnancy plasma. However, in a recent study pregnancy plasma was absorbed to a PAI-2 monoclonal antibody coupled gel which removed approximately 50% of total PAI activity, demonstrating that both PAI-1 and PAI-2, are responsible for the depressed fibrinolytic activity during pregnancy (Lecander *et al.*, 1988). The placenta has been cited the source responsible for increased PAI-1 and PAI-2 during pregnancy (Phillips *et al.*, 1986). Astedt *et al.* (1986) localised PAI-2 in the trophoblastic epithelium using immunohistochemical techniques. Subsequently, Feinberg *et al.* (1989) observed PAI-1 in invading trophoblasts with an accumulation of PAI-2 predominant in villous syncytiotrophoblasts.

3.1.2 Fibrinolysis in the Uteroplacental Compartment

Changes in the Uteroplacental Vasculature

In normal pregnancy the growth and health of the foetus is dependent upon an increasing supply of blood reaching the intervillous space of the placenta. The uterine blood flow to the conceptus gradually increases from less than 50ml/min in early pregnancy to about 800ml/min in late pregnancy (Bonnar, 1987). The placenta is supplied by maternal blood through the spiral arteries. These spiral arteries undergo extensive morphological changes during pregnancy both in the decidua and myometrium of the placental bed. The elastic lamina and smooth muscle of the artery is removed and replaced with a matrix containing cytotrophoblast and varying amounts of fibrin (Brosens *et al.*, 1967; De Wolf *et al.*, 1973; Sheppard & Bonnar, 1974; 1977; 1988). Electron microscopy has shown that the endothelium of the spiral arteries is replaced largely by an intimal layer of cytotrophoblast (Sheppard & Bonnar, 1974a, b). These uteroplacental arteries are sinusoidally distended, extending from their origin in the myometrium through the decidua into the intervillous space. The changes in the uteroplacental arteries allow expansion of the lumen to accommodate the increased blood flow as pregnancy advances, and also reduce the arterial blood flowing to the placenta.

The physiologic alterations to the uteroplacental arteries appear to be

mediated through the activity of the trophoblastic tissue (Brosens *et al.*, 1967; Robertson *et al.*, 1973). Deposition of both intravascular and extravascular fibrin involves the local activation of the coagulation system in the uteroplacental circulation. Trophoblast cells lining decidual spiral arteries are known to have a reduced capacity for fibrinolysis in normal pregnancy (Sheppard & Bonnar, 1976). Sheppard & Bonnar (1978) demonstrated a reduced fibrinolytic capacity within the uteroplacental spiral artery and suggested a release of fibrinolytic inhibitors from the trophoblast.

Fibrin deposition within the uteroplacental vasculature is a normal physiological response of pregnancy. Following the removal of the elastic lamina and smooth muscle, fibrin provides a temporary support within the media of the uterine arteries, which is required in the vasculature as the arteries expand to accommodate the increasing blood flow. Furthermore, fibrin is an ideal substance to provide support during the sudden collapse of the vessels following delivery and placental separation (Sheppard and Bonnar, 1974; 1978). Perivillous deposits of fibrin are observed in term human placentas delivered after normal pregnancy (Grosser, 1925; Fox, 1967). At least three patterns of perivillous fibrin deposits have been described in the human placenta; layering of fibrin near the chorionic and basal plates and around stem villi; agglutination by fibrin and fibrinoid necrosis of villi (Fox, 1978).

3.1.3 Fibrinolysis in Uteroplacental Tissues

Numerous studies have been undertaken to determine the source and concentration of plasminogen activators and their inhibitors in uteroplacental tissue. In general, the fibrinolytic enzymes are found in the placenta and the placental bed.

Plasminogen Activators

The presence of fibrinolytic enzymes in the endometrium of the nonpregnant uterus has been reported (Albrechtsen, 1956a, 1956b, 1957; Rybo, 1966). Rijken *et al.* (1979) demonstrated that tPA from the human uterus was identical to that produced by blood vessel walls. Casslen and Astedt (1983) showed that the endometrium could produce both tPA and uPA; tPA was the major contributor to plasminogen activation in the endometrium while uPA, in smaller concentrations, did not appear to contribute to plasminogen activation.

Uszsynski and Uszynska-Folejewska (1967) reported highest concentrations of plasminogen activator present in the myometrium of non-pregnant women, with a lower concentration by the end of pregnancy and a very low concentration during the advanced stage of labour. However, they calculated levels of total plasminogen activator content ten-fold greater in the last trimester compared with non-pregnant myometrium and concluded that the myometrium is an important source of plasminogen activator in the pregnant woman. They suggested that the sudden fall in the complete

plasminogen activator content during labour suggested the role of uterine contractile activity in releasing the activator into the bloodstream. In a subsequent study the effect of myometrial and placental tissue on plasma fibrinolytic tissue was investigated (Uszsynski and Uszynska-Folejewska, 1968). The myometrium contained the highest concentration of plasminogen activator, whereas the highest concentration of urokinase inhibitor was the placenta. They concluded that the pregnant myometrium is an additional source of plasminogen activator, over and above that of the non-pregnant uterus. In addition the activity of myometrium in fibrinolysis is balanced by that of the placenta, a rich source of fibrinolytic inhibitors and assumed that both tissues took part in the regulation (activation and inhibition) of local and general fibrinolysis during pregnancy (Uszynski & Uszynski-Folejewska, 1968).

Several studies were unable to demonstrate the tissue plasminogen activator in the placenta or decidua (Albrechsten, 1956b; Phillips *et al.*, 1956; Beller *et al.*, 1962). More recently, several groups have reported the presence of tPA and uPA in placental homogenates (Kanfer *et al.*, 1996; Kolben *et al.*, 1996).

In 1976, the synthesis and secretion of plasminogen activator was demonstrated in invading trophoblasts from mice (Strickland & Reich, 1976). They concluded that the trophoblastic expression of plasminogen activator was a contributing factor in embryo implantation. In a subsequent study Martin & Arias (1982) demonstrated that human

trophoblasts from early pregnancy, maintained under tissue culture conditions, produced plasminogen activator. They assumed that variations in the production of trophoblastic plasminogen activator might be critical for the success and failure of placentation (Martin & Arias, 1982). In 1987, Queenan *et al.* reported that cultured human cytotrophoblasts synthesised and secreted urokinase-type plasminogen activator (uPA). They assumed their results accounted for the capacity of placental cells to break down extracellular matrix proteins such as trophoblast implantation to the uterine wall (Queenan *et al.*, 1987).

In an earlier study, Sheppard *et al.* (1978), using electron microscopy and the fibrin slide technique, demonstrated that cytotrophoblasts as components of the intimal lining of decidual spiral arteries showed no fibrinolytic activity. In contrast, endothelial cells, also lining the intima of these arteries exhibited a considerable degree of fibrinolytic activity (Sheppard *et al.*, 1978).

Plasminogen Activators Inhibitors

Inhibitors of plasminogen activation have repeatedly been associated with the placenta (Kawano *et al.*, 1968; Astedt *et al.*, 1970; Uszynski and Abildgaard, 1971; Holmberg *et al.*, 1978). A specific placental-type plasminogen activator inhibitor, PAI-2, has since been purified from the placenta and characterised (Astedt *et al.*, 1985). Astedt *et al.* (1986), using immunohistochemical techniques, localised PAI-2 in the trophoblastic

epithelium of the placenta. Similarly, Sheppard *et al.* (1990) localised PAI-1 to the vascular endothelium and PAI-2 to the trophoblastic epithelium in placenta. Furthermore, they localised PAI-2 in trophoblast cells of decidual spiral arteries that had undergone the physiological changes of normal pregnancy (Sheppard *et al.*, 1990). In the same study, they reported a significantly higher level of PAI-2 as compared to PAI-1 in placental homogenates (Sheppard *et al.*, 1990). Other studies have since reported similar findings (Grancha *et al.*, 1998; Estelles *et al.*, 1998). PAI-1 and PAI-2 concentrations have also been identified in the decidual and myometrial segments of the placental bed, with significantly higher levels of PAI-1 when compared to PAI-2 in the decidual segment (Sheppard *et al.*, 1990). The placenta is the sole source of PAI-2 during pregnancy. Although PAI-1 has also been demonstrated in the placenta (Phillips *et al.*, 1986; Jonasson *et al.*, 1989) it is unlikely to be an exclusive source.

The contribution of the endothelial cells in the maternal vasculature to the increasing levels of PAI-1 during pregnancy is not known.

3.1.4 Trophoblast Invasion

Trophoblast invasion is not due to passive growth pressure but to an active biochemical process. Mossman (1937) first proposed the possibility that the invasiveness of trophoblast might be associated with the proteolytic activity. Later, Blandau (1949) published experimental evidence relating trophoblast invasiveness and proteolysis. That report showed that guinea-pig

trophoblast was capable of digesting a dried gelatin film. The same observation was made with rabbit trophoblast but the proteolytic activity was detected in the abembryonic pole of the blastocyst (Denker, 1972,1982). This proteolytic activity was progesterone dependent and inhibited by endometrial protease inhibitors.

The study of proteases and trophoblast implantation really began with the paper by Strickland, Reich and Sherman (1976). They showed that the trophoectoderm of mouse blastocyst produced plasminogen activator (PA) *in vitro* and that the maximal rate of production of this protease was observed at the time of implantation. Furthermore, PA production was observed in the parietal endoderm of these blastocysts. This lead to the conclusion that PA production by blastocysts was an important factor in tissue remodelling during implantation and embryogenesis. The importance of PA in implantation of the mouse blastocyst was further documented in mutant mice with implantation deficiencies. The blastocysts of these mice attached and grew *in vitro* but showed decreased invasiveness and decreased PA production (Axelrod, 1985).

The production and regulation of PA in human trophoblast has been documented by several studies. It was shown that trophoblast cell cultures produce PA and that PA activity leads to the digestion of a fibrin matrix (Martin and Arias, 1982). Oestradiol or progesterone does not modulate the activity of the enzyme. Purified cytotrophoblast cells in culture produce uPA, while cAMP transiently increases uPA mRNA. These cells also produced

fibronectin but in an inverse relationship to uPA, thus suggesting a possible coupling of fibronectin and uPA gene expression (Queenan *et al.*, 1987).

It has been suggested that the plasminogen activator system plays an important role in mediating trophoblast invasion of the uterus (Yagel *et al.*, 1988; Sappino *et al.*, 1989) and, through its effect on fibrinolysis, in the regulation of uteroplacental blood flow (Multhaupt *et al.*, 1994; Graham and McCrae, 1996). Plasminogen activators are thought to play an important role in cellular invasiveness by virtue of their ability to generate plasmin. Unlike plasminogen activators, plasmin has broad substrate specificity. While the "classical" substrate for plasmin is fibrin, plasmin can cleave most proteins of the ECM. Furthermore, plasmin has been shown to activate a variety of matrix metalloproteinases (Grant *et al.*, 1987; He *et al.*, 1989 Kleiner *et al.*, 1993; Mignatti and Rifkin, 1996; Saarinen *et al.*, 1994) known to be directly involved in the breakdown of the ECM and hence invasion. Indeed, Yagel *et al.* (1988) showed that plasminogen activators, plasmin and metalloproteinases are important for invasion of first trimester human cytotrophoblasts. Furthermore, it has been reported that MMP-9 (92-kDa gelatinase; gelatinase B) is the main proteinase required for first trimester human trophoblast invasion (Librach *et al.*, 1991). Incidentally, MMP-9 can be activated directly by plasmin (Mignatti and Rifkin, 1996).

Plasmin itself may also be involved in the maintenance of blood fluidity within the uteroplacental vessels and intervillous spaces by preventing the formation of fibrin clots. Recently, Graham (1997) showed that TGF-ß1 might

down-regulate PA activity in cultured first trimester human cytotrophoblasts by reducing uPA secretion and increasing PAI-1 production and secretion. This result suggests that TGF-ß may also play a role in the control of trophoblast invasion.

3.1.5 Trophoblast Fibrinolytic Activity

Cultured human trophoblast cells have been demonstrated to secrete uPA with proteolytic activity (Martin and Arias, 1982; Queenan *et al.*, 1987). In addition, Feinberg *et al.* (1989) localised both PAI-1 and PAI-2 in cultured trophoblast, with PAI-1 being present in the cytoplasm and cell surface membrane while PAI-2 occurs only in the cytoplasm. Immunohistological staining of placental sections localised PAI-2 only to villous syncytiotrophoblast, suggesting a possible role of this inhibitor in regulating fibrinolysis within the intervillous space. In contrast, PAI-1 is located mainly among the invading population of extravillous cytotrophoblast, which could reflect a role in trophoblast migration. A recent study claims to detect uPA as well as both PAI-1 and PAI-2 in all trophoblast populations from ectopic pregnancies (Hoffmann *et al.*, 1994). They suggested that their results supported a role for uPA and/or PAI-1, PAI-2 in the controlled invasion of the maternal decidua by the trophoblast during human implantation.

Zini *et al.* (1992) demonstrated the existence of uPA receptors (uPAR) on cultured human trophoblast cells. These trophoblast uPAR appear to be

similar in many respects to that described on other cell types, such as U937 cells, macrophages and endothelial cells. Urokinase binds to uPAR with high affinity via its growth factor domain and binding is largely reversible. Trophoblast uPAR are completely saturated with endogenous uPA, which enables these cells in culture to convert plasminogen to plasmin in the absence of an endogenous source of uPA. Studies have indicated that the trophoblast cells have no net uPA activity due to the high levels of PAI-1 and PAI-2 which are present in the immediate vicinity of the cells but cell-associated uPA activity does occur when the inhibitors are removed (Zini *et al.*, 1992; Graham and McCrae, 1996). Multhaupt *et al.* (1994) detected that uPAR is expressed in placental bed giant cells in a polarised fashion with increased expression at the migrating edge. This suggests that uPAR may play an important role in the mediation of trophoblast invasion.

3.1.6 Aims and Objectives

The first aim of this section of the study was to examine the secretory levels of PAs and PAIs by the placenta and placental bed. As stated above, many studies have identified the placenta as a source of PAs and PAIs. However, the contribution of the placenta and placental bed to the uteroplacental circulation is unknown. By maintaining placental and placental bed biopsies in organ culture, this study wished to increase our knowledge of the residual and secreted levels of PAs and PAIs by

the placenta and placental bed.

Many studies have mimicked trophoblast invasion *in vivo* by isolating and culturing trophoblast cells from placenta. As stated earlier, the secretion of PAs and PAIs by trophoblasts may be critical in regulating their ability to degrade ECM. This study isolated placental and placental bed trophoblast cells. One objective of the study was to compare PA and PAI activity by placental trophoblasts with that of placental bed trophoblasts. As these cells come from different tissues, there may be changes in the levels of secretion of PAs and PAIs. Furthermore, to this author's knowledge, no other study has examined PA and PAI levels in isolated placental bed trophoblasts.

Changes in fibrinolytic activity by placental and placental bed trophoblasts may be of importance in terms of their ability to successfully migrate during pregnancy. In this study, a 'fingerprint' of their migratory abilities is determined by invasion assays. The aim of this section of the study is to determine the overall migrationary ability of placental and placental bed trophoblasts through fibrin and matrigel in order to determine a basic level for further experiments regarding trophoblast cells from pre-eclamptic and IUGR pregnancies.

3.2 RESULTS

3.2.1 Normal Pregnancy: Tissue Levels of Fibrinolytic Activators and Inhibitors in Placental and Placental Bed Extracts

The following results are based on 19 placental and 19 placental bed biopsies.

Plasminogen Activator Inhibitor Antigen (PAI-1): (Fig 3.1) Significantly higher levels were found of PAI-1 in the placenta than in the placental bed ($p < 0.001$).

Plasminogen Activator Inhibitor Antigen (PAI-2): (Fig 3.2) Antigenic PAI-2 was detected in both the placenta and the placental bed extracts. Compared with the placental bed PAI-2 was significantly higher in the placenta ($p<0.001$).

Urokinase Plasminogen Activator Antigen: (Fig 3.3) Antigenic u-PA was detected in both the placenta and the placental bed. Placental u-PA levels were significantly higher than the levels found in the placental bed ($p<0.001$).

Comparable Antigenic Levels: (Fig 3.4) Within the placental extracts PAI-1 and PAI-2 were present in significantly greater amounts than u-PA ($p<0.0001$; PAI-1) ($p<0.005$; PAI-2)

Comparable Antigenic Levels: (Fig 3.5) Within the placental bed extracts PAI-1 and PAI-2 levels are significantly higher than u-PA levels ($p<0.0001$; PAI-1) ($p< 0.0005$; PAI-2).

3.2.2 Normal Pregnancy: Tissue Levels of Fibrinolytic Activators and Inhibitors in Placental and Placental Bed Trophoblasts

Time Course for the Differentiation of Placental Trophoblasts: (Fig 3.6) During culture the cells undergo morphological changes during culture. After 48 hours approximately 20% of the cells are single cells while the remainder is made up of mainly aggregates of trophoblast cells.

Time Course for the Differentiation of Placental Bed Trophoblasts: (Fig 3.7)
Morphological changes occur for placental bed trophoblasts in culture. After 48 hours 90% of the cells are single cells and aggregates.

Time Course for hCG Production: (Fig 3.8) hCG levels are used as a measure of trophoblast viability. In this study the trophoblast cells secreted increasing amounts of hCG as culture progressed. This suggests that the cells are thriving.

Time Course for u-PA Production in Trophoblasts: (Fig 3.9) Placental trophoblasts actively secreted u-PA into the culture supernatant. No significant changes in the levels of secretion are seen over the 4 days of culture.

Time Course for PAI-1 Production in Trophoblasts: (Fig 3.10) PAI-1 secretion increases over the first 48 hours after which the levels secreted reach a plateau. No significant changes can be seen at any time point.

Time Course for PAI-2 Production in Trophoblasts: (Fig 3.11) Levels of PAI-2 secretion by the placental trophoblasts remain constant over the 4 days of culture.

Time Course for u-PA Production in Placental Bed Trophoblasts: (Fig 3.12) Placental bed trophoblasts grow in culture in a similar manner to placental trophoblasts. No significant changes occur in u-PA levels are seen over the time course.

Time Course for PAI-1 Production in Placental Bed Trophoblasts: (Fig 3.13) PAI-1 is actively secreted by the cells and no significant changes in its production are seen over the time course.

Time Course for PAI-2 Production in Placental Bed Trophoblasts: (Fig 3.14)
There were no significant changes in the production of PAI-2 by placental bed trophoblasts over the time course.

Comparison of u-PA, PAI-1 and PAI-2 in Placental Trophoblasts: (Fig 3.15) PAI-1 and PAI-2 secretion are significantly greater than u-PA ($p<0.01$). PAI-1 is the major inhibitor produced by placental trophoblast cells.

Comparison of u-PA, PAI-1 and PAI-2 in Placental Bed Trophoblasts: (Fig 3.16) PAI-2 production by placental bed trophoblasts is significantly higher than for PAI-1 and u-PA. This suggests that alterations have occurred in placental bed trophoblasts which are not present in placental trophoblasts.

3.2.3 Normal Pregnancy: Comparative Changes in Invasion Abilities of Placental and Placental Bed Trophoblasts

Fibrin Degradation by Trophoblasts: (Fig 3.17) Approximately 75% of all trophoblast populations managed to lyse fibrin and pass through the fibrin clot. No significant differences are seen between placentally-derived trophoblasts and placental bed trophoblasts.

Matrigel Degradation by Trophoblasts: (Fig 3.18) Approximately 65% of trophoblast cells successfully invaded the matrigel (an artificial basement membrane matrix). However, no significant changes are seen in the % invasion of the two groups of cells.

Cell Surface Plasminogen Activator Activity: (Fig 3.19) No significant changes in activity levels were seen between the two cell types.

3.2.4 Normal Pregnancy: Comparative Changes in Secretion Levels of Placental and Placental Bed Biopsies in Organ Culture

Secretion of Fibrinolytic Factors: (Table 3.1) The placenta secretes higher amounts of PAI-1 and PAI-2 than the placental bed. After 48 hours these differences are only significant for PAI-2. No comparable changes are seen for u-PA.

3.2.5 Comparison of Fibrinolytic Factors in Control and Normal Pregnancy

19 placental biopsies and 17 controls were used for this study of fibrinolytic factors in placental extracts.

Secretion of Fibrinolytic Factors: (Table 3.2) No significant changes could be found for t-PA, u-PA, PAI-1 or PAI-2 when comparing normal pregnancy to controls. This suggests that placental fibrinolytic is unchanged during the last few weeks of pregnancy.

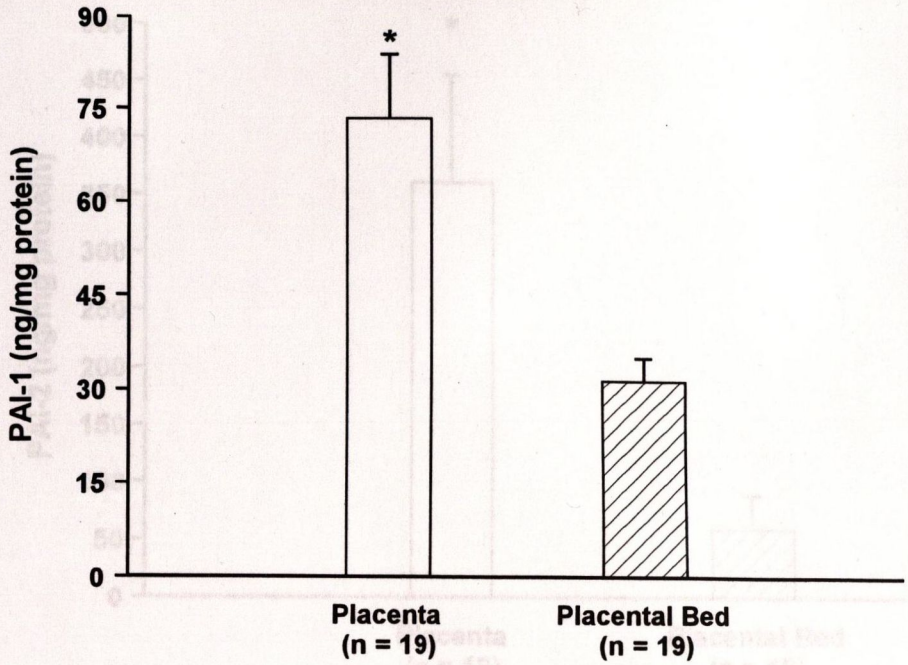


Fig 3.1: Normal Pregnancy: Comparative tissue levels of PAI-1 in the placenta and placental bed.
* $p<0.001$
Values are mean $\pm$ SD. Statistical test is Student's t-test.

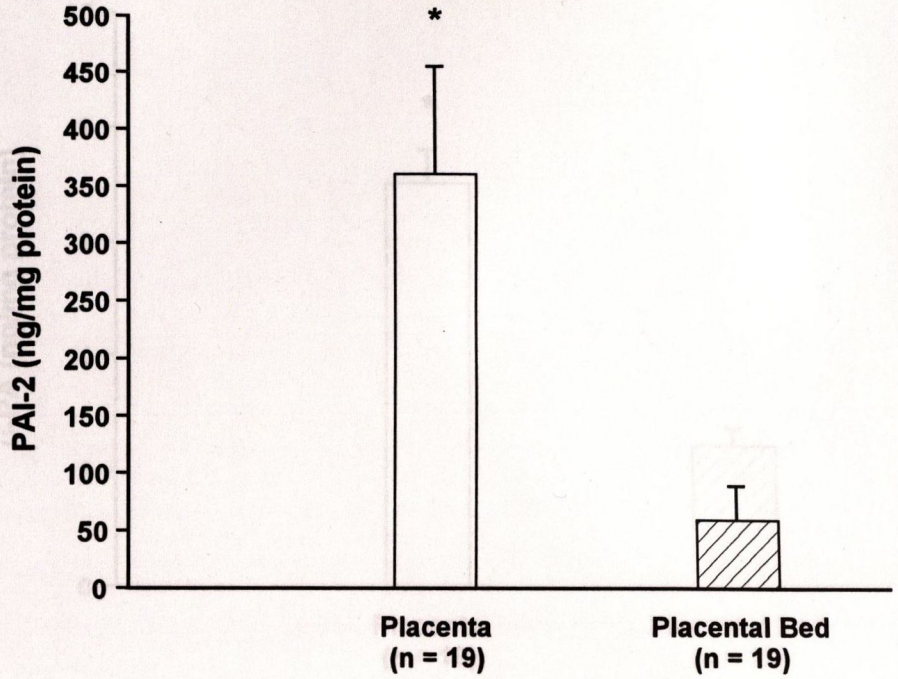


Fig 3.2: Normal Pregnancy: Comparative tissue levels of PAI-2 in the placenta and placental bed.
* $p<0.001$
Values are mean $\pm$ SD. Statistical test is Student's t-test.

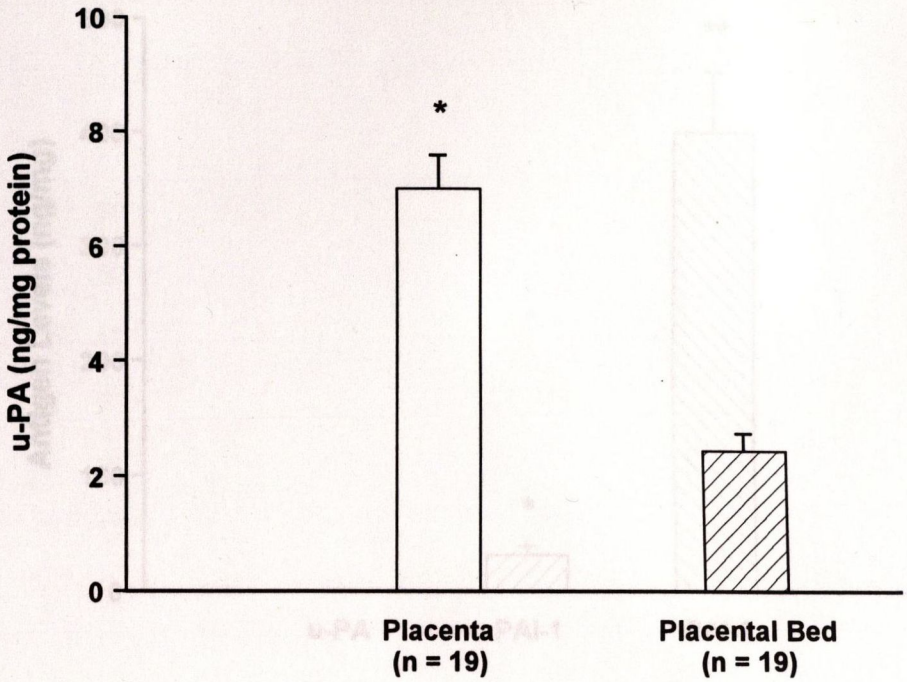


Fig 3.3: Normal Pregnancy: Comparative tissue levels of u-PA in the placenta and placental bed.
* $p<0.001$
Values are mean $\pm$ SD. Statistical test is Student's t-test.

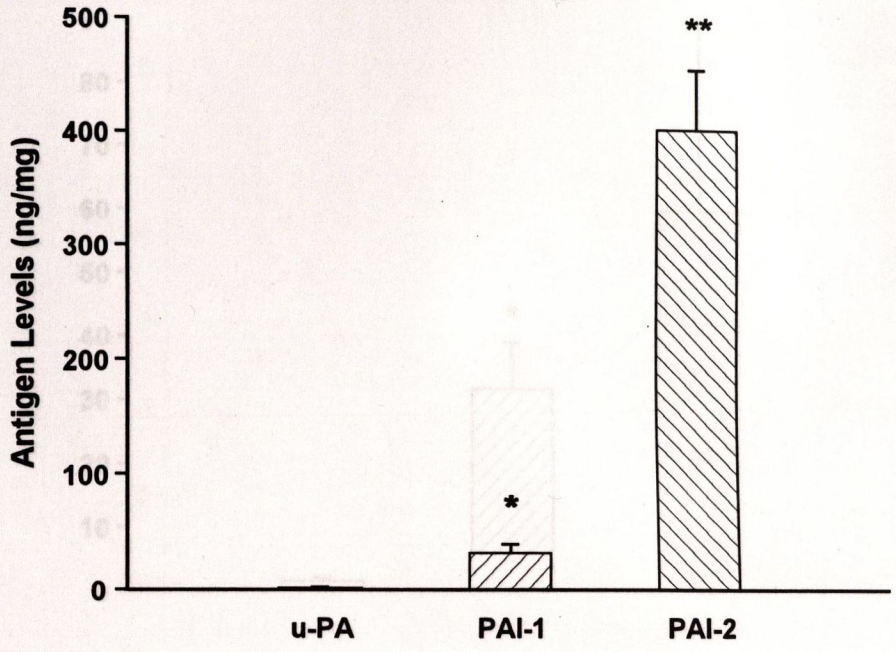


Fig 3.4: Normal Pregnancy: Comparison of u-PA, PAI-1 and PAI-2 antigen levels in the placenta.
* $p<0.0001$ (PAI-1 vs. u-PA)
** $p<0.0005$ (PAI-2 vs. u-PA)
Values are mean $\pm$ SD. Statistical test is Student's t-test.

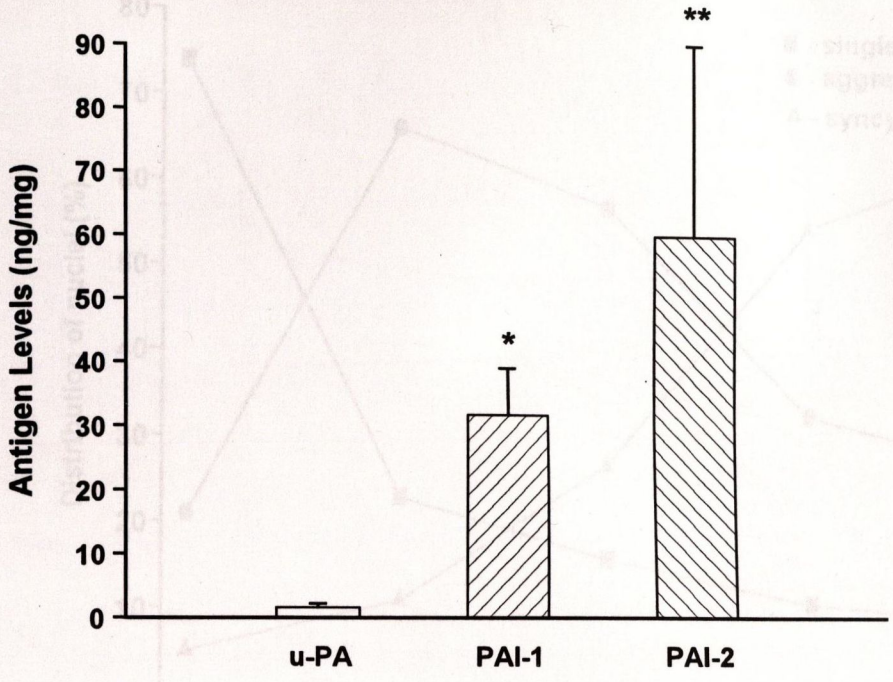


Fig 3.5: Normal Pregnancy: Comparative tissue levels of u-PA, PAI-1 and PAI-2 antigen levels in the placental bed.
* $p<0.0001$ (PAI-1 vs. u-PA)
** $p<0.0005$ (PAI-2 vs. u-PA)
Values are mean $\pm$ SD. Statistical test is Student's t-test.

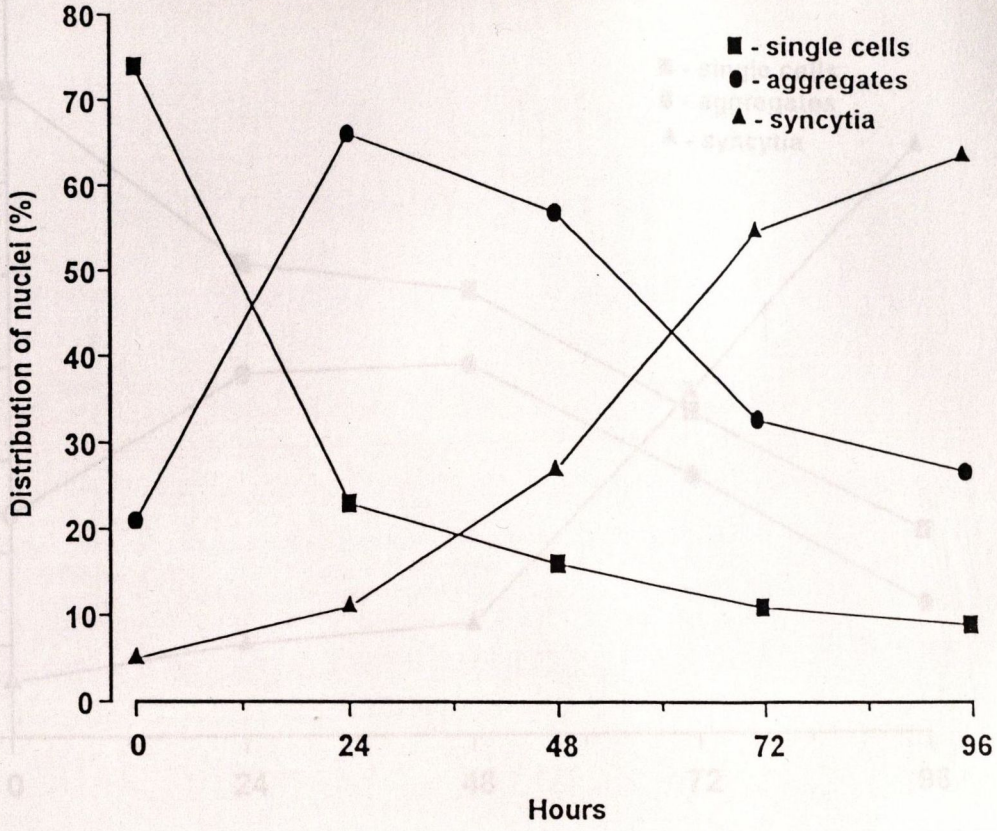


Fig 3.6: Normal Pregnancy: An example time course for the differentiation of placental trophoblast cells in culture. (n = 1).

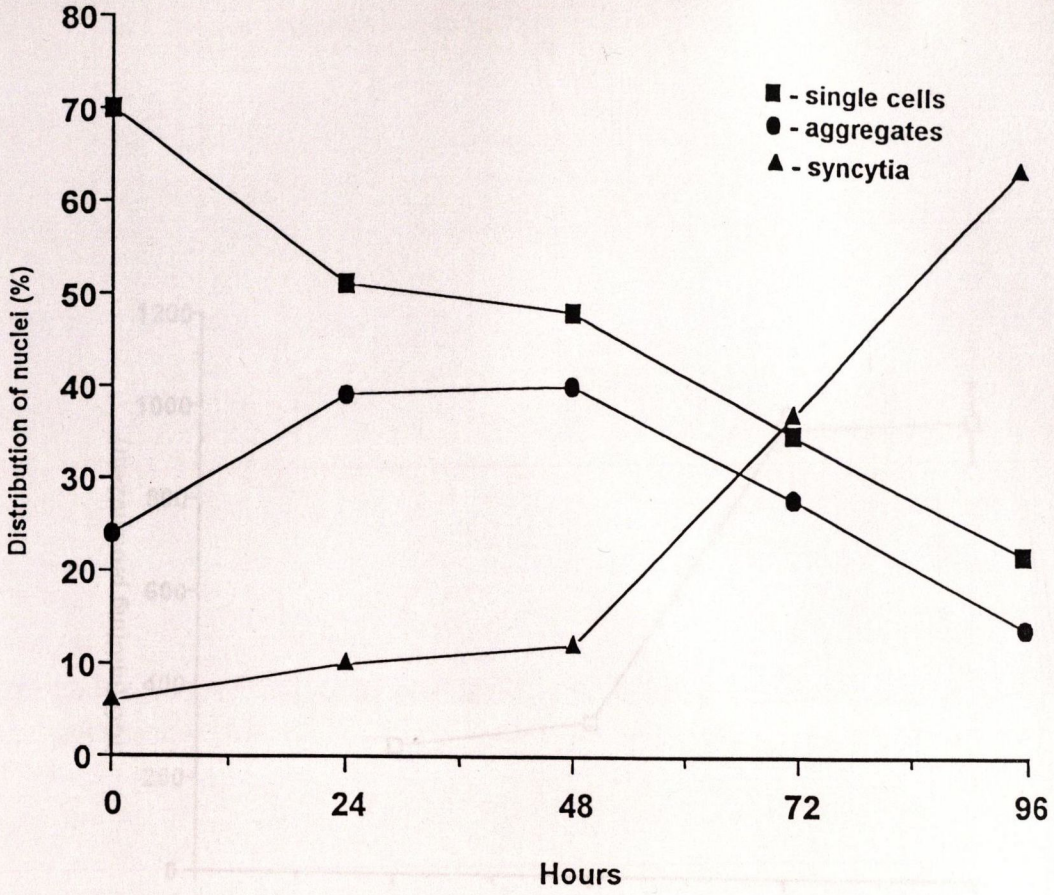
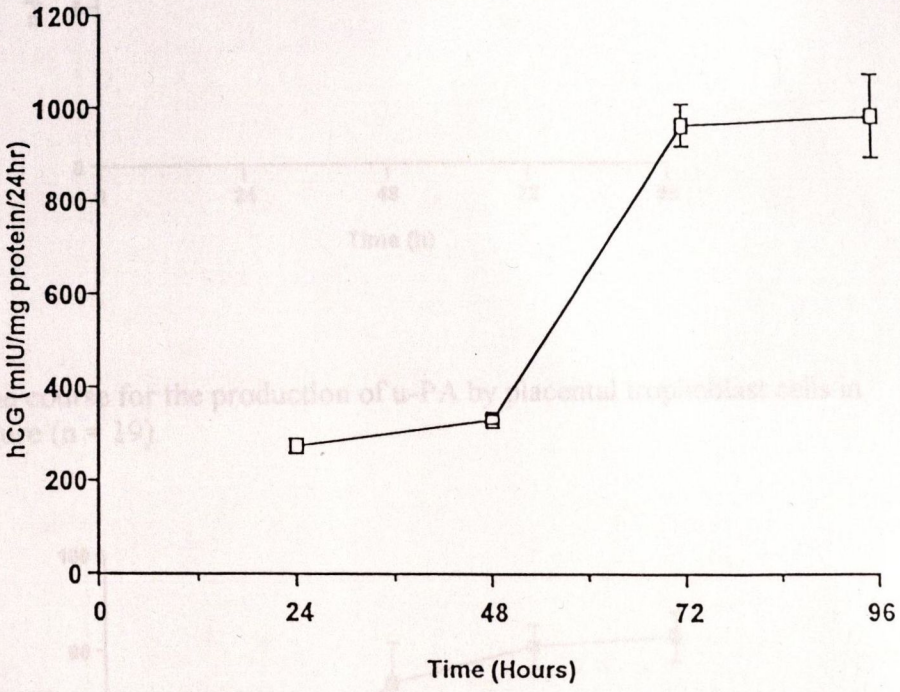


Fig 3.7: Normal Pregnancy: An example time course for the differentiation of placental bed cells in culture. (n = 1)



. Fig 3.8: Example of a time course for the production of hCG by trophoblast cells in culture (n = 5).

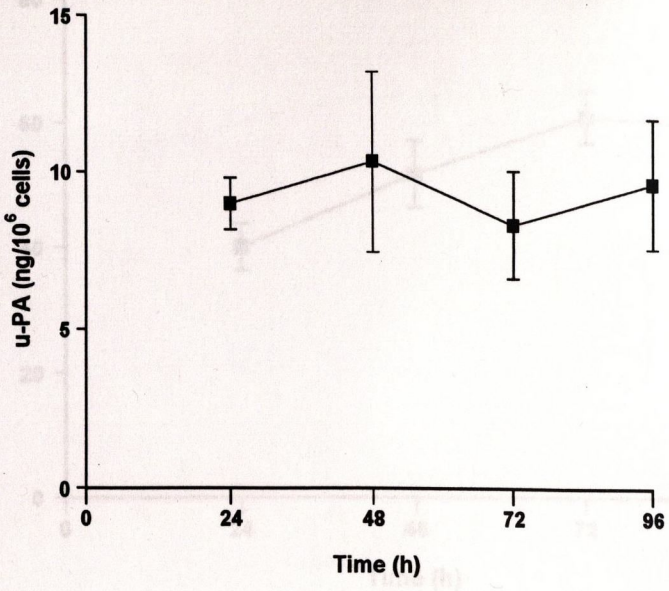


Fig 3.9: Time course for the production of u-PA by placental trophoblast cells in culture (n = 19).

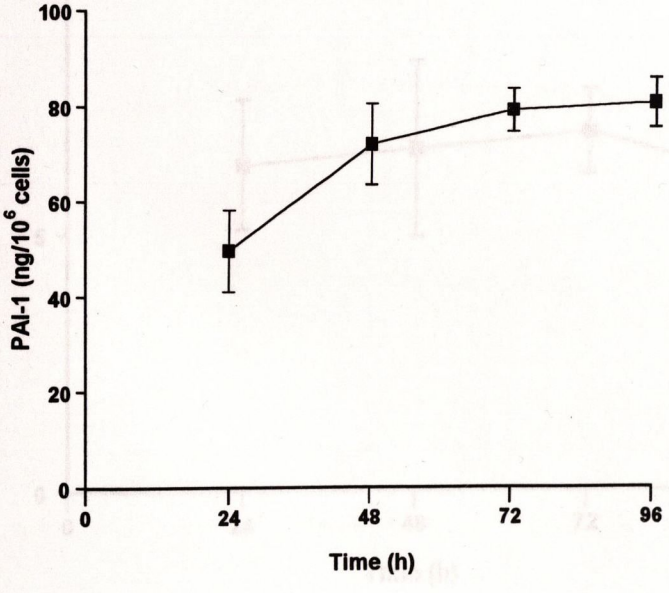


Fig 3.10: Time course for the production of PAI-1 by placental trophoblast cells in culture (n = 19).

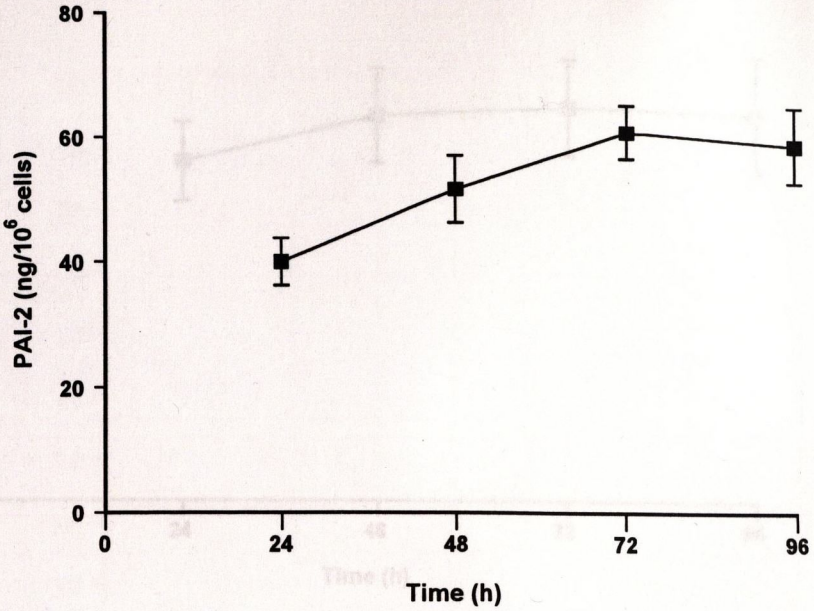


Fig 3.11: Time course for the production of PAI-2 by placental trophoblast cells in culture (n = 19).

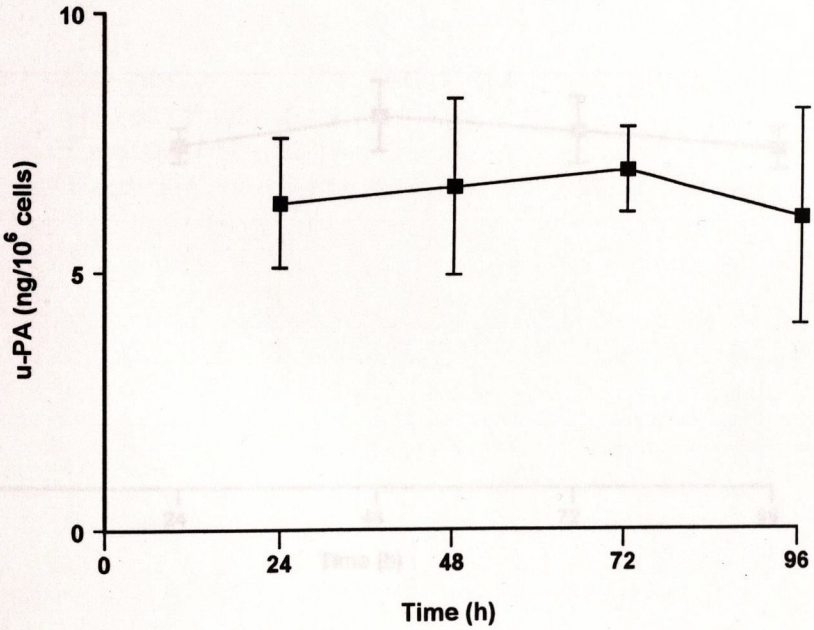


Fig 3.12: Time course for the production of u-PA by placental bed trophoblast cells in culture (n = 19).

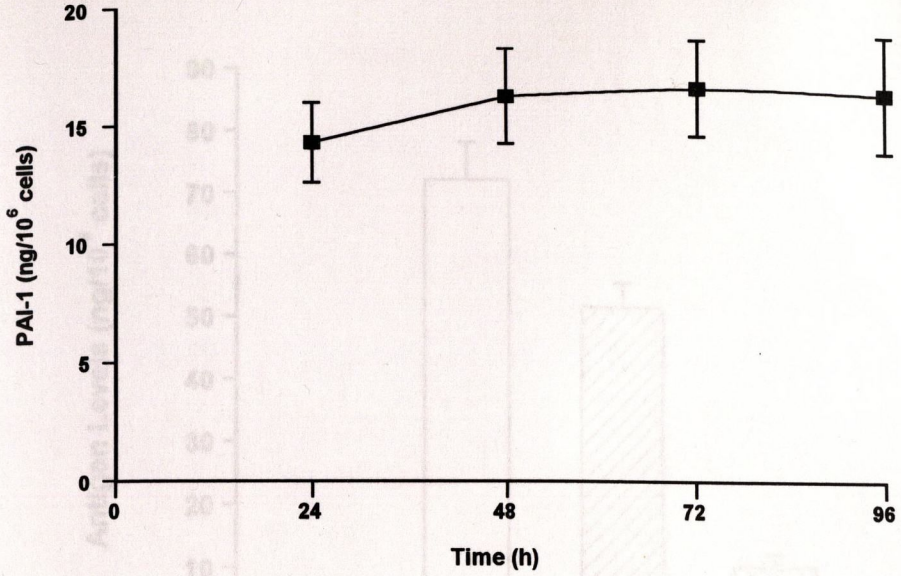


Fig 3.13: Time course for the production of PAI-1 by placental bed trophoblast cells in culture (n = 19).

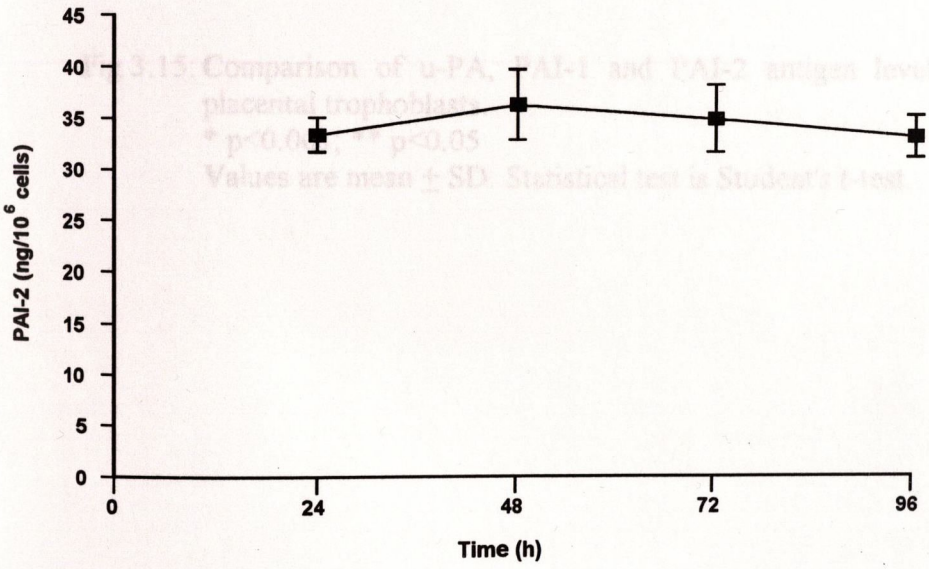


Fig 3.14: Time course for the production of PAI-2 by placental bed trophoblast cells in culture (n = 19).

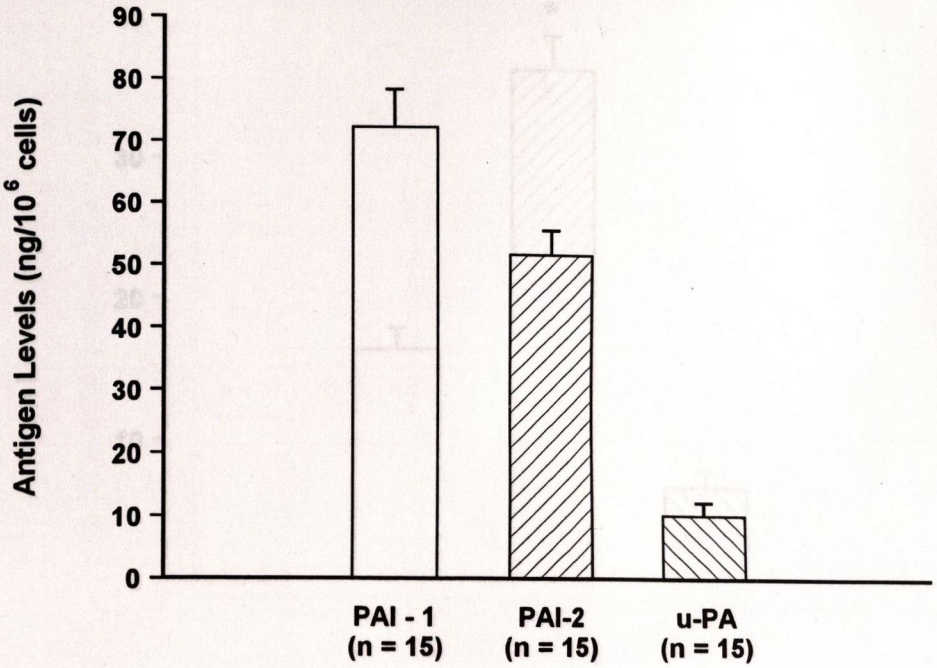


Fig 3.15: Comparison of u-PA, PAI-1 and PAI-2 antigen levels secreted by placental trophoblasts.
* $p<0.001$; ** $p<0.05$
Values are mean $\pm$ SD. Statistical test is Student's t-test.

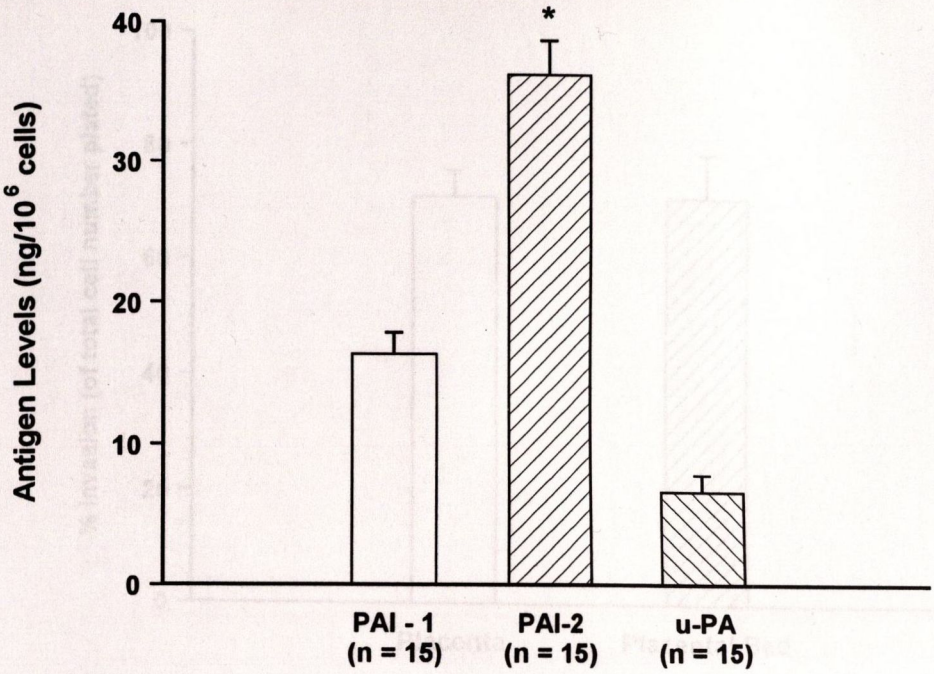


Fig 3.16: Comparison of u-PA, PAI-1 and PAI-2 antigen levels secreted by placental bed trophoblasts.
* $p<0.001$
Values are mean $\pm$ SD. Statistical test is Student's t-test.

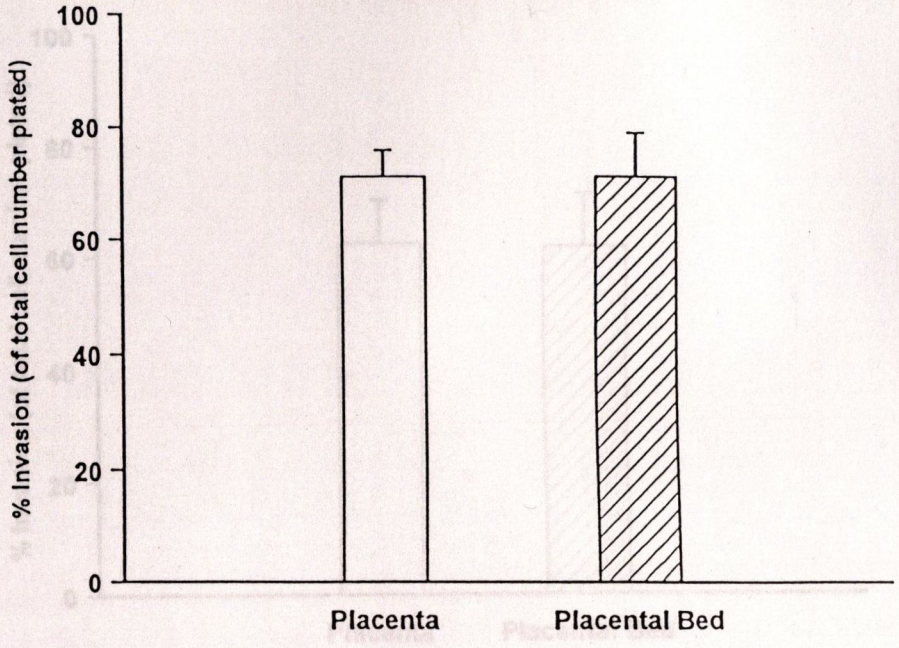


Fig 3.17: Comparison of fibrin invasion by placental and placental bed trophoblasts.
Values are mean ± SD. Statistical test is Student's t-test.

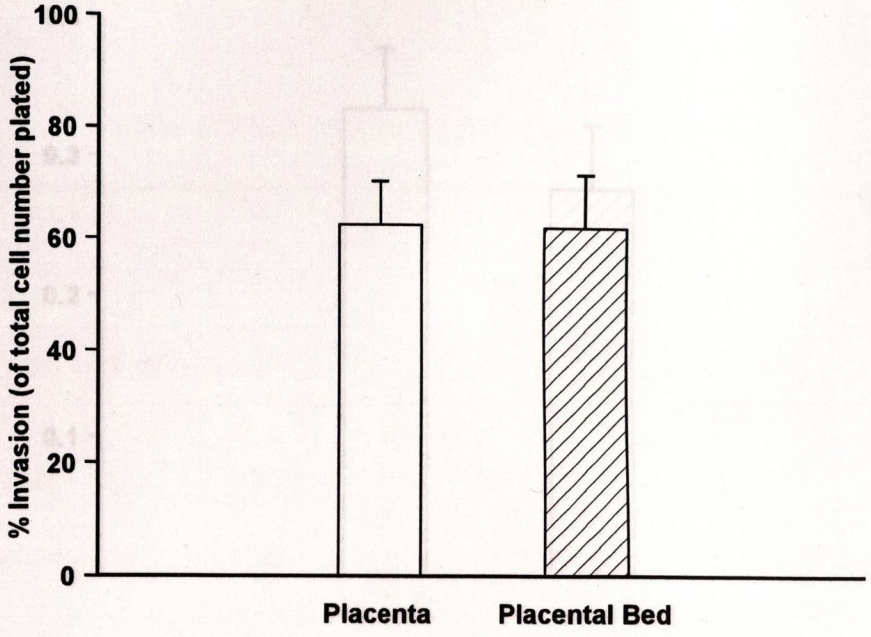


Fig 3.18: Comparison of matrigel invasion by placental and placental bed trophoblasts.
Values are mean $\pm$ SD. Statistical test is Student's t-test.

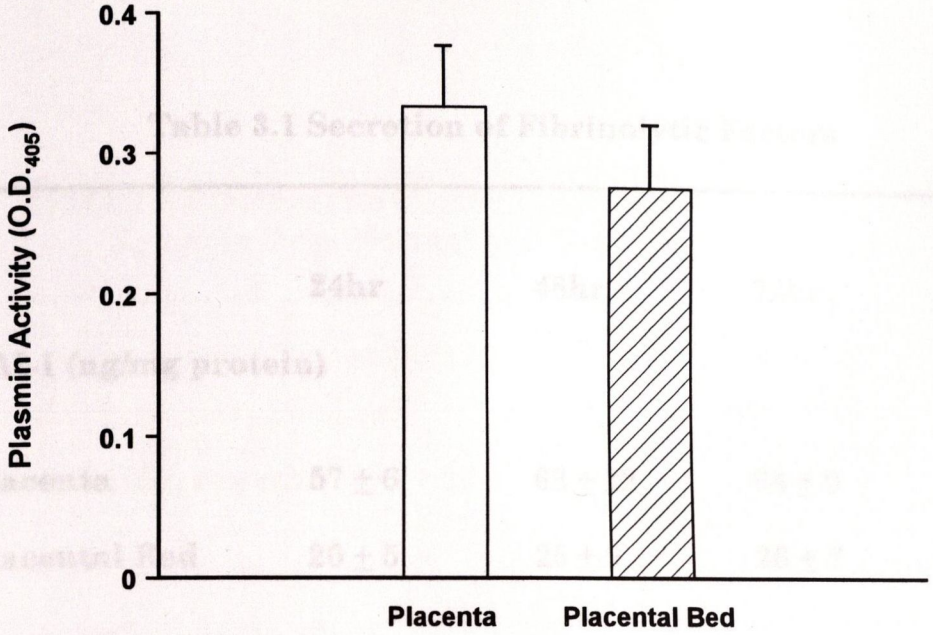


Fig 3.19: Comparison of cell associated plasminogen activator activity in placental and placental bed trophoblasts.
Values are mean $\pm$ SD. Statistical test is Student's t-test.

Table 3.1 Secretion of Fibrinolytic Factors

	24hr	48hr	72hr
PAI-1 (ng/mg protein)			
Placenta	57 ± 6	63 ± 12	64 ± 9
Placental Bed	20 ± 5	25 ± 9	26 ± 7
PAI-2 (ng/mg protein)			
Placenta	45 ± 9	53 ± 12 *	54 ± 11
Placental Bed	10 ± 6	12 ± 3	13 ± 8
u-PA (ng/mg protein)			
Placenta	8 ± 3	7 ± 2	8 ± 4
Placental Bed	3 ± 1.1	5 ± 2.8	6 ± 2.4

*$p < 0.001$ (Placenta vs. Placental Bed) n = 15
Values are mean ± SD. Statistical test is Student's t-test.

Table 3.2 Fibrinolytic Factor Concentrations in Placental Extracts from Normal and Control Pregnancies

	NORMAL (n = 19)	CONTROL (n = 17)
PAI-1 (ng/mg protein)	63 ± 12	78 ± 23
PAI-2 (ng/mg protein)	63 ± 12	64 ± 12
u-PA (ng/mg protein)	7.6 ± 3	8.9 ± 4.3

Values are mean ± SD. Statistical test is Student's t-test.

3.3 DISCUSSION

Profound modifications of the fibrinolytic system are known to occur during pregnancy. This study measured the behaviour of several fibrinolytic activators and their inhibitors of the uteroplacental tissues in a group of nineteen patients.

Fibrinolysis in Uteroplacental Tissues

In the present study tissue-type plasminogen activator (t-PA) was found in the placenta and placental bed. No significant differences in antigen levels or activity could be found when comparing the placenta to the placental bed. In accordance with the present results several studies have detected minimal traces of t-PA in placental extracts (Jonasson *et al.*, 1989; Kolben *et al.*, 1996; Kanfer *et al.*, 1996). However, other studies were unable to demonstrate t-PA in the placenta or decidua (Albrechsten, 1956b; Philips *et al.*, 1956; Beller *et al.*, 1962; Boyle, 1992). Uszynski and Usynska – Folejewska (1967) calculated a total plasminogen activator content in the myometrium ten-fold greater in the last trimester compared with non-pregnant myometrium. With the sudden fall in plasminogen activator during labour it was suggested that uterine contractile activity played a role in releasing the activator into the bloodstream. The presence of t-PA in tissues of the placental bed would contribute to the controlled laying down of fibrin within the uteroplacental spiral arteries and on release during labour provide fibrinolytic control during the departure of the placenta from the

maternal interface. The source of t-PA in the placenta and placental bed may be explained by partly from endothelial cells and partly from blood platelets that are activated and consumed in the uteroplacental microcirculation.

Urokinase plasminogen activator (u-PA) was found in both the placenta and the placental bed. Levels were significantly higher in the placenta. The relatively higher levels of u-PA compared with the placental bed suggests a placental source. Shimada *et al.* (1989) detected higher levels of u-PA antigen in uterine blood than peripheral blood and in accordance with the present study suggested that the placenta was the main source of this activator during pregnancy. The levels of u-PA seen in the placenta and placental bed may be due to secretion of this fibrinolytic activator by trophoblasts which are present in the tissue. Hoffmann *et al.* (1994), using immunohistochemical techniques, demonstrated the presence of u-PA in early implantation sites and localised its expression to the cytoplasm of cytotrophoblasts and the cytoplasm and plasma membranes of intermediate trophoblasts and syncytiotrophoblasts. Furthermore, Liu *et al.* (1998) have identified the u-PA mRNA in trophoblasts and decidual cells in foetal membranes.

Trophoblastic expression of plasminogen activator is involved in embryo implantation (Strickland and Reich, 1976; Martin and Arias, 1982). Cytotrophoblasts have been shown to synthesise and secrete the urokinase plasminogen activator (Queenan *et al*, 1987). In addition

Jensen *et al.* (1989), on demonstrating that u-PA binds to a PAI-2 type molecule in placental microvillous membranes, suggested that this might regulate urokinase activity in the microenvironment.

Significantly higher levels of PAI-1 were found in the placenta than in the placental bed. PAI-1 has been demonstrated in the placenta and within the invading trophoblast (Philips *et al.*, 1986; Feinberg *et al.* 1989; Sheppard *et al.*, 1990). The presence of PAI-1 in the placental bed suggests that the placenta is not the only source of increased PAI-1 during pregnancy. Schatz and Lockwood (1993) demonstrated the secretion of PAI-1 by decidual cells which suggests active secretion of PAI-1 into the microenvironment of the placental bed. In addition endothelial cells of the maternal vasculature and possibly the activation of platelets during pregnancy may complement the placental production of this inhibitor.

In this study significantly higher levels of PAI-2 were found in the placenta than in the placental bed. The placental source of PAI-2 has been confirmed (Astedt *et al.*, 1985). PAI-2 has been localised to the trophoblastic epithelium of the placenta (Astedt *et al.*, 1986; Feinberg *et al.*, 1989). PAI-2 has been identified in the placental bed. Sheppard *et al.* (1990) demonstrated PAI-2 in the trophoblast cells of decidual spiral arteries which had undergone the physiological changes of normal pregnancy. In addition PAI-2 has been localised to the cytoplasm of cytotrophoblasts and in the cytoplasm and plasma membranes of

intermediate trophoblasts and syncytiotrophoblasts (Hoffmann *et al.*, 1994).

In this study placental extracts contain significantly greater levels of PAI-1 and PAI-2 than t-PA and u-PA. Similar results were found for the placental bed. This suggests decreased fibrinolytic potential within the uteroplacental tissues. This may account for the physiological fibrin deposition seen in the placenta and placental bed during normal pregnancy.

In this study the secretion of plasminogen activators and their inhibitors by the placenta and the placental bed were studied by organ culture. Astedt *et al.* (1972) showed that placental extracts released inhibitors which inhibited the activation of plasminogen by urokinase, and inhibited the plasminogen activators released by kidney and foetal vessel explants. The results show that PAI-1 secretion is significantly greater in the placenta compared to the placental bed. However no significant changes are seen for t-PA, u-PA and PAI-2. This suggests that the increase in PAI-1 levels seen in the systemic circulation during pregnancy may in part be due to increased placental production of PAI-1. Sorensen *et al.* (1995) reported increases in t-PA, PAI-1 and PAI-2 in plasma during the course of normal pregnancy and suggest that endothelial cells are the main source of t-PA and PAI-1 during pregnancy. PAI-2 plasma levels have been shown to increase during the course of pregnancy. This study suggests that, although the placenta

secretes similar amounts of PAI-2 as the placental bed, the increase in PAI-2 levels seen in pregnancy may be reflected by increased placental size as pregnancy progresses. The main role of t-PA, u-PA and PAI-2 in the placenta and placental bed may be the control of localised fibrinolysis in the uteroplacental circulation. Within the placental bed this may be of importance in the preservation of fibrin in the uteroplacental vessels following delivery and so securing haemostasis in the highly vascular placental site.

During normal pregnancy trophoblast cells from the placenta invade the placental bed where they are involved in physiological changes to the spiral arteries. These changes include fibrin deposition in the spiral arteries and the surrounding decidua. Electron microscopic studies have shown that cytotrophoblast remains as part of the intimal lining of the decidual spiral artery in late pregnancy, as well as in the media of the vessel (Sheppard and Bonnar, 1974). In the media, fibrin is the predominant feature of the matrix surrounding the cytotrophoblast (de Wolf *et al.*, 1973; Sheppard and Bonnar, 1974).

Cytotrophoblasts may be responsible for the fibrin in the media of spiral arteries by directly inhibiting fibrinolysis. Sheppard and Bonnar (1978) demonstrated that cytotrophoblasts within the decidual spiral arteries have a reduced fibrinolytic activity compared to endothelial cells. The role of fibrin in the media of uteroplacental arteries may be the structural support in vessels expanding progressively to accommodate an

increasing blood flow. A structural support of fibrin in the arterial wall would be especially important in late pregnancy when the elastic tissue and smooth muscle of the media have been removed.

Fibrin would also be the ideal tissue to provide vascular support in a situation where sudden collapse of the vessels was required during and after placental separation. This reduced ability of placental bed trophoblast cells to degrade fibrin *in vitro* confirms the observation that cytotrophoblasts lining the intima of decidual spiral arteries limit localised fibrinolysis (Sheppard and Bonnar, 1978).

Trophoblastic expression of plasminogen activator is involved in embryo implantation and placentation (Strickland and Reich, 1976; Martin and Arias, 1982). Many studies have examined the expression of proteolytic enzymes by normal human trophoblasts. Some of these studies have revealed that cytotrophoblasts secrete prourokinase, PAI-1 and PAI-2 (Feinberg *et al.*, 1989; Zini *et al.*, 1992). Extravillous trophoblasts express urokinase in a polarised manner at the leading edge of the invading cells closest to the myometrium which suggests that this localisation might serve to direct trophoblast invasion and migration into the uterus (Multhaupt *et al.*, 1994). Urokinase receptors are also expressed on the apical surface of both cytotrophoblast and syncytiotrophoblast. This suggests a mechanism by which urokinase receptors localise plasmin generation to the villous surfaces, thereby preventing fibrin deposition within the intervillous space. Growing

evidence supports a physiological role for the plasminogen activator/plasmin system in implantation (Strickland and Richards, 1977). In this system plasminogen is degraded to plasmin by plasminogen activators and activates a system of degradation of basement membrane components of the placental bed. Therefore the activator/inhibitor balance in trophoblasts may influence their ability to invade through the placental bed.

In this study placental trophoblasts secrete significantly higher levels of PAI-1 and PAI-2 than u-PA. This suggests an inhibition of fibrinolytic activity on the surface of these cells. Placental bed trophoblasts secrete PAI-1 and PAI-2 in a different pattern as PAI-2 is the prominent plasminogen activator inhibitor produced by these cells. The differences seen between these cells may be explained by increased PAI-2 production due to an external influence within the placental bed vasculature. However the results may also be explained by increased PAI-1 production and secretion by the placental cells in contrast to the placental bed trophoblasts. The results show that u-PA antigen and PAI-2 antigen levels are similar in the two cell types. The increase in PAI-1 may reflect the change in trophoblasts as they undergo changes in phenotype from villous to invading trophoblast cells.

The ability of placental and placental trophoblast cells to degrade fibrin was examined in this study. As described earlier placental bed trophoblast cells have a reduced ability to lyse fibrin compared to

endothelial cells (Sheppard and Bonnar, 1978). However, in this study, no significant differences could be found in their abilities to lyse a fibrin clot. Approximately 25% of trophoblast cells were unable to migrate through the fibrin clot which demonstrates an overall decrease in fibrinolytic potential within the two populations of cells. These findings reflect the findings of increased PAI-1 and PAI-2 associated with these cells.

The ability of placental and placental bed trophoblasts to invade through matrigel, an artificial basement membrane matrix, was examined. If fibrinolysis is activated in these two cell types, the cells, through the plasminogen activator/plasmin system, should be able to invade through the matrigel matrix. The results show approximately 60% invasion by the two cell types. These results suggest that there are no differences in the two cell types in their abilities to lyse fibrin and invade basement membranes. The cell associated plasminogen activator activity of the two cell groups was studied and the findings show no significant difference in activity. This shows that the fibrinolytic potential of the cells are the same and that any change in fibrinolytic activity seen may be due to changes in the levels of associated plasminogen activator inhibitors. In summary, no significant differences are seen in placental and placental bed trophoblasts in terms of their ability to invade. The secretion of PAI-1 and PAI-2 may regulate their invasive abilities and their ability to lyse fibrin.

CHAPTER 4
FIBRINOLYSIS IN PRE-ECLAMPSIA

4.1 INTRODUCTION

No complication of human pregnancy is both so common and potentially so dangerous for mother and child as the syndrome of pre-eclampsia (Redman, 1982). Pre-eclampsia, pre-eclamptic toxaemia, gestosis, pregnancy induced and pregnancy-associated hypertension are all terms used to describe hypertension which appears between the first and third trimester of pregnancy. Hypertension unique to pregnancy is best termed pre-eclampsia because it identifies the end-point of the problem, namely eclampsia.

Pre-eclampsia affects between 2.6 and 10% of all pregnancies (Chesley, 1974; Gifford *et al.*, 1990; MacGillivray, 1958; Roberts, 1989; Saftlas *et al.*, 1990), and is the most common cause of maternal mortality in the United Kingdom and second most common in the Unites States of America (Chesley, 1974; Department of Health, 1994; Kaunitz *et al.*, 1985).

Pre-eclampsia is a multisystem disease. In pre-eclampsia there is an increased incidence of abruptio placentas (Abdella *et al.*, 1984), disseminated intravascular coagulation, microangiopathic haemolytic anaemia, liver dysfunction, thrombocytopenia (Gifford *et al.*, 1990), cerebral haemorrhage and acute renal failure, together with the development of eclampsia (Gifford *et al.*, 1990; Roberts, 1989; Redman and Roberts, 1993; Sibai *et al.*, 1985). The foetus is at risk from a reduction in uteroplacental blood flow (Dixon *et al.*, 1963), possibly leading to intrauterine growth retardation (Steel *et al.*, 1990) and stillbirth (Gifford *et al.*, 1990). In

addition, the foetus is at risk from preterm birth necessitated by delivery required to protect the mother, and as a consequence, increased perinatal mortality (Gifford *et al.*, 1990; Roberts, 1989; Department of Health, 1994; Sibai *et al.*, 1985, 1990).

4.1.1 Aetiology of Pre-eclampsia

Although pre-eclampsia has been studied extensively, its aetiology is not fully understood. In 1916, Zweifel declared that 'pre-eclampsia is a disease of theories' (Zweifel, 1916). In more recent years, research studies have begun to focus on the pathogenesis of the disease. A possible model explaining the pathophysiology of pre-eclampsia is that secondary to a genetic predisposition there is abnormal placentation characterised by shallow trophoblastic invasion of the maternal spiral arteries. This inadequate placentation results in placental ischaemia, a consequence of which is the release of a factor or factors into the maternal circulation that brings about widespread endothelial cell damage. This in turn leads to the multisystem dysfunction that characterises this disease.

Genetic Disposition in Pre-eclampsia

In 1993, Cooper *et al.* reported that mothers, daughters and sisters of women with pre-eclampsia have a greatly increased frequency of the condition compared with relatives by marriage which suggested a strong genetic component in the aetiology of pre-eclampsia. However, the genetic analysis of pre-eclampsia presents several difficulties. Pre-eclampsia

probably involves a maternal-foetal interaction and this implies that genetic susceptibility could depend on the maternal genotype alone, the foetal genotype alone or some interaction between the two. Furthermore, the different classifications and definitions of pre-eclampsia in general use make any collated interpretation of published results from different pedigrees difficult. Recent genetic research has focussed on linkage studies from large pedigrees. Arngrimmsson *et al.* (1997) reported evidence for the localisation of a familial preeclampsia-susceptibility locus in the region of chromosome 7, encoding the endothelial nitric oxide synthase (eNOS) gene, which would be in keeping with several reports of a role for nitric oxide in the pathophysiology of pre-eclampsia (Lyall and Greer, 1996). In addition, pre-eclampsia has been linked with an abnormal variant of the angiotensinogen gene (Arngrimmsson *et al.*, 1993; Ward *et al.*, 1993). Furthermore, Harrison *et al.* (1997) have reported a linkage between the long arm of chromosome 4 and pre-eclampsia. However, in order to confirm these findings further linkage studies need to be performed in other pedigrees.

Defective Placentation

The placenta plays a central role in pre-eclampsia. This is supported by the observation that the condition resolves itself when the placenta is removed and that pre-eclampsia can occur without the presence of the foetus, as in cases of hydatidiform mole (Newman and Eddy, 1988). In pre-eclampsia, shallow endovascular trophoblast invasion occurs which results in reduced

uteroplacental blood flow. The regulation of trophoblast invasion has been the subject of many research studies. Proteinases, adhesion molecules and HLA antigens have all been suggested to play a role in trophoblast regulation (Graham and Lala, 1995). The 92 kDa type IV collagenase has been shown to play a role in trophoblast invasion (Librach *et al.*, 1991) as has serine proteases such as uPA (Lala *et al.*, 1989). The distribution pattern of adhesion molecules on trophoblast cells is modulated extensively during differentiation along the invasion process ((Damsky *et al.*, 1992). Recently, it was reported that invasive trophoblasts undergo a dramatic transformation of their phenotype in which they acquire endothelial cell characteristics. These trophoblasts thus mimic the maternal vascular endothelial cells that they invade and ultimately replace (Zhou *et al.*, 1997).

In 1990, extravillous trophoblasts that invade the uterus were shown to express a Class I HLA antigen of the non-classical HLA-G locus (Kovats *et al.*, 1990; Ellis *et al.*, 1990). This antigen is not present on any other cells in the uterus, and is thought to confer resistance to natural killer cell activity in the first trimester decidua (Kovats *et al.*, 1991; Loke, 1994).

A recent study has suggested that the shallow endovascular invasion of trophoblasts in pre-eclampsia may be due to a defect in the switch from the epithelial phenotype to the endothelial phenotype in trophoblast cells seen in normal invasion (Zhou *et al.*, 1997). Furthermore, Graham and McCrae (1996) reported a decrease in cell-associated uPA activity in pre-eclamptic trophoblast cells when compared to normal trophoblast cells. This suggests

that in pre-eclampsia, the trophoblast cells are unable to invade the decidua in a normal fashion.

Endothelial Dysfunction

Endothelial dysfunction is considered to play a key role in the pathophysiology of pre-eclampsia. This contention is supported by morphological, biochemical and clinical observations consistent with endothelial damage or activation. The most consistent morphological abnormality in pre-eclamptic women is the renal lesion, glomerular endotheliosis, in which glomerular capillary endothelial cells are engorged with intracellular inclusions (Roberts, 1989). The prominent clinical feature of pre-eclampsia, oedema, and the glomerular capillary protein leak are consistent with loss of normal endothelial transport functions.

The biochemical evidence of endothelial cell injury in pre-eclampsia involves increased levels of fibronectin and factor VIII antigen both of which are known to be released from damaged endothelial cells.

One of the most striking pathophysiological abnormalities in pre-eclampsia is an increased sensitivity of these women to pressor agents (Chesley, 1978; Roberts, 1989). Disruption of vascular endothelium eliminates the source of the potent vasodilator prostacyclin and other less characterised agents (Roberts, 1989).

4.1.2 Definition of Pre-eclampsia

Research in pre-eclampsia has been plagued by the lack of clear, relevant

and uniform diagnostic criteria for the disease. In 1955, Nelson defined pre-eclampsia as a condition in which diastolic blood pressure rises to 90 mmHg on at least two occasions separated by at least 24 hours after 26 weeks gestation or if diastolic blood pressure rises progressively during labour. In severe pre-eclampsia, albuminuria exceeding 0.25 g/L is present whereas in mild pre-eclampsia, albuminuria is absent (Nelson, 1955). However, since diastolic blood pressure has been found to rise more than 10 mmHg in late pregnancy (MacGillivray *et al.*, 1969), Nelson's definition would also include women with mild chronic hypertension. Redman and Jeffries (1988) suggested that a combination of a high maximum diastolic blood pressure with a large increase from baseline was better for identifying a group with pre-eclampsia than either measurement on its own. They suggested that a first diastolic blood pressure of below 90 mmHg, a subsequent increase of at least 25 mmHg, and a maximum reading of at least 90 mmHg gave appropriate criteria for defining pre-eclampsia. Davey and MacGillivray (1986) defined pre-eclampsia as the existence of both hypertension and proteinuria (at least 0.3 g/L per day) in pregnancy. Hypertension was defined as the existence of either one measurement of diastolic blood pressure of 110 mmHg or more, on at least one occasion or, at least 90 mmHg or more on two consecutive measurements taken at least four hours apart. This definition is employed by the International Society for the Study of Hypertension in Pregnancy.

Because the primary cause of pre-eclampsia is not known, treating the

disease and preventing its occurrence has proved difficult. On diagnosis, early hospital admission and delivery of the placenta remain the key principles of management.

4.1.3 Fibrin Deposition in Pre-eclampsia

As early as 1893 fibrin deposits and thrombi were found in vessels of the various organs of women who died from pre-eclampsia (Schmorl, 1893). In pre-eclampsia, fibrin deposition has been demonstrated in the liver (Sheehan and Lynch, 1973), kidney (Vassalli *et al.*, 1963) and brain (Sheehan and Lynch, 1973). Considerable fibrin deposition has been demonstrated within the uterine spiral arteries supplying the placenta in pregnancies complicated by pre-eclampsia (Robertson *et al.*, 1967; Sheppard and Bonnar, 1976).

A haemostatic imbalance between fibrin deposition and fibrinolysis is now known to occur in pre-eclampsia (Hathaway and Bonnar, 1987). The origin of these haemostatic changes remains unknown. As yet, the possible role of the fibrinolytic system in the pathogenesis of pre-eclampsia is not fully understood.

4.1.4 Fibrinolysis in the Systemic Circulation in Pre-eclampsia

Despite the evidence of a greater level of fibrinolytic inhibition in pre-eclampsia, most studies have shown that the levels of fibrin degradation products are increased (Bonnar, 1971; Estelles *et al.*, 1988; Giles, 1982). In

addition, the levels of fibrinopeptide A and B - ß peptides are also increased (Borok *et al.*, 1984; Douglas, 1982). Overall fibrinolytic activity as measured by euglobulin clot lysis time (Bonnar *et al.*, 1971) or by a whole blood clot lysis method (Arias *et al.*, 1979) is decreased in patients with pre-eclampsia as compared with normal pregnancy. However, other investigators (Gow *et al. 1984; Howie* et al., 1977; Thornton *et al.*, 1988) found that there was no significant difference between the fibrinolytic activity levels in normal pregnancy and those in pre-eclampsia. Certainly, most studies in pre-eclampsia have reported increased levels of fibrinogen-fibrin degradation products in serum or urine (Bonnar *et al.*, 1969; Hedner and Astedt, 1970; Howie *et al.*, 1971; Dube *et al.*, 1975), though in a few, levels of fibrinogen-fibrin degradation products have remained unchanged (Sher *et al.*, 1975; Wallmo *et al.*, 1984). Chromographic study of soluble fibrinogen-fibrin complexes in plasma showed levels to be increased in pre-eclampsia compared with normal pregnancy (McKillop *et al.*, 1976). Such complexes have been shown to be composed of non-cross-linked dimers formed from fibrin and fibrinogen and probably due to increased thrombin generation (Edgar *et al.*, 1977).

Aznar *et al.* (1986), using an enzyme immunoassay, observed a slight increase in tPA antigen in pre-eclamptic patients as compared with normal pregnant women at similar gestational age. Subsequent studies confirmed an elevation in levels of tPA antigen associated with pre-eclampsia (Lorient Roudant *et al.*, 1986; Estelles *et al.*, 1987; Daly, 1989; Carron *et al.*, 1991; Ho

and Yang, 1992).

Balleger *et al.* (1987) reported comparable antigenic levels of free and total tPA between pre-eclampsia and normal pregnancy, however patient numbers in this study were small. In a subsequent larger study, the same author reported higher levels of tPA antigen before 20 weeks gestation in pre-eclampsia compared with normal pregnancy and this effect persisted until delivery (Balleger *et al.*, 1989). Estelles *et al.* (1991) observed an increase in tPA antigen in pre-eclamptic pregnancies with or without IUGR as compared with a normotensive pregnant group. Furthermore, Halligan *et al.* (1994) confirmed that tPA antigen levels are increased in pre-eclamptic patients when compared to normotensive patients. In contrast, the levels of activatable uPA plus active uPA activities were lower in preeclampsia (Estelles *et al.*, 1991). Furthermore, Lindoff and Astedt (1994) found that the plasma concentrations of uPA antigen were significantly lower in women with severe pre-eclampsia than those in women with normal pregnancies. They demonstrated that uPA antigen levels in plasma could be positively correlated to birth weight and placental and suggested that this implies that uPA may be important as a marker of placental function.

In conclusion, it can be stated that the consistently higher levels of tPA antigen observed suggests higher fibrinolytic potential in pre-eclampsia compared with normal pregnancy.

Plasminogen Activator Inhibitors

Bonnar *et al.* (1971) first reported higher levels of a urokinase inhibitor in the plasma of pre-eclamptic women compared with normal healthy women matched for age and gestation. Terao and Kobayashi (1983) suggested that hypofibrinolysis of "toxemic" pregnant urine resulted from increased levels of urokinase inhibitor in the urine. In contrast to the previous report by Bonnar *et al.*, (1971), Gow *et al.* (1984) described similar plasma levels of inhibitory activity against both urokinase and tissue activator between preeclamptic and normal pregnancy.

Over the last 14 years considerably more reliable results on the functional and antigenic levels of these inhibitors have been obtained due to the advent of chromogenic substrate assays and immunoassays. In a group of high risk pregnancies including patients with pre-eclampsia, Wiman *et al.* (1984) reported a considerably wider range of tPA inhibitor concentration in the last trimester, as compared with healthy pregnant women.

A decrease in plasma fibrinolytic activity caused by increased PAI-1 levels has been reported in pre-eclamptic patients (Estelles *et al.*, 1989; Estelles *et al.*, 1991; Estelles *et al.*, 1994; Balleger *et al.*, 1991; Caron *et al.*, 1991; Reith *et al.*, 1993; Sheppard *et al.*, 1990; Halligan *et al.*, 1994; Friedman *et al.*, 1995). In 1976, Elder suggested that the altered placenta may be the source of increased PAI-1 (Elder, 1976). This hypothesis is supported by the data in Estelles *et al.* (1989) which indicates that pre-eclamptic patients with extensive areas of placental infarction had increased plasmatic levels of

PAI-1.

de Boer *et al.* (1988) observed low plasma levels of PAI-2 in pre-eclampsia compared with matched controls. PAI-2 levels were significantly decreased, in particular in pregnancies with a poor foetal outcome (de Boer *et al.*, 1988). Similarly, lower levels of both antigenic and functional PAI-2 in severe pre-eclampsia compared with a normal pregnant group have been reported (Estelles *et al.*, 1989; Estelles *et al.*, 1991; Reith *et al.*, 1993; Halligan *et al.*, 1994; He *et al.*, 1996). Recent studies have found a positive correlation between PAI-2 levels and birth weight and placental weight (Lindoff and Astedt, 1994; He *et al.*, 1996). These studies suggest that PAI-2 can be used as a marker for placental function. In addition, *et al.* (1996) could find no difference in PAI-2 levels between mild and severe preeclamptic groups.

4.1.5 Fibrinolysis in the Uteroplacental Compartment in Pre-eclampsia

Several studies have shown that uteroplacental blood flow is impaired in pre-eclampsia (Browne and Veall, 1953; Lunnell *et al.*, 1982; Trudinger *et al.*, 1985; Leiberman *et al.*, 1988). A failure of physiological changes to extend into the spiral arteries of the placental bed has been well documented in pregnancies complicated by pre-eclampsia (Brosens *et al.*, 1972; Sheppard and Bonnar, 1976, 1981, 1988; Gerretsen *et al.*, 1981; Robertson and Khong, 1987). The absence of these physiological changes is a prerequisite for the development of pathological lesions termed "acute

atherosis" (Zeek and Assali, 1950). The lesion is characterised by fibrinoid necrosis, accumulation of lipophages in the damaged arterial walls and plaques on the intimal surface of the vessels containing large amount of lipids, smooth muscle cells and fibrin (Brosens *et al.*, 1972; Sheppard and Bonnar, 1976; Sheppard and Bonnar, 1988). Vascular occlusion ultimately occurs and the blood flow to the placenta, and the supply of essential nutrients to the developing baby, is compromised.

In pre-eclampsia, the defective physiological changes of the spiral arteries are due to a failure of complete trophoblastic invasion (Robertson *et al.*, 1975; Khong *et al.*, 1986). It remains unclear whether the invading trophoblast subsequently contributes to the vascular lesions described. Thrombosis is a feature often associated with these lesions. Pathological deposition of fibrin contributes to the thrombotic occlusion of spiral arteries in pre-eclampsia (Sheppard and Bonnar, 1976).

Trophoblast cells lining decidual spiral arteries are known to have a reduced capacity for fibrinolysis in normal pregnancy (Sheppard and Bonnar, 1978). Feinberg *et al.* (1989) showed the presence of PAI-1 in extravillous trophoblast and PAI-2 in villous trophoblast. Further studies have revealed that cultured cytotrophoblasts secrete uPA, uPAR, PAI-1 and PAI-2 (Feinberg *et al.*, 1989; Zini *et al.*, 1992).

In a recent study, the *in vitro* degradative properties of trophoblast cells isolated from placentas of pre-eclamptic pregnancies were compared with trophoblast cells isolated from normal placentas. This study revealed that

the trophoblast cells from normal and preeclamptic placentas secreted similar amounts of uPA and PAI-1. However, the pre-eclamptic trophoblast cells expressed significantly less cell surface plasminogen activator activity (Graham and McCrae, 1996).

Several studies have examined plasminogen activator activity and plasminogen activator inhibitor activity in placental extracts of pre-eclamptic pregnancies. It was reported (Yoshimura *et al.*, 1985) that placental tissue from pre-eclamptic women contained a diminished plasminogen activator activity, measured by a chromogenic method. In contrast, Kanfer *et al.* (1996) found no differences between control and pre-eclamptic placentas for plasminogen activator antigens, assessed with an ELISA which recognised plasminogen activators complexed with plasminogen activator inhibitors (inactive) as well as free (active) plasminogen activators.

Sheppard *et al.* (1990) found that the placental extracts of normal, preeclamptic and IUGR pregnancies contained significantly higher levels of PAI-2 than PAI-1. Furthermore the levels of PAI-1 were significantly higher in preeclampsia than in normal pregnancy. In addition, they showed the presence of PAI-1 and PAI-2 in the decidual and myometrial segments of the placental bed although no significant differences in PAI-1 and PAI-2 could be identified between the clinical groups.

A significant increase in PAI-1 antigen levels and a significant decrease in PAI-2 antigen levels in placental extracts from pre-eclamptic pregnancies

complicated with IUGR have been reported (Estelles *et al. 1994, 1998).* In contrast, Kanfer *et al.*(1996) could find no differences in PAI-1 antigen levels while the PAI-2 content was significantly increased. The discrepancy may be explained by the fact that the latter study recruited patients with hypertension / preeclampsia without the added complication of IUGR.

Different intensities of PAI-1 and PAI-2 immunohistochemical staining between normal and preeclamptic placentas have been reported. Estelles *et al.* (1994) demonstrated weak PAI-1 immunoreactivity in the syncytiotrophoblast cells from normal placenta. In contrast, prominent staining of these cells was observed in the preeclamptic placentas. Furthermore, they demonstrated that the intensity of PAI-2 staining decreased in preeclamptic placentas (Estelles *et al.*, 1994).

4.1.6 Objectives of the Study

In this section of the study the main aim is to compare trophoblast cells from pre-eclamptic and/or IUGR with normal pregnancy. A normal characteristic of pre-eclampsia and IUGR is reduced trophoblast invasion through the placental bed. There are many possible reasons for this event, but this study is exclusively interested in the role of fibrinolytic factors in the occurrence of this situation. During normal pregnancy, the plasma levels of plasminogen activators and their inhibitors increase during pregnancy. This results in a reduced fibrinolytic potential in the uteroplacental circulation.

In pregnancies complicated with pre-eclampsia and IUGR, there is

increased PAI levels in the circulatory system and increased fibrin deposition in the placenta and the placental bed. Furthermore, pre-eclampsia and IUGR is associated with impaired trophoblast invasion in the placental bed. As discussed above, these changes may be due to changes in expression of fibrinolytic factors by the placental and placental bed trophoblasts. The aim of this chapter is to compare the overall production and secretion of fibrinolytic factors by the placenta and placental bed in these pregnancies against normal pregnancies.

Furthermore, placental and placental bed trophoblasts were examined to determine there ability to invade and their expression of PAs and PAIs related to normal pregnancy. If significant changes are seen, then the importance of the fibrinolytic systemic in trophoblastic invasion and fibrin deposition within the uteroplacental circulation may be further enhanced.

4.2 RESULTS

4.2.1 Tissue Levels of Fibrinolytic Activators and Inhibitors in Placental and Placental Bed Extracts from Pre-eclamptic and Pre-eclamptic with IUGR Pregnancies

The following results are based on 19 placental and 19 placental bed biopsies for normal pregnancy and 17 placental and placental biopsies for the other two groups.

Tissue Plasminogen Activator Antigen: (Fig 4.1) Tissue levels of antigenic t-PA were extracted from the placenta. There was no significant difference between the levels seen in the placenta for any of the patient groups.

Urokinase Plasminogen Activator Antigen: (Fig 4.2) Antigenic u-PA was detected in the placenta. Placental u-PA levels were similar in all three patient groups.

Plasminogen Activator Inhibitor Antigen (PAI-1): (Fig 4.3) Significantly higher levels were found of PAI-1 in the PET and PET/IUGR groups when compared to normal pregnancy ($p<0.001$).

Plasminogen Activator Inhibitor Antigen (PAI-2): (Fig 4.4) Antigenic PAI-2 levels significantly decreased in the PET and the PET/IUGR groups ($p<0.001$).

t-PA in the Placental Bed: (Fig 4.5) Comparable levels of t-PA were found in the placental bed extracts for all the group types.

u-PA in the Placental Bed: (Fig 4.6) No significant differences were found for u-PA levels in the placental bed of the patient groups studied.

PAI-1 in the Placental Bed: (Fig 4.7) PAI-1 levels in the placental bed for the three groups studied were not significantly different.

PAI-2 in the Placental Bed: (Fig 4.8) PAI-2 levels are higher in PET and PET/IUGR. However they do not reach significance.

4.2.2 Tissue Levels of Fibrinolytic Activators and Inhibitors in Placental and Placental Bed Trophoblasts in Normal, PET and PET/IUGR Pregnancies

PAI-1 Secretion by Placental Trophoblasts: (Fig 4.9) Placental trophoblasts from PET and PET/IUGR secrete significantly greater amounts of PAI-1 into the culture supernatant over a 24 hour period than placental trophoblasts from normal pregnancies ($p<0.001$).

PAI-2 Secretion by Placental Trophoblasts: (Fig 4.10) No significant differences are seen in PAI-2 production and secretion by placental trophoblasts from the three patient groups.

u-PA Secretion by Placental Trophoblasts: (Fig 4.11) No significant differences are seen in u-PA production and secretion by placental trophoblasts from the three patient groups.

PAI-1 Secretion by Placental Bed Trophoblasts: (Fig 4.12) No significant differences are seen in PAI-1 production and secretion by placental bed trophoblasts from the three patient groups.

PAI-2 Secretion by Placental Bed Trophoblasts: (Fig 4.13) No significant differences are seen in PAI-2 production and secretion by placental bed trophoblasts from the three patient groups.

u-PA Secretion by Placental Bed Trophoblasts: (Fig 4.14) No significant differences are seen in PAI-2 production and secretion by placental bed trophoblasts from the three patient groups.

Cell Associated Plasminogen Activator Activity: (Fig 4.15) Cell associated plasminogen activator activity is significantly reduced in PET and PET/IUGR placental trophoblasts when compared to normal controls ($p<0.001$).

Cell Associated Plasminogen Activator Activity: (Fig 4.16) Cell associated plasminogen activator activity is significantly reduced in PET and PET/IUGR placental bed trophoblasts when compared to normal controls ($p<0.001$).

4.2.3 Comparative Changes in Invasion Abilities of Placental and Placental Bed Trophoblasts in PET and PET/IUGR

Fibrin Degradation by Placental Trophoblasts: (Fig 4.17) Significant decreases in the ability of placental trophoblasts from PET and PET/IUGR were seen ($p<0.001$).

Fibrin Degradation by Placental Bed Trophoblasts: (Fig 4.18) PET and PET/IUGR placental bed trophoblasts have a significantly reduced ability to lyse fibrin clots when compared to normal pregnancy ($p< 0.001$).

Matrigel Degradation by Placental Trophoblasts: (Fig 4.19) Significant decreases in matrigel invasion are seen for both PET and PET/IUGR groups compared to normal placental trophoblasts ($p<0.001$).

Matrigel Degradation by Placental Bed Trophoblasts: (Fig 4.20) PET and PET/IUGR placental bed trophoblasts have significant less ability to migrate through the matrigel matrix compared to normal placental bed trophoblasts ($p<0.001$).

4.2.4 Comparative Changes in Secretion Levels of Placental and Placental Bed Biopsies in Organ Culture for PET and PET/IUGR Compared to Normal Pregnancy

Secretion of Fibrinolytic Factors: (Table 4.1) The placenta secretes higher amounts of PAI-1 and PAI-2 than the placental bed. In pregnancies complicated by PET and PET/IUGR, significant increases in PAI-1 levels secreted by the placenta are seen compared to normal pregnancy ($p<0.001$).

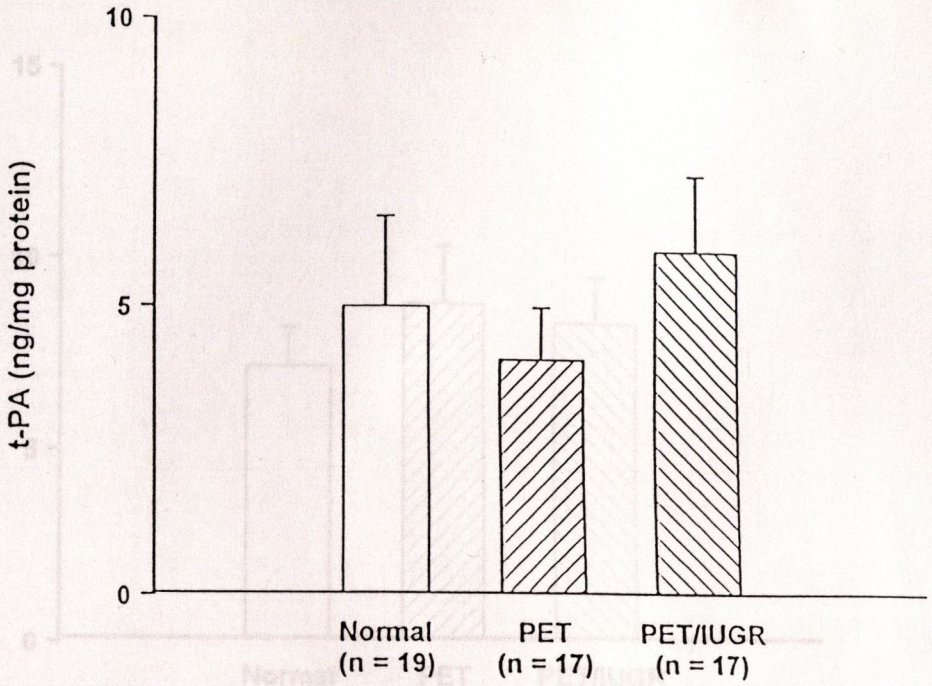


Fig 4.1: Comparative tissue levels of t-PA antigen in the placenta from normal, PET and PET/IUGR pregnancies.
Values are mean ± SD. Statistical test is Student's t-test.

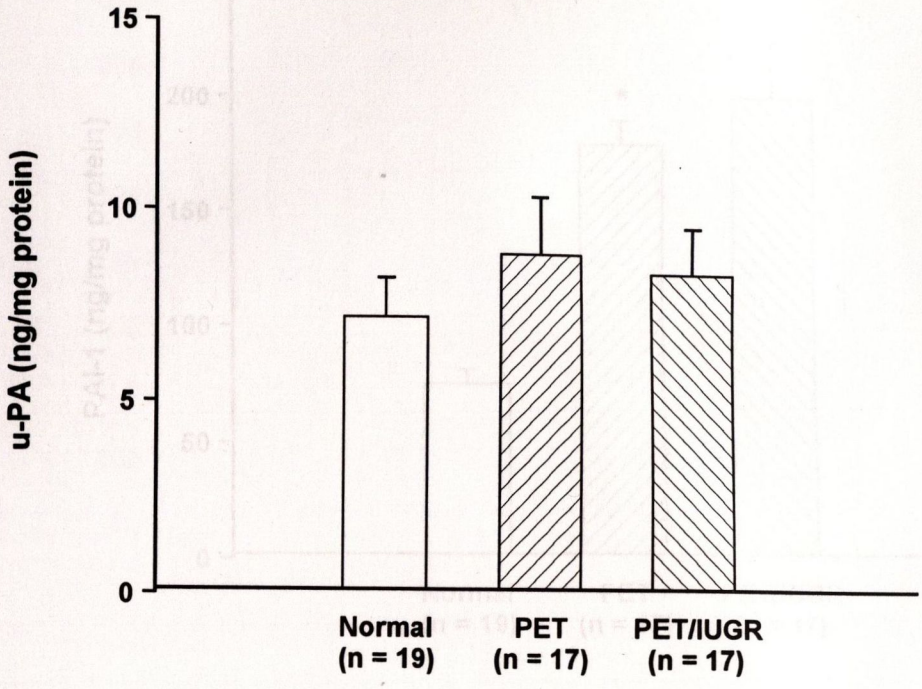


Fig 4.2: Comparative tissue levels of u-PA antigen in the placenta from normal, PET and PET/IUGR pregnancies.
Values are mean $\pm$ SD. Statistical test is Student's t-test.

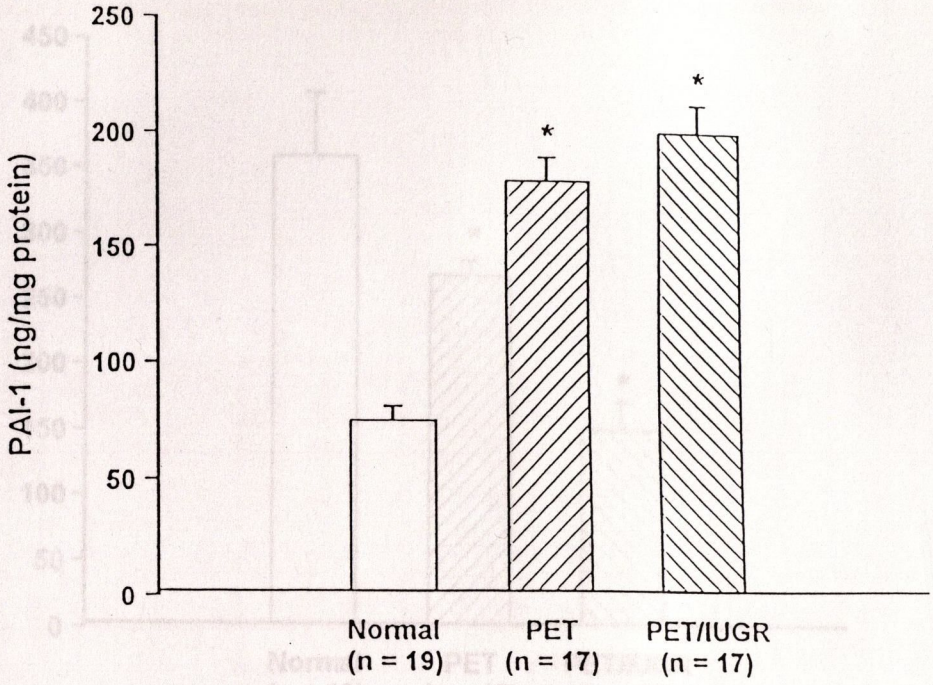


Fig 4.3: Comparative tissue levels of PAI-1 antigen in the placenta from normal, PET and PET/IUGR pregnancies.
* $p < 0.01$ PET and PET/IUGR vs. Normal.
Values are mean $\pm$ SD. Statistical test is Student's t-test.

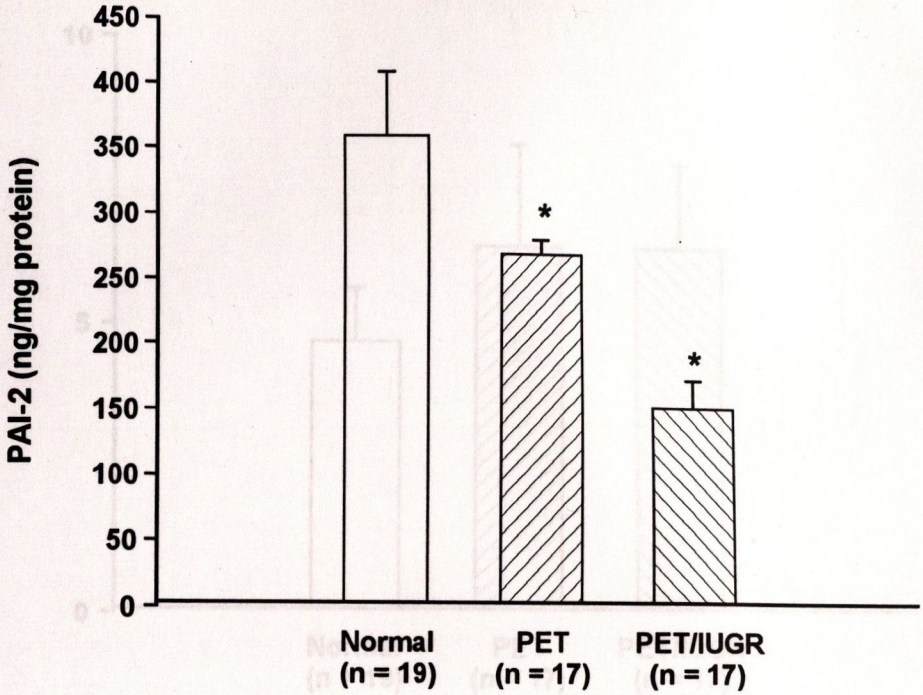


Fig 4.4: Comparative tissue levels of PAI-2 antigen in the placenta from normal, PET and PET/IUGR pregnancies.
* $p<0.001$ (PET vs. normal and PET/IUGR vs. normal)
Values are mean $\pm$ SD. Statistical test is Student's t-test.

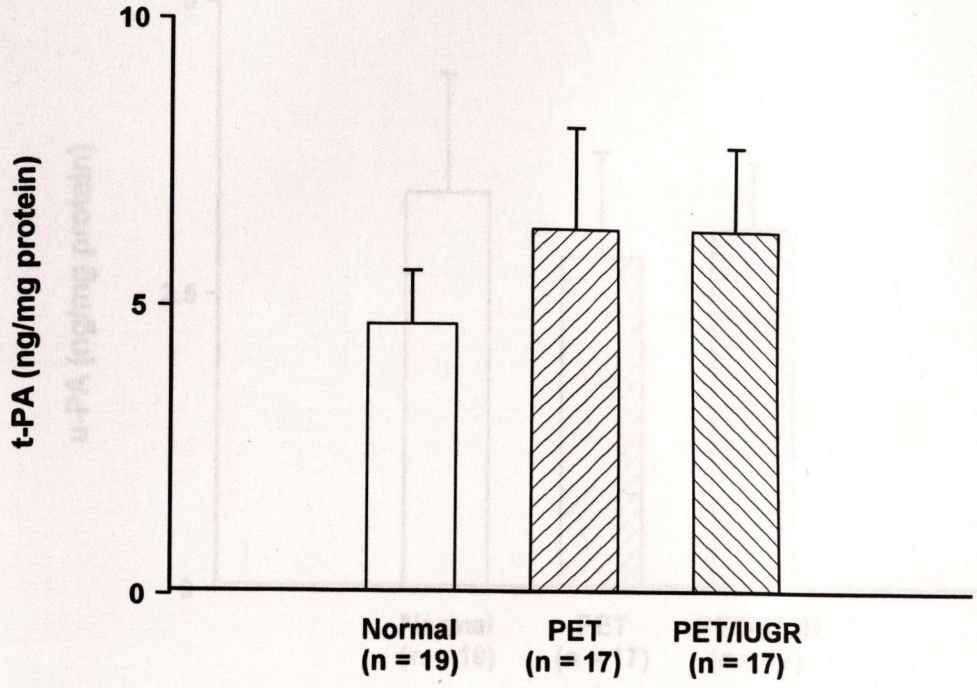


Fig 4.5: Comparative tissue levels of t-PA antigen in the placental bed from normal, PET and PET/IUGR pregnancies.
Values are mean $\pm$ SD. Statistical test is Student's t-test.

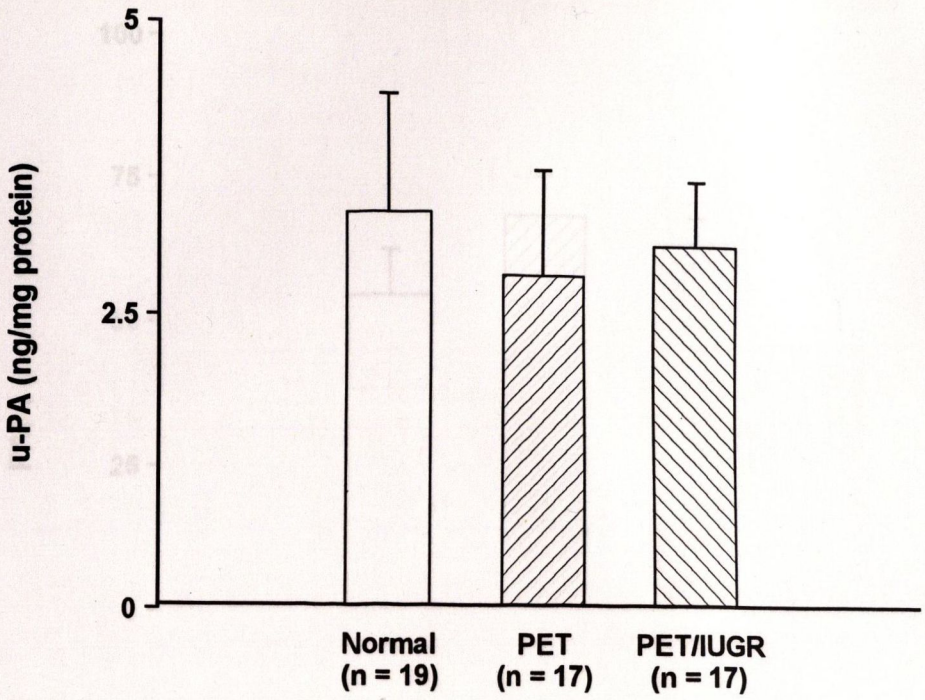


Fig 4.6: Comparative tissue levels of u-PA antigen in the placental bed from normal, PET and PET/IUGR pregnancies.
Values are mean $\pm$ SD. Statistical test is Student's t-test.

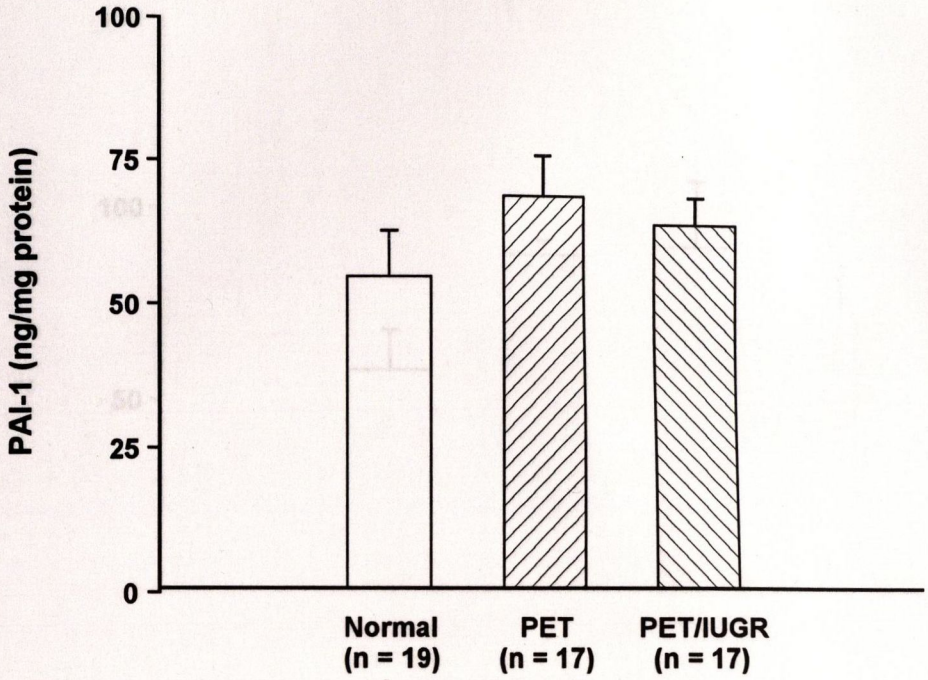


Fig 4.7: Comparative tissue levels of PAI-1 antigen in the placental bed from normal, PET and PET/IUGR pregnancies.
Values are mean $\pm$ SD. Statistical test is Student's t-test.

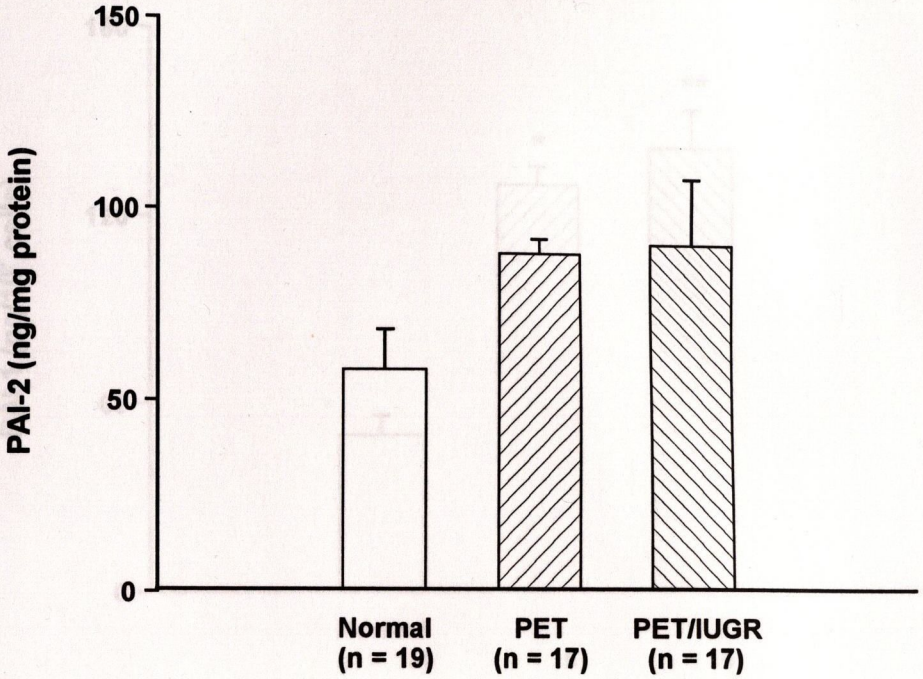


Fig 4.8: Comparative tissue levels of PAI-2 antigen in the placental bed from normal, PET and PET/IUGR pregnancies.
Values are mean ± SD. Statistical test is Student's t-test.

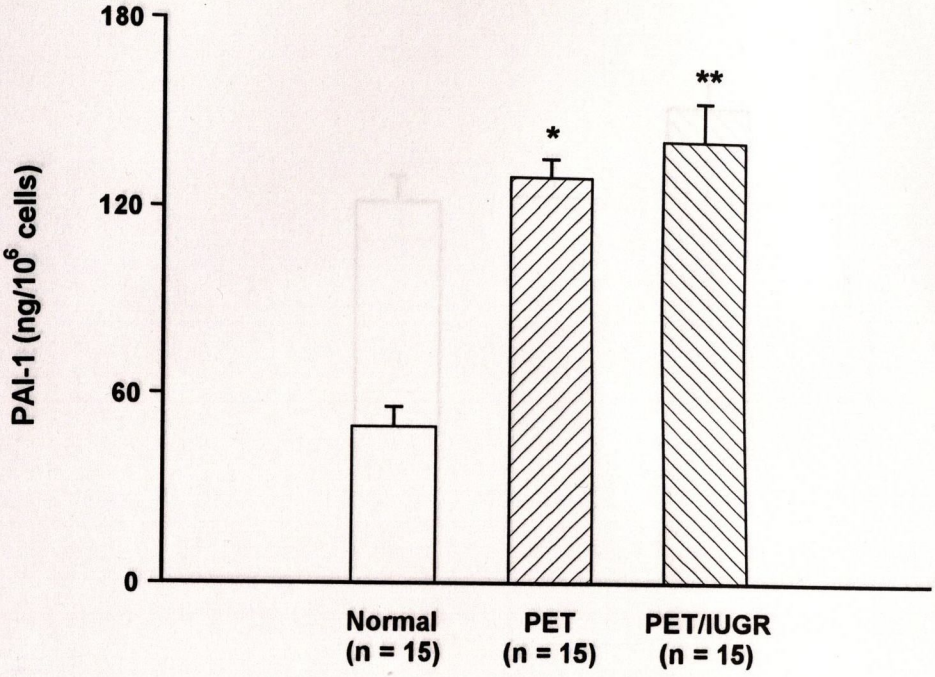


Fig 4.9: Comparison of PAI-1 production by placental trophoblasts in normal, PET and PET/IUGR pregnancies.

* $p < 0.001$ (PET vs. normal)
** $p < 0.001$ (PET/IUGR vs. normal)
Values are mean $\pm$ SD. Statistical test is Student's t-test.

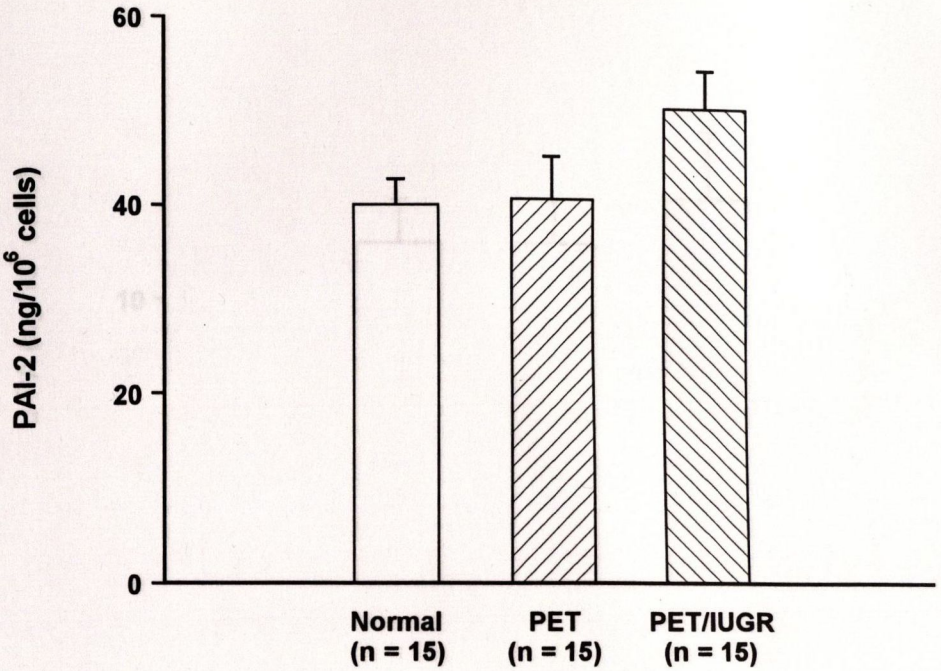


Fig 4.10: Comparison of PAI-2 production by placental trophoblasts in normal, PET and PET/IUGR pregnancies.
Values are mean $\pm$ SD. Statistical test is Student's t-test.

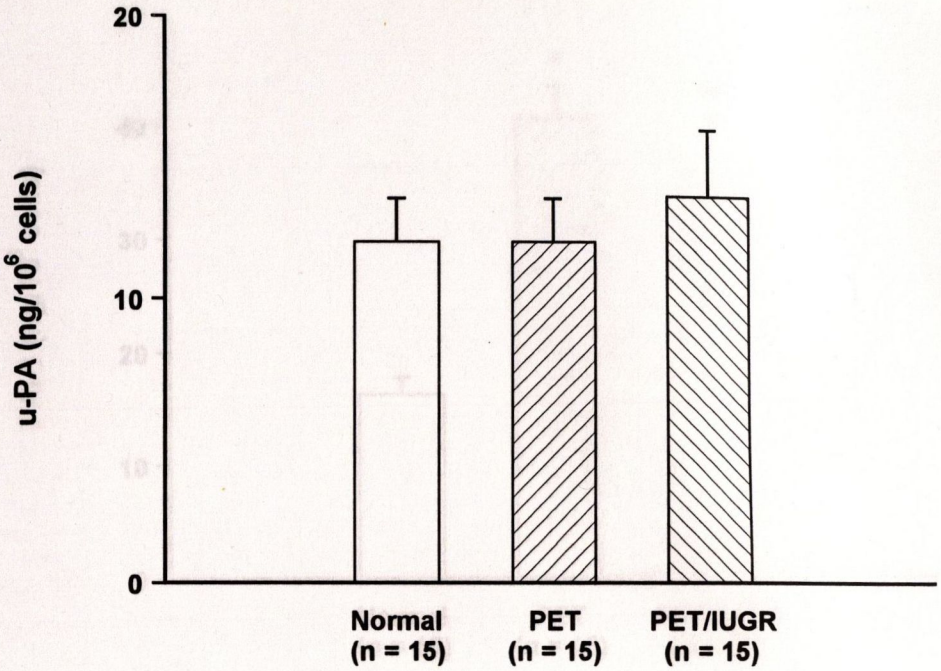


Fig 4.11: Comparison of u-PA production by placental trophoblasts in normal, PET and PET/IUGR pregnancies.
Values are mean ± SD. Statistical test is Student's t-test.

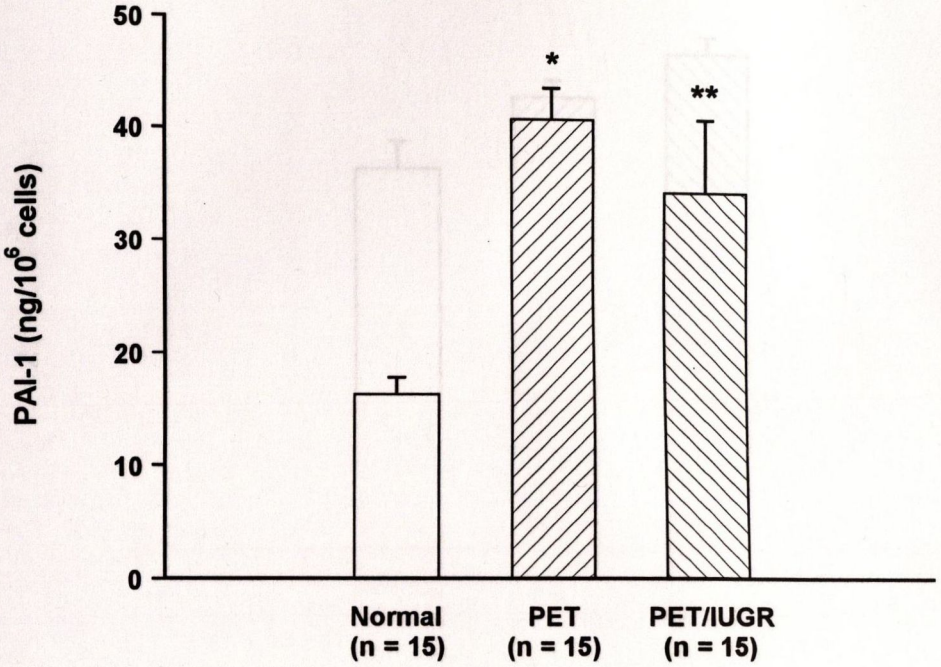


Fig 4.12: Comparison of PAI-1 production by placental bed trophoblasts in normal, PET and PET/IUGR pregnancies.

* $p<0.001$ (PET vs. Normal)

** $p<0.001$ (PET/IUGR vs. Normal)

Values are mean $\pm$ SD. Statistical test is Student's t-test.

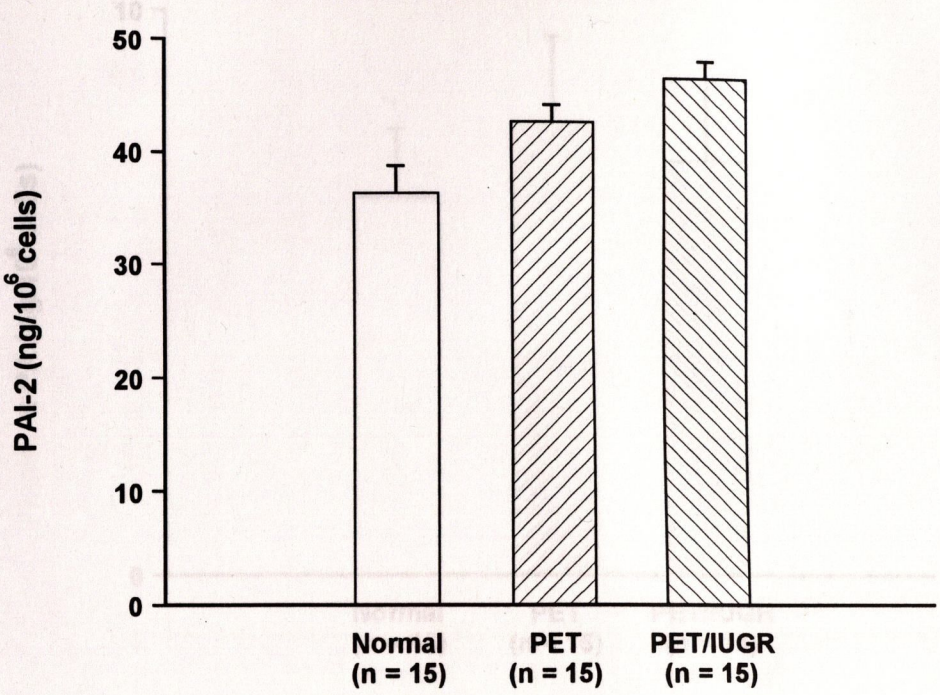


Fig 4.13: Comparison of PAI-2 production by placental bed trophoblasts in normal, PET and PET/IUGR pregnancies.
Values are mean $\pm$ SD. Statistical test is Student's t-test.

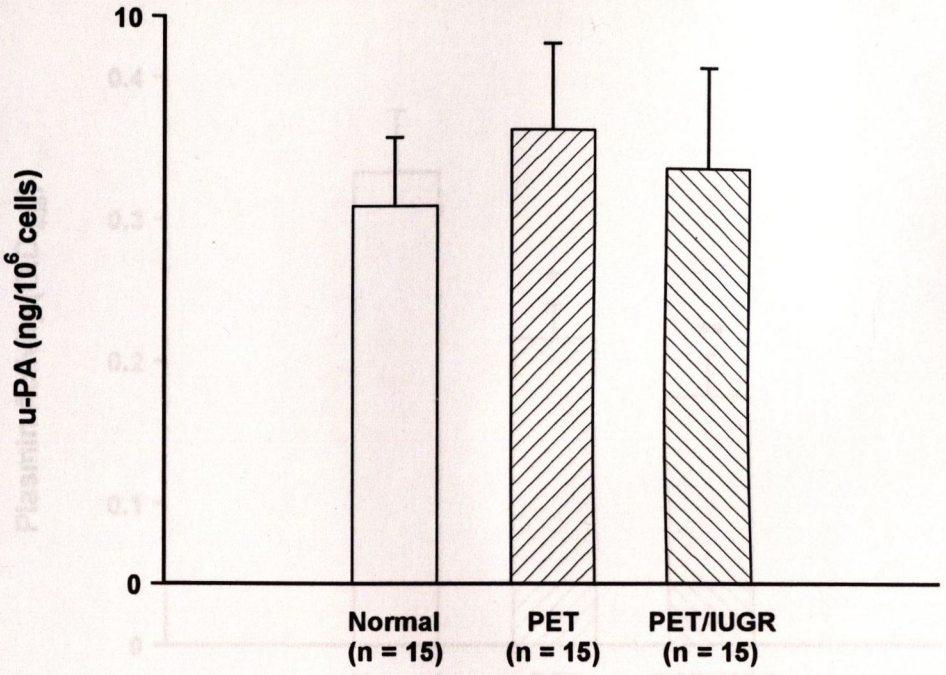


Fig 4.14: Comparison of u-PA production by placental bed trophoblasts in normal, PET and PET/IUGR pregnancies.
Values are mean $\pm$ SD. Statistical test is Student's t-test.

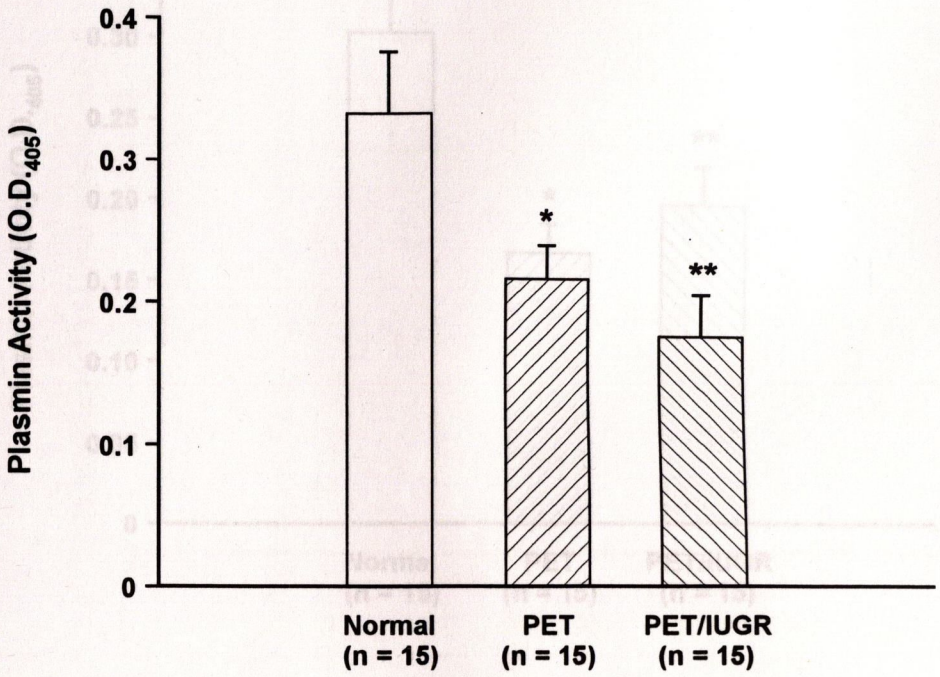


Fig 4.15: Comparison of cell associated plasminogen activator activity by placental trophoblasts in normal, PET and PET/IUGR pregnancies.

* $p < 0.001$ (PET vs. normal)
** $p < 0.001$ (PET/IUGR vs. Normal)
Values are mean $\pm$ SD. Statistical test is Student's t-test.

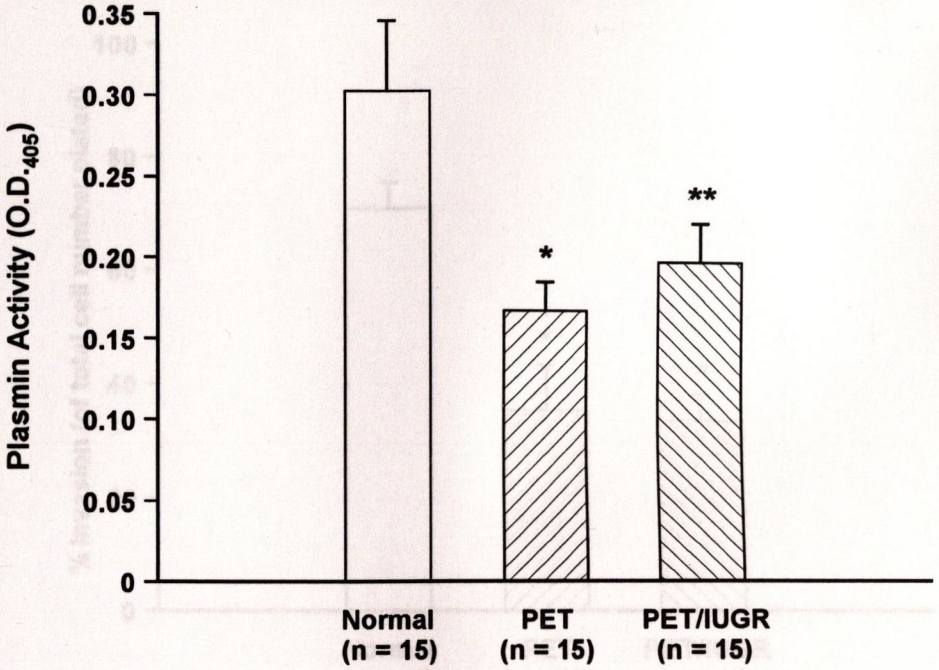


Fig 4.16: Comparison of cell associated plasminogen activator activity by placental bed trophoblasts in normal, PET and PET/IUGR pregnancies.

* $p < 0.001$ (PET vs. normal)
** $p < 0.001$ (PET/IUGR vs. Normal)
Values are mean $\pm$ SD. Statistical test is Student's t-test.

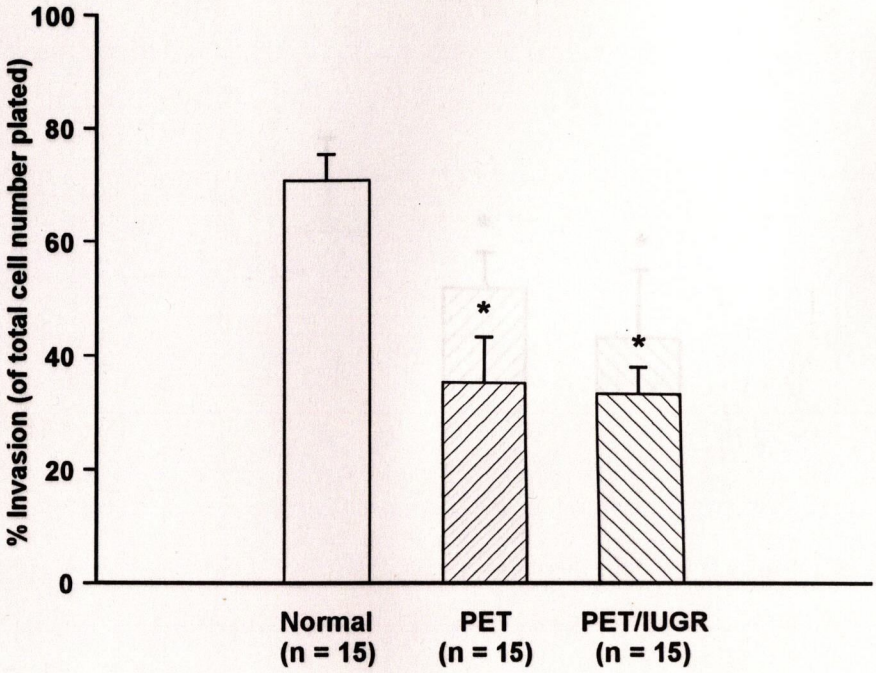


Fig 4.17: Comparison of fibrin invasion by placental trophoblasts in Normal, PET and PET/IUGR pregnancies.
*$p<0.001$ (PET and PET/IUGR vs. Normal)
Values are mean $\pm$ SD. Statistical test is Student's t-test.

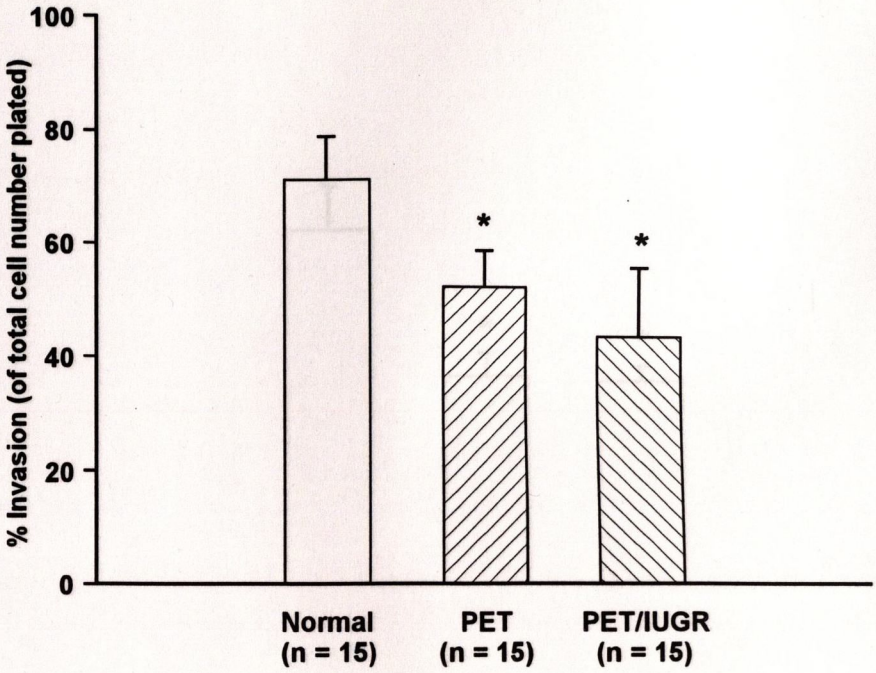


Fig 4.18: Comparison of fibrin invasion by placental bed trophoblasts in normal, PET and PET/IUGR pregnancies.
*$p<0.001$ (PET and PET/IUGR vs. Normal)
Values are mean $\pm$ SD. Statistical test is Student's t-test.

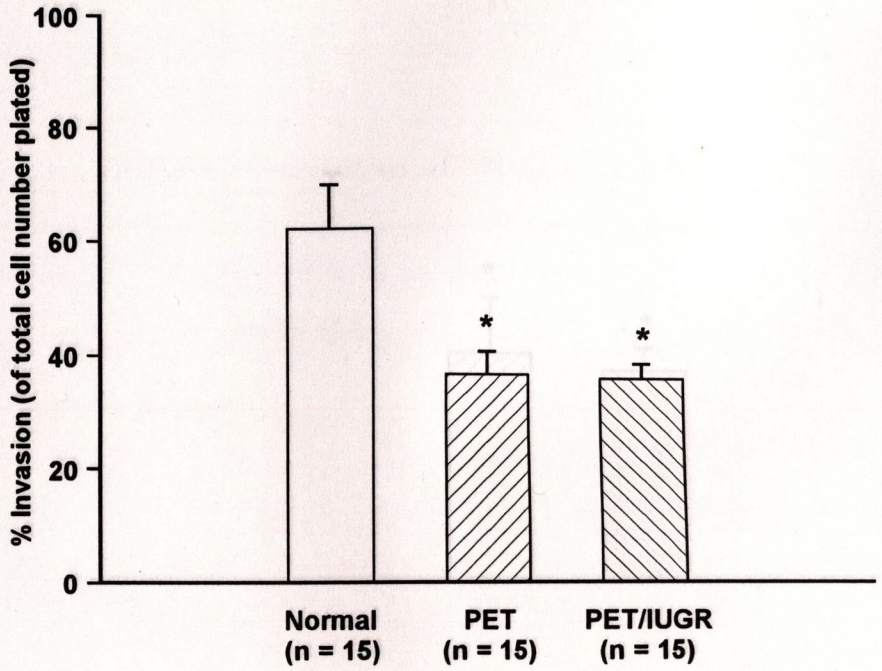


Fig 4.19: Comparison of matrigel invasion by placental trophoblasts in normal, PET and PET/IUGR pregnancies.
*$p<0.001$ (PET and PET/IUGR vs. Normal)
Values are mean $\pm$ SD. Statistical test is Student's t-test.

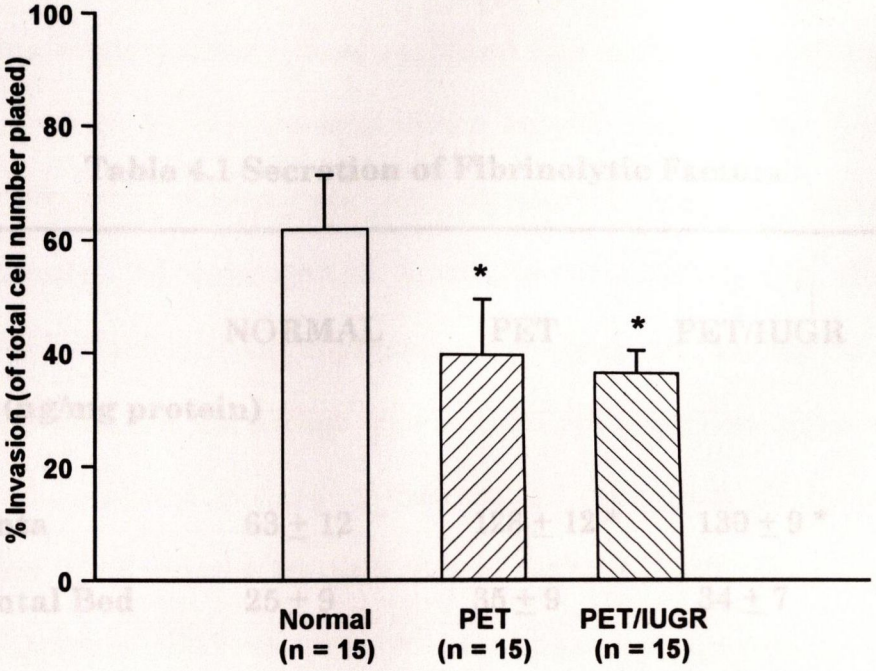


Fig 4.20: Comparison of matrigel invasion by placental bed trophoblasts in normal, PET and PET/IUGR pregnancies.
*p<0.001 (PET and PET/IUGR vs. Normal)
Values are mean ± SD. Statistical test is Student's t-test.

Table 4.1 Secretion of Fibrinolytic Factors

	NORMAL	PET	PET/IUGR
PAI-1 (ng/mg protein)			
Placenta	63 ± 12	128 ± 12 *	130 ± 9 *
Placental Bed	25 ± 9	35 ± 9	34 ± 7
PAI-2 (ng/mg protein)			
Placenta	53 ± 12	73 ± 9	67 ± 9
Placental Bed	12 ± 3	17 ± 5	14 ± 8
u-PA (ng/mg protein)			
Placenta	7.6 ± 2	6.6 ± 2.7	8.2 ± 3.1
Placental Bed	5.7 ± 2.8	5.4 ± 2.8	6.2 ± 2.4

*$p < 0.001$ (PET and PET/IUGR vs. Normal). n = 15
Values are mean ± SD. Statistical test is Student's t-test.

4.3 Discussion

In this study patients were recruited and subdivided into two groups according to the birth weight of the baby: (i) pure pre-eclampsia (baby birth weights appropriate for gestational age), (ii) pre-eclampsia complicated by intrauterine growth retardation (IUGR) (baby birth weight below the tenth centile according to sex and gestational age).

To the author's knowledge this is the first report on organ culture of biopsies and the culture of trophoblast cells from the placenta and placental bed in pre-eclampsia with or without IUGR.

No change in t-PA levels were found when comparing pre-eclampsia with normal pregnancy. Some studies have reported a change in u-PA antigen levels in pre-eclampsia. As the placenta is a major source of u-PA in pregnancy it could be assumed that these changes might be due to altered expression of u-PA by trophoblast cells (Shimada *et al.*, 1989). However, in this study no alteration in u-PA expression could be found in cultured placental trophoblasts. It could be argued that this alteration in u-PA is only seen in syncytiotrophoblasts and that the cells used in this study are more representative of cytotrophoblasts. However *in vitro* studies could not find any significant change in u-PA secretion as cultured cells differentiated from cytotrophoblasts into syncytiotrophoblasts.

In this study isolated placental trophoblasts secreted significantly larger

amounts of PAI-1 in pre-eclampsia compared to normotensive. Graham and McCrae (1996) investigated u-PA and PAI-1 antigen in normal and pre-eclamptic placental trophoblasts. However they were unable to find any significant differences in u-PA and PAI-2 levels between normotensive and pre-eclamptic trophoblasts.

The levels of PAI-2 did not differ significantly between trophoblasts from pre-eclamptic and normotensive placenta. Cell surface plasminogen activator activity significantly decreases in pre-eclamptic trophoblast. This is in agreement with a study by Graham and McCrae (1996) that investigated cell surface plasminogen activator activity in normal and pre-eclamptic placental trophoblasts. They concluded that pre-eclamptic placental trophoblasts display a significantly reduced cell surface plasminogen activator activity than trophoblasts from normal placenta.

The higher levels of PAI-1 in placental trophoblasts may contribute to the higher levels of PAI-1 reported in plasma levels of the uterine vein (Sheppard *et al.*, 1990). Furthermore the increase in PAI-1 reported here is reflected in increased immunoreactivity for PAI-1 within the syncytiotrophoblast layer in pre-eclamptic placenta (Estelles *et al.*, 1994; 1998).

Placental bed trophoblasts show an overall decrease in fibrinolytic activity in pre-eclampsia. The levels of PAI-1 secretion are significantly greater in pre-eclampsia compared to normotensive pregnancy. In addition PAI-2 levels are increased, although not significantly. Cell

surface plasminogen activator activity is also significantly reduced.

The overall effect of these changes in the levels of fibrinolytic factors is reduced fibrinolytic potential and activity within the microenvirons of placental and placental bed trophoblasts. This reduced fibrinolytic activity in these cells may be of importance in the pathogenesis of pre-eclampsia.

The invasion assays reported in this study show that placental and placental trophoblasts from pre-eclamptic patients have a reduced ability to breakdown basement membrane components. This decrease in proteolytic activity may be reflected by the significant changes in PAI-1 production cell surface plasminogen activator activity in these trophoblasts.

The reduced ability of pre-eclamptic placental and placental bed trophoblasts to degrade fibrin is demonstrated in the fibrin invasion assays. As stated earlier, pre-eclampsia is associated with large deposition of fibrin in the spiral arteries of the placental bed. The reduced fibrinolytic activity of these trophoblast cells may, in part, account for the fibrin formation within the spiral arteries of the placental bed.

A failure of physiological changes to extend into the spiral arteries of the placental bed has been well documented in pregnancies complicated by pre-eclampsia (Brosens *et al.*, 1972; Sheppard and Bonnar, 1976, 1981; Gerretsen *et al.*, 1981; Robertson and Khong, 1987; Sheppard and Bonnar,

1988). The absence of these physiological changes is a prerequisite for the development of pathological lesions termed "acute atherosis" (Zeek and Assali, 1950). The lesion is characterised by fibrinoid necrosis, accumulation of lipophages in the damaged arterial walls and plaques on the intimal surface of the vessels containing large amount of lipids, smooth muscle cells and fibrin (Brosens *et al.*, 1972; Sheppard and Bonnar, 1976; Sheppard and Bonnar, 1988). Vascular occlusion ultimately occurs and the blood flow to the placenta, and the supply of essential nutrients to the developing baby, is compromised.

In pre-eclampsia, the defective physiological changes of the spiral arteries are due to a failure of complete trophoblastic invasion (Robertson *et al.*, 1975; Khong *et al.*, 1986). This impaired trophoblastic invasion may, in part, be due to the decrease in cell surface plasminogen activity and decreased fibrinolytic activity of trophoblasts as found in this study. Trophoblast cells from the placenta and placental show a decrease in ability to migrate through matrigel and fibrin.

The abnormal plasma levels of fibrinolytic components, in particular PAI-1 and PAI-2 have been suggested to be influenced by the presence of placental infarction (de Boer *et* al., 1988; Estelles *et* al., 1989). Estelles *et al.* (1994) have reported an increase in PAI-1 protein and mRNA in the placentas of pre-eclamptic patients and decreased PAI-2 protein and mRNA in the placentas of patients with pre-eclampsia and IUGR. A more recent study has shown that PAI-1 mRNA is increased not only in

syncytiotrophoblast and infarction areas, but also in fibroblasts and some endothelial cells of foetal vessels in placentas from patients with pre-eclampsia and IUGR (Estelles *et al.*, 1998). This suggests an altered release of fibrinolytic factors by the placenta in pre-eclampsia can occur. The placenta biopsies obtained during the course of this study were removed from areas of the placenta which macroscopically appear healthy. The increase in concentration and secretion of PAI-1 levels in pre-eclamptic placentas demonstrates that the placenta may contribute to the high circulating plasma levels of PAI-1 reported in pre-eclampsia. Placental extracts from normotensive pregnancies with IUGR have an increased capacity to inhibit urokinase-induced fibrinolysis (Elder and Myatt, 1976). The depression of fibrinolysis in normotensive IUGR may explain the pathological presence of fibrin in the uteroplacental spiral arteries (Sheppard and Bonnar, 1976). This occlusive vascular disease leads to placental infarction which correlates with the severity of foetal growth retardation (Bonnar and Sheppard, 1977).

CHAPTER 5
FIBRINOLYSIS IN INTRAUTERINE GROWTH RETARDATION

5.1 INTRODUCTION

In 1954, Clifford described the dysmature, wasted foetus associated with prolonged pregnancy (Clifford, 1954). Further studies laid the groundwork for the recognition of the growth-retarded infant through population studies that established the relationship between gestational age and weight. Greunwald highlighted the important differences among the neonate with low birth weight secondary to prematuruity, the infant born too soon, and the infant who was small when compared with other new-borns of the same gestational age, the infant born too soon (Greunwald, 1963). These babies were clearly identified as being at high risk for perinatal morbidity and mortality.

5.1.1 Definition and Classification

Intrauterine growth retardation (IUGR) is not a common problem, complicating 3 to 7 percent of all pregnancies. However, IUGR is the second most important cause of perinatal mortality after preterm delivery (Wolfe and Gross, 1989). The perinatal mortality rate for growth-retarded infants may be six to ten times that for a normally grown population. The incidence of intrapartum asphyxia in cases complicated by IUGR has been reported to be 50 percent (Low and Boston, 1972). These infants also suffer an increased incidence of hypoglycaemia, polycythemia and hypothermia in the neonatal period.

Early observers appreciated the need to separate low-birth-weight babies who were premature but normally grown from those who had failed to achieve the size expected for their gestational age. They recognised the disproportionate morbidity and mortality rates of these growth- retarded infants. Infants who at birth are below the tenth percentile in weight for their gestational age are considered growth retarded and this is the definition most widely used in describing IUGR.

Using the tenth percentile cut-off, approximately 70 percent of the infants designated as growth retarded are normally or constitutionally small, so-called light for gestational age or small for gestational age (SGA) (Ott, 1988). These neonates are not at increased risk for poor outcome. However, this group below the tenth percentile also includes infants who are truly growth retarded in terms of their growth potential, and these babies may suffer marked increased perinatal morbidity and mortality.

5.1.2 Aetiology of Intrauterine Growth Retardation

Absolute identification of IUGR has largely remained a postpartum event. The aetiologies of IUGR must be considered when considering the definition and detection of IUGR. IUGR is a heterogeneous group with a variety of pathological mechanisms. The possible causes of foetal growth retardation however, are often interrelated and difficult to separate.

Intrauterine infections, chromosomal abnormalities and congenital

malformations accounts for less than 10 percent of all cases. Intrauterine infections produce early disruption of foetal growth during the stage of cell hyperplasia and are associated with a poor prognosis for normal development.

Placental abnormalities such as a reduction in mass and area, altered function and abnormal placental location are known causes of growth retardation (Baur, 1977; Hoogland *et al.*, 1980).

Exogenous conditions such as infections, cigarette smoking (Haworth *et al.*, 1980), maternal drug ingestion and alcohol consumption (Mills *et al.*, 1984) have long been recognised as risk factors for IUGR.

A previous pregnancy complicated by IUGR is often associated with the subsequent birth of a growth retarded infant (Galbraith *et al.*, 1979; Tejani, 1982).

Maternal vascular disease whether it is chronic hypertension, pre-eclampsia or diabetes has been associated with impaired foetal growth (Long *et al.*, 1980; Katz *et al.*, 1980). Long *et al.* (1980) observed an increased incidence of IUGR in early-onset pre-eclampsia and suggested that foetal growth retardation preceded clinical signs of pre-eclampsia. Other studies however have denied a special relationship between foetal growth retardation and pre-eclampsia (Beaudry and Sutherland, 1960; de Souza *et al.*, 1976).

Despite the many causes of intrauterine growth retardation, many IUGR cases arise which cannot be attributed to foetal abnormality, maternal disease or other obvious complications of pregnancy.

5.1.3 Uteroplacental Blood Flow

Idiopathic IUGR is associated with reduced blood placental perfusion. At least 35% of cases of IUGR are attributable to a disturbance in the uteroplacental circulation (Dawes, 1974). A decrease in perfusion pressure and/or an increase in vascular resistance has been associated with the chronic uteroplacental circulatory insufficiency which generally results in foetal growth retardation (Wallenburg, 1975). Scintigraphic studies have shown that uteroplacental blood flow index in patients with IUGR was less than half that of normal pregnancies (Nylund *et al.*, 1983).

5.1.4 Fibrin in the Uteroplacental Compartment

Wallenburg (1968) reported a higher incidence of placental infarcts in cases of IUGR compared with pregnancies resulting in normal birth weight infants. This placental infarction was believed to signal a disturbance in the uteroplacental circulation (Wallenburg, 1971, 1975).

In 1976, Sheppard and Bonnar described an uteroplacental vasculopathy in normotensive IUGR, a feature previously believed to be pathognomonic to the placental beds of hypertensive pregnancies with proteinuria (Brosens and Renaer, 1972; Robertson *et al.*, 1975). Using electron microscopy, the spiral arteries showed occlusive atheromatous lesions with considerable fibrin deposition in both the intima and media of the vessel walls (Sheppard and Bonnar, 1976). These pathological lesions within the spiral arteries resulting in a narrowing of the vascular lumen causing a decrease in

uteroplacental blood flow. Indeed, the severity of foetal growth retardation has been correlated with the degree of placental infarction produced by the occlusive vascular disease (Bonnar and Sheppard, 1977).

Detailed studies of the occlusive thrombi found in the uteroplacental circulation showed clumps of aggregated platelets enmeshed in strands of fibrin (Wallenburg, 1971; Sheppard and Bonnar, 1976). Abnormal platelet-vessel and platelet-platelet interaction may explain these pathological processes within the uteroplacental environment (Stuart *et al.*, 1981). In addition, placentas from normotensive pregnancies with IUGR not only have a greatly reduced ability to produce prostacyclin, a potent vasodilator and platelet anti-aggregator (Joggee *et al.*, 1983), but also have an increased ability to inhibit urokinase-induced fibrinolysis compared with normal placentas (Elder and Myatt, 1976).

The pathological role of fibrin, or the factor(s) that stimulates its excessive deposition within the uteroplacental vasculature of these complicated pregnancies, is as yet unknown. Furthermore, the vasculopathy in both normotensive and hypertensive pregnancies with IUGR implies a possible common aetiology. Haemostatic changes and endothelial damage, in both the systemic and the uteroplacental circulations, are associated with pre-eclampsia. If pre-eclampsia and IUGR share a common aetiology, this suggests that haemostatic changes also occur in intrauterine growth retardation.

5.1.5 Systemic Haemostasis in Intrauterine Growth Retardation

Several studies have reported only slight changes in the levels of components of the haemostatic mechanism in normotensive patients with intrauterine growth retardation when compared with normal pregnancy (Frigoletto *et al.*, 1971; Howie *et al.*, 1971; Elder and Myatt, 1976; Persson *et al.*, 1982). McKillop *et al.* (1978) suggested that increased fibrin-fibrinogen complexes might reflect increased local intravascular coagulation within the placenta in pregnancies complicated by IUGR. However, Thornton *et al.* (1989) were unable to establish a haemostatic predictive index at 28 weeks gestation for the development of either IUGR or pre-eclampsia.

Marked alterations in the levels of systemic haemostatic parameters are known to occur in pre-eclampsia. In contrast to this, there is little evidence to support the involvement of generalised intravascular coagulation in IUGR. It is generally believed in IUGR, disordered activity of platelets and the coagulation system is confined to the uteroplacental circulation (Howie *et al.*, 1971; Howie, 1977; McKillop *et al.*, 1978; Wallenburg and van Kessel, 1979; Persson *et al.*, 1982).

5.1.6 Systemic Fibrinolysis in Intrauterine Growth Retardation

Fibrinolytic activity

Howie *et al.* (1971) were unable to detect any abnormalities in either euglobulin or dilute blood clot lysis times in patients delivering "small for

dates" infants. In contrast, Uszynski *et al.* (1980) demonstrated, using euglobulin lysis times, a weaker fibrinolytic response in women with retarded foetal development compared with normal pregnancies. Thornton *et al.* (1989) were unable to confirm a difference in fibrinolytic activity in IUGR.

Plasminogen Activators

Ballegeer *et al.* (1987) observed a marked reduction in tPA release during late pregnancy in four patients with IUGR. In contrast, Estelles *et al.* (1991) reported similar tPA antigen levels in normotensive IUGR and normal pregnancy. However, they found that tPA antigen levels were significantly higher in severe pre-eclampsia, with or without IUGR (Estelles *et al.*, 1991). In addition, Boyle (1992) found similar levels of tPA antigen and activity in normotensive IUGR compared with normal pregnancy.

On detecting lower levels of urokinase plasminogen activator activity in both normotensive and pre-eclamptic IUGR, Estelles *et al.* (1991) suggested that uPA concentrations might correlate with placental function. They were unable however, to find a significant correlation between activator levels and foetal weight. Later, Lindoff and Astedt (1994) reported a significant decrease in uPA antigen in plasma from IUGR pregnancies in comparison to normal pregnancy. Furthermore, they reported that uPA antigen were significantly correlated with placental and foetal growth and suggested that uPA plasma levels appear to reflect placental remodelling and function.

Plasminogen Activator Inhibitors

PAI-1 and PAI-2 are produced by the placenta during pregnancy. Estelles *et al.* (1991) studied the levels of these inhibitors in the systemic circulation of both normotensive and pre-eclamptic IUGR patients. Similar levels of both PAI activity and PAI-1 antigen were found in the normotensive IUGR group and the normal pregnancy group. In contrast, levels of PAI activity and PAI-1 antigen were found to be significantly higher in pre-eclamptic patients with IUGR (Estelles *et al.*, 1991). In a recent study, Schjetlein *et al.* (1997) found a lower mean PAI-1 activity in pre-eclamptic women with IUGR than in those who delivered babies >2.5 weight percentile.

Estelles *et al.* (1990) detected lower levels of PAI-2 antigen in amniotic fluid from pregnancies complicated by IUGR compared with normal pregnancy. It was suggested that these decreased levels represented abnormal placental function. In a subsequent study plasma levels of PAI-2 antigen were also decreased in both hypertensive and normotensive IUGR groups (Estelles *et al.*, 1991). The reduced circulating levels of this pregnancy-specific inhibitor were proposed as an adequate marker of impaired placental function.

Lindoff and Astedt (1994) reported reduced plasma PAI-2 concentrations in IUGR pregnancies in comparison to normal pregnancies. In 1996, He *et al.* reported that PAI-2 levels were significantly decreased in patients with pre-eclampsia complicated with IUGR when compared to normal pregnancy. They suggested that plasma PAI-2 levels are more closely related to placental function and foetal growth than to the severity of pre-eclampsia.

In a recent report, Schjetlein *et al.* (1997) reported that patients with pre-eclampsia and IUGR had significantly lower PAI-2 antigen plasma levels than patients with pre-eclampsia alone.

5.1.7 Fibrinolysis in the Uteroplacental Circulation

Bonnar *et al.* (1990) reported that tPA antigen was significantly higher in pregnancies complicated by IUGR in peripheral and uterine vein blood and cord blood. In pre-eclamptic patients, the tPA levels were higher in the peripheral vein alone.

PAI-1 was higher in IUGR and pre-eclamptic pregnancies in both peripheral and uterine vein blood. PAI-2 levels were much lower in pre-eclamptic and IUGR pregnancies in both peripheral and uterine vein blood (Bonnar *et al.*, 1990).

During the same year Sheppard *et al.* (1990) reported significantly higher PAI-1 levels in peripheral vein blood from pre-eclamptic and IUGR pregnancies and PAI-2 levels significantly lower in IUGR than in normal pregnancies. In addition, they reported a significant increase in PAI-1 levels and a significant decrease in PAI-2 levels in the uterine vein blood of IUGR pregnancies compared to normal pregnancies (Sheppard *et al.*, 1990).

5.1.8 Fibrinolysis in the Uteroplacental Tissue

Estelles *et al.* (1994) reported a significant increase in PAI-1 antigen levels in placental extracts in pre-eclamptic pregnancies complicated with IUGR

compared to normal pregnancy. Furthermore, they could find no significant changes in PAI-1 antigen levels in normotensive IUGR compared to normal pregnancy. These results were confirmed in a later study (Grancha *et al.*, 1996).

Using immunohistochemical techniques, Estelles *et al.* (1994) demonstrated strong PAI-1 staining in areas of placental infarcts and syncytiotrophoblast cells of pre-eclamptic/IUGR placentas. Weaker PAI-1 staining was seen in syncytiotrophoblast cells was seen in normotensive IUGR placentas.

In a recent study, the same author (Estelles *et al.*, 1998) again demonstrated, by ELISA, that PAI-1 antigen levels are significantly greater in pre-eclampsia/IUGR placentas. Furthermore, they detected a three-fold increase in the mean value of PAI-1 mRNA which agrees with the antigenic levels (Estelles *et al.*, 1998).

In addition to looking at PAI-1, Estelles *et al.* (1994) measured PAI-2 antigen levels in normotensive and pre-eclamptic IUGR placentas. In both groups they found a significant decrease in PAI-2 antigen levels when compared to normal placentas. This result was reflected in the immunohistochemical study where a decrease in PAI-2 immunoreactivity was noted in the syncytiotrophoblast cells in placentas from both groups.

In 1996, Grancha *et al.* reported a significant decrease in PAI-2 antigen levels in normotensive and pre-eclamptic IUGR placentas. In addition they suggested that this decrease is due to altered expression of PAI-2 mRNA in the syncytiotrophoblast cells.

5.1.9 Objectives of the Study

The main aims of this part of the study are identical to that of Chapter 4. The study wants to determine the contribution of the placenta and the placental bed to the overall uteroplacental circulation seen in pregnancies complicated by IUGR. As stated, these pregnancies are associated with increases in PAI activity and increased fibrin deposition. One aim of this study is to determine the contribution of the placenta and placental bed to the overall decreased fibrinolytic activity reported in these pregnancies. In addition, the expression and release of fibrinolytic factors by placental and placental bed trophoblasts is examined by cell culture. Furthermore, the invasive abilities of these cells are examined by invasion assays. If changes in fibrinolytic factors are involved in fibrin deposition and invasive behaviour in pregnancies associated with IUGR then differences in one or more factors will be able to be identified.

5.2 RESULTS

5.2.1 Tissue Levels of Fibrinolytic Activators and Inhibitors in Placental and Placental Bed Extracts from PET/IUGR and IUGR Pregnancies

The following results are based on 19 placental and 19 placental bed biopsies for normal pregnancy and 17 placental and placental biopsies for the other two groups.

Tissue Plasminogen Activator Antigen: (Fig 5.1) Tissue levels of antigenic t-PA were extracted from the placenta. There was no significant difference between the levels seen in the placenta for any of the patient groups.

Urokinase Plasminogen Activator Antigen: (Fig 5.2) Antigenic u-PA was detected in the placenta. Placental u-PA levels were similar in all three patient groups.

Plasminogen Activator Inhibitor Antigen (PAI-1): (Fig 5.3) Significantly higher levels were found of PAI-1 in the PET/IUGR and IUGR groups when compared to normal pregnancy ($p<0.001$).

Plasminogen Activator Inhibitor Antigen (PAI-2): (Fig 5.4) Antigenic PAI-2 levels significantly decreased in the PET/IUGR and the IUGR groups ($p<0.001$).

t-PA in the Placental Bed: (Fig 5.5) Comparable levels of t-PA were found in the placental bed extracts for all the group types.

u-PA in the Placental Bed: (Fig 5.6) No significant differences were found for u-PA levels in the placental bed of the patient groups studied.

PAI-1 in the Placental Bed: (Fig 5.7) PAI-1 levels in the placental bed for the three groups studied were not significantly different.

PAI-2 in the Placental Bed: (Fig 5.8) PAI-2 levels are higher in PET/IUGR and IUGR. However they do not reach significance.

5.2.2 Tissue Levels of Fibrinolytic Activators and Inhibitors in Placental and Placental Bed Trophoblasts in Normal, PET/IUGR and IUGR Pregnancies

PAI-1 Secretion by Placental Trophoblasts: (Fig 5.9) Placental trophoblasts from PET/IUGR and IUGR secrete significantly greater amounts of PAI-1 into the culture supernatant over a 24 hour period than placental trophoblasts from normal pregnancies ($p<0.001$).

PAI-2 Secretion by Placental Trophoblasts: (Fig 5.10) No significant differences are seen in PAI-2 production and secretion by placental trophoblasts from the three patient groups.

u-PA Secretion by Placental Trophoblasts: (Fig 5.11) No significant differences are seen in u-PA production and secretion by placental trophoblasts from the three patient groups.

PAI-1 Secretion by Placental Bed Trophoblasts: (Fig 5.12) placental bed trophoblasts in both PET/IUGR and IUGR groups secrete significantly greater amounts of PAI-1 compared with normal placental bed trophoblasts ($p<0.001$).

PAI-2 Secretion by Placental Bed Trophoblasts: (Fig 5.13) No significant differences are seen in PAI-2 production and secretion by placental bed trophoblasts from the three patient groups.

u-PA Secretion by Placental Bed Trophoblasts: (Fig 5.14) No significant differences are seen in PAI-2 production and secretion by placental bed trophoblasts from the three patient groups.

Cell Associated Plasminogen Activator Activity: (Fig 5.15) Cell associated plasminogen activator activity is significantly reduced in PET/IUGR and IUGR placental trophoblasts when compared to normal controls ($p<0.001$).

Cell Associated Plasminogen Activator Activity: (Fig 5.16) Cell associated plasminogen activator activity is significantly reduced in PET/IUGR and IUGR placental bed trophoblasts when compared to normal controls ($p<0.001$).

5.2.3 Comparative Changes in Invasion Abilities of Placental and Placental Bed Trophoblasts in PET/IUGR and IUGR

Fibrin Degradation by Placental Trophoblasts: (Fig 5.17) Significant decreases in the ability of placental trophoblasts from PET/IUGR and IUGR to lyse fibrin clots were seen ($p<0.001$).

Fibrin Degradation by Placental Bed Trophoblasts: (Fig 5.18) PET/IUGR and IUGR placental bed trophoblasts have a significantly reduced ability to lyse fibrin clots when compared to normal pregnancy ($p< 0.001$).

Matrigel Degradation by Placental Trophoblasts: (Fig 5.19) Significant decreases in matrigel invasion are seen for both PET/IUGR and IUGR groups compared to normal placental trophoblasts ($p<0.001$).

Matrigel Degradation by Placental Bed Trophoblasts: (Fig 5.20) PET/IUGR and IUGR placental bed trophoblasts have significant less ability to migrate through the matrigel matrix compared to normal placental bed trophoblasts ($p<0.001$).

5.2.4 Comparative Changes in Secretion Levels of Placental and Placental Bed Biopsies in Organ Culture for PET/IUGR and IUGR Compared to Normal Pregnancy

Secretion of Fibrinolytic Factors: (Table 5.1) The placenta secretes higher amounts of PAI-1 and PAI-2 than the placental bed. In pregnancies complicated by PET/IUGR and IUGR, significant increases in PAI-1 levels secreted by the placenta are seen compared to normal pregnancy ($p<0.001$).

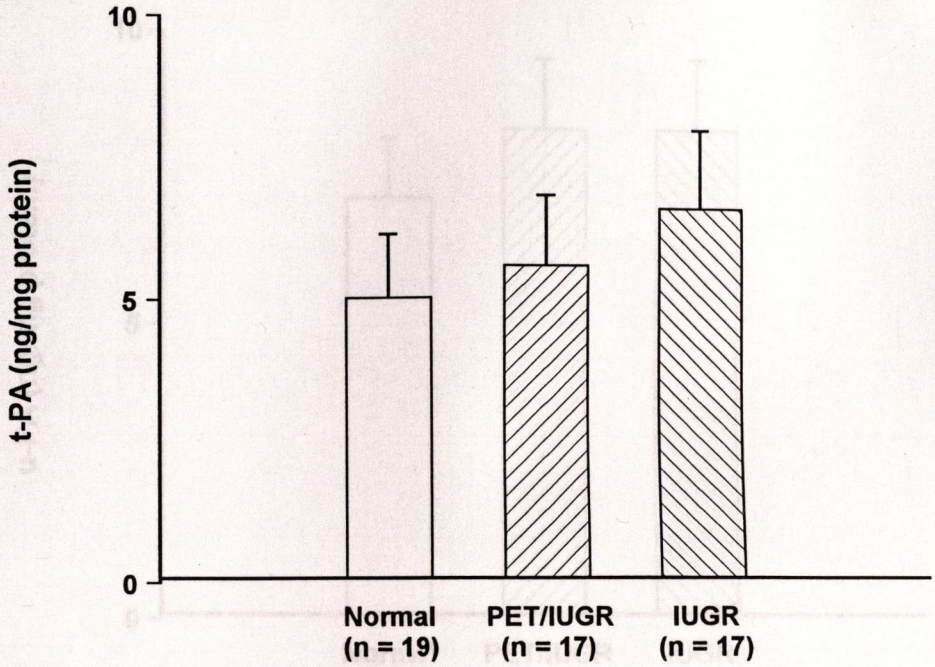


Fig 5.1: Comparative tissue levels of t-PA antigen in the placenta from normal, PET/IUGR and IUGR pregnancies.
Values are mean ± SD. Statistical test is Student's t-test.

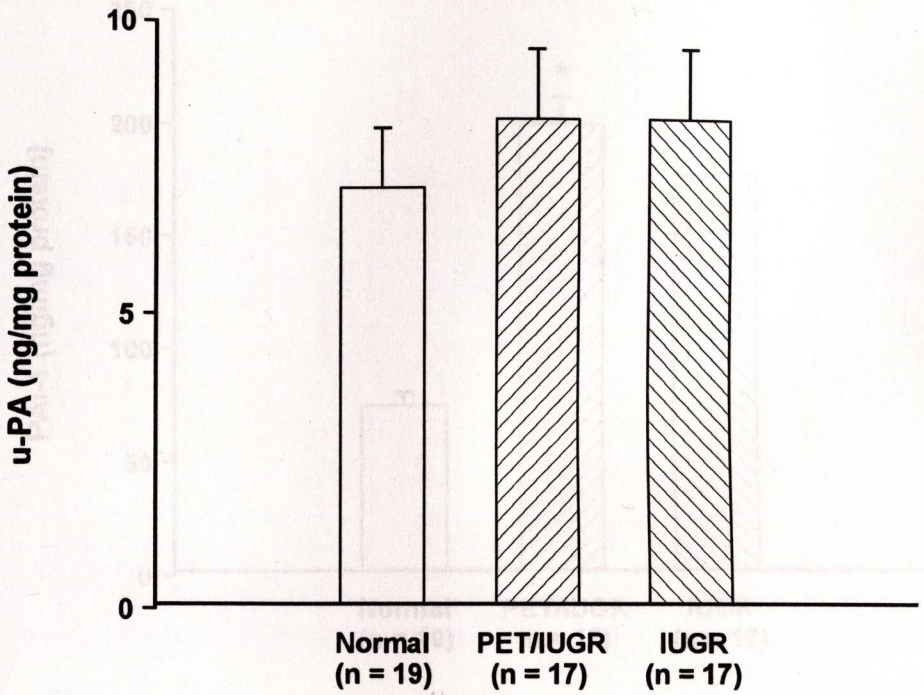


Fig 5.2: Comparative tissue levels of u-PA antigen in the placenta from normal, PET/IUGR and IUGR pregnancies.
Values are mean $\pm$ SD. Statistical test is Student's t-test.

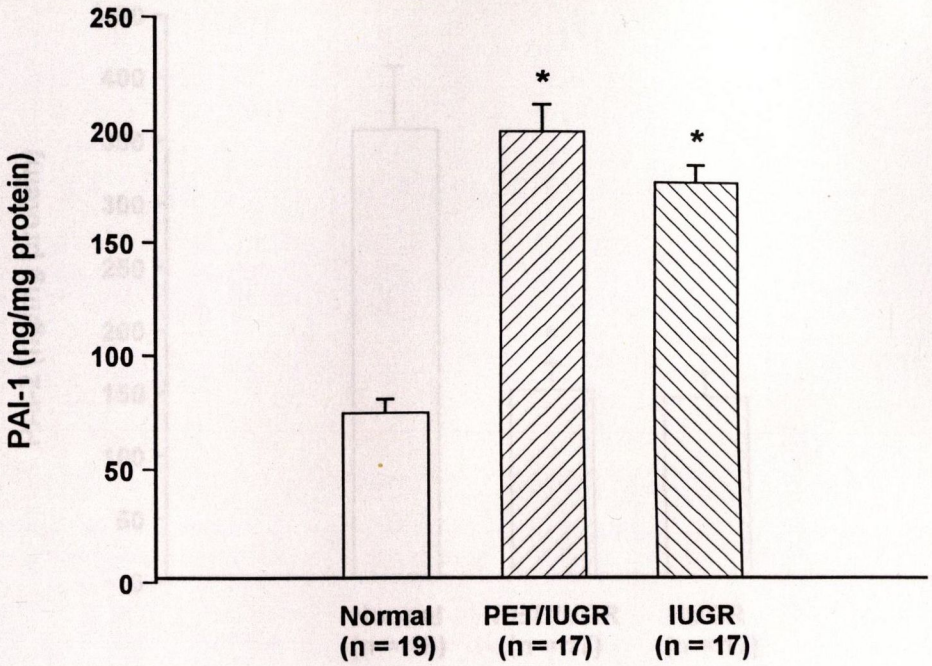


Fig 5.3: Comparative tissue levels of PAI-1 antigen in the placenta from normal, PET/IUGR and IUGR pregnancies.
* $p<0.001$ PET/IUGR and IUGR vs. Normal
Values are mean $\pm$ SD. Statistical test is Student's t-test.

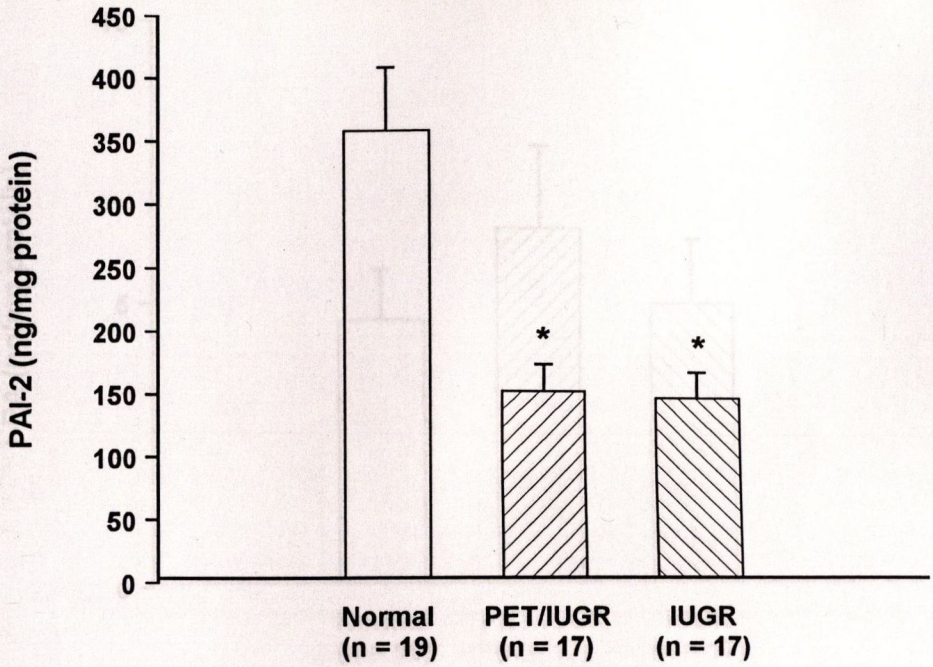


Fig 5.4: Comparative tissue levels of PAI-2 antigen in the placenta from normal, PET/IUGR and IUGR pregnancies.
*$p<0.001$ PET/IUGR and IUGR vs. Normal
Values are mean $\pm$ SD. Statistical test is Student's t-test.

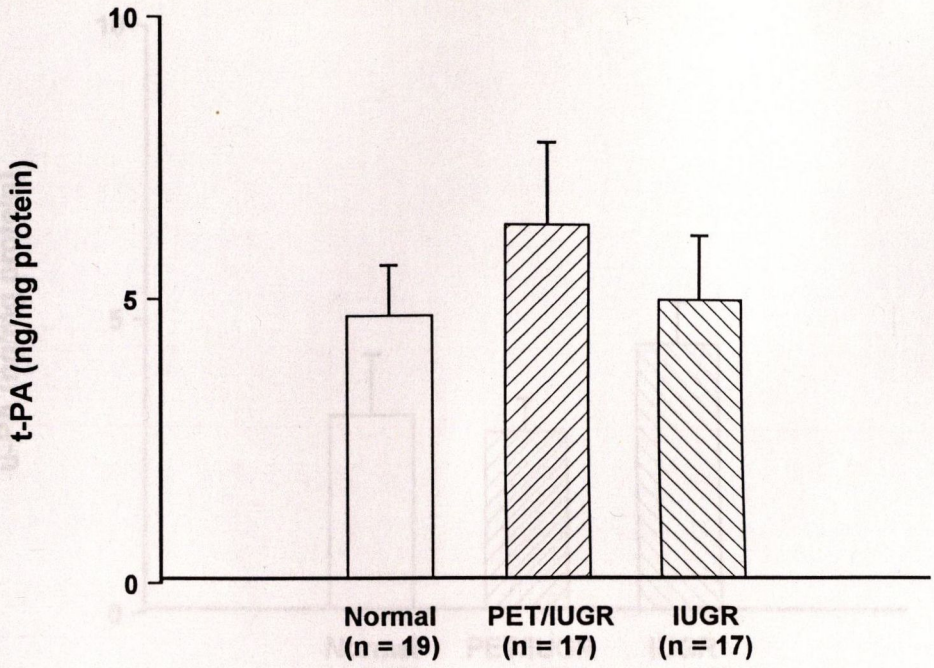


Fig 5.5: Comparative tissue levels of t-PA antigen in the placental bed from normal, PET/IUGR and IUGR pregnancies.
Values are mean $\pm$ SD. Statistical test is Student's t-test.

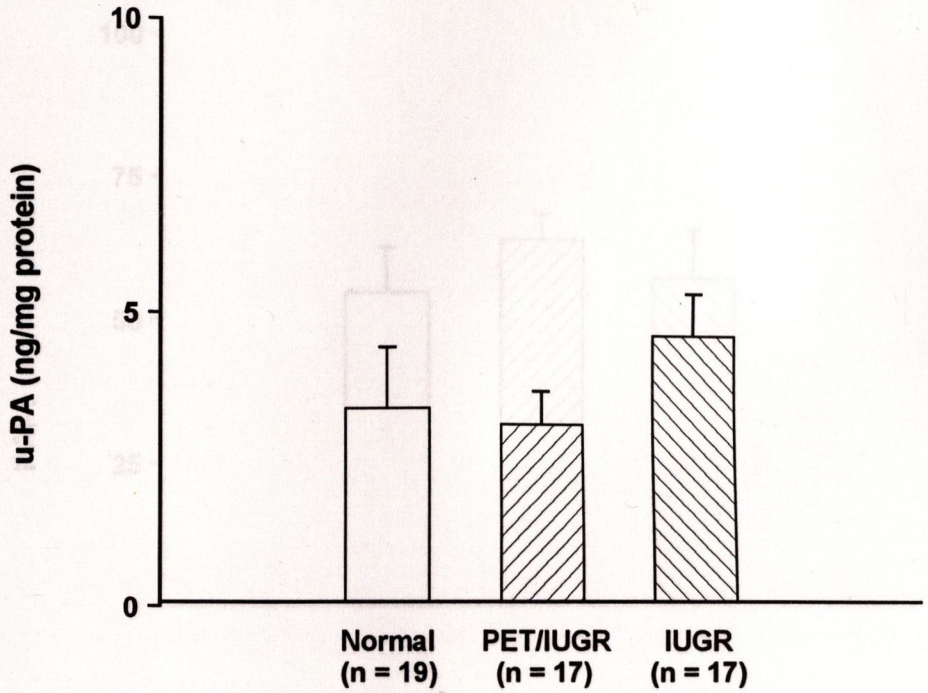


Fig 5.6: Comparative tissue levels of u-PA antigen in the placental bed from normal, PET/IUGR and IUGR pregnancies.
Values are mean $\pm$ SD. Statistical test is Student's t-test.

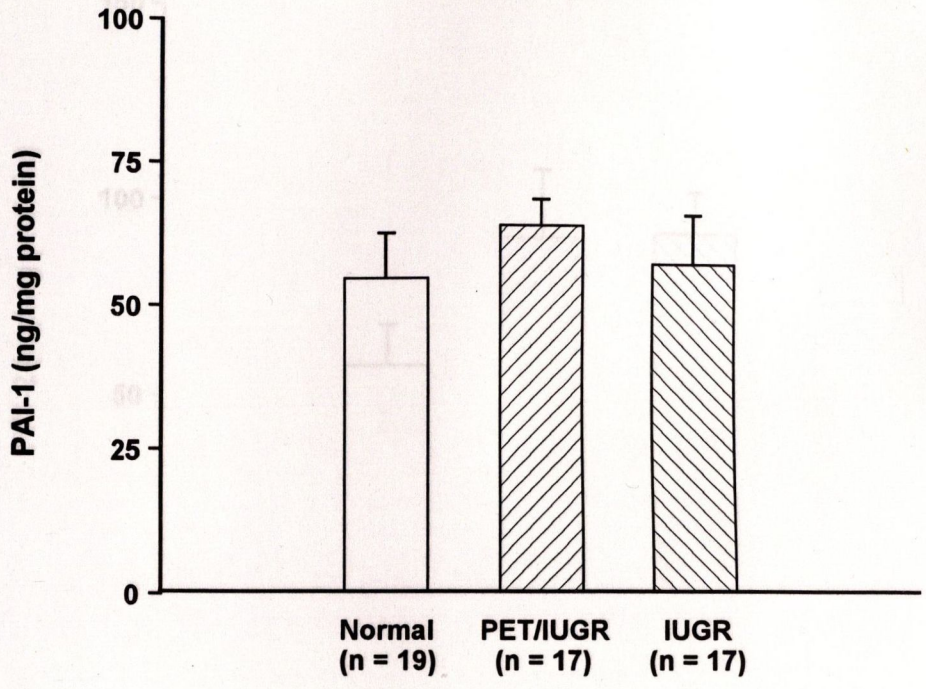


Fig 5.7: Comparative tissue levels of PAI-1 antigen in the placental bed from normal, PET/IUGR and IUGR pregnancies.
Values are mean $\pm$ SD. Statistical test is Student's t-test.

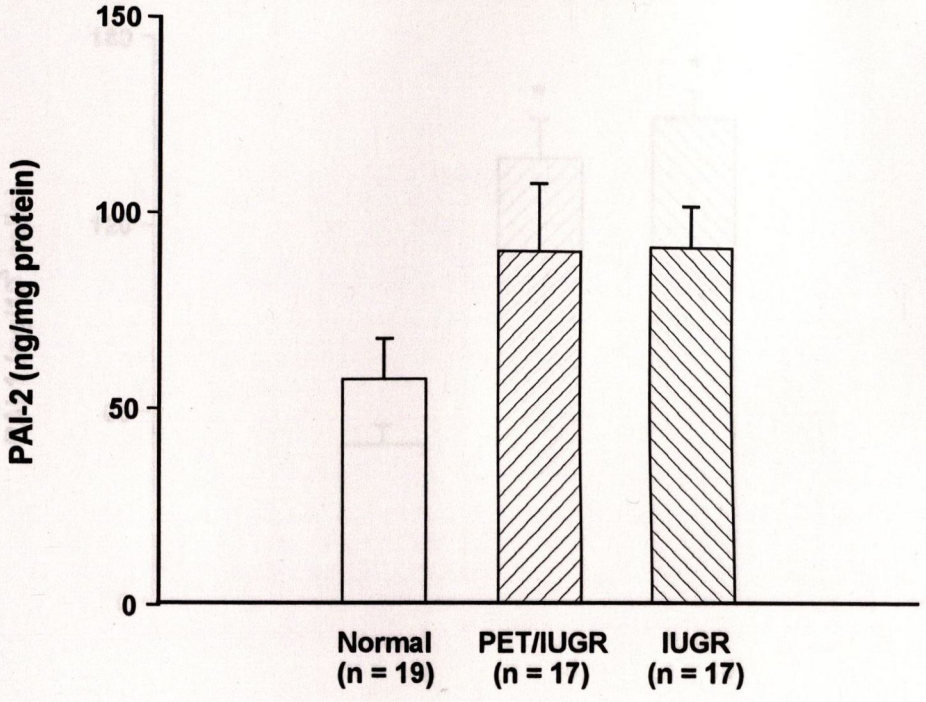


Fig 5.8: Comparative tissue levels of PAI-2 antigen in the placental bed from normal, PET/IUGR and IUGR pregnancies.
Values are mean $\pm$ SD. Statistical test is Student's t-test.

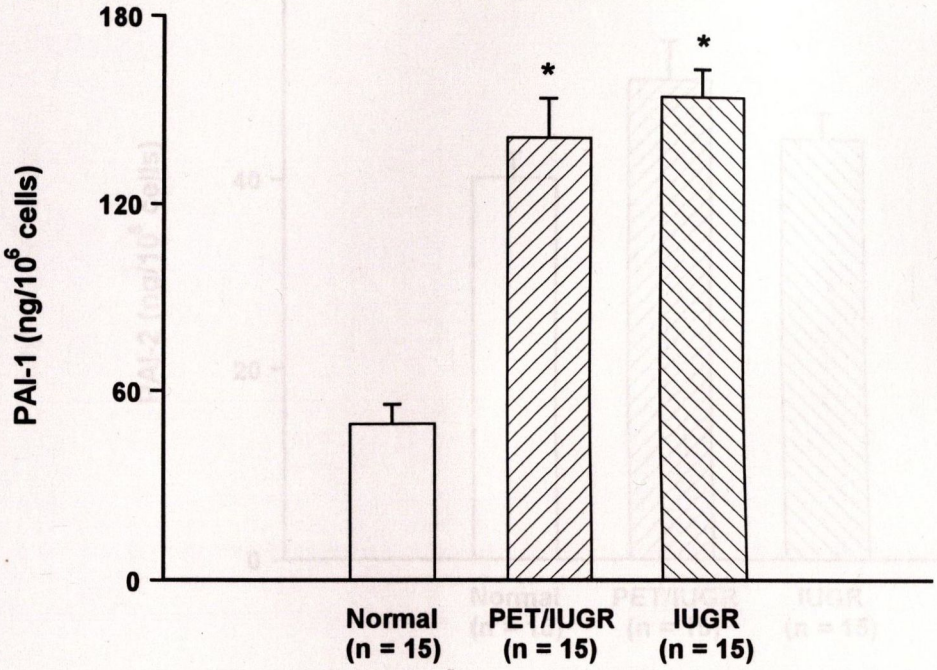


Fig 5.9: Comparative levels of PAI-1 antigen produced by placental trophoblasts in Normal, PET/IUGR and IUGR pregnancies.
* $p<0.001$ (PET/IUGR and IUGR vs. Normal)
Values are mean $\pm$ SD. Statistical test is Student's t-test.

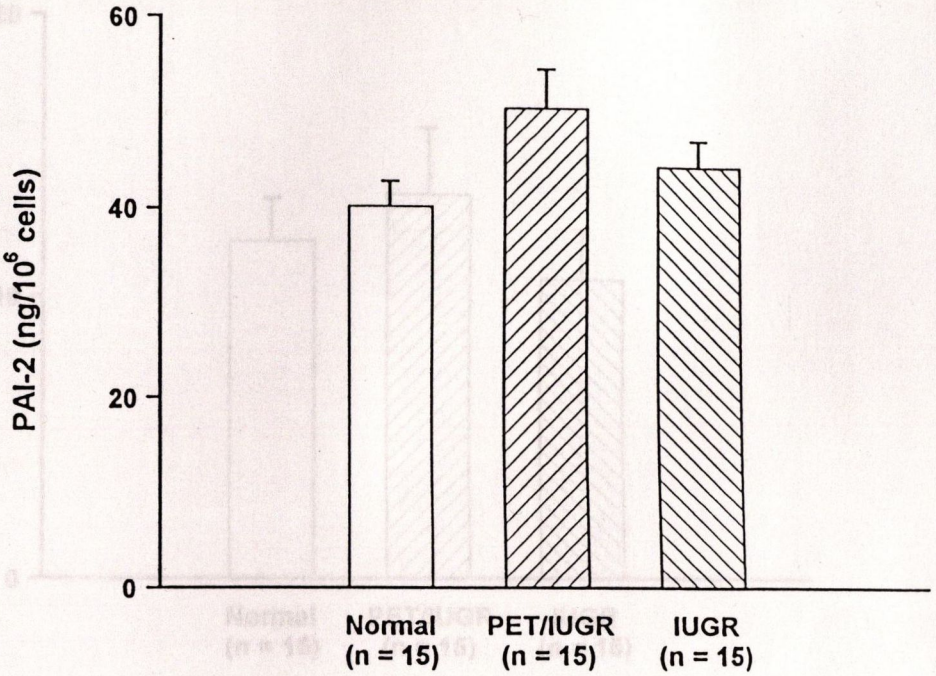


Fig 5.10: Comparative levels of PAI-2 antigen produced by placental trophoblasts in normal, PET/IUGR and IUGR pregnancies.
* $p<0.001$ (PET/IUGR and IUGR vs. Normal)
Values are mean $\pm$ SD. Statistical test is Student's t-test.

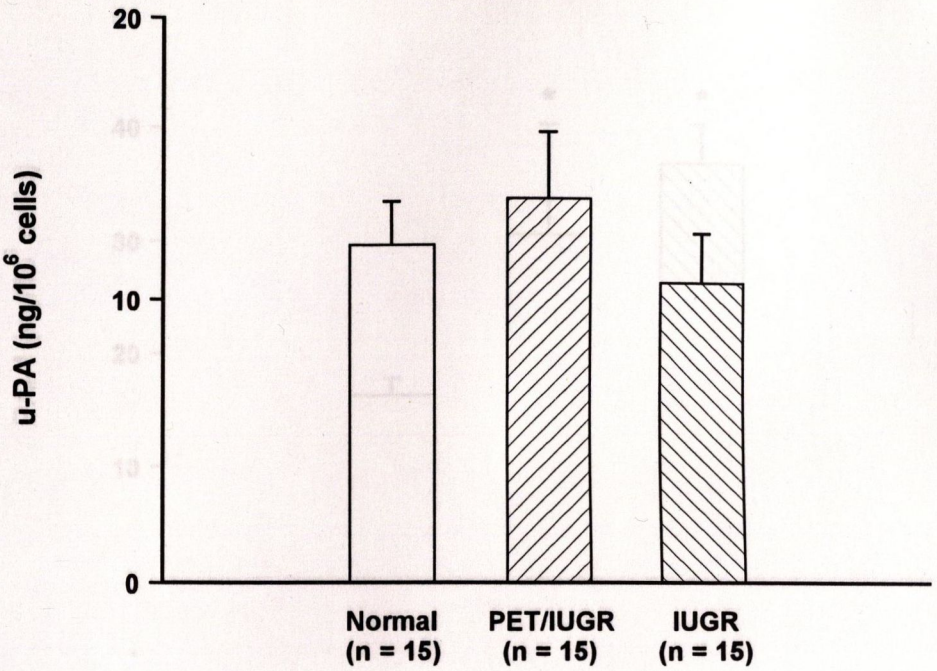


Fig 5.11: Comparative levels of u-PA antigen produced by placental trophoblasts in normal, PET/IUGR and IUGR pregnancies.
Values are mean $\pm$ SD. Statistical test is Student's t-test.

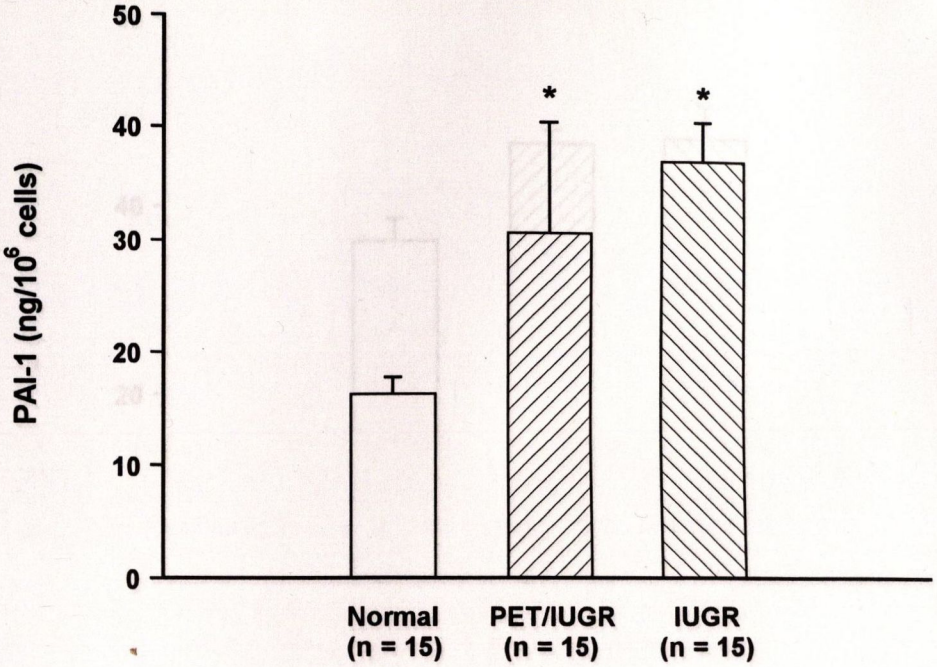


Fig 5.12: Comparative levels of PAI-1 antigen produced by placental bed trophoblasts in normal, PET/IUGR and IUGR pregnancies.
* $p<0.001$ (PET/IUGR and IUGR vs. Normal)
Values are mean $\pm$ SD. Statistical test is Student's t-test.

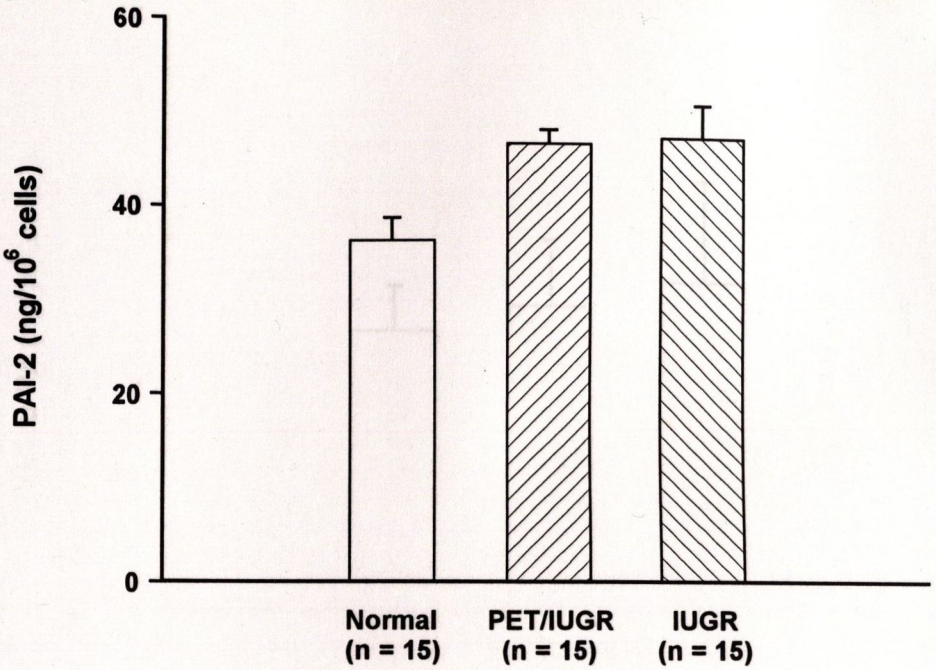


Fig 5.13: Comparative levels of PAI-2 antigen produced by placental bed trophoblasts in normal, PET/IUGR and IUGR pregnancies.
Values are mean $\pm$ SD. Statistical test is Student's t-test.

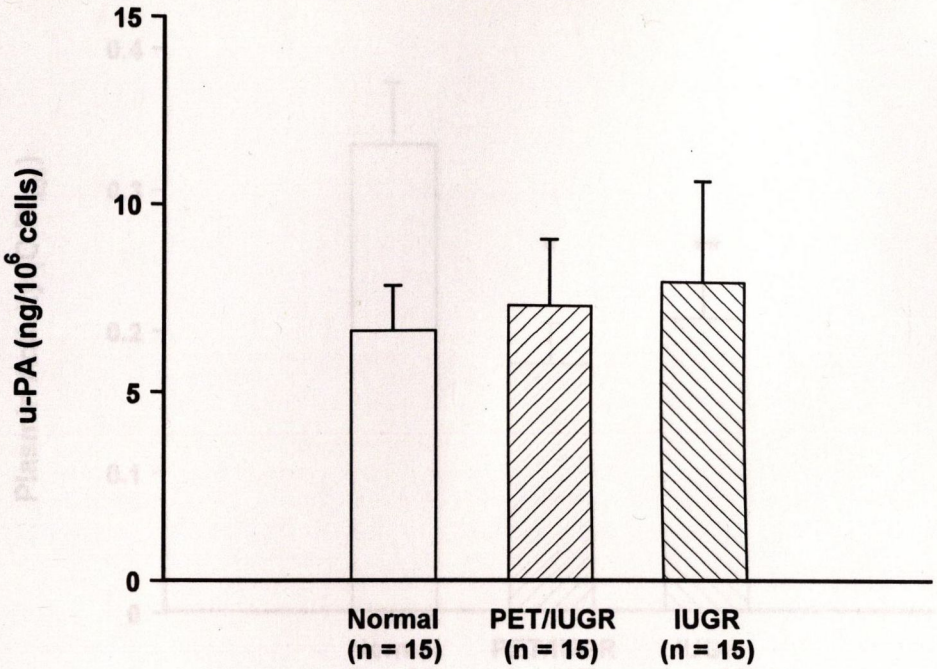


Fig 5.14: Comparative levels of u-PA antigen produced by placental bed trophoblasts in normal, PET/IUGR and IUGR pregnancies.
Values are mean $\pm$ SD. Statistical test is Student's t-test.

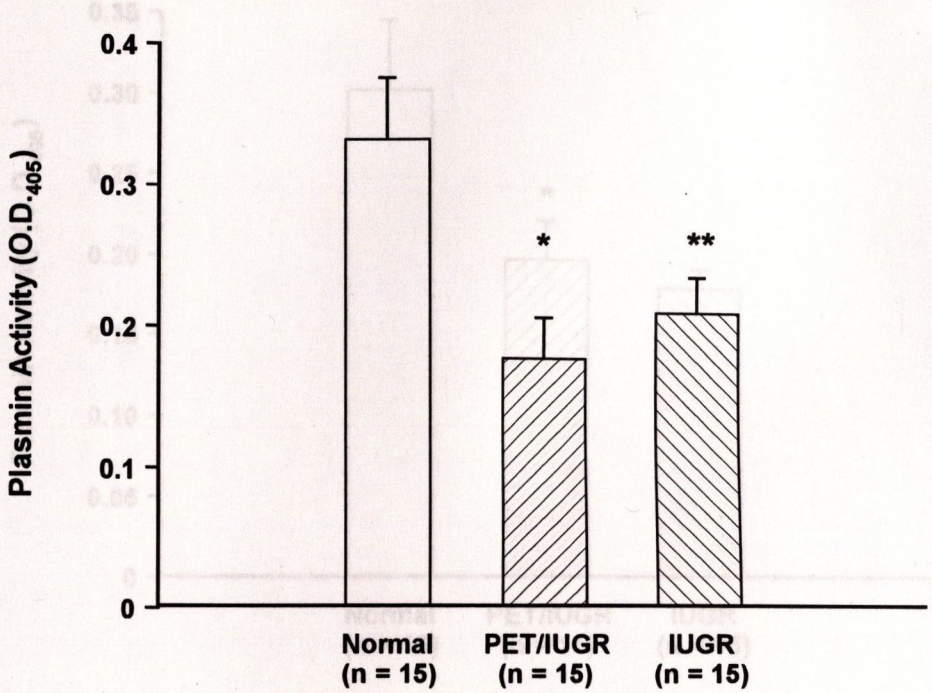


Fig 5.15: Comparison of cell associated plasminogen activator activity by placental trophoblasts in normal, PET/IUGR and IUGR pregnancy.

* $p < 0.001$ (PET/IUGR vs. Normal)
** $p < 0.001$ (IUGR vs. Normal)
Values are mean $\pm$ SD. Statistical test is Student's t-test.

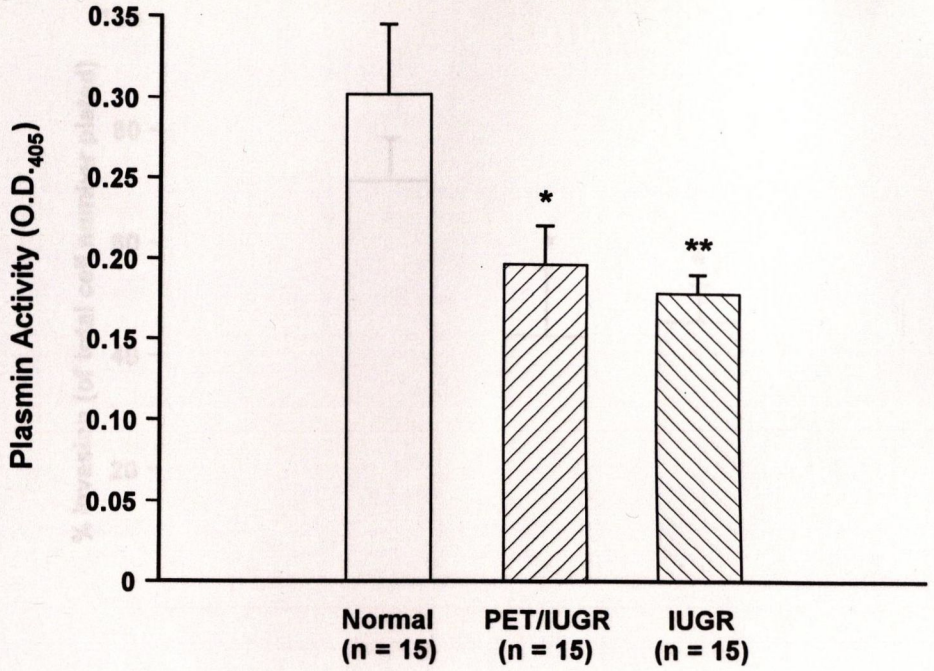


Fig 5.16: Comparison of cell associated plasminogen activator activity by placental bed trophoblasts in normal, PET/IUGR and IUGR pregnancies.

* $p < 0.001$ (PET/IUGR vs. Normal)
** $p < 0.001$ (IUGR vs. Normal)
Values are mean $\pm$ SD. Statistical test is Student's t-test.

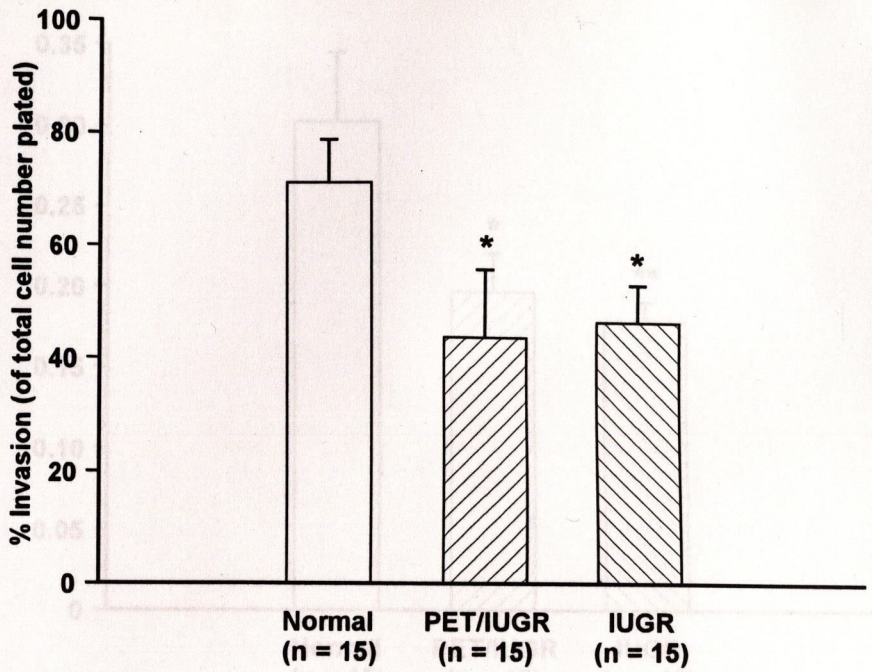


Fig 5.17: Comparison of fibrin invasion by placental trophoblasts in normal, PET/IUGR and IUGR pregnancies.
* $p<0.001$ (PET/IUGR and IUGR vs. Normal)
Values are mean $\pm$ SD. Statistical test is Student's t-test.

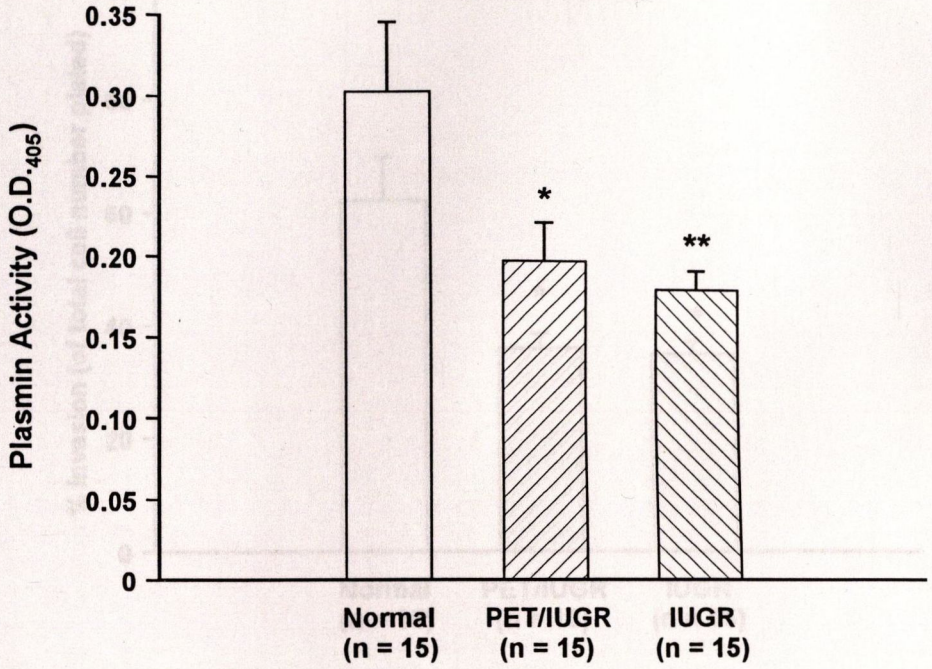


Fig 5.18: Comparison of cell associated plasminoger activator activity by placental bed trophoblasts in normal, PET/IUGR and IUGR pregnancies.
* $p<0.001$ (PET/IUGR and IUGR vs. Normal)
Values are mean $\pm$ SD. Statistical test is Student's t-test.

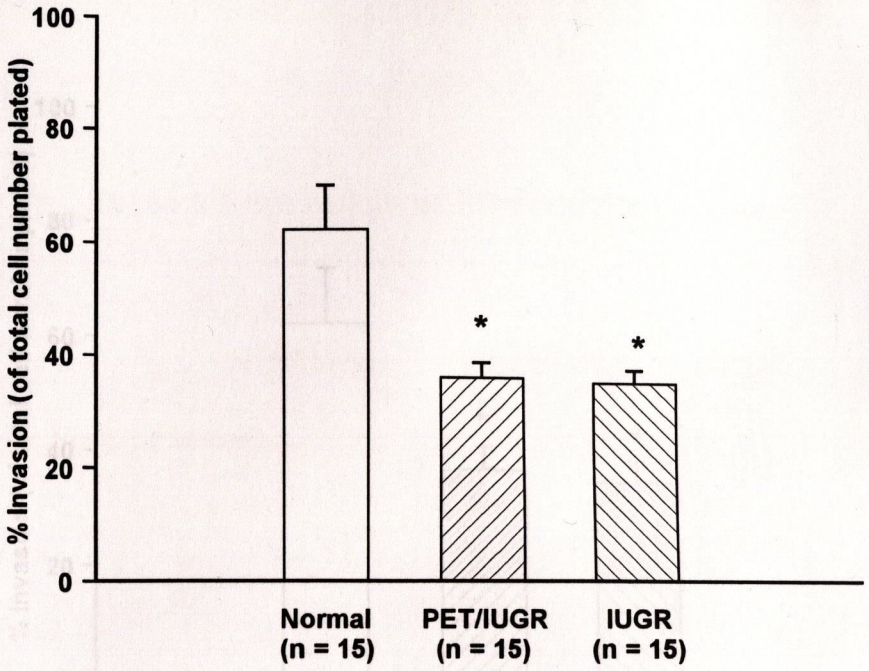


Fig 5.19: Comparison of matrigel invasion by placental trophoblasts in normal, PET/IUGR and IUGR pregnancies.
* $p<0.001$ (PET/IUGR and IUGR vs. Normal)
Values are mean $\pm$ SD. Statistical test is Student's t-test.

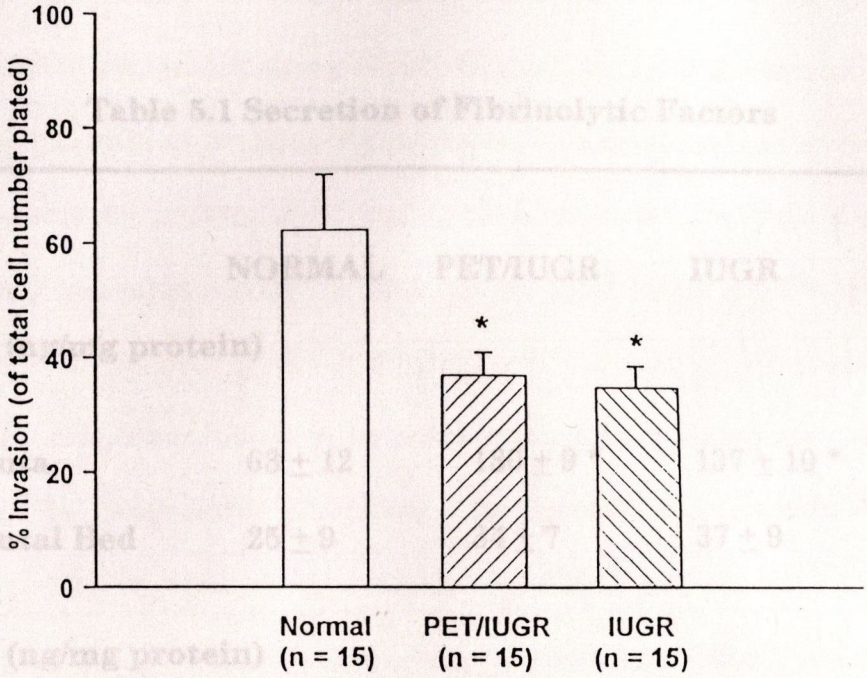


Fig 5.20: Comparison of matrigel invasion by placental bed trophoblasts in normal, PET/IUGR and IUGR pregnancies.
* $p < 0.001$ (PET/IUGR and IUGR vs. Normal)
Values are mean $\pm$ SD. Statistical test is Student's t-test.

Table 5.1 Secretion of Fibrinolytic Factors

	NORMAL	PET/IUGR	IUGR
PAI-1 (ng/mg protein)			
Placenta	63 ± 12	130 ± 9 *	137 ± 10 *
Placental Bed	25 ± 9	34 ± 7	37 ± 9
PAI-2 (ng/mg protein)			
Placenta	53± 12	67 ± 9	54 ± 7
Placental Bed	12 ± 3	14 ± 8	16 ± 7
u-PA (ng/mg protein)			
Placenta	7.6 ± 2	8.2 ± 3.1	7.3 ± 3.3
Placental Bed	5.7 ± 2.8	6.2 ± 2.4	5.9 ± 3.1

*$p < 0.001$ (PET/IUGR and IUGR vs. Normal). n = 15
Values are mean ± SD. Statistical test is Student's t-test.

5.3 Discussion

In pregnancy maternal blood is supplied to the intervillous space of the placenta by way of the uterine spiral arteries and the growth of the foetus is dependent on an increasing supply of blood reaching the intervillous space of the placenta as pregnancy progresses. In normal pregnancy the modified spiral arteries contain fibrin and trophoblast cells. The fibrin deposition in normal pregnancy may be related to trophoblast cell function since fibrinolytic activity of the trophoblast, in contrast to endothelium in the vessel, has been shown to be markedly reduced (Sheppard and Bonnar, 1978). In pregnancies complicated by severe foetal growth retardation, irrespective of whether hypertension is present or not, the uteroplacental vessels show occlusive lesions with an increased fibrin deposition and accumulation of lipid in the vessel walls (Sheppard and Bonnar, 1976). The placental villi directly distal to these arteries show degrees of ischaemia and infarction related to the degree of occlusion of the spiral arteries while the cell structure of the decidual tissue surrounding the spiral appears normal (Sheppard and Bonnar, 1980).

Failed trophoblastic invasion of trophoblasts into the myometrium occurs in normotensive IUGR (Sheppard and Bonnar, 1976). Furthermore, placental tissue in normotensive IUGR exhibits altered haemostatic functions with an increased ability to inhibit urokinase-induced fibrinolysis (Elder and Myatt, 1976).

IUGR is commonly associated with pre-eclampsia. Marked alterations in

various haemostatic parameters are known to occur in pre-eclampsia irrespective of whether the pregnancy is complicated by IUGR.

Normotensive IUGR and pre-eclampsia without IUGR share common fibrinolytic changes compared with normal pregnancy. Fibrinolytic factors, u-PA and PAI-2, both produced by the placenta, circulate in reduced concentration. Placental infarction, common to each of these conditions, reduces the amount of normal functioning placental tissue and may explain the low levels of these fibrinolytic factors.

In pre-eclampsia a systemic disorder of coagulation and fibrinolysis, extensive endothelial damage and fibrin occluded spiral arteries may all be associated with the development of maternal hypertension. Raised levels of t-PA and PAI-1 are believed to be a secondary response to the increased generation of fibrin and endothelial damage characteristic of the hypertensive state. In normotensive IUGR however, endothelial damage is confined to the uteroplacental vasculature. Despite depressed fibrinolysis in normotensive IUGR, systemic haemostasis appears secure and vascular integrity is intact.

Comparable levels of t-PA and u-PA antigen were found in tissue extracts of the placenta and placental bed in normotensive, pre-eclampsia/IUGR, pre-eclampsia without IUGR and normal pregnancy. Similar results are shown to occur for the placenta and placental bed maintained in organ culture. Fibrinolytic factor levels of placenta were significantly higher than placental bed extracts which suggests a placental origin for u-PA. Endothelial cells

within the placental villous structures may account for the increased t-PA antigen level while u-PA antigen levels are directly linked to the syncytiotrophoblast in the placenta. The similar levels of u-PA antigen seen in this study contradict the theory that u-PA levels can be correlated with placental function (Estelles *et al.*, 1991). One possible explanation for this discrepancy is that the biopsies may not give a representation of the total u-PA antigen levels produced and secreted by the placenta. This may be due to the biopsies containing less infarction areas than the normal distribution seen *in vivo*.

The production and secretion of PAI-1 is significantly higher for pre-eclampsia with IUGR compared with normal pregnancy. Levels of PAI-1 fall for normotensive IUGR (IUGR) versus pre-eclampsia with IUGR (PET/IUGR). This concurs with the changes seen in plasma levels of PAI-1 in PET/IUGR and IUGR compared with normal pregnancy (Grancha *et al.*, 1996).

In the placenta no significant differences between the relevant groups can be seen for PAI-2 production and secretion although there is a decrease in IUGR compared PET/IUGR. Other studies have shown that the PAI-2 plasma levels decrease in IUGR compared to normal and have suggested that this is due to a decrease in placental function and placental size (Grancha *et al.*, 1996). The levels of PAI-2 found in this study coupled with a decrease in placental size would produce a decrease in the circulating plasma levels of PAI-2.

Comparing PAI-2/PAI-1 ratios for the production and secretion of these fibrinolytic factors suggests that PAI-2 secretion from the placenta to plasma is lower than PAI-1. An increase in the PAI-2 levels in the placenta may be involved in controlling the fibrin deposition seen in IUGR. The production and secretion of PAI-1 in the placenta is significantly increased in normotensive IUGR when compared to normal placenta.

In the placental bed no significant changes are found for PAI-1 and PAI-2 in the groups studied. However, the concentrations of PAI-1 and PAI-2 are significantly greater than u-PA and t-PA. This suggests that within the placental bed a depressed fibrinolytic state exists. As the placental bed is made up of a mixed cell population, this decrease in fibrinolytic potential in all patient groups may be due to altered PAI expression by any number of cells. These results do not rule out an important role for trophoblast cells in the fibrin deposition seen in the spiral arteries in IUGR.

In summary, the production and secretion of PAI-1 in this study is closely related to levels seen in plasma and placental extracts (Grancha, 1996). The differences in the

Cultured trophoblast cells have been shown to produce u-PA, PAI-1 and PAI-2 (Queenan *et al.*, 1987; Feinberg *et al.*, 1989). Their ability to migrate through the placental bed during pregnancy is thought to be controlled by a plasminogen activator/plasmin system. Shallow invasion by the trophoblasts, as seen in pre-eclampsia and IUGR, leads to a reduction in the number of spiral arteries which undergo physiological changes. In this

study, the production by placental trophoblasts of u-PA, PAI-1 and PAI-2 is investigated. Comparable levels in the secretion of u-PA and PAI-2 by normal, PET/IUGR and IUGR were found. PAI-1 levels in IUGR are significantly higher than for normal placental trophoblasts. Comparable levels were found for pre-eclampsia, PET/IUGR and IUGR. Furthermore, comparable levels of cell associated plasminogen activator activity where found in pre-eclampsia, PET/IUGR and IUGR. Invasion assays through matrigel and fibrin yielded similar results. All the data suggests that in pre-eclampsia, PET/IUGR and IUGR compared to normal pregnancy, there is a reduced fibrinolytic capability of these cultured placental cells to utilise the plasminogen activator/plasmin system which would allow them to migrate through the basement membrane of the placental bed. Cell associated plasminogen activation activity is lower which suggests that these cells have been 'switched off'. Their inability to migrate through fibrin can be attributed to this reduced cell surface plasminogen activator activity and also to the large concentration of PAI-1 and PAI-2 produced by the cells. PAI-1 has been associated with invading trophoblasts and therefore the significant increases in its secretion by these trophoblasts (pre-eclamptic, PET/IUGR and IUGR) compared to normal trophoblasts may be the mechanism by which their migratory ability is curtailed.

Placental bed trophoblast cells secrete fibrinolytic factors in a similar manner to placental trophoblasts. Trophoblast cells from IUGR placental beds secrete significantly higher levels of PAI-1 than normal placental bed

trophoblasts. Comparable levels for PET/IUGR were found when compared to IUGR. Cell surface plasminogen activator activity is significantly reduced. Their ability to lyse fibrin and migrate through matrigel diminishes. The results suggest that increased PAI-1 production and secretion by these cells regulates their invasive and fibrinolytic ability. In addition, the reduced fibrinolytic ability could account for the fibrin deposits which are present in the spiral arteries of pre-eclampsia, PET/IUGR and IUGR.

CHAPTER 6
FIBRINOLYSIS IN ENDOTHELIAL AND DECIDUAL CELLS

6.1 Introduction

The endothelium is a dynamic, heterogeneous, disseminated organ that possesses vital secretory, synthetic, metabolic and immunologic functions (see Cines *et al.* (1998) for review). The endothelial cell surface in the adult human is composed of approximately 1 to 6 x 10^{13} cells, weighs approximately I Kg and covers a surface area of approximately 1 to 7 m^2 (Augustin *et al.*, 1994). Endothelial cells line vessels in every organ system and regulate the flow of nutrient substances, diverse biologically active molecules and the blood cells themselves. This gate-keeping role of endothelium is effected through the presence of membrane-bound receptors of numerous molecules including proteins (*e.g.*, growth factors, coagulant and fibrinolytic proteins), lipid transporting particle (*e.g.*, low-density lipoprotein [LDL]), metabolites (*e.g.*, nitrous oxide and serotonin), and hormones (*e.g.*, endothelin-1), as well as through specific junctional proteins and receptors that govern cell-cell and cell-matrix interactions.

The endothelium also plays a pivotal role in regulating blood flow. In part, this results from the capacity of quiescent endothelial cells to generate an active antithrombotic surface that facilitates transit of plasma and cellular constituents throughout the vasculature. Perturbations, such as those that may occur at sites of inflammation or high hydrodynamic shear stress, disrupt these activities and induce

endothelial cells to create a prothrombotic and antifibrinolytic microenvironment. Blood flow is also regulated, in part, through secretion and uptake of vasoactive substances by the endothelium that act in a paracrine manner to constrict and dilate specific vascular beds in response to stimuli such as endotoxin. Detailed study of endothelial function has become increasingly feasible with the development in the 1970s of techniques to culture endothelial cells *in vitro* (Jaffe *et al.*, 1973; Gimbrone *et al.*, 1974; Lewis *et al.*, 1973). Recently, techniques have been reported which allow the isolation of endothelial cells from placental and decidual tissues (Leach *et al.*, 1994; Schutz and Friedl, 1996; Drake and Loke; 1991; Gallery *et al.*, 1995; Grimwood *et al.*, 1995).

6.1.1 Endothelial Cells and Fibrinolysis

Over the years a concept has arisen that the endothelial surface is profibrinolytic and thus helps maintain blood in its fluid state (Hirsh *et al.*, 1994). However, it has become clear that the contribution of endothelial cells to fibrinolysis varies with their metabolic status (*i.e.* quiescent or activated). Their vascular derivation and the concentration of other haemostatically active molecules in the local plasma milieu.

Plasminogen Activators

Studies with endothelial cells cultured from various tissues have lead to the inference that t-PA production and secretion is a property of all

endothelial cells (Hirsh *et al.*, 1994). However, recent studies suggest that t-PA production remains an exclusive property of microvascular endothelial cells (Levin and Osborn, 1997).

The production of t-PA by cultured endothelial cells is regulated by a variety of external stimuli at the level of gene transcription and cellular release (Levin *et al.*, 1989). Measurements of plasma t-PA levels suggest such regulated production/secretion occurs *in vivo* as well (Cugno *et al.*, 1989). Intracellular signalling pathways operate in stimulated t-PA release have been described *in vitro* (Levin and Santell, 1991).

u-PA appears not to be produced by most quiescent endothelial cells (Wojta *et al.*, 1989). Rather, it is expressed by endothelial cells involved in wound repair or angiogenesis (Bacharach *et al.*, 1992), consistent with the hypothesised importance of u-PA in cell migration and tissue remodelling. Yet, u-PA is obviously important to vascular homeostasis, because mice genetically deficient in u-PA develop inflammation-induced thrombi (Carmeliet *et al.*, 1994) and manifest thrombotic tissue injury in response to liposaccharide (LPS) (Yamamoto and Loskutoff, 1996). However, the extrarenal source of u-PA in physiologic states has not been established.

Plasminogen Activator Receptors

Several groups have reported the presence of t-PA receptors (Barnathan *et al.*, 1988; Beebe *et al.*, 1989; Fukao *et al.*, 1992; Ramakrishnan *et al.*,

1990; Cheng *et al.*, 1996; Hajjar *et al.*, 1990). Binding of t-PA to endothelial cells has been reported to promote its fibrinolytic activity (Barnathan *et al.*, 1988; Hajjar and Hamel, 1990), and to stimulate cell proliferation (Weling *et al.*, 1996). Recently, one such receptor for t-PA, called annexin II, has been reported. It is expressed on endothelial cells and binds t-PA in a specific and saturable manner *in vitro* (Hajjar *et al.*, 1994; Hajjar and Hamel, 1990). However, the expression of annexin II on endothelial cells *in vivo* has yet to be demonstrated.

The u-PA receptor (uPAR) appears to be identical to that expressed on other cell types (Barnathan *et al.*, 1990a; Barnathan *et al.*, 1990b). uPAR is a three-domain protein linked to surfaces by a glycerophosphatidyl inositol anchor. Single-chain u-PA bound to cells via uPAR exhibits increased plasminogen activating efficiency (Barnathan *et al.*, 1990a; Manchanda and Schwartz, 1991) and is relatively protected from inhibition by PAI-1 and PAI-2 (Schwartz, 1994; Higazi *et al.*, 1996). uPAR may be expressed primarily on the surface of migrating endothelial cells participating in angiogenesis, rather than on quiescent endothelial cells lining normal vessels (Pepper *et al.*, 1993).

Cells express diverse binding sites for plasminogen. Plasminogen binds to endothelial cells *in vitro* with an affinity that would predict receptor occupancy at physiologic plasma concentrations (Miles *et al.*, 1988). Cell-associated plasmin may be relatively protected from inhibition by α_2-plasmin inhibitor (Plow *et al.*, 1986). Lp(a) competes for the binding of

plasminogen to endothelial cells (Miles *et al.*, 1989), which may contribute to the prothrombotic effects of this lipoprotein (Grainger *et al.*, 1995).

Plasminogen Activator Inhibitors (PAIs)

Endothelial cells in culture produce abundant PAI-1 that is associated primarily with its extracellular matrix, resulting in stabilisation of its activity (Levin and Santell, 1987). PAI-1 synthesis is stimulated by several agents, including thrombin, endotoxin, various cytokines, Lp(a) and oxidised LDL, amongst others (Loskutoff *et al.*, 1989).

PAI-2 is found normally in plasma only during pregnancy (Astedt *et al.*, 1986) and is not synthesised by endothelial cells to an appreciable extent. However, multiple passaged endothelial cells express PAI-2 in response to some agonists that may point to a local effect in select settings (Scarpati and Sadler, 1989).

PAI-3 (also known as the protein C inhibitor) has a much lower affinity for t-PA and u-PA than does PAI-1, but is present in plasma at much higher concentrations (Suzuki *et al.*, 1983). Production of PAI-3 by endothelial cells has not been reported, but PAI-3 antigen can bind to heparan sulphate proteoglycan on the lumeral surface of endothelial cells, thereby increasing its activity (Geiger *et al.*, 1991).

Thrombomodulin

Binding of thrombin to thrombomodulin accelerates its capacity to activate a protein known as thrombin-activatable fibrinolysis inhibitor (TAFI) (Bajzar *et al.*, 1996). TAFI is a procarboxypeptidase B-like molecule that, when activated, cleaves basic carboxyterminal residues within fibrin and other proteins. This results in the loss of plasminogen/plasmin and t-PA binding sites on fibrin such that fibrinolysis is retarded (Bajzar *et al.*, 1996). Thus, through the regulated expression of thrombomodulin, endothelial cells can control the rate of intravascular fibrinolysis.

6.1.2 Preeclampsia

Pre-eclampsia is the most common medical disorder of pregnancy, affecting 5% to 13% of all primaras. Although the clinical manifestations of this order are not evident until the third trimester, its pathogenesis may involve a deficiency in placentation (Khong *et al.*, 1987). Alterations in endothelial cells morphology occur within the placenta (Khong *et al.*, 1992) and in glomerular capillaries (glomerular endotheliosis), the latter being characterised by endothelial cell swelling and lipid accumulation (see Ferris (1995) for review). Fibrin deposition in microvasculature is common.

Affected women show increased responsiveness to the pressor effects of angiotensin II (Stamler *et al.*, 1993), increased amounts of thromboxane A_2 relative to PGI_2 in their urine (Fitzgerald *et al.*, 1987), elevated

plasma levels of endothelin (Taylor *et al.*, 1990) and their umbilical vessels demonstrate less PGI_2 synthesis and decreased Nitric Oxide release in response to bradykinin (See Ferris (1995) for review). Additional evidence suggesting endothelial damage is the reported findings of elevated plasma levels of endothelial cell–derived fibronectin (Lockwood and Peters, 1990; Friedman *et al.*, 1995), vWF and PAI-1 in affected women (Friedman *et al.*, 1995).

The pathophysiology of endothelial cell damage remains unclear. Some (Rogers *et al.*, 1988; Tsukimori *et al.*, 1992), but not all groups (Endresen *et al.*, 1995; Zammit *et al.*, 1996), have reported that plasma from affected women is cytotoxic for cultured endothelial cells. Pre-eclamptic sera have also been reported to impair endothelial cell proliferation (Smarason *et al.*, 1996), stimulate fibronectin release (Taylor *et al.*, 1991), increase triglyceride accumulation (Lorentzen *et al.*, 1991) and increase PDGF synthesis (Roberts *et al.*, 1992). A role for abnormalities of lipid peroxidation (Poranen *et al.*, 1996), for immunologic factors and for underlying maternal vascular disease (see Ness and Roberts (1996) for review) have also been advanced.

6.1.3 Decidual Cells of the Placental Bed

The decidua at term is made up of a number of cell types, stromal cells, epithelial cells, endothelial cells, vascular smooth muscle cells and leukocytes. These cell types are known to secrete soluble factors such as

cytokines into the local environment which may control trophoblast invasion during pregnancy. Stromal cells secrete the basement membrane which consists of type IV collagen, laminin, heparan sulphate proteoglycan and glycoproteins (Kisalus, Herr and Little, 1987; Aplin, Charlton and Ayad, 1988).

During pregnancy, extravillous trophoblasts migrate through the decidua and myometrium to the spiral arteries where they are involved in the setting up of an increased blood supply to the placenta. In pre-eclamptic pregnancies inadequate trophoblast invasion occurs in the placental bed resulting in a reduced blood flow to the placenta. As discussed earlier, plasminogen activators and their inhibitors may be involved in this inadequate invasion. A decreased ability of trophoblast cells to generate plasmin due to increased levels of PAI-1 and PAI-2 may result in decreased basement membrane degradation and thus result in impaired invasion into the placental bed. The presence of plasminogen activator inhibitors within the placental bed could yield the same result. Furthermore, the presence of PAI-1 and PAI-2 in the placental bed may affect fibrinolytic activity in the spiral arteries and result in increased fibrin deposition in these vessels.

Sheppard *et al.* (1990) studied the levels of PAI-1 and PAI-2 in the placental bed decidua. They showed that the decidual extracts contained greater amounts of PAI-1 than PAI-2. In both pre-eclampsia and IUGR the levels of both PAI-1 and PAI-2 increase. However, these increases are

not significant. Schatz *et al.* (1995) showed that during decidualisation of stromal cells *in vitro*, t-PA and u-PA antigen and activity levels decrease as PAI-1 antigen levels increase. The results suggest that PAI-1 expression by the decidual cells may play a decisive role in regulating proteolysis in the decidua.

6.1.4 Objectives of the Study

The main aim of this study is to identify changes in the expression of fibrinolytic factors in decidual cells and endothelial cells in normal, pre-eclamptic and IUGR pregnancies. The depth of trophoblast invasion and therefore pregnancy outcome may depend on the levels of fibrinolytic secretion by endothelial cells and endothelial cells in the placental bed. This study examined and measured fibrinolytic activity of endothelial cells and decidual cells in normal pregnancy, pre-eclampsia and /or IUGR. As discussed earlier, pre-eclampsia and IUGR are associated with diminished trophoblast invasion of the placental bed. This may be due to increased secretion of PAIs by the trophoblast. However, the decrease in invasion may be due to increased secretion of PAIs by the endothelial and decidual cells of the placental bed. This study examines the secretion of PAIs by these cells in the different patient groups in order to see if significant changes in fibrinolytic activity occur in high-risk pregnancies.

6.2 Results

6.2.1: Fibrinolytic Activity of Cultured Placental Endothelial Cells

t-PA Antigen: (Table1) No significant increases in t-PA antigen levels were found for cultured endothelial cells amongst the four patient groups. However, in PET and PET/IUGR t-PA levels do rise almost to significance.

u-PA Antigen: (Table 1) Levels of u-PA antigen were not significantly different between the patient groups studied.

PAI-1 Antigen: (Table 1) Antigen levels increased significantly for PET, PET/IUGR and IUGR when compared to normal pregnancy ($p < 0.01$).

PAI-2 Antigen: (Table 1) no significant changes were seen between the patient groups.

6.2.2: Fibrinolytic Activity of Cultured Placental Bed Endothelial Cells

t-PA Antigen: (Table2) No significant increases in t-PA antigen levels were found for cultured endothelial cells amongst the four patient groups.

u-PA Antigen: (Table 2) Levels of u-PA antigen were not significantly different between the patient groups studied.

PAI-1 Antigen: (Table 2) Antigen levels increased significantly for PET, PET/IUGR and IUGR when compared to normal pregnancy ($p < 0.01$).

PAI-2 Antigen: (Table 2) no significant changes were seen between the patient groups.

6.2.3: Fibrinolytic Activity of Cultured Decidual Cells

t-PA Antigen: (Table3) No significant increases in t-PA antigen levels were found for cultured endothelial cells amongst the four patient groups. However, in PET and PET/IUGR and IUGR t-PA levels do rise although not to significance.

u-PA Antigen: (Table 3) Levels of u-PA antigen were not significantly different between the patient groups studied.

PAI-1 Antigen: (Table 3) Antigen levels increased significantly for PET, PET/IUGR and IUGR when compared to normal pregnancy ($p < 0.01$).

PAI-2 Antigen: (Table 3) no significant changes were seen between the patient groups although the levels for PET, PET/IUGR and IUGR are higher than that for normal pregnancy.

Table 6.1 Fibrinolytic Activity of Placental Endothelial Cells

	Normal	PET	PET/IUGR	IUGR
(ng/10^6 cells)				
t-PA	4.3 ± 2.1	7.9 ± 3.5	8.5 ± 3.5	5.2 ± 2.3
u-PA	3.2 ± 1.1	3.4 ± 2.0	4.1± 2.2	3.9 ± 2.7
PAI-1	7.8 ± 3.0	15.0 ± 4.4*	17.8 ± 4.9*	17.6 ± 5.7*
PAI-2	4.1 ± 3.1	4.7 ± 3.4	4.5 ± 2.8	6.3 ± 2.6

Values are mean ± SD for five experiments performed in triplicate.

* $p < 0.01$ (vs. Normal; Student's t-test).

Table 6.2 Fibrinolytic Activity of Placental Bed Endothelial Cells

	Normal	PET	PET/IUGR	IUGR
(ng/10^6 cells)				
t-PA	6.5 ± 3.0	10.6 ± 3.4	9.9 ± 4.0	8.6 ± 2.7
u-PA	3.8 ± 2.9	6.4 ± 2.6	5.1± 2.2	3.7 ± 0.9
PAI-1	10.5 ± 4.7	21.8 ± 3.2*	22.4 ± 5.3*	19.5 ± 4.7*
PAI-2	6.8 ± 2.2	8.0 ± 4.7	7.7 ± 2.9	8.5 ± 3.1

Values are means ± SD for five experiments performed in triplicate.

* $p < 0.01$ (vs. Normal; Student's t-test).

Table 6.3 Fibrinolytic Activity of Placental Bed Decidual Cells

	Normal	PET	PET/IUGR	IUGR
(ng/10^6 cells)				
t-PA	6.9 ± 3.4	11.2 ± 2.4	12.5 ± 4.3	11.7 ± 4.0
u-PA	3.8 ± 2.9	6.4 ± 2.6	5.1± 2.2	3.7 ± 0.9
PAI-1	8.4 ± 2.6	25.3 ± 6.5*	24.7 ± 5.7*	22.2 ± 6.6*
PAI-2	6.4± 3.2	9.7± 5.0	8.1 ± 3.8	10.4± 4.3

Values are mean ± SD for five experiments performed in triplicate.

* $p < 0.01$ (vs. Normal; Student's t-test).

6.3 Discussion

During pregnancy fibrin deposition occurs in the spiral arteries of the placental bed. Sheppard and Bonnar (1978) showed that endothelial cells in the spiral arteries had greater fibrinolytic activity than trophoblast cells. In addition placental endothelial cells may play a role in controlling fibrin deposition within the placenta. In pre-eclampsia (PET) and IUGR, increased fibrin deposition is found in the placenta and placental bed. Furthermore, plasma concentrations of PAI-1 increase while PAI-2 levels decrease. A decrease in overall fibrinolytic activity in the uteroplacental tissue and therefore, increased fibrin deposition may, in part, be due to excess PAI-1 production by endothelial cells. In this study endothelial cells from the placenta and placental bed secrete significantly higher levels of PAI-1 in pregnancies complicated by PET and IUGR. It has been suggested that pre-eclampsia is associated with endothelial dysfunction. This study agrees with other studies in showing that fibrinolytic activity in endothelial cells is altered in pre-eclampsia and PET/IUGR.

In decidual cells PAI-1 levels increased significantly for PET, PET/IUGR and IUGR compared to normal pregnancy. This result suggests that within the placental bed tissue there is decreased fibrinolytic activity. This may facilitate fibrin deposition in the spiral arteries in PET and IUGR.

Decidual cells may also play a part in the regulation of trophoblast invasion by decreasing the fibrinolytic potential in the placental bed. Previous studies have shown that PAI-1 levels increase as the myometrium undergoes decidualisation (Schatz and Lockwood, 1993). Increases in PAI-1 levels in the decidua may affect blastocyst attachment and trophoblast invasion. In addition, high antifibrinolytic potential may be involved in controlling blood loss following the removal of the placenta.

The increase in PAI-1 levels in the placental bed in PET, PET/IUGR and IUGR may explain the reduced trophoblast invasion associated with these pregnancies. Increased PAI-1 levels in the placental bed could affect the plasminogen activator/plasmin system utilised by trophoblast cells to migrate through the placental bed.

CHAPTER 7
GENERAL DISCUSSION AND CONCLUSIONS

7.1 Discussion

In the normal healthy individual the enzymatic systems of fibrinolysis and coagulation are held in a dynamic haemostatic balance. The role of fibrinolysis is to control the deposition of fibrin at the site of a developing thrombus. In addition, ongoing fibrinolytic activity prevents the unnecessary laying down of fibrin and allows the blood to flow freely throughout the vascular system. Defective fibrinolysis, whether in the form of an inadequate response or excessive activity, could predispose the individual to either thromboembolic or haemorrhagic episodes, which may prove fatal.

During pregnancy the haemostatic balance is shifted towards coagulation. It is thought that enhanced coagulation is further offset by a depressed fibrinolytic system.

Normal Pregnancy

In the present study the placenta and placental bed contain equal amounts of t-PA. This suggests that t-PA may play a role in the regulation of fibrin deposition in the uteroplacental tissue. This study shows that endothelial cells and decidual cells both secrete t-PA. u-PA is present in both the placenta and the placental bed. However, the results demonstrate that the placenta is the main source of u-PA in the uteroplacental tissue. The placenta contains significantly greater amounts of PAI-1 and PAI-2 than the placental bed. Comparisons of

fibrinolytic factor concentration show that the placenta contains larger amounts of plasminogen activator inhibitors compared to plasminogen activators. This suggests a decreased fibrinolytic level in the uteroplacental vasculature.

During pregnancy trophoblast cells invade the placental bed and are involved in the restructuring of the spiral arteries. The trophoblasts which line the spiral arteries are associated with fibrin within the vessel wall. A previous study demonstrated that these cytotrophoblasts have a decreased fibrinolytic activity compared to endothelial cells. In this study placental and placental bed trophoblasts were studied. The placental bed trophoblasts secrete larger amounts of PAI-1 and PAI-2 than u-PA. This suggests decreased fibrinolytic activity. In addition, placental trophoblasts secrete large amounts of plasminogen activator inhibitors with the emphasis being on increased PAI-1 production. Many studies have used placental trophoblasts in researching the control of trophoblast invasion. Invading trophoblasts utilise a plasminogen activator/plasmin system to enable them to migrate through the basement membrane. The increase in plasminogen activator inhibitor concentrations suggests that this invasive behaviour is tightly controlled. Furthermore, the placental secretes large amounts of plasminogen activator inhibitor and this may be a mechanism of controlling the depth of invasion by trophoblast cells into the placental bed. In this study the secretion pattern of fibrinolytic factors by the placenta and the placental

bed was studied. PAI-1 and PAI-2 are secreted in much larger quantities than u-PA. This adds further evidence to a decrease in fibrinolysis in the uteroplacental circulation.

Pre-eclampsia

Excessive fibrin deposition within vascular compartments of certain target organs is commonly associated with pre-eclampsia. An ineffective fibrinolytic response in the presence of increased coagulation has been proposed as the mechanism by which this occurs. In this study t-PA and u-PA concentrations were not significantly changed in PET and PET/IUGR pregnancies. However, PAI-1 levels increased while PAI-2 levels decreased. The PAI-1 increase may be due to increased PAI-1 secretion by endothelial cells in the placenta. The decrease in PAI-2 concentrations may, in part, be accounted for by increases in the number of infarct areas in the placenta and a decrease in the placental weight especially in PET/IUGR. No changes were identified in the placental bed. Placental and placental bed trophoblasts showed significant increases in PAI-1 concentrations in PET and PET/IUGR compared to normal pregnancy. These increases may explain the increase in fibrin deposition seen in the placenta and spiral arteries seen in PET and IUGR. Furthermore, the increase in PAI-1 may account for the decrease in trophoblast invasion which is associated with these pregnancies.

Intrauterine Growth Retardation

Like pre-eclampsia, a pathological amount of fibrin is a common finding in the spiral arteries of pregnancies complicated by normotensive intrauterine growth retardation (IUGR). In this study significantly higher levels of PAI-1 is coupled with a significant decrease in PAI-2 antigen levels. This may reflect placental damage with decreased PAI-2 production by trophoblasts in areas of placental villi damaged coupled with normal PAI-1 release by the endothelium of the villous blood vessels.

In common with pre-eclampsia, reduced trophoblast invasion is associated with IUGR. This study shows that trophoblast cells from the placenta and the placental bed of IUGR pregnancies have a reduced ability to lyse fibrin and invade matrigel, an artificial basement membrane protein. In addition, their cell associated plasminogen activator activity decreases. These results suggest that the trophoblast, through decreased cell surface plasminogen activator activity and increased PAI-1 production, may not be able to utilise the plasminogen activator/plasmin system and hence invasion of the placental bed is compromised.

Endothelial and Decidual Cells

The contribution of endothelial cells and decidual cells to the overall levels of plasminogen activators and their inhibitors in pregnancy.

Increases in PAI-1 were found in all cell types examined. The increase production of PAI-1 by endothelial cells observed in PET, PET/IUGR and IUGR pregnancies may be due to increased endothelial cell activation which is commonly associated with pre-eclampsia. The increase in PAI-1 observed by endothelial cells may be in response to increased fibrin deposition in the placenta. The increase in PAI-1 production by decidual may be of more importance to the regulation of trophoblast invasion. Increased PAI-1 in the placental bed would lead to a possible reduction in trophoblast invasion which is common with PET and IUGR.

7.2 Conclusions

The placenta is a rich source of plasminogen activators and their inhibitors during pregnancy. Increased production of PAI-1 and PAI-2 results in a tight regulation of fibrin deposition within the uteroplacental circulation. Placental trophoblast cells are known to be a source of PAI-1 and PAI-2. In addition, this study observed PAI-1 secretion by endothelial cells isolated from the placenta and the placental bed. In addition, decidual cells isolated from the placental bed secreted PAI-1. This suggests that the placenta is not the only source of PAI-1 during pregnancy.

During normal pregnancy cytotrophoblast cells invade the spiral arteries of the placental bed and remodel these vessels resulting in increased blood flow to the placenta. The trophoblasts in these spiral arteries are associated with fibrin which suggests reduced fibrinolytic activity. In this study trophoblast cells are shown to secrete vast amounts of PAI-1 and PAI-2 which could account for the reduced fibrinolytic activity of these cells.

Pre-eclampsia (PET) and IUGR are associated with decreased trophoblast invasion and increased fibrin deposits in the uteroplacental vasculature. The increase in production of fibrinolytic inhibitors by trophoblasts may explain how the process of invasion is controlled. In addition to the trophoblast, decidual cells secrete higher levels of PAI-1

in the placental bed in PET and IUGR and this may be a mechanism of controlling the invasive abilities of trophoblast cells.

A study of plasminogen activators and their inhibitors in placental and placental extracts shows that in PET and IUGR, increased PAI-1 production is coupled with a decrease in PAI-2 levels. Trophoblast cells are shown to secrete larger amounts of PAI-1 in these pregnancies which suggests an important role for the placenta in controlling PAI-1 levels and hence fibrin deposition in the placental bed. The decrease in PAI-2 levels in the placenta suggests placental dysfunction. Organ culture experiments show that PAI-2 is secreted normally by the placenta in PET and IUGR. The natural conclusion is that the decrease in PAI-2 levels is due to infarcts within the placenta itself.

In summary, uteroplacental fibrinolytic activity is reduced in the uteroplacental circulation. It is due to an increase in PAI-1 and PAI-2 production and secretion by the placenta and the placental bed. Endothelial cells and decidua cells secrete PAI-1 and PAI-2 in the placental bed. Trophoblast cells are a major source of these inhibitors. PAI-1 and PAI-2 may not only restrict the fibrinolytic activity of these cells in the placental bed but may affect their ability to invade the decidua successfully.

Future Work

Further work will need to be done to improve the isolation techniques used to isolate placental and placental bed trophoblasts. These cells are notoriously difficult to isolate and culture. The cells differentiate within a few days of isolation and hence their characteristics change. The yields from the isolation techniques differ from patient to patient so making it difficult to carry out a comprehensive study of the cells. In addition, the cells need to be characterised in more detail in order to determine if the cells are 'true' trophoblasts or a subset of the family.

Future experiments need to be performed to correlate the changes seen in the levels of plasminogen activators and inhibitors secreted by trophoblasts with levels of metalloproteinases secreted by the cells. This would give us a better understanding of the plasmin/plasminogen system in these cells.

Invasion assays using antibodies and reagents directed at plasminogen activators and inhibitors would give us a better insight into the role of each factor in invasion. Initial results (not published due to small patient numbers) indicate that trophoblast invasion is diminished in the presence of an inhibitor of u-PA.

The organ culture system used in this study is of extreme importance. The system is able to maintain the viability of placental and placental bed tissue for many days. It may be able to adapt the procedure in order to isolate trophoblast cells from the tissue after a number of days in

culture. They could then be used to correlate trophoblast secretion of fibrinolytic factors with the overall secretion by the tissue in question. Co-culturing of trophoblasts with endothelial cells or decidual cells followed by immunohistochemical analysis may give us a better understanding of the effect of these cells on trophoblast expression of fibrinolytic factors. These results could be correlated to the effects seen on trophoblasts in the placental bed *in vivo*.

CHAPTER 8
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