

STUDY OF PLASMINOGEN ACTIVATION
BY HUMAN TROPHOBLASTS

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ISTVAN JOJART



STUDY OF PLASMINOGEN ACTIVATION BY HUMAN TROPHOBLASTS

by

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in partial fulfilment of the requirements for
the degree of Doctor of Philosophy**

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ABSTRACT

Successful implantation and placentation during pregnancy depend on the regulated expression of trophoblast-associated protease activity. One of the proteolytic mechanisms utilized by trophoblasts is plasmin generation which is thought to be involved in trophoblast invasion and control of placental fibrin deposition. In this study, the production and regulation of plasminogen activator (PA) activity by human villous trophoblasts were investigated.

Primary trophoblast cultures were established from normal human term placentas. Highly purified villous cytotrophoblasts were isolated through sequential trypsin-DNase digestions of the selected villous tissue, followed by Percoll density gradient centrifugation and subsequent removal of contaminating cells using monoclonal antibodies to monomorphic determinants of HLA class I and class II antigens. When placed in culture, the isolated cells were characterized as being cytokeratin positive, vimentin, HLA-ABC, HLA-DR and CD68 negative. In about 48 hours the majority of the cytotrophoblasts transformed into syncytiotrophoblasts. The cultures were functionally viable as assessed by their capacity for hCG secretion and reductive cleavage of MTT. No significant cytotrophoblast cell proliferation could be demonstrated in ³H-thymidine uptake experiments, and trophoblasts at their syncytiotrophoblastic stage did not proliferate at all.

When the expression of plasminogen activators and their specific inhibitors in vitro was compared with in vivo findings, distinct differences were found. Immunocytochemical studies in term placental tissue showed strong syncytial PAI-2

immunoreactivity and faint to moderate PAI-1 staining. No staining could be demonstrated for either u-PA or t-PA. In contrast, the majority of cultured trophoblasts expressed u-PA during the first 24 hours of culture. All forms of trophoblast (mononuclear trophoblasts, multinuclear aggregates and syncytiotrophoblasts) expressed PAI-1, but only occasional syncytiotrophoblasts stained positively for PAI-2 and no t-PA immunoreactive trophoblasts were observed. Trophoblast-secreted PAI-1 could also be detected in conditioned media by ELISA.

Activation of plasminogen was measured by monitoring the conversion of exogenously added Glu-plasminogen to plasmin in serum-free trophoblast cultures, using a chromogenic plasmin substrate H-D-Val-Leu-Lys-pNA (S-2251). Plasminogen activation by trophoblasts followed Michaelis-Menten type kinetics and produced parabolic progress curves of pNA accumulation that could be transformed to linear progress curves of plasmin generation, the slopes of which yielded constant rates of plasmin generation (i.e. PA activity). Trophoblast-associated plasminogen activation was found to originate from u-PA but not t-PA, because plasmin generation was markedly reduced in the presence of a neutralizing antibody to u-PA but not appreciably in the presence of a neutralizing antibody to t-PA. In addition, trophoblasts enhanced plasminogen activation induced by exogenous pro-u-PA, suggesting that cell surface binding of pro-u-PA potentiates the generation of plasmin. The basal level of endogenous trophoblast PA activity was specifically augmented by a neutralizing antibody to PAI-1, indicating that trophoblast-secreted PAI-1 regulates PA activity in an autocrine manner. Consistent with this finding,

physiological concentrations of exogenous PAI-1 and PAI-2 resulted in a dose-dependent inhibition of PA activity.

In order to define modulators of trophoblast-associated PA activity, several types of molecules were tested. Activated protein C (APC) increased, whereas thrombin decreased PA activity, demonstrating that the coagulation system has the potential to modulate trophoblast plasminogen activation. Among growth factors/cytokines, EGF and its structural analog TGF- α proved to be potent stimulators. A neutralizing antibody to EGF did not have an effect, suggesting that term trophoblast cultures do not produce a sufficient quantity of EGF to modulate PA activity. TGF- β , IGF-II and IL-1 β did not change PA activity appreciably, nor did LPS, a known stimulator of IL-1 secretion. With respect to hormones, dexamethasone and hCG had an inhibitory effect. Agents that elevate intracellular cAMP levels (8-bromo-cAMP and forskolin) inhibited plasminogen activation, whereas PMA had no appreciable effect.

Taken together, this study demonstrates that the human term villous trophoblast possesses a highly regulated plasminogen activation system, and various types of physiologically important molecules including enzymes, growth factors, steroid and peptide hormones modulate the generation of trophoblast-associated plasmin.

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TABLE OF CONTENTS

ABSTRACT	ii
ACKNOWLEDGEMENTS	v
LIST OF TABLES AND FIGURES	xi
LIST OF ABBREVIATIONS	xv
CHAPTER 1 INTRODUCTION: PLASMINOGEN ACTIVATION AND THE PLACENTAL TROPHOBLAST	
1.1 The plasminogen activation system	1
1.1.1 Plasminogen and plasmin	1
1.1.2 Plasmin inhibitors	3
1.1.3 Plasminogen activators	5
1.1.4 Inhibitors of plasminogen activators	9
1.1.5 Receptors	11
1.1.5.1 Plasminogen receptors	12
1.1.5.2 The urokinase receptor	13
1.1.5.3 t-PA receptors	15
1.2 Major mechanisms in plasminogen activation	16
1.2.1 Fibrinolysis	16
1.2.2 Plasminogen activation at the cell surface	20
1.2.2.1 Pericellular distribution of the plasminogen activation system	21
1.2.2.2 u-PA dependent cell surface plasminogen activation	22
1.2.2.3 t-PA dependent cell surface plasminogen activation	24
1.2.2.4 Inhibition of cell surface plasminogen activation	26
1.3 Regulation of plasminogen activation	28
1.4 The placental trophoblast: invasiveness and proteolytic activity	29
1.4.1 Development and structural organization of the human placenta	29
1.4.2 Pathways of trophoblast differentiation: heterogeneity of the trophoblast	36

1.4.3	Trophoblast invasion during implantation and placental development	39
1.4.4	Trophoblast invasiveness: involvement of proteases and protease inhibitors	42
1.4.4.1	Matrix degradation by the periimplantation blastocyst	46
1.4.4.2	Expression of matrix-degrading proteinases and their inhibitors by human trophoblasts	47
1.4.4.3	Degradation and invasion of extracellular matrices by human cytotrophoblasts	50
1.4.4.4	Control of trophoblast invasiveness: role of factors modulating trophoblast proteinase activity	53
1.4.5	Plasminogen activation in normal and preeclamptic pregnancy: role of the trophoblast	57
1.5	Rationale and objectives	60
CHAPTER 2 MATERIALS AND METHODS		
2.1	Materials	61
2.1.1	Materials for cell culture	61
2.1.2	Antibodies	61
2.1.3	Fibrinolysis and coagulation products	62
2.1.4	Miscellaneous chemicals	62
2.2	Methods	63
2.2.1	Isolation and culture of cytotrophoblasts	63
2.2.2.1	Mechanical processing of the placental tissue	63
2.2.1.2	Enzymatic disaggregation	63
2.2.1.3	Percoll density gradient centrifugation	64
2.2.1.4	Immunomagnetic separation of villous cytotrophoblasts	65
2.2.1.5	Primary trophoblast culture	66
2.2.2	Immunocytochemical procedures	67
2.2.2.1	Immunocytochemical staining of placental tissue sections and trophoblast cell pellets	67
2.2.2.2	Immunocytochemical staining of trophoblast cultures	70
2.2.2.3	Ultrastructural immunocytochemistry	71

2.2.3	[³ H]thymidine incorporation	71
2.2.4	MTT assay	72
2.2.5	Quantitation of hCG	72
2.2.6	Measurement of PAI-1 antigen	73
2.2.7	Measurement of trophoblast-associated plasminogen activation	73
2.2.7.1	Plasminogen activation assay	73
2.2.7.2	Analysis and kinetic treatment of data	75
 CHAPTER 3 ISOLATION, CHARACTERIZATION AND CULTURE OF VILLOUS CYTOTROPHOBLASTS FROM HUMAN TERM PLACENTAS		
3.1	Introduction	78
3.2	Results	79
3.2.1	Immunocytochemical identification of various cell types in the placenta	79
3.2.2	Characterization of Percoll purified cells	82
3.2.3	Characteristics and culture of purified villous cytotrophoblasts	103
3.3	Discussion	107
 CHAPTER 4 ACTIVATION OF PLASMINOGEN BY HUMAN TERM TROPHOBLASTS		
4.1	Introduction	129
4.2	Results	129
4.2.1	Expression of plasminogen activators and their inhibitors by trophoblasts in vivo and in vitro	129
4.2.2	Kinetics of plasminogen activation by cultured trophoblasts	130
4.2.3	Characterization of trophoblast PA activity	142
4.2.4	Trophoblast enhance plasminogen activation initiated by single-chain u-PA (pro-u-PA)	153
4.2.5	Inhibition of trophoblast PA activity by PAIs	156

4.2.6	Regulation of trophoblast PA activity by endogenous PAIs	156
4.2.7	Modulation of trophoblast PA activity by various compounds	165
4.2.7.1	Effects of components of the blood coagulation system	165
4.2.7.2	Effects of growth factors/cytokines	165
4.2.7.3	Effects of hormones	185
4.2.7.4	Effect of 8-bromo-cAMP, forskolin and PMA	185
4.3	Discussion	185
CHAPTER 5 CONCLUSION AND PERSPECTIVES		218
REFERENCES		222

LIST OF TABLES AND FIGURES

Table 3.1	Immunocytochemical characterization of various cell types in placental villi	80
Fig. 3.1	Immunostaining for cytokeratin in placenta and Percoll purified cells	84
Fig. 3.2	Immunostaining for vimentin in placenta and Percoll purified cells	86
Fig. 3.3	Localization of HLA antigens in the placenta	88
Fig. 3.4	Immunostaining for hCG in placenta and Percoll purified cells	90
Fig. 3.5	Immunostaining for hPL in placenta and Percoll purified cells	92
Fig. 3.6	Immunostaining for ACT in placenta and Percoll purified cells	94
Fig. 3.7	Immunostaining for CD68 in placenta and Percoll purified cells	96
Fig. 3.8	Ultrastructural localization of CD68 immunoreactivity in Hofbauer cells	98
Fig. 3.9	Double immunocytochemical staining of placental sections for CD68 and ACT	100
Fig. 3.10	Separation of cytotrophoblasts by density gradient centrifugation	102
Fig. 3.11	Time course of ³ H-methyl-thymidine uptake by trophoblast cultures	105
Fig. 3.12	Micrographs of trophoblast cultures immunostained for cytokeratin	109

Fig. 3.13	Micrographs of trophoblast cultures immunostained for vimentin	111
Fig. 3.14	Immunostaining of trophoblast cultures for HLA-DR and CD68	113
Fig. 3.15	Immunocytochemical staining of trophoblast cultures for class I HLA antigens	115
Fig. 3.16	Stimulation of hCG secretion by 8-bromo-cAMP in trophoblast culture	117
Fig. 3.17	Dose-dependent stimulation of hCG secretion by 8-bromo-cAMP in trophoblast culture	119
Fig. 3.18	MTT cleavage by cultured trophoblasts	121
Fig. 4.1	Immunostaining for PAIs and u-PA in the placenta	132
Fig. 4.2	Immunocytochemical staining of trophoblast cultures for u-PA and PAIs	134
Fig. 4.3	Secretion of PAI-1 by cultured trophoblasts	136
Fig. 4.4	Effect of seed cell density on plasminogen activation by trophoblasts	139
Fig. 4.5	Plasminogen-independent hydrolysis of S-2251 by trophoblasts	141
Fig. 4.6	Kinetics of Glu-plasminogen activation by trophoblasts	144
Fig. 4.7	Inhibition of trophoblast-associated plasminogen activation by aprotinin	146
Fig. 4.8	Inhibition of trophoblast plasminogen activation by a neutralizing antibody to u-PA	148
Fig. 4.9	Effect of a neutralizing antibody to t-PA on plasminogen activation by trophoblasts	150

Fig. 4.10	Effect of normal goat IgG on plasminogen activation by trophoblasts	152
Fig. 4.11	Effect of trophoblasts on exogenous pro-u-PA induced plasminogen activation	155
Fig. 4.12	Inhibition of trophoblast plasminogen activation by PAI-1	158
Fig. 4.13	Inhibition of trophoblast plasminogen activation by PAI-2	160
Fig. 4.14	Enhancement of trophoblast plasminogen activation by anti-PAI-1	162
Fig. 4.15	Effect of anti-PAI-2 on plasminogen activation by trophoblasts	164
Fig. 4.16	Inhibition of trophoblast plasminogen activation by thrombin	167
Fig. 4.17	Effect of APC on plasminogen activation by trophoblasts	169
Fig. 4.18	Stimulation of trophoblast plasminogen activation by EGF	172
Fig. 4.19	Stimulation of trophoblast plasminogen activation by TGF- α	174
Fig. 4.20	MTT reductive cleavage by cultured trophoblasts following EGF stimulation of plasminogen activation	176
Fig. 4.21	Effect of anti-EGF on plasminogen activation by trophoblasts	178
Fig. 4.22	Effect of TGF- β_1 on plasminogen activation by trophoblasts	180
Fig. 4.23	Effect of IGF-II on plasminogen activation by trophoblasts	182
Fig. 4.24	Effect of IL-1 β on plasminogen activation by trophoblasts	184
Fig. 4.25	Effect of LPS on plasminogen activation by trophoblasts	187

Fig. 4.26	Inhibition of trophoblast plasminogen activation by hCG	189
Fig. 4.27	Inhibition of trophoblast plasminogen activation by dexamethasone	191
Fig. 4.28	Inhibition of trophoblast plasminogen activation by 8-bromo-cAMP	193
Fig. 4.29	Inhibition of trophoblast plasminogen activation by forskolin	195
Fig. 4.30	Effect of PMA on plasminogen activation by trophoblasts	197

LIST OF ABBREVIATIONS

8-bromo-cAMP	8-bromoadenosine 3':5'-cyclic monophosphate
ABC	Avidin-biotin complex
ACT	Alpha-1-antichymotrypsin
APC	Activated protein C
ATF	Amino terminal fragment of urokinase
BSA	Bovine serum albumin
CD68	Cluster of differentiation 68
D-PBS	Dulbecco's Phosphate-buffered saline
DAB	3,3'-diaminobenzidine tetrahydrochloride
DFP	Diisopropyl fluorophosphate
DMEM	Dulbecco's modified Eagle medium
DMF	N, N-dimethyl formamide
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
FPA	Fibrinopeptide A
FPB	Fibrinopeptide B
FSH	Follicle-stimulating hormone
GPI	Glycosyl-phosphatidylinositol
HB-EGF	Heparin-binding EGF-like growth factor
HBSS	Hanks' balanced salt solution
hCG	Human chorionic gonadotropin
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
HLA	Human leukocyte antigen
HMW u-PA	High molecular weight u-PA
hPL	Human placental lactogen
HRP	Horseradish peroxidase
HUVEC	Human umbilical vein endothelial cell
IGF-I	Insulin-like growth factor-I
IGF-II	Insulin-like growth factor-II
IGFBP	IGF-binding protein
IL-1 β	Interleukin-1 β
KSCN	Potassium thiocyanate
LBS	Lysine binding site
LIF	Leukaemia inhibitory factor

LMW u-PA	Low molecular weight u-PA
Lp(a)	Lipoprotein (a)
LPS	Lipopolysaccharide
MMP	Matrix metalloproteinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PA	Plasminogen activator
PAI-1	Plasminogen activator inhibitor type 1
PAI-2	Plasminogen activator inhibitor type 2
PAI-3	Plasminogen activator inhibitor type 3
PAP	Peroxidase-antiperoxidase
PBS	Phosphate-buffered saline
PI-PLC	Phosphatidylinositol-specific phospholipase C
PMA	Phorbol 12-myristate 13-acetate
pNA	P-nitroaniline
RT	Room temperature
S-2251	H-D-Val-Leu-Lys-pNA
sct-PA	Single-chain tissue-type plasminogen activator
scu-PA	Single chain urokinase-type plasminogen activator
SDS	Sodium dodecyl sulfate
t-PA	Tissue-type plasminogen activator
t-PAR	Receptor for tissue-type plasminogen activator
tct-PA	Two-chain tissue-type plasminogen activator
tcu-PA	Two-chain urokinase-type plasminogen activator
TGF- α	Transforming growth factor- α
TGF- β	Transforming growth factor- β
TIMP	Tissue inhibitor of metalloproteinases
u-PA	Urokinase-type plasminogen activator
u-PAR	Receptor for urokinase-type plasminogen activator

CHAPTER 1

INTRODUCTION: PLASMINOGEN ACTIVATION AND THE PLACENTAL TROPHOBLAST

1.1 THE PLASMINOGEN ACTIVATION SYSTEM

Plasminogen activators (PAs) are serine proteases that convert the inactive plasminogen to plasmin. Two types of physiological PAs are known: tissue-type (t-PA) and urokinase-type (u-PA). The PAs are involved in a variety of biological processes associated with fibrinolysis and/or degradation of the extracellular matrix, such as thrombolysis, postlactation involution, ovulation, trophoblast invasion, cell migration, inflammation, angiogenesis, tumor invasion and metastasis etc. (Dano et al., 1985; Mayer, 1990). Plasminogen activation occurs as a result of concerted interactions between the components of the plasminogen activation system. The system includes: (1) plasminogen and plasmin, (2) PAs, (3) inhibitors of plasmin and PAs, and (4) receptors for plasmin(ogen) and PAs (Mayer, 1990).

1.1.1 Plasminogen and plasmin

Plasminogen is an inactive proenzyme (zymogen) of the fibrinolytic enzyme plasmin. It is widely distributed in various extracellular fluids and in plasma where its concentration has been found to be about 1.5 to 2 μM (Lijnen and Collen, 1982; Dano et al., 1985). Plasminogen is primarily produced in the liver (Saito et al., 1980; Raum et al., 1980), albeit other sites for its synthesis have also been reported (Valinsky and Reich, 1981; Saksela and Vihko, 1986).

Native human plasminogen (also called Glu-plasminogen after its N-terminal

glutamic acid) is a single chain glycoprotein with a molecular mass of 92 kDa that consists of 791 amino acids (Halkier, 1991). It exists in two differently glycosylated forms denoted Glu-plasminogen I and Glu-plasminogen II (Castellino and Powell, 1981). From the N-terminus, Glu-plasminogen is built up of a preactivation peptide, five sequential kringle domains, a connecting strand followed by the serine proteinase domain. The connecting strand contains the peptide bond Arg561-Val562 cleaved by plasminogen activators (t-PA and u-PA) when plasminogen is activated to plasmin (Halkier, 1991; Lijnen et al., 1994). The kringle domains possess lysine binding sites (LBS) that mediate the binding of plasminogen to lysine residues of fibrin or cell surfaces (Anglés-Cano, 1994). During plasminogen activation, Glu-plasminogen may be converted to Lys-plasminogen by plasmin-catalyzed release of the preactivation peptide (Lijnen et al., 1994). This conversion alters the conformation (Violand et al., 1978) and binding characteristics (Lucas et al., 1983) of the native molecule and thus, activation of Lys-plasminogen to Lys plasmin progresses at a much higher rate than that of Glu-plasminogen (Hoylaerts et al., 1982). Glu-plasmin may also be converted to Lys-plasmin by autocatalysis (Dano et al., 1985).

Plasmin is a broad-spectrum trypsin-like serine protease capable of degrading fibrin as well as several extracellular matrix and basement membrane proteins such as fibronectin, laminin and thrombospondin. Part of its proteolytic effect is exerted through activation of other enzymes such as latent metalloproteinases and prourokinase (Pöllänen et al., 1991).

Plasmin consists of two polypeptide chains held together by two disulfide bridges. The heavy chain or A-chain originating from the N-terminal part of the plasminogen molecule contains the kringles with the LBS and therefore is responsible for binding to fibrin or cells. The light chain or B-chain corresponding to the C-terminal part of plasminogen contains the active site that is composed of His602, Asp645 and Ser740 (Pöllänen et al., 1991; Collen, 1980).

1.1.2 Plasmin inhibitors

Plasmin is inhibited by several protease inhibitors occurring in plasma. Among them the most important is α_2 -antiplasmin (α_2 -plasmin inhibitor), a 67 kDa single chain glycoprotein consisting of 452 amino acids (Halkier, 1991), which migrates as an α_2 -globulin on electrophoresis (Wiman, 1981). It is synthesized in the liver and has a plasma concentration of approximately 1 μ M (Aoki and Harpel, 1984). Alpha₂-antiplasmin belongs to the serine proteinase inhibitor superfamily (serpins). The reactive site of the inhibitor is at Arg364-Met365 (Halkier, 1991). Alpha₂-antiplasmin has two forms in normal human plasma (Clemmensen et al., 1981): the native form binds plasminogen, whereas another modified form does not, because it lacks a C-terminal peptide (Sasaki et al., 1986) that contains a second site for interaction with plasmin(ogen) in addition to the reactive center. Alpha₂-antiplasmin establishes stable 1:1 stoichiometric complexes with plasmin leading to extremely efficient inhibition. The high inhibition rate is the result of interactions between (1) the reactive site of the inhibitor and the active site of plasmin, and (2) the carboxy terminal end of α_2 -antiplasmin and the LBS of kringle 1 of plasmin

(Anglés-Cano, 1994). Due to the latter interaction, α_2 -antiplasmin is much less effective towards the enzyme in the presence of lysine analogues (Wiman and Collen, 1978; Christensen and Clemmensen 1978). Although α_2 -antiplasmin rapidly inhibits plasmin in solution, plasmin bound to fibrin or cell surfaces appears to be resistant to inhibition. In this situation, the LBS of kringles in plasmin are occupied by lysine residues of fibrin or cell membranes preventing α_2 -antiplasmin from binding to and inhibiting plasmin (Anglés-Cano, 1994). Fibrinogen possesses a crosslinking site for α_2 -antiplasmin. Crosslinking occurs between Gln-2 of α_2 -antiplasmin and a lysine residue of the α -chain of fibrin(ogen) and is catalyzed by factor XIIIa (Kimura and Aoki, 1986).

Another inhibitor of plasmin is α_2 -macroglobulin. Human α_2 -macroglobulin is a glycoprotein of Mr 725,000. The purified protein consists of a tetramer of identical subunits of Mr ~185,000, each containing approximately 1450 amino acids linked in pairs by disulfide bonds. Two pairs associate by noncovalent binding to form the native, tetrameric molecule (Travis and Salvesen, 1983). Alpha₂-macroglobulin is synthesized by liver cells, mononuclear phagocytes and fibroblasts (Saksela, 1985). Its plasma concentration is 2.5 g/L (3 μ M). It has a very broad specificity. With respect to the fibrinolytic system, it inactivates tcn-PA, t-PA, u-PA and plasmin at relatively slow rates (Bachmann, 1994a). Alpha₂-macroglobulin is the slower-reacting plasmin inhibitor in plasma and is thought to have a significant role in the inactivation of plasmin under conditions when α_2 -antiplasmin is depleted (Collen, 1980).

1.1.3 Plasminogen activators

Plasminogen activators are serine proteases that convert the zymogen plasminogen to the active protease plasmin. Two types of specific plasminogen activators have been defined: urokinase-type (u-PA) and tissue-type (t-PA) plasminogen activators (Danø et al., 1985).

t-PA has been named tissue plasminogen activator because it was found in tissue sections and extracts of various tissues. It has also been identified in blood plasma and body fluids (Danø et al., 1985). t-PA is synthesized as a 72 kDa single-chain glycoprotein. From the N-terminus, the t-PA molecule is composed of a finger domain, a growth factor domain, two kringle domains, a connecting strand, and a serine protease domain with the catalytic site comprised of His47, Asp96 and Ser203. Conversion of single-chain t-PA (sct-PA) to two-chain t-PA (tct-PA) occurs upon plasmin catalyzed cleavage at Arg278-Ile279 and at Arg3-Ser4. This cleavage results in a 36 kDa heavy chain derived from the N-terminus, and a light chain with a molecular mass of 32 kDa derived from the carboxy terminal part (Halkier, 1991). Current evidence indicates that unlike most single chain forms of serine proteases, single chain t-PA possesses significant catalytic activity (Andreasen et al., 1991).

Both forms of t-PA bind to fibrin. This binding is mediated by the second kringle and finger domains of t-PA. There is, however, disagreement on whether kringle 1 is also implicated in fibrin binding, and some authors claim that kringle 1 has a fibrin binding capability comparable to that of kringle 2 (Gaffney and Longstaff, 1994). The affinity of

t-PA for fibrin increases significantly in the presence of plasminogen. In this situation, formation of a ternary complex comprising t-PA, plasminogen and fibrin is likely to occur (Hoylaerts et al., 1982; Collen and Lijnen, 1991).

Fibrin strikingly enhances the activation rate of plasminogen by both single-chain and two-chain t-PA (Hoylaerts et al., 1982; Andreasen et al., 1991; Collen and Lijnen, 1991). Thus, a fast activation can take place on the fibrin clot, while no efficient activation occurs in plasma. Enhancement of t-PA catalytic activity is not exclusive to fibrin, because other compounds such as fibrin(ogen) fragments, polylysine, heparin and some denatured proteins are also able to bind and stimulate t-PA (Mayer, 1990).

u-PA was discovered first in human urine, but was soon found in other body fluids and tissues (Danø et al., 1985). It is synthesized and secreted as a 50-54 kDa single-chain glycoprotein (scu-PA) containing 411 amino acid residues (Halkier, 1991). Scu-PA (also called pro-u-PA or pro-urokinase), unlike t-PA, is considered to be a proenzyme. It appears to be the predominant extracellular form of u-PA (Skriver et al., 1984; Kielberg et al., 1985), has little or no measurable activity (Gaffney and Longstaff, 1994), and does not react with serine protease inhibitors (Andreasen et al., 1986) or the active site titrant diisopropyl fluorophosphate (DFP) (Gurewich et al., 1984; Pannell and Gurewich, 1986). The low but significant intrinsic plasminogen activator activity (0.4% to 6% of the activity of two-chain u-PA) and amidolytic activity (ranging 0.1% to 0.4% of that of two-chain u-PA) reported by some authors (Gurewich et al., 1984; Pannell and Gurewich, 1986, 1987; Ellis et al., 1987; Lijnen et al., 1989, 1990) may arise from small amounts of two-

chain u-PA generated from scu-PA by traces of contaminating proteases (Husain, 1991).

The human u-PA gene is 6.4 kb long and located on chromosome 10. Starting from the N-terminus, scu-PA is composed of an epidermal growth factor domain, a single kringle domain, a connecting peptide, and a C-terminal serine proteinase domain (Bachmann, 1994a). A low molecular weight variant of scu-PA (32 kDa) is produced by a metalloproteinase that cleaves the peptide bond between Glu143-Leu144 while the Lys158-Ile159 bond remains intact (Stump et al., 1986; Marcotte et al., 1992). Conversion of scu-PA to the active two-chain u-PA (synonyms: tcu-PA or high molecular weight (HMW) urokinase) occurs upon cleavage of the peptide bond Lys158-Ile159 by any of several proteases such as plasmin, trypsin, plasma kallikrein, cathepsin B and L, and factor XIIa (Bachmann, 1994a, 1994b). HMW urokinase which has a molecular weight of about 50-54 kDa (the same as that of scu-PA) possesses a 20 kDa light or A-chain (residues 1-157) and a 30 kDa heavy or B-chain (residues 159-411) held together by a single disulfide bond. The A-chain holds the growth factor and kringle domains, whereas the B-chain contains the serine protease domain with the catalytic triad of the active center (His46, Asp97 and Ser198 corresponding to His204, Asp255 and Ser356 in scu-PA) (Günzler et al., 1982; Bachmann, 1984a; Halkier, 1991; Gaffney and Longstaff, 1994). Further processing of HMW u-PA leads to A-chain terminal heterogeneity: fully active HMW u-PA in urine and tissue culture contains a C-terminal Phe157 (Günzler et al., 1982a; Lenich et al 1991). In addition, plasmin cleavage of HMW urokinase at Lys135-Lys136 results in a low molecular weight form (33 kDa) of two-chain urokinase

(LMW urokinase) whose B-chain is identical with that of HMW urokinase (residues 159-411), but the 'A-chain no longer contains the amino terminal fragment (ATF; residues 1-135) (Günzler et al., 1982b; Gaffney and Longstaff, 1994).

Different molecular forms of u-PA possess distinct differences with respect to their activities and binding properties. In contrast to scu-PA, HMW u-PA and LMW u-PA are highly active enzymes, whereas ATF that lacks the serine proteinase domain is catalytically inactive. Scu-PA, HMW u-PA and LMW u-PA are all used as therapeutical thrombolytics, but only scu-PA can induce clot-specific activation of plasminogen. The active urokinases have no fibrin specificity and activate fibrin-bound and circulating plasminogen relatively indiscriminately (Gurewich, 1989; Gaffney and Longstaff, 1994). The mechanism of clot-specific plasminogen activation by scu-PA is not clear. It is known that neither scu-PA nor tzu-PA have an affinity for fibrin. scu-PA, however, binds with high affinity to plasminogen (Longstaff et al., 1992; Gaffney and Longstaff, 1994) which, in turn, can bind to fibrin. Fibrin enhances scu-PA or tzu-PA induced plasminogen activation, but this stimulation remains far below that observed for t-PA (Takada et al., 1989; Liu and Gurewich, 1991), the primary plasminogen activator for physiological fibrinolysis. All forms of u-PA having the epidermal growth factor domain (scu-PA, HMW u-PA and ATF) bind to the cellular u-PA receptor. LMW u-PA, however, has no receptor binding capacity due to the lack of this domain (Vassalli et al., 1985; Blasi et al., 1986).

1.1.4 Inhibitors of plasminogen activators

One way of regulating plasminogen activator enzyme activity is through plasminogen activator inhibitors (PAIs). The PAIs are members of the serpin family of proteins (Kruithof, 1988). Currently, four distinct PAIs are known. These include (1) plasminogen activator inhibitor type 1 (PAI-1), (2) plasminogen activator inhibitor type 2 (PAI-2), (3) plasminogen activator inhibitor type 3 (PAI-3 or protein C inhibitor) and (4) protease nexin (Loskutoff et al., 1989; Kruithof, 1988; Schleef and Loskutoff, 1988). PAI-1 and PAI-2 are considered to be the most important, highly specific PAIs, whereas PAI-3 and protease nexin have broader specificities.

PAI-1 was first described in endothelial cell culture (Loskutoff and Edgington, 1977; Loskutoff et al., 1983). Since then its occurrence has been reported in numerous cell types (Kruithof, 1988). It is also present in plasma. PAI-1 is a Mr ~ 50 kDa single-chain glycoprotein (Ericson et al., 1984; van Mourik et al., 1984; Andreasen, 1986) encoded by 3.0 and 2.2 kb mRNAs (Ny et al., 1986; Ginsburg et al., 1986). The human PAI-1 gene is 12.2 kb long and located on chromosome 7. The PAI-1 molecule contains 379 amino acid residues and some heterogeneity may exist at the amino terminus. Its reactive center is formed by the Arg346-Met347 bond. The presence of methionine in the reactive center may explain its unusual sensitivity to oxidants such as chloramine T, N-chlorosuccinimide and hydrogen peroxide (Lawrence and Loskutoff, 1986; Loskutoff et al., 1989). Another important feature is that PAI-1 activity can still be detected after SDS-polyacrylamide gel electrophoresis under reducing conditions (van Mourik et al., 1984).

PAI-1 is secreted in an active form. Active PAI-1 is unstable and spontaneously converted into a stable inactive latent state unless its active conformation is stabilized (Loskutoff et al., 1989). Vitronectin is a binding protein for PAI-1 and appears to stabilize PAI-1 activity in both the plasma and extracellular matrix (Declerck et al., 1988; Wiman et al., 1988; Mimuro and Loskutoff, 1989). Latent PAI-1 can be reactivated with denaturants including KSCN, guanidine hydrochloride, SDS and urea (Hekman and Loskutoff, 1985). However, oxidatively inactivated PAI-1 cannot be converted to active PAI-1 by treatment with SDS, suggesting that latency does not result from oxidants (Lawrence and Loskutoff, 1986; Loskutoff et al., 1989). PAI-1 inhibits sct-PA, tct-PA and tcu-PA by forming 1:1 molar complexes that resist dissociation by SDS, but it does not react with scu-PA (Andreasen et al., 1990).

PAI-2 was discovered originally in placenta. It has also been found in cells of the monocyte/macrophage lineage. High levels of PAI-2 have been detected in pregnancy plasma, but it is not present in the circulation under normal conditions. PAI-2 exists in two molecular forms. Intracellular PAI-2 is a 47 kDa non-glycosylated protein, whereas the secreted form is glycosylated and has a molecular mass of about 60 kDa. The PAI-2 gene is located on chromosome 18 and PAI-2 cDNA encodes a protein consisting of 415 amino acids. The intracellular and secreted forms have a single mRNA of 2 kb. The reactive centre of PAI-2 is formed by Arg380-Thr381. In contrast to PAI-1, PAI-2 is a stable inhibitor and has not been shown to occur in a latent inactive form. PAI-2 primarily inhibits u-PA. It forms covalently bonded equimolar complexes with tcu-PA,

but does not react with scu-PA. PAI-2 also inhibits tct-PA. It is a very poor inhibitor of sct-PA (Andreasen et al., 1990; Pöllänen et al., 1991; Booth, 1994).

PAI-3 has been identified in human urine and is also found in plasma. It is a single-chained 50 kDa glycoprotein that inhibits tct-PA and thrombin at comparable rates, but reacts very slowly with tct-PA. PAI-3 has been demonstrated to be immunologically identical with the heparin dependent inhibitor of activated protein C. It is unlikely to be a relevant physiological plasminogen activator inhibitor. However, PAI-3 may have a more significant function during thrombolytic therapy when the plasma concentration of u-PA is high (Kruithof, 1988; Loskutoff et al., 1989; Pöllänen et al., 1991).

Protease nexin I is a 45 kDa glycoprotein containing 392 amino acid residues. It is produced by many cell types but is not found in plasma. In fact, it is a broad specificity inhibitor of trypsin-like serine proteases rather than a true plasminogen activator inhibitor. It inhibits trypsin, thrombin, u-PA, sct-PA, tct-PA and plasmin while forming 1:1 complexes with these proteases. Complex formation is promoted by heparin. Complexes of protease nexin I with thrombin and u-PA bind to and are internalized by fibroblasts. A role for protease nexin I in controlling cell surface protease activity has been proposed (Sprengers and Kluft, 1987; Pöllänen et al., 1991).

1.1.5 Receptors

Pericellular plasminogen activation is a localized and directed process requiring assembly of the components at specific sites on the cell surface. Receptors for plasminogen and plasminogen activators play important roles in focusing plasminogen

activator activity in a spatially and temporally regulated manner (Pöllänen et al., 1991; Blasi, 1993).

1.1.5.1 Plasminogen receptors

Most cells bind plasminogen with very similar characteristics. The binding is of low affinity (K_d is approximately $1 \mu\text{M}$) and high capacity. The number of plasminogen binding sites can exceed 10^7 sites per cell and more than half of them should be occupied if the physiological plasma concentration of plasminogen ($2 \mu\text{M}$) is considered. Plasminogen binding to cells can be inhibited by lysine and lysine analogs (6-aminohexanoic acid, tranexamic acid). It appears that both the high affinity LBS of kringle 1 and the low affinity LBS of kringle 5 are involved in the interaction, and this can lead to a heterogenous orientation of bound plasminogen molecules on the cell surface that might influence their activation (Plow and Miles, 1990; Plow et al., 1991).

Conflicting data have been reported as to whether Lys-plasminogen and Glu-plasminogen bind to cells with similar or markedly different affinities. The state of glycosylation of plasminogen can also affect affinity, but the data obtained from different laboratories are controversial (Plow and Miles, 1990; Plow et al., 1991).

Plasminogen and plasmin bind to the same sites on cells. Some authors found that plasmin binds to cells with much higher affinity than plasminogen. This is, however, not generally accepted (Plow and Miles, 1990). An important functional consequence of plasminogen binding and subsequent cell surface plasmin generation is the protection of cell bound plasmin from its inhibitors, α_2 -antiplasmin and α_2 -macroglobulin (Plow et al.,

1986; Ellis et al., 1991; Hall et al., 1991; Duval-Jobe and Parmely, 1994).

No single molecule has yet been identified as a plasminogen receptor. Even though plasminogen binds to a single class of sites on cells with respect to affinity, considering the very high binding capacity, it is unlikely that a single receptor molecule serves as an exclusive receptor. Cell surface proteins with carboxy-terminal lysines that bind plasminogen via its LBS may function as plasminogen receptors. Representatives of this category of plasminogen receptors are α -enolase and annexin II. Other plasminogen receptor candidates include glutaraldehyde 6-phosphate dehydrogenase, amphoterin, GPIIb-IIIa from platelets, a GPIIb-IIIa-related molecule from fibroblasts, gangliosides and glycosaminoglycans. Thus, both protein and non-protein molecules may function as plasminogen receptors (Plow et al., 1995).

1.1.5.2 The urokinase receptor

Specific cell surface receptors for u-PA (u-PAR) were first reported on human blood monocytes and on cells of the monocyte line U937 (Vassalli et al., 1985; Stoppelli et al., 1985), and they have been found on various normal and neoplastic cells as well. u-PAR is a heavily glycosylated, single-chain protein with an apparent Mr of 55,000 to 60,000. The Mr has been reduced to 35,000 upon deglycosylation with N-glycanase (Nielsen et al. 1988; Behrendt et al., 1990). The protein sequence has been deduced from the structure of cDNA encoding the entire human receptor (Roldan et al., 1990). The nascent molecule contains 313 amino acid residues with five potential attachment sites for N-linked carbohydrate. The length of the mature u-PAR protein is somewhat shorter due

to COOH-terminal processing (Ploug et al., 1991). The u-PAR is organized in three domains of about 90 amino acids each. The region which is responsible for the specific binding of u-PA is located in the NH₂-terminal domain (Behrendt et al., 1991).

u-PAR is an integral membrane protein anchored to the cell membrane by a COOH-terminal glycosyl-phosphatidylinositol (GPI) moiety (Ploug et al., 1991). The GPI anchor can be cleaved by bacterial phosphatidylinositol-specific phospholipase C (PI-PLC), thus releasing the receptor. Unoccupied u-PAR released to the medium retains its binding capability. Small amounts of water soluble u-PAR can be detected in conditioned media of U937 cells even without PI-PLC treatment, suggesting a regulatory action by putative human phospholipases.

u-PAR binds both pro-u-PA and u-PA through their EGF-like domain. The binding is of high affinity (K_d 10^{-9} to 10^{-11} M) and low capacity (1000 to 250,000 sites per cell) (Blasi, 1988). Catalytic activity of the plasminogen activator is not required for the interaction, because inactivation of u-PA by DFP does not abolish binding (Vassalli et al., 1985). Furthermore, the catalytically inactive ATF has retained its capability for cellular binding (Stoppelli et al., 1985). In contrast, LMW u-PA, which is proteolytically active but lacks the NH₂-terminal fragment, cannot bind to the u-PA receptor (Vassalli et al., 1985). Preformed u-PA/PAI-1 complexes also interact specifically with the receptor and require the NH₂-terminal fragment of u-PA for binding (Cubellis et al., 1989).

Pro-u-PA can be activated to u-PA while bound to the receptor (Cubellis et al., 1986). Bound u-PA retains its activity (Vassalli et al., 1985), dissociates very slowly from

the cell surface (Stoppelli et al., 1985) and is neither internalized nor degraded (Cubellis et al., 1990). Receptor bound u-PA is accessible to and inhibited by PAI-1 and PAI-2 (Cubellis et al., 1989; Ellis et al., 1990; Pöllänen et al., 1990), and u-PA/PAI-1 complexes bound to u-PAR are internalized and degraded (Cubellis et al., 1990).

1.1.5.3 t-PA receptors

In human umbilical vein endothelial cells (HUVEC) high (K_d 28.7 pM) and low (K_d 18.1 nM) affinity binding sites for t-PA have been described. Binding of t-PA to HUVEC was specific, saturable, reversible and did not require lysine binding sites (Hajjar et al., 1987). HUVEC binding sites for t-PA were identified in a plasma membrane enriched endothelial cell fraction as a single saturable site (K_d 9.1 nM). Ligand binding studies demonstrated a 40 kDa t-PA binding membrane protein that might represent the functional t-PA receptor (Hajjar and Hamel, 1990). A t-PA binding protein isolated from detergent extracts of human placenta has been found to be identical with the endothelial t-PA receptor. t-PA receptor (t-PAR) is postulated to be a unique dual ligand receptor, because it binds specifically both t-PA and plasminogen. The two ligands did not exhibit cross-competition, which suggests that t-PAR assists juxtaposition of t-PA and plasminogen thus promoting cell surface plasminogen activation (Hajjar, 1991). Recently, it has also been pointed out that the 40 kDa t-PAR is an annexin II related protein (Hajjar and Jacovina, 1993).

1.2 MAJOR MECHANISMS IN PLASMINOGEN ACTIVATION

1.2.1 Fibrinolysis

Fibrinolysis, an integral part of hemostasis, involves plasminogen activation at the fibrin surface followed by plasmin-catalyzed degradation of the insoluble fibrin clot into readily soluble fibrin fragments. Abnormalities in controlling fibrin degradation may have serious pathological consequences including bleeding or thrombosis (Ránby and Brändström, 1988; Halkier, 1991).

Plasminogen activators are normally present in the blood, yet very little if any plasminogen activation occurs (Collen, 1980; Pannell and Gurewich, 1986). t-PA, which is thought to be the principal plasminogen activator of fibrinolysis, activates plasminogen very inefficiently when fibrin is not present. Fibrin has several important roles in fibrinolysis. It triggers and regulates its own degradation by enhancing the activation rate of plasminogen. Generation of plasmin is focused on and restricted to the fibrin clot which provides a solid phase with favorable enzyme kinetics and is also a substrate for plasmin. In addition, plasmin generated on the fibrin surface is partly protected from inactivation, thus ensuring high-efficiency fibrinolysis (Collen and Lijnen, 1991; Anglés-Cano, 1994).

The soluble precursor of fibrin is fibrinogen, a glycoprotein composed of two symmetric half molecules, each possessing three polypeptide chains named $A\alpha$, $B\beta$ and γ . The two half-molecules are joined in a central amino terminal domain (E domain). Formation of a fibrin clot is the final phase of blood coagulation. Fibrinogen is converted

to fibrin by thrombin which cleaves the A α and B β chains at specific amino terminal sites and releases fibrinopeptide A (FPA) and fibrinopeptide B (FPB) molecules from fibrinogen. Fibrin monomers polymerize into a fibrin polymer clot, which subsequently undergoes covalent stabilization through crosslinking of monomers by activated factor XIII (Mosesson, 1990; Halkier, 1991).

Binding of t-PA and plasminogen to fibrin dramatically increases the rate of plasminogen activation as compared to catalytic rates obtained in the absence of fibrin. Conversion of fibrinogen to fibrin exposes t-PA binding sites and fibrin-bound sct-PA activates fibrin-bound plasminogen. Plasmin initially generated unveils carboxy-terminal lysine residues on the surface of partially degraded fibrin that provide additional binding sites for both t-PA and plasminogen resulting in the acceleration of plasmin-catalyzed fibrinolysis. Initial plasmin degradation is thus an important positive feedback mechanism in fibrinolysis (Halkier, 1991; Anglés-Cano, 1994; Lijnen et al., 1994). During fibrin clot lysis sct-PA is converted to tct-PA on the fibrin surface by the proteolytic action of plasmin (Halkier, 1991). This conversion is, however, unlikely to contribute to positive feedback amplification of fibrinolysis, because the two forms of t-PA are equally active in the presence of both intact and degraded fibrin (Suenson and Petersen, 1986; Urano et al., 1989; Lijnen et al., 1990; Andreassen et al., 1991).

The role of u-PA in fibrinolysis is not clearly established. In the presence of fibrin scu-PA but not tcu-PA induces fibrin-specific proteolysis (Gurewich et al., 1984; Zamarron et al., 1984). Scu-PA is not an efficient activator in the presence of intact

fibrin, but it activates plasminogen bound to partially degraded fibrin with an efficiency comparable to that of t-PA (Fleury et al., 1993a). Scu-PA which has no affinity for fibrin may induce fibrin-specific plasminogen activation by interaction with plasminogen bound to carboxy-terminal lysine residues of partially digested fibrin (Pannel et al., 1988; Fleury et al., 1993b).

Whether plasmin-mediated conversion of Glu-plasminogen to Lys-plasminogen occurs during fibrinolysis is still debated. Lys-plasminogen demonstrates improved fibrin binding and activation rate over Glu-plasminogen (Wohl et al., 1980; Hoylaerts et al., 1982; Nesheim et al., 1990; Suenson et al., 1990) and thus, formation of Lys-plasminogen could theoretically contribute to the positive feedback amplification of fibrinolysis. α_2 -antiplasmin might have a role in regulating Lys-plasminogen formation, inasmuch as it has been shown that in the presence of this plasmin inhibitor Lys-plasminogen is not an intermediate in Glu-plasminogen activation (Rouy and Anglés-Cano, 1990), whereas the absence of α_2 -antiplasmin permits Lys-plasminogen and Lys-plasmin to form (Rouy and Anglés-Cano, 1990).

Lipoprotein (a) (Lp(a)), a plasma lipoprotein appears to attenuate fibrinolysis. The human apolipoprotein(a) shows remarkable homology to plasminogen (McLean et al., 1987). Lp(a) competes with both t-PA and plasminogen for binding sites on fibrin (Loscalzo et al., 1990). Furthermore, Lp(a) binding to fibrin is enhanced by plasmin-mediated partial degradation of the fibrin surface (Harpel et al., 1989). As a result, fibrin-dependent activation of plasminogen by t-PA is inhibited (Loscalzo et al., 1990). Elevated

concentrations of Lp(a) may thus significantly impair physiological fibrinolysis.

Fibrinolysis may be inhibited at the level of plasminogen activators or at the level of plasmin. The most important inhibitors involved in fibrinolysis are PAI-1 and α_2 -antiplasmin (Rånby and Brändström, 1988). PAI-1 has been shown to bind to fibrin and fibrin-bound PAI-1 forms SDS-stable complexes with t-PA (Wagner et al., 1989; Reilly and Hutzelmann, 1992). PAI-1 bound to fibrin inhibits t-PA mediated fibrinolysis in a dose-responsive manner (Reilly and Hutzelmann, 1992). Inactive PAI-1/t-PA complexes dissociate from the fibrin matrix and may rebind to fibrin through the finger and kringle 2 domains of t-PA. Hence, t-PA/PAI-1 complexes are likely to compete with free t-PA for the same binding sites on fibrin leading to further inhibition of t-PA dependent fibrinolysis (Wagner et al., 1989). Thus, fibrin-bound PAI-1 at sites of thrombus formation may help prevent thrombi from premature dissolution. However, other data indicate that PAI-1 fails to inhibit fibrin-bound t-PA significantly (Chmielewska et al., 1988; Masson and Angles-Cano, 1988). A possible explanation for the divergent results may be the difference in the order of appearance and assembly of t-PA and PAI-1 on the fibrin surface, arising from the different experimental set-ups.

The physiological plasmin inhibitor, α_2 -antiplasmin has two modes of action. Alpha₂-antiplasmin in fluid phase is inefficient at inhibiting fibrin bound plasmin. Free plasmin dissociated from the clot is, however, rapidly inactivated. This mechanism ensures that plasmin remains effective only if bound at the fibrin surface (Wiman and Collen, 1978; Collen, 1980; Rånby and Brändström, 1988). Contrary to the fluid phase

inhibitor, α_2 -antiplasmin crosslinked to fibrin appears to inhibit bound plasmin since α_2 -antiplasmin deficient plasma clots undergo rapid fibrinolysis even if the clot is immersed in α_2 -antiplasmin containing normal plasma (Sakata and Aoki, 1982). Hence, the significance of inhibition by crosslinked α_2 -antiplasmin probably lies in preventing premature clot lysis.

1.2.2 Plasminogen activation at the cell surface

It is now realized that many cell types utilize a concerted cascade-like action of proteolytic enzymes on or near the cell surface to degrade extracellular matrices (ECM) (Moscatelli and Rifkin, 1988; Chen, 1992). Major ECM degrading cell surface proteases include three classes of secreted proteases (serine proteases, cathepsins and matrix metalloproteases) and integral membrane proteases (Chen, 1992). Assembly of relevant proteases and their inhibitors at specific sites on the cell surface provides the cells with the potential of a highly efficient and regulated proteolysis that is strictly localized in nature. Central to proteolytic mechanisms on the cell surface is plasminogen activation. The serine protease plasmin and, to a lesser degree, u-PA are themselves able to degrade certain components of ECM proteins (Liotta et al., 1981; Saksela and Rifkin, 1988; Gold et al., 1989). In addition, and perhaps even more importantly, these enzymes can process and activate latent metalloproteinases (Matrisian, 1990; Keski-Oja et al., 1992), which are amongst the most powerful ECM degrading enzymes currently known. Thus, the plasminogen activation system plays a major part in the regulation of the activity of metalloproteinases and in pericellular ECM degradation.

1.2.2.1 Pericellular distribution of the plasminogen activation system

Complex molecular interactions resulting in cellular adhesion are an important part of the mechanisms that maintain the normal phenotype of cells. Adhesive interactions between the cell surface and extracellular matrix focus on discrete contact sites where modulation of such interactions by cell-surface proteolysis can lead to cell migration and invasion (Pöllänen et al., 1991; Blasi, 1993).

In cultures of fibroblasts three types of cell-substratum contact sites are known (Chen and Singer, 1982; Pöllänen et al., 1991): (1) focal contacts (synonyms: focal adhesions or adhesion plaques), (2) close contacts, and (3) fibronexus contacts (extracellular matrix contact sites). Focal contacts are the sites of closest apposition between the ventral cell surface and growth substratum (~15 nm). They are frequently seen close to the leading edge of moving cells associated with lamellopodia and microspikes. Close contacts are adhesions with 30-50 nm spacings between the cell surface and substratum, whereas in fibronexus contacts the ventral cell surface is far removed from the substratum, though sporadically connected to it by filamentous aggregates of the extracellular matrix.

In actively growing HT-1080 fibrosarcoma and human embryonic lung fibroblast cultures u-PA has been detected in focal contacts on all parts of the ventral cell surface, particularly at leading lamellae, lamellopodia and microspikes. Extracellular u-PA has also been found at cell-cell contact sites. By contrast, no u-PA could be demonstrated at

close contacts and fibronexus contact sites characteristic of normal stationary fibroblasts (Pöllänen et al., 1987, 1988; Vaheri et al., 1990).

Distribution of extracellular PAI-1 is quite distinct from that of u-PA. It is widely distributed on the growth substratum beneath the ventral cell surface (Pöllänen et al., 1987). The extracellular distribution of PAI-1 appears identical with that of vitronectin, a binding protein for PAI-1. In the vitronectin-PAI-1 complex PAI-1 remains active and vitronectin retains its cell adhesion properties. Furthermore, cellular vitronectin receptors are known to be localized at focal contacts. Thus, interactions between vitronectin and its receptor may place vitronectin-bound PAI-1 in an appropriate position to inhibit receptor bound u-PA in focal contacts, further promoting cellular adhesion (Vaheri et al., 1990; Pöllänen et al., 1991).

Plasminogen is distributed all over the ventral cell surface, whereas the dorsal surface does not bind plasminogen (Pöllänen et al., 1991).

1.2.2.2 u-PA dependent cell surface plasminogen activation

Most cells capable of cell surface plasminogen activation synthesize both scu-PA (pro-u-PA) and u-PA, but do not usually produce plasminogen. After secretion, pro-u-PA binds to u-PA by autocrine/paracrine mechanisms. Subsequently, activation of bound pro-u-PA to active scu-PA can take place (Stoppelli et al., 1986; Stephens et al., 1989; Blasi, 1993). This process is clearly dependent on cellular binding of the ubiquitous plasminogen. Plasminogen binding occurs through interactions with plasminogen receptors on the cell surface and is stimulated by interactions between u-PA and its

receptor (Plow et al., 1986). The initiating event for pro-u-PA activation is, however, still not known (Ellis and Danø, 1991). It may be provided by trace amounts of active proteases. Plasmin is capable of catalyzing receptor bound pro-u-PA activation (Cubellis et al., 1986; Stephens et al., 1989) as may be other proteases such as cathepsin B (Kobayashi et al., 1991). Induction of proteolytic activity in pro-u-PA upon binding to its receptor has been also proposed as a possible alternative mechanism (Manchanda and Schwartz, 1991).

Generation of active u-PA results in rapid activation of cell bound plasminogen. Plasmin formed can lead, in turn, to further activation of pro-u-PA and plasminogen, enabling more plasmin to form, and so on. Positive feedback regulation resulting in progressive increases in the rates of plasmin generation can occur at various levels of the plasminogen activation pathway in both solution and in the presence of cells. Conversion of pro-u-PA to active u-PA is obviously subject to feedback activation by plasmin. After initiation of plasminogen activation increasing concentrations of newly formed plasmin enhance the rate of active u-PA generation, leading to increases in the plasmin generation rate (Collen et al., 1986; Ellis et al., 1987, 1989; Stephens et al., 1989; Liu et al., 1992). Another feedback amplification in plasminogen activation may be initiated at the level of conversion of Glu- to Lys-plasminogen. It has been demonstrated that activation of cell-bound Lys-plasminogen proceeds with more favorable kinetics than that of Glu-plasminogen (Ellis et al., 1991). Moreover, conversion of Glu-plasminogen to Lys-plasminogen has been detected on the cell surface and this conversion could be inhibited

by aprotinin. It is, therefore, reasonable to assume that plasmin contributes to the feedback amplification of plasminogen activation at yet another level, by catalyzing Lys-plasminogen formation. However, conversion of Glu- to Lys-plasminogen was not affected by specific inhibitors of plasmin and plasminogen activation, suggesting that serine proteases other than plasmin were likely to be involved. Still, the involvement of plasmin remains a strong possibility.

It is now well established that plasminogen activation at the cell surface can proceed at a much higher rate than in the fluid phase (Ellis et al., 1989, 1991; Duval-Jobe and Parmely, 1994). Potentiation of plasminogen activation in the presence of cells could be abolished by ATF that competes with u-PA binding to u-PAR or lysine analog plasminogen binding antagonists (6-aminohexanoic acid, tranexamic acid), thus demonstrating the requirement of specific u-PA and plasminogen binding to their respective cellular receptors. Cell surface bound u-PA, in fact, activates fluid phase plasminogen with kinetics virtually identical with those obtained by u-PA and plasminogen in solution. Enhanced feedback activation of pro-u-PA is likely to be the most important mechanism of cellular potentiation of plasminogen activation. The increase in the rate of two-chain u-PA generation appears to be mediated by cell-bound plasmin (Ellis et al., 1989; Duval-Jobe and Parmely, 1994).

1.2.2.3 t-PA dependent cell surface plasminogen activation

While it is abundantly clear that many cell types utilize u-PA dependent cell surface plasminogen activation, only a few cases of t-PA induced activation have been

reported (Hajjar et al., 1987; Hajjar and Nachmann, 1988; Bizik et al., 1990; Nachmann and Hajjar, 1991).

Intravascular plasminogen activation is controlled to a great extent by vascular endothelial cells through their ability to produce plasminogen activators (t-PA and u-PA) (van Hinsbergh, 1988) as well as plasminogen activator inhibitors (PAI-1 and PAI-2) (Schleef and Loskutoff, 1988). t-PA and PAI-1 appear to be the predominant components of the vascular plasminogen activation system. They are released into the circulating blood (van Hinsbergh et al., 1991), but are also found bound to the surface of endothelial cells (Barnathan et al., 1988). Both plasminogen (Hajjar et al., 1986; Hajjar and Nachman, 1988) and t-PA (Hajjar et al., 1987; Barnathan et al., 1988) bind specifically to HUVEC and furthermore, an endothelial cell t-PA receptor which binds both t-PA and Lys-plasminogen has also been identified (Hajjar, 1991). Activation of plasminogen by HUVEC bound t-PA proceeds with a considerably higher catalytic activity compared to fluid phase activation (Hajjar et al., 1987). Enhancement of catalytic efficiency by endothelial cells seems to result, at least in part, from the conversion of Glu- to Lys-plasminogen on the cell surface (Hajjar and Nachman, 1988). t-PA bound to the cell surface is protected from PAI-1 (Hajjar et al., 1987), and this is in contrast to the effective inhibition of cell surface bound u-PA by PAIs (Ellis et al., 1990). Lp(a), by competing at the endothelial cell surface for plasminogen binding sites down-regulates generation of cell-associated plasmin (Nachman and Hajjar, 1991). Overall, the above findings strongly suggest the existence of a powerful endothelial plasminogen activation system whose main

role probably lies in controlling physiological non-thrombogenicity at the vascular surface.

Several human melanoma cell lines also favor t-PA mediated cell surface plasminogen activation (Bizik et al., 1990). These cells produce primarily t-PA and very little u-PA. Exogenous t-PA binds to these melanoma cells, but the binding of u-PA is insignificant indicating that functional binding sites for u-PA are poorly expressed. In the presence of plasminogen, cell surface bound plasmin activity could be recovered from tranexamic acid eluates and was inhibited by an anticatalytic antibody to t-PA, while an anticatalytic u-PA antibody had no effect. These findings suggest t-PA mediated cell surface plasminogen activation. The reason why some melanoma cell lines employ t-PA rather than u-PA to promote cell surface plasmin generation remains unclear and awaits further investigation.

1.2.2.4 Inhibition of cell surface plasminogen activation

Inhibition of the plasminogen activation system at the cell surface can occur at the level of plasminogen activators or of plasmin. Activation of plasminogen at focal contacts is regulated through inhibition of receptor-bound u-PA by PAIs. PAI-1 freshly secreted from cells or bound to vitronectin in the extracellular matrix is active and has the capacity to rapidly control cell surface plasminogen activator activity (Pöllänen et al. 1991). In cell culture, neutralizing antibodies to PAI-1 have been shown to increase the amount of active u-PA recovered from the cell surface, indicating that u-PA activity is subject to inhibition by endogenous active PAI-1 (Stephens et al., 1989). PAI-2, which is not associated with the ECM, also has the potential to regulate cell-surface u-PA activity (Ellis et al., 1990).

Exogenous recombinant PAI-2 has been found to bind to and inhibit active u-PA at focal adhesion sites (Pöllänen et al., 1990). Because it does not bind to ECM, it is a suitable probe for mapping active u-PA sites on the cell surface.

In U937 promonocyte cultures both PAI-1 and PAI-2 inhibited receptor bound u-PA with association rate constants that were approximately 40% lower in each case when compared to solution phase inhibition of unbound active u-PA (Ellis et al., 1990). These constants ($4.5 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ and $3.3 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ for PAI-1 and PAI-2, respectively), however, still represent rapid inhibition demonstrating that u-PA bound to its cellular receptor remains available for efficient inhibition by PAIs.

Receptor bound active u-PA dissociates slowly and is neither internalized nor degraded (Vassalli et al., 1985; Stoppelli et al., 1985; Cubellis et al., 1990). However, u-PA:PAI-1 complexes undergo u-PA receptor mediated internalization, followed by degradation (Cubellis et al., 1990; Olson et al., 1992) and the internalized receptors are recycled back to the cell surface (Conese and Blasi, 1995). Similarly, u-PA:PAI-2 complexes are subject to u-PA receptor-dependent internalization and degradation (Estreicher et al., 1990). Recently, involvement of the α_2 -macroglobulin receptor/low density lipoprotein receptor-associated protein (α_2 -MR) and epithelial glycoprotein 330 (gp330) in the internalization and degradation of u-PA:PAI-1 complexes has been also suggested (Conese and Blasi, 1995).

While plasmin is readily inhibited in solution, cell-bound plasmin is protected from inactivation by its inhibitors α_2 -antiplasmin and α_2 -macroglobulin (Plow et al., 1986; Ellis

et al., 1991; Hall et al., 1991; Duval Jobe and Parmely, 1994). Due to the low affinity interaction between plasmin and its receptor, dissociation of plasmin relative to u-PA can occur more easily. In serum-containing cultures, dissociated plasmin is rapidly inhibited by its inhibitors, thus confining plasmin activity to the cell surface. In addition, due to the presence of inhibitors, plasmin is not taken up from serum medium onto the cell surface, ensuring that cell bound plasmin results exclusively from activation of bound plasminogen (Stephens et al., 1989).

In summary, cell surface plasminogen activation is a highly regulated and localized process that requires complex interactions between the components of the plasminogen activation system. This process involves a complete cycle of u-PA from synthesis, secretion and autocrine/paracrine binding through cell surface activation and inhibition to internalization and degradation. Recycling of u-PAR to the cell surface favors formation of new focal adhesion sites required for cell migration and breakdown of physical barriers. Thus, the cell surface can be regarded as being composed of proteolytically active and adhesion promoting areas that rearrange continuously in the process of cell migration and adhesion (Blasi, 1993).

1.3. REGULATION OF PLASMINOGEN ACTIVATION

Plasmin activity available for various biological functions is spatially and temporally controlled through regulatory mechanisms acting at several levels of the plasminogen activation system. Finely tuned mechanisms exist that regulate the synthesis, release, binding characteristics, activity and elimination of several components of the

system. Hence, net plasminogen activation is a balance of complex interactions between fibrinolytic components each regulated by a variety of mechanisms (For reviews see Danø et al., 1985; Saksela and Rifkin, 1988; Loskutoff et al., 1989, 1991; Mayer, 1990; Van Hinsbergh et al., 1991; Plow et al., 1995).

1.4 THE PLACENTAL TROPHOBLAST: INVASIVENESS AND PROTEOLYTIC ACTIVITY

1.4.1. Development and structural organization of the human placenta

Human development commences at fertilization that normally occurs in the ampulla of the uterine tube (Sadler, 1985; Moore, 1988). As the fertilized ovum (zygote) passes along the uterine tube it undergoes a series of mitotic cell divisions (cleavage) resulting in the formation of daughter cells called blastomeres. Approximately three days after fertilization a mulberry-shaped mass of 12-16 blastomeres (morula) still surrounded by the protective layer zona pellucida enters the uterine cavity. About four days after fertilization embryonic development results in a blastocyst consisting of a fluid-filled cavity (blastocoele) enclosed by a group of centrally located cells (inner cell mass or embryoblast), and a flattened outer cell layer known as the trophoblast. The embryoblast is the primordium of the embryo, while the trophoblast will eventually give rise to the fetal part of the placenta. Initially, the blastocyst floats freely in the uterine secretions, and during this period the zona pellucida gradually degenerates and disappears. About six days after fertilization the human blastocyst attaches to the endometrial epithelium via its embryonic pole (that part of the blastocyst where the embryoblast is located). This

attachment marks the beginning of the process of implantation, which comprises a series of important events (Schlafke and Enders, 1975). Usually, the blastocyst implants in the endometrium of the posterior or anterior wall of the body of the uterus.

The trophoblast at the implanting embryonic pole proliferates rapidly and differentiates into two layers: (1) an inner cytotrophoblast (cellular trophoblast), and (2) an outer syncytiotrophoblast (syncytium) (Sadler, 1985; Moore, 1988). The latter is a continuous mass of fused trophoblastic cells without cellular boundaries that has lost its capability to divide. The syncytiotrophoblast continues to expand by proliferation and subsequent fusion of neighboring cytotrophoblasts. It invades the uterine epithelium and endometrial stroma, and the blastocyst slowly embeds itself in the endometrium. Meanwhile, stromal cells of the endometrium at the implantation site proliferate and enlarge, thus transforming to decidual cells.

During further development isolated lacunae appear in the syncytium that soon communicate with eroded endometrial blood vessels, and thus become filled with maternal blood (Moore, 1989). By the tenth day after fertilization the blastocyst is completely implanted in the superficial compact layer of the endometrium. At this stage the outer surface of the blastocyst is completely transformed into syncytiotrophoblast. The syncytiotrophoblast lacunae soon become partially confluent to form lacunar networks, establishing a primitive uteroplacental circulation. Lacuna formation subdivides the trophoblast covering of the implanted blastocyst into (1) primary chorionic plate, (2) lacunar system and (3) trophoblastic shell (Benirschke and Kaufmann, 1990).

The primary chorionic plate separates the lacunar system from the blastocyst cavity. Initially, it consists only of cytotrophoblast and syncytiotrophoblast. It becomes triple-layered when the extraembryonic mesenchyme spreads over the cytotrophoblastic surface of the blastocyst cavity. Toward the lacunae the cytotrophoblast is covered by syncytiotrophoblast. The lacunae are separated from each other by trabeculae of the syncytiotrophoblast. These trabeculae are invaded longitudinally by cytotrophoblasts derived from the primary chorionic plate. Where the peripheral ends of the trabeculae join together, they form the trophoblastic shell facing the endometrium (decidua). At the beginning, it is purely syncytiotrophoblastic in nature. As soon as, however, the cytotrophoblasts reach the shell it becomes triple-layered, consisting cytotrophoblasts in the middle and syncytium on the fetal as well as maternal aspects (Benirschke and Kaufmann, 1990).

At the end of the second week increased cytotrophoblastic proliferation and syncytial fusion in the trabeculae take place (Moore, 1988; Benirschke and Kaufmann, 1990). As locally growing cytotrophoblast masses extend into the syncytiotrophoblast primary chorionic villi protruding into the lacunae (henceforth called intervillous space) are formed. These villi consist of a core of cytotrophoblasts surrounded by an outer layer of syncytiotrophoblast. Branching of the primary villi initiate the development of villous trees, the stems of which are derived from the former trabeculae. When the stem villi keep their contact with the trophoblastic shell they are called anchoring villi. Villi that do not contact the trophoblastic shell are free (floating) villi. At around day 15

mesenchymal cells derived from the extraembryonic mesoderm of the primary chorionic plate grow into the primary chorionic villi transforming them to secondary villi. These villi have a core of mesoderm covered by a single layer of cytotrophoblastic cells, which, in turn, are surrounded by the outer syncytium. After blood vessels have appeared in the villi they are called tertiary chorionic villi. Capillaries in tertiary villi come into contact with vessels in the mesoderm of the chorionic plate and in the connecting stalk that later develops into the umbilical cord, forming the connection between placenta and embryo. By the end of the third week embryonic blood begins to circulate through the capillaries of the chorionic villi. Until about the eighth week villi cover the entire surface of the chorion. As development advances, villi at the embryonic pole continue to expand giving rise to the villous chorion (chorion frondosum). Chorionic villi at the abembryonic pole degenerate and disappear. This villous-free part of the chorion is called the smooth chorion (chorion laeve). The functional layer of the gravid endometrium known as decidua is found at three locations: (1) over the villous chorion (decidua basalis), (2) over the abembryonic pole (decidua capsularis), and (3) on the surface of the uterus opposite to the growing embryo (decidua parietalis).

The placenta has two components: (1) a fetal portion, and (2) a maternal portion (Moore, 1988). The fetal portion is formed by the villous chorion, consisting of the chorionic plate and the villi. The latter project into the intervillous spaces containing maternal blood. The maternal portion is formed by the decidua basalis.

Development of the chorionic plate begins with the formation of the primary chorionic plate (Benirschke and Kaufmann, 1990). When the amnion comes into close contact with and attaches to the primary chorionic plate, the latter is transformed to the definitive (mature) chorionic plate. It serves as the base from which the villous trees are suspended into the intervillous space. The mature chorionic plate is composed of several layers. At the fetal aspect three layers of the amnion are found, i.e. amnionic epithelium, compact layer consisting of a lattice of collagen fibers, and amnionic mesoderm. Undemeath the latter there is a spongy layer, separating the amnion and the chorionic mesoderm. The next layer consists of primary cytotrophoblasts (remnants of the original cytotrophoblast layer of the blastocyst), which rests on the subjacent Langhans fibrinoid. This layer contains X cells which are derived from either primary chorionic cytotrophoblasts or villous cytotrophoblasts of peripheral villi attached to and encased by the fibrinoid. Finally, the intervillous surface of the chorionic plate is covered by remnants of the syncytiotrophoblast.

The placental (chorionic) villi exhibit a characteristic cellular organization. They possess a central mesenchymal core (stroma) covered by an outer layer of syncytiotrophoblast and mononuclear cytotrophoblasts (Langhans' cells) underneath. The syncytiotrophoblast (syncytium) is a continuous multinucleated trophoblastic layer without separating cell borders. It lines the intervillous space and thus faces the maternal blood directly. The Langhans' cells which form the inner layer of the trophoblastic covering of the placental villi are joined to each other and the syncytium by desmosomes and tight

junctions. The basal surface of the Langhans' cells rest on the trophoblastic basal lamina that separates the trophoblast from the villous core. At the sites where the Langhans' layer becomes discontinuous the syncytium establishes direct contact with the basal lamina. Villous cytotrophoblasts function as stem cells that proliferate and subsequently differentiate into villous syncytiotrophoblast by fusion. During early pregnancy, villous cytotrophoblasts form a nearly complete layer which later becomes discontinuous, and at term these cells are found beneath only about 20% of the syncytiotrophoblast (Moore, 1988; Benirschke and Kaufmann, 1990).

The stroma of the placental villi consists of fibroblasts, macrophages, fetal vessels and connective tissue fibers.

The villi serve as the site where the exchange of materials between fetus and mother takes place. Oxygenated maternal blood enters the intervillous space via the eroded ends of decidual spiral arteries, while deoxygenated maternal blood leaves through endometrial veins. Fetal deoxygenated blood reaches the placenta in two umbilical arteries that divide into numerous branches in the chorionic plate before entering the villi. The blood vessels form an extensive arterio-capillary-venous network in the villi. Oxygenated fetal blood leaves the placenta via the umbilical vein. The maternal and fetal circulations are separated by the placental membrane (barrier) consisting of (1) syncytium, (2) cytotrophoblast, (3) trophoblastic basal lamina, (4) connective tissue, and (5) fetal endothelium surrounded by its basal lamina. As pregnancy advances the syncytium becomes thinner, the number of cytotrophoblasts is diminished and the fetal capillaries

come closer to the villous surface. Consequently, the barrier becomes significantly reduced in thickness, and in many places it encompasses only syncytium, fused trophoblastic and capillary basal lamina, and fetal capillary endothelium (Moore, 1988; Benirschke and Kaufmann, 1990).

On the maternal aspect of the delivered placenta the basal plate borders on the intervillous space. The term basal plate is applied to the fused contact zone between the trophoblastic shell and the decidua basalis, where cytotrophoblasts derived from the trophoblastic shell invade and intermingle with the endometrial stroma so that the border separating trophoblastic shell and decidua can no longer be defined. The deeper tissue layers of the placental site that remain in utero after placental separation are referred to as the placental bed. *In situ* the basal plate and placental bed are collectively called the junctional zone, which include all admixed fetal and maternal tissues basal to the intervillous space. The mature basal plate is about 100 μm to 1.5 mm in thickness. Its inner surface facing the intervillous space is covered with focal residues of the syncytiotrophoblast of the former trophoblastic shell. Where the syncytiotrophoblast is absent it is replaced by Rohr's fibrinoid believed to result at least from tissue necrosis. The syncytiotrophoblast rests on an inconsistent connective tissue layer followed by the principal layer of the basal plate consisting of a mixture of decidual cells, extravillous cytotrophoblasts, fibrinoid and remnants of buried villi and cell columns. The next layer is called the Nitabuch' fibrinoid. Separation of placenta from the placental bed usually takes place just below this layer (Benirschke and Kaufmann, 1990).

During early stages of pregnancy anchoring villi are attached to the intervillous surface of the basal plate via cytotrophoblastic cell columns. The cell columns are composed of a multilayered core of extravillous cytotrophoblasts demarcated sharply by a basal lamina from the adjacent stroma of the anchoring villi. Invasion of the basal plate by columnar cytotrophoblasts is accompanied by cellular differentiation. Cytotrophoblast stem cells located near the basal lamina proliferate and either fuse to form the syncytiotrophoblast covering of the anchoring villi or invade, and thus extensively colonize the basal plate and placental bed. During later stages of pregnancy the cell columns are not conspicuous but rather exist incorporated into the basal plate at the base of the anchoring villi (Benirschke and Kaufmann, 1990).

The basal plate is protruded into the intervillous space in the form of placental septa which divide the fetal part of the placenta into several cotyledons. Characteristic cellular elements of these septa are decidual cells and extravillous cytotrophoblasts. Similar to placental septa, cell islands are also non-villous parts of the placenta connected to villous tips or the chorionic plate. They are composed of mainly extravillous cytotrophoblasts encased in fibrinoid (Benirschke and Kaufmann, 1990).

1.4.2 Pathways of trophoblast differentiation: heterogeneity of the trophoblast

During the villous stage of placental development trophoblasts are found in the chorionic villi and at extravillous locations (Benirschke and Kaufmann, 1990). The villous syncytiotrophoblast forms the superficial layer of the villi, overlying the inner cytotrophoblasts (Langhans' cells). The remaining extravillous trophoblast, classified into

extravillous cytotrophoblast and extravillous syncytiotrophoblast, is present outside of the villi at several locations, such as (1) chorionic plate, (2) chorion laeve, (3) marginal zone, (4) trophoblastic shell and basal plate, (5) cell columns, (6) cell islands and (8) placental bed . Various forms of extravillous trophoblast have been described and designated as extravillous cytotrophoblast, interstitial trophoblast, intravascular trophoblast, endovascular trophoblast, intraarterial trophoblast, placental site giant cell, multinucleated trophoblastic giant cell, X cell, primary extravillous cytotrophoblast, secondary extravillous cytotrophoblast, primary chorionic cytotrophoblast, primary and secondary basal trophoblast etc. This diversity of designations suggests that, despite different morphological features and special localizations, some of the different names may represent identical or similar differentiation stages of the trophoblast.

Recently, an alternative classification distinguishing three types of trophoblast has been proposed (Kurman et al., 1984; Yeh and Kurman, 1989; Kurman, 1991). According to the authors, there exist a transitional form of trophoblast (intermediate trophoblast) in addition to the clearly defined cytotrophoblast and syncytiotrophoblast. The intermediate trophoblast is believed to represent an intermediate stage of differentiation between cytotrophoblast and syncytiotrophoblast, and is characterized by distinctive biochemical features and a diverse morphological expression. This cell type occurs at both villous and extravillous locations, and thus it cannot be simply identified as being extravillous cytotrophoblast. On the other hand, extravillous trophoblast is often recognized as a particular location of the intermediate trophoblast. Some authorities believe, however, that

the extravillous cytotrophoblast population is heterogenous (Benirschke and Kaufmann, 1990).

The trophoblast undergoes different pathways of differentiation dependent on the anatomical location and the gestational age. All forms of trophoblast derive from the blastocystic trophoblast, the outer mononuclear cell layer of the preimplantation blastocyst (Hamilton and Boyd, 1960). During early stages of implantation the blastocystic trophoblast differentiates into primitive mononuclear cytotrophoblast and syncytiotrophoblast. The former acts as a stem cell by proliferating and subsequently fusing with the overlying syncytiotrophoblast. Over the course of the lacunar and villous stages of placental development further differentiation of the trophoblast takes place, resulting in various trophoblast subpopulations. Soon after lacunae appear in the primitive syncytium the cytotrophoblasts grow into the syncytiotrophoblastic trabeculae and ultimately give rise to highly invasive intermediate trophoblasts of the trophoblastic shell. Intermediate trophoblasts deriving from the shell subsequently invade the decidua and myometrium. During the villous stage of placental development two different pathways of trophoblast differentiation exist, which give rise to villous and extravillous trophoblast populations (Kurman et al., 1984; Yeh and Kurman, 1989; Kurman, 1991; Damsky et al., 1992). In floating chorionic villi cytotrophoblast stem cells that are mononuclear, undifferentiated trophoblasts displaying proliferating activity fuse into syncytiotrophoblast covering the entire villous surface. The villous syncytiotrophoblast (syncytium) represents a terminally differentiated noninvasive state. In anchoring chorionic villi cytotrophoblast

stem cells enter both differentiation pathways. Again, some stem cells fuse into syncytium. Others break through the syncytium at the tips of the villi and differentiate into intermediate cells of cell columns attached to the uterine wall. Intermediate trophoblasts emanating from the cell columns deeply invade the uterus and display such morphological and functional expressions as interstitial trophoblasts and intravascular trophoblasts. Intermediate trophoblasts may also fuse and form binucleate, trinucleate and multinucleated cells. The latter is also referred to as syncytiotrophoblastic giant cells.

1.4.3 Trophoblast invasion during implantation and placental development

The placenta is a fetomaternal organ developed primarily for an exchange of molecules between fetus and mother. To achieve this goal fetal and maternal circulations are brought into a close proximity by fetal trophoblast invasion of the uterus. The degree of trophoblast invasion, differing widely in extent among various mammalian orders, determines the complexity of the placental barrier separating the two circulations. Epitheliochorial placentas (e.g. in ungulates) are non-invasive, the chorionic trophoblast remains closely attached to the intact endometrial epithelium. In the syndesmochorial type placentas of sheep and goats trophoblast invasion is arrested after destruction of the uterine epithelium. Deeper invasion resulting in the erosion of the endometrial connective tissue establishes the endotheliochorial type placenta characteristic of carnivores. Finally, when the maternal vessels are also eroded and destroyed by the invasive trophoblast, the hemochorial placenta is developed. In this case, the maternal blood is in direct contact with the trophoblast. This relationship occurs for instance in rodents and higher primates

including the human (Benirschke and Kaufmann, 1990).

Trophoblast invasion begins with implantation of the blastocyst. In different mammals implantation varies with regard to the nature of the involvement of the uterine epithelium. Accordingly, three types of implantation can be distinguished: (1) intrusive implantation, characterized by trophoblast penetration between intact uterine epithelial cells, (2) displacement implantation, in which large areas of epithelium are displaced from the underlying basal lamina, and (3) fusion implantation, where the invading syncytiotrophoblast is fused with epithelial cells during penetration of the uterine epithelium (Schlafke and Enders, 1975). In the implantation of the human blastocyst, a combination of the fusion and intrusive types are likely to be involved (Denker, 1990).

After penetration of the luminal endometrial epithelium the expanding syncytiotrophoblast continues to invade the epithelial basal lamina and underlying maternal tissues. Due to the invasive activity of the syncytiotrophoblast, some maternal capillaries become eroded and may be incorporated into the syncytial lacunar system. At this stage, however, there is no communication with the endometrial spiral arteries. During the first month of pregnancy proliferating cytotrophoblasts of the emerging trophoblastic shell extend the implantation site and start to invade the decidua where they are referred to as interstitial cytotrophoblasts. At the same time, the decidual segments of spiral arteries are breached as a result of the first wave of endovascular trophoblast migration. The endovascular trophoblasts appear to be derived from the trophoblastic shell, and migrate in the lumen of decidual spiral arteries. However, they do not cross

the decidual-myometrial junction in the first few months of pregnancy (Pijnenborg, 1990).

At 4-6 weeks the trophoblastic shell begins to regress, but proliferating cytotrophoblasts remain at the tips of the anchoring villi (cell columns) (Pijnenborg, 1990). From 8 weeks these cells migrate into the decidua, and start to invade the inner myometrium (interstitial invasion). The myometrial segments of spiral arteries develop regressive changes, such as endothelial swelling, internal vacuolation, appearance of hypertrophied basophilic cells and disruption of the media. Disintegration of the spiral arterial wall is positively correlated with the emergence of perivascular interstitial cells, and hence the regressive changes may be viewed as a preparation by interstitial trophoblasts for the second wave of endovascular trophoblast invasion, which affects the myometrial segments of spiral arteries, starts at 14-15 weeks and ends by about 18-20 weeks. The endovascular trophoblasts replace the endothelium, and destroy the muscular and elastic elements of the vessel wall. Eventually, the spiral arteries are converted into widened tubes, the walls of which are composed of trophoblasts embedded in an amorphous fibrinoid material. Due to the absence of normal muscular elements, these transformed spiral arteries (also called uteroplacental vessels) can passively accommodate the greatly augmented blood flow as pregnancy advances. Although the primary role has been assigned here to the endovascular trophoblast, some claim that invasion of the spiral arteries is initiated by interstitial trophoblasts from outside the vessels (Kurman et al., 1984).

After 10 weeks of gestation a growing number of multinucleated trophoblastic

giant cells have also been observed in the decidua and myometrium. These cells appear to develop by fusion of interstitial trophoblasts, and probably represent a final noninvasive stage of cytotrophoblastic differentiation (Pijnenborg, 1990).

1.4.4 Trophoblast invasiveness: involvement of proteases and protease inhibitors

During implantation and placental development human trophoblasts invade the uterus and come in direct contact with the maternal circulation. Trophoblast invasion normally continues until about the fourth month of pregnancy and is restricted to the maternal endometrium and the inner third of the myometrium. Various steps of trophoblast invasion, including attachment to and detachment from basement membranes followed by degradation of basement membrane components, utilize mechanisms very similar to those evolved in invasive tumor cells (Lala and Graham, 1990; Graham and Lala, 1992). Yet, the invasive behavior of trophoblasts is markedly different from that of tumor cells, considering that trophoblast invasion is a temporally and spatially regulated process as opposed to the uncontrolled invasiveness of tumors that lead to the malignant destruction of adjacent and distant tissues.

Trophoblast differentiation along the invasive pathway involves the coordinated expression of several classes of molecules, including adhesion receptors, ECM components, HLA-G, matrix degrading proteases and their inhibitors etc. Acquisition of the appropriate adhesion phenotype is critical to trophoblast invasiveness. Trophoblasts express various integrin adhesion receptors for interaction with basement membrane constituents such as laminin, collagen type IV and fibronectin. Human first trimester

cytotrophoblast stem cells in the villi express $\alpha 6 \beta 4$ integrin, a receptor for laminin. As these cells enter the cytotrophoblast cell columns, they downregulate the $\alpha 6 \beta 4$ integrin, and express primarily the fibronectin receptor $\alpha 5 \beta 1$. Cytotrophoblasts in the uterine wall express $\alpha 5 \beta 1$ and $\alpha 1 \beta 1$ integrins. The latter serves as a receptor for laminin and type IV collagen (Damsky et al., 1992). First trimester cytotrophoblasts differentiating along the invasive pathway *in vitro* recapitulate the pattern of their integrin switching observed *in situ*. Term cytotrophoblasts, however, which display greatly reduced invasive potential, are unable to upregulate their $\alpha 1 \beta 1$ expression, suggesting that this molecule is essential for trophoblast invasion. Invasion assays involving antibodies to integrin receptors or their ECM ligands indicated that interactions between laminin or collagen type IV and their $\alpha 1 \beta 1$ receptor promote, whereas fibronectin- $\alpha 5 \beta 1$ interactions inhibit trophoblast invasion (Damsky et al., 1994). The observation that laminin-trophoblast interactions promote invasion is consistent with laminin stimulation of trophoblast type IV collagenolytic activity (Emonard et al., 1990), which is known to be necessary for trophoblast invasiveness (Graham and Lala, 1992). Pathological evidence for the involvement of interactions between trophoblast integrin receptors and ECM components in trophoblast invasion has been obtained by Zhou et al. (1993) who demonstrated that upregulation of $\alpha 1 \beta 1$ integrin by invasive cytotrophoblasts fails to occur in preeclampsia, a condition characterized by decreased trophoblast invasion.

The distribution pattern of ECM ligands is also tightly regulated during trophoblast invasion (Damsky et al., 1992), and this, along with the coordinated expression of integrin

receptors provides a means for trophoblasts to up-, and downregulate their invasive potential. Trophoblasts themselves synthesize fibronectin (Queenan et al., 1987), laminin and type IV collagen (Autio-Harmainen et al., 1991), and during their migration they may even contribute to the formation of maternal cell-associated ECM, thus facilitating trophoblast-ECM interactions.

By analogy to tumor cells, trophoblasts appear to detach from basement membranes by reducing their adhesion to ECM components. Both tumor cells and invasive first-trimester trophoblasts express β 1-6 branched Asn-linked oligosaccharides on the cell surface, which serve to reduce adhesive cell-ECM interactions and thereby facilitate invasion. Hence expression of branched oligosaccharides is likely to be an important part of the invasive trophoblast phenotype (Yagel et al., 1989, 1990; Graham and Lala, 1992).

Another molecule linked to differentiation of cytotrophoblasts along the invasive pathway is HLA-G (McMaster et al., 1995). Expression of HLA-G *in vivo* is confined to invasive cytotrophoblasts of the cell columns and uterine wall. Consistent with this observation, HLA-G was exclusively produced by isolated cytotrophoblasts that invaded Matrigel (Collaborative Research Inc., Bedford, MA), an ECM substrate, in an *in vitro* invasion model. In addition, cytotrophoblasts purified from first-, second-, and third-trimester placentas respectively upregulated their HLA-G production when cultured on a thin layer of Matrigel, but the mRNA levels remained unchanged all over the culture period suggesting postranscriptional regulation. High levels of HLA-G mRNA were

detected in first- and second trimester cytotrophoblasts, whereas only trace amounts were found in term cytotrophoblasts committed at least *in vivo* to the noninvasive pathway of differentiation.

Breakdown of ECM barriers during trophoblast invasion depends on trophoblast-secreted proteolytic enzymes. In particular, urokinase-type plasminogen activator (u-PA) and matrix-degrading metalloproteinases appear to endow trophoblasts with degradative properties necessary for invasiveness (Graham and Lala, 1992). u-PA may cleave ECM components, including fibronectin (Gold et al., 1989), but it is not considered to be an efficient direct mediator of matrix degradation. By activating plasminogen, however, it can trigger a proteolytic cascade leading to the activation of metalloproteinases that directly degrade numerous matrix components (Matrisian, 1992). Moreover, it may proteolytically process the 72 kDa type IV collagenase (Keski-Oja et al., 1992). Plasmin may also degrade a variety of matrix proteins, including fibronectin, laminin, proteoglycans and type IV collagen (Liotta et al., 1981; Goldfarb et al., 1986; MacKay et al., 1990). Also, it can activate metalloproteinases, including interstitial procollagenase and prostromelysin (Matrisian, 1992). The matrix-degrading metalloproteinase (MMP) family plays important roles in processes requiring extensive ECM remodelling, such as embryonic development or tumor invasion (Matrisian, 1990). On the basis of substrate specificity metalloproteinases can be divided into three groups: the collagenases, gelatinases, and stromelysins (Matrisian, 1992). The collagenase group includes the interstitial collagenase (MMP-1) and neutrophil collagenase (MMP-5). While both

collagenases share the ability to degrade collagens I, II, and III, interstitial collagenase has somewhat wider specificity as it can also cleave collagens VII and X. Gelatinases are specific for denatured collagens (gelatins), elastin and collagen types IV and V. The gelatinase group has two members that have been designated under various names: (1) gelatinase A also known as 72 kDa type IV collagenase or MMP-2, and (2) gelatinase B (92 kDa type IV collagenase, MMP-9). The third group is the stromelysins that include stromelysin 1 (MMP-3), stromelysin 2 (MMP-10), and matrilysin (MMP-7). They all degrade a number of substrates, such as proteoglycans, fibronectin, laminin, gelatins, elastin and collagen IV.

1.4.4.1 Matrix degradation by the periimplantation blastocyst

It has long been known that blastocyst-associated proteases are essential for the success of implantation (Denker, 1977). Cultured mouse blastocysts and trophoblasts have been shown to secrete plasminogen activator, with a pattern of enzymatic activity that corresponds closely with the limited period of trophoblast invasiveness in vivo (Strickland et al., 1976). Trophoblast outgrowth by mouse blastocysts cultured on monolayers of decidual cells could be inhibited by plasminogen-depleted serum or nitrophenol-p-guanidino benzoate (NPGB) that prevented trophoblast plasminogen activation (Kubo et al., 1981). It has also been reported that implantation-defective mouse blastocysts produce less fibrinolytic activity than control blastocysts (Axelrod, 1985). Zymographic and Northern blot analyses of extracts of trophoblasts isolated from cultured blastocysts identified u-PA as the exclusive plasminogen activator synthesized by mouse trophoblasts

in vitro, and furthermore, expression of the u-PA gene has been demonstrated by invasive trophoblast cells in vivo (Sapino et al., 1989). The accumulated results thus appear to suggest that activation of plasminogen by trophoblasts is implicated in the process of implantation. Other reports, in turn, do not support the view that trophoblast-secreted u-PA has a major role in ECM degradation. Matrix degradation by mouse trophoblast outgrowths has not been significantly affected by plasminogen depleted serum or inhibitors of plasminogen activation (Glass et al., 1983; Behrendson et al., 1992), and in addition, u-PA and u-PA-R deficient mice can apparently implant normally (Carmeliet et al., 1994; Bugge et al., 1995).

Recently, cultured mouse blastocysts have been shown to express matrix-degrading metalloproteinases, including 92-kD gelatinase and stromelysin, and tissue inhibitors of metalloproteinases (TIMP-1,-2,-3) (Brenner et al., 1989; Behrendtsen et al., 1992; Harvey et al., 1995). Degradation of ECM by trophoblast outgrowths in blastocyst culture was inhibited by neutralizing antibodies to 92-kD gelatinase, and TIMP (Behrendtsen et al., 1992). These findings point to metalloproteinases as essential mediators of matrix degradation by trophoblast cells.

1.4.4.2 Expression of matrix-degrading proteinases and their inhibitors by human trophoblasts

Martin and Arias (1982) demonstrated the secretion of plasminogen activator by cultures of human first trimester trophoblasts, and Queenan et al. (1987) reported that the plasminogen activator activity measured in term trophoblast cultures was originated from

u-PA production. Zini et al. (1992) provided immunocytochemical evidence for the expression of u-PA by cultured term trophoblasts, and detected secreted u-PA, PAI-1 and PAI-2 antigens in trophoblast conditioned media. Furthermore, synthesis of PAI-1 and PAI-2 by cultured trophoblasts have been confirmed by immunocytochemistry and Northern blot hybridization (Feinberg et al., 1989). Cellular localization of plasminogen activator inhibitors have also been studied in vivo. PAI-1 was primarily localized in trophoblasts invading the decidua and myometrium, while only weak immunoreactivity was found in the villous syncytiotrophoblast, and cytotrophoblasts did not stain. PAI-2 staining was characteristic of the syncytium, but there was no obvious staining in villous cytotrophoblasts and extravillous invading trophoblasts. It thus appears that PAI-1 is a marker of invasive extravillous (intermediate) trophoblasts in vivo (Feinberg et al., 1989). Differentiation of cytotrophoblasts into syncytiotrophoblasts in culture is associated with gradually declining u-PA secretion, u-PA mRNA expression and u-PA binding. Urokinase receptors on cultured trophoblasts are saturated with endogenous prourokinase, which is converted into active two-chain urokinase by a mechanism largely, but perhaps not exclusively, dependent on the presence of plasminogen. First-trimester cytotrophoblasts express more urokinase receptors than cytotrophoblasts isolated from term placentas. In addition, the ability of first trimester cytotrophoblasts to generate plasmin appears to exceed that of term cytotrophoblasts (Zini et al., 1992). Urokinase receptors in vivo are strongly expressed by villous cytotrophoblasts and syncytiotrophoblast in the first and second trimesters, whereas their expression is markedly diminished at term.

Extravillous cytotrophoblasts expressed u-PAR predominantly at their leading edge consistent with a role for u-PA in directing plasminogen activation during trophoblast invasion (Mulhaupt et al., 1994). In conclusion, these findings taken together indicate that human trophoblasts express several elements of the plasminogen activation system in a developmentally regulated manner.

Several cell-associated and secreted gelatin-degrading metalloproteinases have been detected by zymographic analysis of human trophoblast cultures. Cytotrophoblasts derived from early or term placentas differed in their ability to express distinct types of these proteases. First trimester trophoblasts produced a complex array of metalloproteinases (Fisher et al., 1989; Bischof et al., 1991), whereas second- and third trimester trophoblasts displayed a simple pattern of expression (Fisher et al., 1989). Furthermore, first trimester trophoblasts produced significant 92 kD and 72 kD type IV collagenolytic activities. At term, the synthesis of the 92 kD type IV collagenase was greatly reduced, and only minimal 72 kD activity was detectable (Fisher et al., 1989; Shimonovitz et al., 1994). Secretion of the 92 kD type IV collagenase antigen (Librach et al., 1991) and the mRNA expression of both type IV collagenases (Shimonovitz et al., 1994) followed similar temporal pattern. There was also a fairly good correlation between the production of these gelatinases and temporal expression of invasiveness in vivo, suggesting that the 72 kD and 92 kD type IV collagenases are needed for trophoblast invasion. Distribution of metalloproteinases and their inhibitors has been recently mapped in the human placenta. Interstitial collagenase (Moll and Lane, 1990) and 72 kD type IV collagenase (Fernandez

et al., 1992) were localized in first-trimester trophoblasts of both villous and extravillous (intermediate) types. Polette et al. (1994) documented the expression of gelatinase A and B and their inhibitors by immunohistochemistry, Northern blot analysis and *in situ* hybridization. Gelatinase A (72 kD type IV collagenase) was primarily present in first-trimester intermediate trophoblasts. Gelatinase B was also expressed in villous trophoblasts of floating villi where it was found codistributed with TIMP-2. In term placentas, gelatinase B was no longer detected, whereas TIMP-2 expression continued until term.

1.4.4.3 Degradation and invasion of extracellular matrices by human cytotrophoblasts

Fisher et al. (1985) demonstrated that cytotrophoblast outgrowths of isolated first-trimester placental villi degraded extracellular matrices containing basement membrane proteins. By contrast, no evidence of matrix degradation was obtained if second- or third-trimester villi were tested. Consistent with these findings, isolated first trimester cytotrophoblasts also degraded the matrices on which they were cultured (Fisher et al., 1989; Bischof et al., 1991), but again degradation was not observed by cytotrophoblasts prepared from second- or third-trimester placentas (Fisher et al., 1989). Kliman and Feinberg (1990) reported, however, that both first- and third-trimester cytotrophoblasts displayed degradative activity depending on the ECM thickness.

Several authors assayed trophoblast invasiveness, employing *in vitro* invasion models, based on the ability of cytotrophoblasts to invade natural and artificial basement membranes (Yagel et al., 1988; Kliman and Feinberg, 1990; Librach et al., 1991; Graham

and Lala, 1991; Shimonovitz et al., 1994). The *in vitro* invasive capability of cytotrophoblasts isolated from placentas of various gestational ages is a question that has given rise to some controversy. While it is abundantly clear that first-trimester cytotrophoblasts are highly invasive (Kliman and Feinberg, 1990; Librach et al. 1991; Shimonovitz et al., 1994), there is no agreement on whether term cytotrophoblasts retain their invasive capacity *in vitro*. Librach et al. (1991) showed that term cytotrophoblasts did not penetrate an artificial basement membrane (Matrigel), whereas others pointed out that isolated cytotrophoblasts at term possess some invasive potential (Kliman and Feinberg, 1990; Graham and Lala, 1992; Shimonovitz et al., 1994).

Considerable progress has been made towards elucidation of the proteolytic mechanisms utilized by trophoblasts to invade basement membranes (Yagel et al., 1988; Lala and Graham, 1990; Librach et al., 1991; Graham and Lala, 1992). Yagel et al. (1988) examined the role of serine and metalloproteinases in mediating the invasiveness of the malignant human JAR choriocarcinoma and murine B16F10 melanoma lines and of normal trophoblast lines established from first-trimester human placentas. Normal trophoblasts and metastatic malignant cells demonstrated similar capacity for the invasion of an epithelium-free human amniotic membrane and had some invasion-promoting proteolytic mechanisms in common. Inhibitors of metalloproteinases (1,10-phenanthroline, TIMP) or serine proteases (aprotinin, epsilon aminocaproic acid, and an antiplasminogen antibody) almost completely blocked the invasiveness of both the normal trophoblast and malignant cell lines. The collagenase activator Mersalyl (Sigma, St. Louis, MO) enhanced

the invasion by all the three lines investigated, and this effect could not be prevented by simultaneous administration of aprotinin. Lala et al. (1990) also reported that collagenase activity was reduced in trophoblast cultures grown in plasminogen-deficient (serum-free) media and inhibited by aprotinin in the presence of serum.

Librach et al. (1991) showed that metalloproteinase inhibitors (TIMP and TIMP-2) and a neutralizing antibody to the 92 kD type IV collagenase completely abrogated the ability of first-trimester cytotrophoblasts to invade Matrigel. In contrast, an antibody that inhibits interstitial collagenase had no effect on cytotrophoblast invasiveness. Plasminogen activator inhibitors (PAI-1 and PAI-2), a neutralizing antibody to u-PA and aprotinin only partially (20-40%) inhibited invasion, whilst α_2 -antiplasmin had negligible effect. These results contrast those of Yagel et al. (1988) who reported a nearly complete blockade of cytotrophoblast invasion by inhibitors of the plasminogen activation system. In a review on the mechanisms of trophoblast invasion, Graham and Lala (1992) proposed that serum-free media lacking plasminogen may account for the moderate anti-invasive effect observed in the Matrigel system of Librach et al. (1991). In this case, small amounts of plasmin(ogen) remaining associated with the trophoblast membrane could only take part in the proteinase cascade.

Overall, the above studies suggest that a proteinase cascade involving metallo- and serine proteinases mediate trophoblast invasiveness and the 92 kD type IV collagenase is rate-limiting for invasion. Serine proteinases appear to play a role by turning on metalloproteinase activation, although direct stimulation of invasiveness by plasmin or u-

PA cannot be completely ruled out either.

1.4.4.4 Control of trophoblast invasiveness: role of factors modulating trophoblast proteinase activity

Mouse trophoblasts turned out to be more invasive when developed in non-pregnant uteri or extrauterine sites than in pregnant or pseudopregnant decidualized uteri (Kirby, 1965). Similarly, placenta accreta or ectopic pregnancy in humans, conditions with little or no decidual tissue, are associated with increased trophoblast invasion (Billington, 1971). These findings indicate that trophoblasts are intrinsically invasive and their invasiveness is under decidual control.

Conditioned media from first trimester human decidual cells (DCM) inhibited trophoblast invasiveness *in vitro* (Graham and Lala, 1991; Graham et al., 1993). This inhibition was completely prevented by either a neutralizing antibody to TGF- β or TIMP-1, and reproduced by TGF- β_1 alone. The same antibodies also stimulated invasiveness above control levels, suggesting that the decidua controls trophoblast invasiveness by producing TGF- β and TIMP-1 which are also elaborated, however, by trophoblasts themselves. Results from another laboratory (Bass et al., 1994) are in apparent contrast to those just quoted. Accordingly, TGF- β_1 had no effect in a Matrigel invasion assay. The reason for the lack of effect may lie in the relatively high TGF- β content of Matrigel (Vukicevic et al., 1992). TGF- β activity measurements demonstrated that decidual cells release latent TGF- β , while trophoblasts produce this growth factor in the active form. TGF- β_1 reduced collagenase type IV activity, and anti-TGF- β resulted in a significant

decrease in TIMP mRNA level as well as activity in first trimester trophoblast cultures. Moreover, TIMP activity was completely abrogated by addition of anti-TGF- β to decidual cultures. Thus, decidual and trophoblast derived TGF- β inhibits trophoblast invasion by inducing TIMP-1 production in both decidual cells and trophoblasts. TIMP-1, in turn, can directly inhibit type IV collagenases that are critical to trophoblast invasion. Consistent with these findings, TGF- β_1 upregulated the levels of both TIMP-1 and the 3.5 kb TIMP-2 messages in normal first trimester trophoblasts (Graham et al., 1994).

An additional mechanism through which TGF- β can exert its anti-invasive effect is downregulation of u-PA activity, as demonstrated by casein zymography of first trimester trophoblast-conditioned media (Graham et al., 1993, 1994). Whether this effect is due to upregulation of PAI-1 activity or downregulation of u-PA synthesis remains to be investigated. Moreover, the trophoblast plasminogen activation system may turn on the TGF- β -dependent, invasion-inhibiting pathway by plasmin-mediated activation of the decidual-derived latent TGF- β (Graham and Lala, 1992; Strickland and Richards, 1992). This may be one way by which trophoblasts can possibly interact with decidual cells to control their own invasiveness. Autocrine inhibition of invasion via production of endogenous TGF- β_1 also remains a possibility (Lala and Graham, 1990). TGF- β also inhibits trophoblast invasiveness by other mechanisms, including increased differentiation of invasive mononuclear trophoblasts into non-invasive multinuclear cells (Graham et al., 1992), and reduction of trophoblast migration via modulation of cell surface integrin expression (Irving and Lala, 1995). Whether these mechanisms are independent of the

TGF- β -induced downregulation of u-PA and type IV collagenase activities are not known and therefore await clarification.

In addition to TGF- β , several other maternally and fetally derived molecules have been shown to regulate trophoblast protease activity as well as invasiveness. Librach et al. (1994) reported that IL-1 β stimulated MMP-9 (92 kDa type IV collagenase) protein production and activity by first-trimester human cytotrophoblasts. It also enhanced cytotrophoblast invasion. LPS, which upregulated cytotrophoblast production of IL-1 β , mimicked these effects. Conversely, dexamethasone and cortisol decreased IL-1 β production and inhibited MMP-9 secretion and trophoblast invasiveness. These results suggest that trophoblast-derived IL-1 β may act as an autocrine mediator in controlling trophoblast invasiveness via modulation of pericellular metalloproteinase activity. As decidual production of IL-1 β is known (Romero et al., 1989; Kauma et al., 1990), paracrine stimulation of trophoblast invasiveness by IL-1 β is also highly probable.

During early murine development u-PA and MMP-9 are strongly expressed by invasive trophoblasts (Strickland et al., 1976; Sappino et al., 1989; Behrendtsen et al., 1992; Harvey et al., 1995). Trophoblast outgrowths of cultured mouse blastocysts on day 7 of development, which coincides with the period of trophoblast invasion of the uterus *in vivo*, responded to EGF or leukaemia inhibitory factor (LIF) stimulation by increased production of both u-PA and MMP-9 activities (Harvey et al., 1995). Uterine expression of EGF and LIF is triggered by maternal estrogen at the time of implantation (Huet-Hudson et al., 1990; Bhatt et al., 1991; Cross et al., 1994). It thus appears that the uterus

controls trophoblast proteinase expression and invasiveness by estrogen dependent secretion of LIF and EGF. This mechanism may turn out to be critical to implantation, considering that blastocysts of mice lacking a functional LIF gene fail to implant (Stewart et al., 1992), and mouse blastocysts having no EGF receptors die shortly after initiation of implantation (Harvey et al., 1995).

hCG treatment of human trophoblast cultures has been reported to suppress urokinase and general serine protease as well as the 92 kDa collagenase activities in conditioned media (Milwidsky et al., 1993; Yagel et al., 1993). In addition, nonphysiologically high concentrations of hCG or FSH, a structurally related glycoprotein hormone, also caused a partial direct inhibition of purified urokinase preparations. hCG-mediated inhibition of neither u-PA nor collagenase activities could be ascribed to a decreased synthesis of these enzymes or an increase in the synthesis of metalloproteinase inhibitors, because hCG had no effect on u-PA, collagenase or TIMP mRNAs and TIMP protein secretion. As hCG did not inhibit collagenase IV or plasmin preparations either, it has been suggested that hCG controls trophoblast metalloproteinase activity via a urokinase-mediated cascade that involves the generation of plasmin. In accordance with the proposed role of proteinases in invasiveness (Mignatti and Rifkin, 1993), hCG downregulation of enzyme activities was accompanied by inhibition of trophoblast invasion through amniotic basement membranes (Yagel et al., 1993).

Dexamethasone that inhibits trophoblast invasion and MMP-9 activity (Librach et al., 1994) also caused a dose-dependent decrease in plasminogen activator production by

first-trimester human trophoblast cultures (Martin and Arias, 1982). Therefore, plasmin-dependent control of trophoblast metalloproteinase activity and invasion by maternal glucocorticoids is a possible scenario *in vivo*.

Extracellular matrix (ECM) components are also involved in regulating trophoblast protease activity. Laminin and fibronectin are effective stimulators of uPA production in cultured mouse blastocysts, suggesting that trophoblast-ECM interactions may control trophoblast invasion by regulating PA activity (Zhang et al., 1996). Moreover, both malignant and normal first-trimester human trophoblasts increased their expression of interstitial and type IV collagenase activities when cultured on type I collagen or Matrigel containing various ECM proteins. Laminin, however, selectively increased type IV collagenase activity without having any effect on interstitial collagenase activity (Emonard et al., 1990).

1.4.5 Plasminogen activation in normal and preeclamptic pregnancy: role of the trophoblast

Plasma fibrinolytic activity decreases progressively during normal pregnancy, and is barely detectable at term (Bonnar et al., 1990; Åstedt, 1993). It rapidly returns, however, to non-pregnant values shortly after separation of the placenta, which suggests that this organ is essential for maintaining the depressed fibrinolytic activity in pregnancy plasma. A marked elevation in PAI activity, involving increased secretion of both PAI-1 and PAI-2, has been shown to account for this condition. The pregnancy-induced suppression of fibrinolytic activity may be viewed as a physiologic adaptation which,

however, contributes to the increased risk of venous thromboembolism in pregnancy.

Exaggerated suppression of fibrinolytic activity has been found in preeclampsia (Bonnar et al., 1971; Yoshimura et al., 1985), a common pregnancy-induced syndrome that endangers the lives of both the mother and fetus. Although it is recognized primarily by maternal hypertension and proteinuria, various maternal abnormalities, including those of the liver, brain, blood vessels, coagulation and fibrinolytic system, render the disease polymorphic (Weiner, 1988; Roberts, 1989; Bonnar et al., 1990; Redman, 1990). Preeclampsia occurs with high frequency in hydatidiform mole in which the uterus contains only disordered placental tissue. Removal of the placental tissue cures the disease. This implies that the fetus is not necessary for the disease to develop and suggests that preeclampsia is a trophoblastic disorder with secondary maternal manifestations (Redman, 1990, 1993). According to the current perception the most likely factor underlying the pathogenesis of preeclampsia is placental ischaemia secondary to structural abnormalities of maternal spiral arteries (Redman, 1993). In preeclampsia trophoblast invasion of the myometrial segments of the spiral arteries does not occur (Brosens et al., 1972; Gerretsen et al., 1981) or is deficient (Meekins et al., 1994). Trophoblast invasion may be incomplete in some decidual spiral arteries as well (Khong et al., 1986; Meekins et al., 1994). The partial failure in the conversion of the spiral arteries to uteroplacental vessels is believed to be a major cause of the reduced blood flow to the placenta in preeclampsia (Redman, 1993). The reason for this failure is obscure. It is known, however, that several components of the proteolytic cascade mediating

trophoblast invasiveness is abnormally altered in preeclampsia. Plasminogen activator activity is reduced in placental tissue (Yoshimura et al., 1985) and plasma (Bonnar et al., 1971) of women with preeclampsia. Furthermore, trophoblasts purified from placentas of preeclamptic patients secrete less activated MMP-9 and express reduced cell surface-associated PA activity compared to trophoblasts from normal placentas (Graham and McCrae, 1996). The diminished expression of trophoblast-associated protease activity may restrict trophoblast invasion and is likely to contribute to the widespread fibrin deposition seen in spiral arteries (Bonnar et al., 1990) and the placental villous tissue (Kanfer et al., 1996) in preeclampsia. Excess fibrin deposition may also reduce the uteroplacental blood flow thus causing placental ischaemia. Elevated PAI-1 activity and antigen levels have been found in both the plasma and placental extracts from women with preeclampsia (Estellés et al., 1989, 1994; Bonnar et al., 1990; Reith et al., 1993), suggesting that increased PAI-1 secretion is responsible for the excessive suppression of fibrinolytic activity. By contrast, plasma PAI-2 antigenic levels in preeclampsia are significantly lower than those in normal pregnancy (Reith et al., 1993; Estellés et al., 1994). In preeclampsia, increased production of PAI-1 has been localized in villous syncytiotrophoblasts which produced significantly less PAI-2 compared to syncytiotrophoblasts of placentas from normal pregnancies (Estellés et al., 1994). The reason for the altered synthesis of PAI is, at present, totally unknown. Elucidation of the mechanisms leading to abnormal regulation of the plasminogen activation system in

preeclampsia may bring us one step closer to understanding the pathogenesis of this severe disorder.

1.5 RATIONALE AND OBJECTIVES

The ability of human trophoblasts to invade the uterine wall and possibly regulate fibrin deposition at the sites of invasion and in the placental intervillous spaces appears to be linked to their plasmin generation. Plasminogen activation by trophoblasts must be exquisitely regulated to ensure proper realization of the trophoblast proteolytic potential critical to normal placental development and function. Abnormal regulation of trophoblast plasminogen activation may lead to disordered placentation such as in preeclampsia. Hence, a clear understanding of the normal function as well as regulation of trophoblast plasminogen activation is not only of physiological importance, but may also provide potential clues to elucidating the pathogenesis of this disorder. It is hypothesized that autocrine, paracrine and endocrine regulatory influences control the activation of plasminogen by trophoblasts.

The primary objectives of this thesis are:

- (1) to purify and culture a pure population of human term villous cytotrophoblasts that allows studying trophoblast plasminogen activation in the absence of contaminating cells,
- (2) to examine the expression of plasminogen activators and their inhibitors by trophoblasts *in vivo* and in culture, and
- (3) to define modulators of trophoblast plasminogen activation *in vitro*.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Materials for cell culture

Trypsin, DNase and bovine serum albumin (BSA) were purchased from Sigma Chemical Company (St. Louis, Missouri). Dulbecco's Modified Eagle Media, Dulbecco's Phosphate-Buffered Saline, Hanks' Balanced Salt Solutions (HBSS), HEPES, penicillin, streptomycin, kanamycin, and Fungizone were purchased from Gibco (Burlington, Ontario). Fetal bovine serum was obtained from Cansera (Rexdale, Ontario). A microcarrier spinner flask and magnetic stirrer were obtained from Bellco (Vineland, New Jersey). A stainless steel sieve (104 μm) and 40 μm nylon mesh for vacuum filtration were supplied by Sigma Chemical Company (St. Louis, Missouri) and Small Parts Inc. (Miami Lakes, Florida), respectively. Percoll, density marker beads and peristaltic pump were supplied by Pharmacia (Baie D'urfé, Québec). Tissue culture plates, dishes, tubes and Thermanox coverslips were from Nunc (distributed by Gibco, Burlington, Ontario). Square and round glass coverslips, and culture slides were supplied by Fisher (Dartmouth, Nova Scotia).

2.1.2 Antibodies

Monoclonal mouse antibodies to cytokeratin, vimentin, CD68, HLA-ABC, HLA-DR, and negative control mouse IgG1 and IgG2a were purchased from DAKO (distributed by Dimension Laboratories, Mississauga, Ontario). Swine anti-rabbit immunoglobulins, rabbit PAP, normal rabbit immunoglobulins as well as polyclonal rabbit antibodies to

alpha-1-antichymotrypsin (ACT), beta-2-microglobulin, human placental lactogen (hPL), and human chorionic gonadotropin (hCG) were also supplied by DAKO (distributed by Dimension Laboratories, Mississauga, Ontario). Normal horse serum, biotinylated anti-mouse IgG, biotinylated anti-rabbit IgG, avidin, and biotinylated HRP were from Vector Laboratories (distributed by Dimension Laboratories, Mississauga, Ontario). Normal goat immunoglobulin, antibodies to PAI-1, PAI-2, UK, t-PA were provided by American Diagnostica (Greenwich, Connecticut) and Biopool (Burlington, Ontario). Anti-human EGF neutralizing antibody was obtained from R&D Systems Inc. (Minneapolis, MN). Dynabeads M-450 coated with goat anti-mouse IgG was purchased from Dynal (distributed by Harlan Bioproducts for Science Inc., Indianapolis, Indiana).

2.1.3 Fibrinolysis and coagulation products

Pro-urokinase (scu-PA), activated human protein C (APC), recombinant PAI-1, recombinant PAI-2, and Imubind PAI-1 ELISA kits were purchased from American Diagnostica (Greenwich, Connecticut). Human Glu-plasminogen was obtained from Biopool (Burlington, Ontario). H-D-Valyl-L-Leucyl-L-Lysin-p-Nitroanilin (S-2251) was supplied by Helena Laboratories (Mississauga, Ontario). Human α -thrombin was purchased from Sigma Chemical Company (St. Louis, Missouri).

2.1.4 Miscellaneous chemicals

Human recombinant transforming growth factor- α (TGF- α) and insulin-like growth factor II were obtained from Gibco (Burlington, Ontario). Human recombinant interleukin 1 beta (IL-1 β), transforming growth factor beta 1 (TGF- β_1) were purchased from R&D

Systems Inc. (Minneapolis, Minnesota). Epidermal growth factor (EGF), phorbol 12-myristate 13-acetate (PMA), dexamethasone, human chorionic gonadotropin (hCG), 8-bromoadenosine 3':5'-cyclic monophosphate (8-bromo-cAMP), forskolin, lipopolysaccharide (LPS) from *E. coli* 0111:B4, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and protease type XIV were supplied by Sigma Chemical Company (St. Louis, Missouri). hCG MAIA clone kits were purchased from Serono (distributed by NCS Diagnostics Inc., Etobicoke, Ontario). Tritiated methyl-thymidine was obtained from ICN (Mississauga, Ontario). Filters for cell harvesting were supplied by Skatron (Sterling, Virginia). Liquid scintillation cocktail (Ready Protein+) was purchased from Beckman (Mississauga, Ontario).

2.2 METHODS

2.2.1 Isolation and culture of cytotrophoblasts

2.2.1.1 Mechanical processing of the placental tissue

Fifty-six normal human term placentas were obtained from uncomplicated Cesarean sections or occasional vaginal deliveries. Small blocks of villous tissue dissected from the softest, thickest portions of cotyledons, carefully avoiding the chorionic plate, decidua basalis and fibrous areas, were gently pressed and washed in D-PBS several times to remove as much blood as possible. Finally, the tissue blocks were coarsely chopped into 3-5 mm pieces.

2.2.1.2 Enzymatic disaggregation

Digestion of the chopped villous tissue was performed using the procedure of

Kliman et al. (1986) to which some minor modifications were added. About 80 g of chopped tissue was transferred into a microcarrier spinner flask (Bellco) with 300 ml of Ca^{2+} and Mg^{2+} free HBSS, containing 25 mM HEPES, pH 7.4, 100 units/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin, 100 $\mu\text{g/mL}$ kanamycin, 2.5 $\mu\text{g/mL}$ Fungizone, 13000 units/mL trypsin, and 300 Kunitz units/mL DNase. The mixture was incubated at 37 °C for 30 min under continuous stirring at 40 rpm on a Bellco μ -carrier magnetic stirrer. An additional 30000 Kunitz units of DNase were added after the initial 10 min incubation. After tissue fragments were allowed to settle for 1 min, the supernatant was carefully decanted into 50 mL plastic centrifuge tubes containing 5 mL FCS and mixed by inversion. The tissue fragments were resuspended in 100 mL of HBSS, and the supernatant was obtained as before. Next, the supernatants were centrifuged at 1000 x g for 10 min. Finally, the pellet was resuspended in DMEM containing 20% FCS and allowed to stand until the remaining two digestions were completed. The technique of the second and third digestions was essentially the same as that described above, except that the volume of the trypsin/DNase mixture was adjusted to 200 and 150 mL, respectively, and no extra DNase was admixed following the initial 10 min incubation. The resuspended pellets from all three digestions were filtered through a 104 μm stainless steel sieve (Sigma) and then through a 40 μm nylon mesh (Small Parts Incorporation) by vacuum filtration.

2.2.1.3 Percoll density gradient centrifugation

Separation of cytotrophoblasts at their buoyant densities was performed as reported by Kliman et al. (1986). The placental cell suspension obtained by enzymatic

disaggregation was spun at 1000 g for 10 min. The pellet was resuspended in HBSS to a final volume of 8 mL and then the suspension was divided into two 4 mL aliquots. Each aliquot was carefully loaded onto a preformed, discontinuous Percoll gradient in a 50 mL plastic tube. The gradient was prepared by layering, using a peristaltic pump, 3 mL each of 70% to 5% Percoll diluted with HBSS from 90% Percoll (9 parts (v/v) of Percoll mixed with 1 part (v/v) 10x HBSS) in 5 % gradations. The gradients loaded with the cell suspension were centrifuged at 1200 x g for 20 min and the cells forming a band at densities between 1.051 and 1.063 g/mL were collected. Densities were determined by running an identical gradient loaded with color-coded density marker beads. The collected cells were diluted 5 times in culture medium (DMEM supplemented with 100 units/mL penicillin, 100 µg/mL streptomycin, 100 µg/mL kanamycin, 2.5 µg/mL Fungizone and 20% FCS) and pelleted at 1000 g. The cells were washed once more and kept resuspended in culture medium until further purification.

2.2.1.4 Immunomagnetic separation of villous cytotrophoblasts

Percoll-purified cytotrophoblasts were further purified by immunomagnetic cell separation using a modification of the method of Douglas and King (1989). Non-trophoblastic elements were removed by incubating the cells with anti-HLA antibodies bound to secondary antibody-coated magnetic beads. Briefly, to a mixture of 10 µL (0.94 µg) of monoclonal anti-HLA-ABC (DAKO) and 20 µL (1.06 µg) of anti-HLA-DR (DAKO) antibodies 40 µL (1.6×10^7 beads) Dynabeads M-450 Goat anti-Mouse IgG (DYNAL) were added in sterile microcentrifuge tubes. Control tubes received 9.4 µL

(0.94 μg) mouse IgG2a (DAKO) and 10.6 μL (1.06 μg) mouse IgG1 (DAKO) negative control antibodies in place of the anti-HLA antibodies. The antibody mixtures were incubated for 4 hours at room temperature under rotation for even mixing. The beads were collected with a Dynal Magnetic Particle Concentrator and then washed 4 times for 30 min with Ca^{2+} and Mg^{2+} free HBSS supplemented with 100 units/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin, 100 $\mu\text{g/mL}$ kanamycin, 2.5 $\mu\text{g/mL}$ Fungizone and 1% heat-inactivated FCS (complete HBSS). The preformed antibody-bead complexes were stored in 250 μL of complete HBSS at 4 $^{\circ}\text{C}$ until use. Percoll-purified cytotrophoblasts were washed once with cold complete HBSS and the cell concentration was adjusted to $1.7 \times 10^7/\text{mL}$. Two hundred and fifty μL (4×10^6) cells were then mixed with 250 μL preformed antibody-bead complexes (4:1 bead to cell ratio) and incubated on ice for 45 min under occasional gentle mixing by inversion. Villous cytotrophoblasts were collected by aspirating the supernatant while the microcentrifuge tubes were in the Magnetic Particle Concentrator. Finally, the cells were spun at 400 x g and resuspended in culture medium.

2.2.1.5 Primary trophoblast culture

Cytotrophoblasts were plated out at various densities into 96-well Nunclon plates, 24-well Nunclon plates with or without Thermanox coverslips/12 mm round glass coverslips (Fisher), 35 mm Nunclon culture dishes with 22 x 22 mm glass coverslips or Fisher SuperCell Culture Slides. The cells were cultured in DMEM supplemented with 100 units/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin, 100 $\mu\text{g/mL}$ kanamycin, 2.5 $\mu\text{g/mL}$

Fungizone and 20% FCS in humidified 5% CO₂ - 95% air at 37 °C for up to 14 days.

The culture medium was replaced every 24 hours.

2.2.2 Immunocytochemical procedures

2.2.2.1 Immunocytochemical staining of placental tissue sections and trophoblast cell pellets

Small pieces of intact or trypsin/DNase digested placental tissue as well as pellets of Percoll-purified cytotrophoblasts were fixed in 4% paraformaldehyde with 0.2% picric acid in 0.1 M sodium phosphate buffer [pH 7.4] or Bouin's fluid (75 mL 1% picric acid, 25 mL formalin, 5 mL glacial acetic acid) for 48 hours at 4 °C. After intensive washing in 0.1 M sodium phosphate buffer [pH 7.4], the specimens were dehydrated and embedded in paraffin. Sections cut at 4-5 µm thicknesses were deparaffinized and hydrated through decreasing concentrations of ethanol to deionized water prior to immunocytochemistry. Cryostat sections were cut at 5 µm from placental tissue snap frozen in petroleum ether in a bath of acetone cooled with solid carbon dioxide. After thawing, sections were fixed in acetone for 5 min at room temperature (RT), washed in PBS and then subjected to immunocytochemistry.

For single immunolabelling, sections were stained using the avidin-biotin complex (ABC) immunoperoxidase technique (Hsu et al., 1981). Endogenous peroxidase was inhibited by treating sections with 0.6% H₂O₂ in methanol for 15 min at RT. After washing several times in PBS (0.01 M sodium phosphate buffer containing 0.9% NaCl [pH 7.4]), dependent on the antigen to be localized, the staining intensity and number of

labelled cells were increased by incubation with 0.025% protease type XIV (Sigma) for 15 min at RT. Subsequently, sections were washed and incubated with 5% normal serum, obtained from the species in which the secondary antibody was raised, for 30 min at RT to block nonspecific binding of immunoglobulins. After blotting excess serum, sections were incubated with the primary antibody, diluted in PBS containing 1% normal serum, for 60 min at RT or 24-48 hours at 4 °C. Sections were washed again and incubated with 5 µg/mL biotinylated secondary antibody for 30 min at RT, followed by the Vectastain ABC reagent (Vector) for 60 min at RT. The ABC reagent was prepared by mixing reagent A (avidin) with reagent B (biotinylated HRP) as suggested by the manufacturer. After washing, the peroxidase reaction was developed with 0.05 M Tris-buffered saline [pH 7.6] containing 0.02-0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB), 0.04% nickel chloride and 0.01% H₂O₂. This technique produced a strong blue/black precipitate. To obtain a different brown reaction product the nickel chloride component was omitted from the substrate solution. Finally, sections were washed in deionized water, counterstained for 15 min with 0.25% methyl green when necessary, dehydrated, cleared in xylene and mounted in Permount.

For double immunocytochemistry, the first antigen was localized by means of the ABC immunoperoxidase method, as described above, employing monoclonal mouse primary antibodies. DAB-Ni was used as peroxidase substrate. After washing in deionized water, the second antigen was detected with the peroxidase-antiperoxidase (PAP) method (Steinberger, 1974) as follows. First, peroxidase activity was quenched

with 0.6% H_2O_2 in methanol for 15 min at RT. Sections were then incubated for 30 min with 5% normal serum obtained from the species in which the secondary antibody was raised. After blotting excess serum, sections were incubated with rabbit primary antibody, diluted in PBS containing 1% normal serum, for 60 min at RT or 24 to 48 hours at 4 °C. Next, sections were washed in PBS and incubated with the secondary antibody in large excess for 30 min. After washing, sections were incubated with the peroxidase-antiperoxidase (PAP) complex diluted at 1:100 in PBS containing 1% normal serum. After sections had been washed, the peroxidase reaction was developed as before, using DAB as chromogen. Finally, sections were washed in deionized water, counterstained with 0.25% methyl green for 15 min, dehydrated in n-butanol, cleared in xylene and mounted in Permount.

In all cases, immunocytochemical control experiments included replacement of the primary antibody with normal immunoglobulin of the same isotype and concentration, using adjacent sections. For double immunocytochemistry, several additional controls were employed to determine if false positive double labelling had occurred, due to nonspecific color mixing or cross-reactions between the 1st and 2nd reaction sequences. These included (1) incubation of sections with methanol/ H_2O_2 , followed by DAB/ H_2O_2 after the first reaction had been completed; this sequence had been designed to exclude the possibility that some peroxidase activity remained available before starting the second reaction, (2) testing the possibility that the 2nd secondary antibody cross-reacts with the 1st primary antibody; to this end, sections were sequentially incubated with the 1st

primary antibody, 2nd secondary antibody, PAP and DAB/H₂O₂, (3) testing if cross-reactivity exists between the 2nd primary antibody and 1st secondary, by sequentially incubating with the 2nd primary antibody, 1st secondary antibody, ABC reagent, DAB/H₂O₂, (4) testing the possibility of cross reactions between the secondary antibodies as well as between the 1st secondary antibody and PAP; this was performed by incubating in the order: 1st primary antibody, 1st secondary antibody, 2nd secondary antibody, PAP, DAB/H₂O₂, and (5) sequential incubations by the 1st primary antibody, 2nd secondary antibody, PAP and DAB/H₂O₂ to exclude possible cross-reactivity between the 2nd secondary antibody and PAP.

2.2.2.2 Immunocytochemical staining of trophoblast cultures

Trophoblast cultures were washed twice in PBS and fixed in either acetone/methanol (1:1) for 10 min at RT or Bouin's fluid for 24 hours at 4 °C. After washing several times in PBS, acetone/methanol fixed cultures were treated with 0.6% H₂O₂ in PBS for 15 min to block endogenous peroxidase. Cultures fixed in Bouin's fluid were first permeabilized with 0.2% (v/v) Triton X-100 in PBS for 10 min, washed again and subsequently exposed to 0.6% H₂O₂ in PBS for 15 min. All cultures were immunostained by means of the ABC method as described earlier. Counterstaining was performed using 0.25% methyl-green or Mayer's hematoxylin for 15 min. Finally, cultures were washed, dehydrated, cleared in xylene and mounted in Permount. Control experiments were done as described before.

2.2.2.3 Ultrastructural immunocytochemistry

Placental tissue samples were fixed in 4% paraformaldehyde, 0.2% glutaraldehyde in 0.1 M sodium phosphate buffer [pH 7.4], washed several times with 0.1 M sodium phosphate buffer [pH 7.4], dehydrated in graded alcohols and embedded in Lowicryl. Ultrathin sections were mounted on nickel grids, washed in PBS and incubated with 5% normal goat serum in PBS for 15 min. Grids were then transferred onto drops of the primary antibody diluted in PBS containing 1% normal goat serum and incubated overnight at 4 °C. After washing, sections were incubated for 60 min at RT with goat anti-mouse IgG labelled with colloidal gold particles 10 nm in size. Sections were washed again, exposed to osmium vapor, contrasted with uranyl acetate and lead citrate and viewed under a Philips EM300 electron microscope.

2.2.3 [³H]thymidine incorporation

Cytotrophoblasts (1.6×10^4 in 200 μ L aliquots) were cultured in Nunc 96-well flat-bottomed plates for various periods of time. The culture medium was changed every 24 hours. Six hours before concluding the assay, 10 μ L culture medium containing 1 μ Ci [methyl-³H]thymidine (ICN) was added to each well. At the end of the incubation the cultures were washed with Ca²⁺ and Mg²⁺ free HBSS and then incubated with 0.25% trypsin-1mM EDTA at 37 °C for 30 min. Cells were harvested on microglass filters (Skatron) using a Skatron cell harvester. The filters were airdried at room temperature for a minimum of 2 hours and placed in Beckman Mini Poly-Q vials. To each vial, 5 mL of scintillation cocktail (Ready Protein+, Beckman) was added. Finally, radioactivity was

counted on a Beckman LS3801 liquid scintillation counter.

2.2.4 MTT assay

This was based on the method originally described by Mosmann (1983) and developed further by Denizot and Lang (1986) and Hansen et al. (1989). Cytotrophoblasts were cultured at 2×10^5 /100 μ L/well in 96-well culture plates as described above. The cultures were washed twice with indicator-free DMEM containing 100 units/mL penicillin, 100 μ g/mL streptomycin, 100 μ g/mL kanamycin, 2.5 μ g/mL Fungizone and 1 mg/mL BSA. To each well, 100 μ L of the same medium and then 25 μ L of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dissolved in PBS (0.01 M sodium phosphate [pH 7.4], 0.9% NaCl) at 5 mg/mL were added and the cultures were incubated for 2 hours. After washing with PBS, each well received 100 μ L of lysis buffer (20% (v/v) SDS in a mixture of 50% (v/v) of each DMF and deionized water [pH 4.7]). The MTT formazan reaction product was completely solubilized by incubating the plates overnight at 37 °C. Absorbances were measured at 595 nm using an automated microplate reader (Bio-Rad).

2.2.5 Quantitation of hCG

Supernatants of trophoblast cultures were assayed for hCG using Serono hCG MAIAclone kits. Briefly, 50 μ L of hCG standards and appropriately diluted supernatant samples were pipetted into 12 x 75 mm round-bottom polystyrene tubes (Falcon). To each tube, 500 μ L of 125 I anti-hCG Reagent containing fluorescein and iodine-125 labelled mouse monoclonal antibodies to hCG was added and the tubes were incubated at 37 °C

for 15 min. Subsequently, 200 μ L of sheep antiserum to fluorescein covalently bound to magnetic particles were added. After 5 min incubation at room temperature, the hCG-monoclonal antibody-second antibody complex was sedimented in a MAIA Magnetic Separator, washed once and the radioactivity of the bound fraction was measured for 1 min in a gamma counter (LKB 1277 Gammamaster). Results were obtained in mIU/mL, using the automated data reduction program provided for the gamma counter.

2.2.6 Measurement of PAI-1 antigen

The concentration of PAI-1 in trophoblast conditioned medium were measured by ELISA, using Imubind PAI-1 ELISA kits (American Diagnostica). This ELISA utilizes two different monoclonal antibodies for capture and detection, respectively. The assay was performed as suggested by the manufacturer. Briefly, flat-bottomed Nunc Maxisorp microtitre plates were first incubated with (1) a monoclonal mouse antibody to PAI-1. This and all subsequent incubations were followed by washing to remove excess unreacted reagents. After further incubations with (2) conditioned medium, (3) biotinylated monoclonal mouse anti-human PAI-1 (detection antibody) and (4) horseradish peroxidase conjugated streptavidin, the peroxidase reaction was developed by orthophenilenediamine/ H_2O_2 substrate solution. The reaction was stopped by 1 M H_2SO_4 . Absorbance was read at 492 nm, using an automated microplate reader (Bio-Rad).

2.2.7 Measurement of trophoblast-associated plasminogen activation

2.2.7.1 Plasminogen activation assay

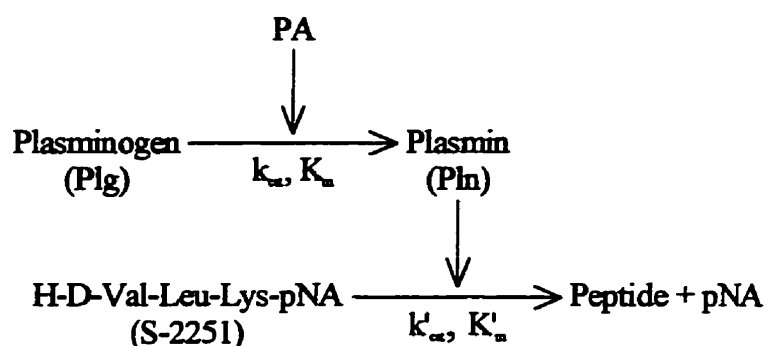
Trophoblast-mediated activation of plasminogen was measured using an adaptation

of the method of Zini et al. (1992). Villous cytotrophoblasts were plated at 2×10^5 into Nunc 96-well plates and cultured in DMEM supplemented with 100 units/mL penicillin, 100 $\mu\text{g/mL}$ each of streptomycin and kanamycin, 2.5 $\mu\text{g/mL}$ Fungizone and 20% FCS. Trophoblast cultures at 36 to 72 hours were then washed twice with indicator- and serum-free DMEM containing 1 mg/mL BSA as well as antibiotics and Fungizone (hereafter called serum-free medium) at concentrations indicated above. Hydrolysis of S-2251 was determined in quadruplicate under various conditions. All reagents were diluted in serum-free medium prior to the assay and filter-sterilized. For measurement of total trophoblast-mediated hydrolysis of S-2251, each well received (1) 50 μL of serum-free medium with or without modulator, (2) 100 μL of 0.8 mM S-2251, and (3) 50 μL of 100 $\mu\text{g/mL}$ Glu-plasminogen. For measurement of trophoblast-mediated plasminogen-independent hydrolysis of S-2251, each well received (1) 50 μL of serum-free medium with or without modulator, (2) 100 μL of 0.8 mM S-2251, and (3) 50 μL of serum-free medium. Spontaneous hydrolysis of S-2251 was determined by adding into empty wells (1) 100 μL of serum-free medium, followed by (2) 100 μL of 0.8 mM S-2251. To assess plasmin activity contaminating the purified plasminogen preparation, empty wells received (1) 50 μL of serum-free medium, (2) 100 μL of 0.8 mM S-2251, and (3) 50 μL of 100 $\mu\text{g/mL}$ Glu-plasminogen. After plasminogen activation had been initiated by adding plasminogen (time zero), cells were continued to culture under assay conditions up to an additional 48 hours in humidified 5% CO_2 - 95% air at 37 °C. Absorbance readings (at 405 nm) were taken regularly at 4 to 8 hours using an automated microplate reader (Bio-Rad).

2.2.7.2 Analysis and kinetic treatment of data

The absorbance value representing plasminogen-dependent hydrolysis of S-2251 (catalyzed by plasmin generated during activation of exogenous plasminogen) was calculated by subtracting the absorbance due to plasminogen-independent (catalyzed by various trophoblast-secreted proteases and by plasmin present at time zero) hydrolysis and plasmin contamination of the plasminogen preparation from the total hydrolysis measured. Subtraction of the absorbance resulting from plasminogen-independent hydrolysis is based on the assumption that plasminogen activation and plasminogen-independent hydrolysis of S-2251 are simply independent and additive.

In the plasminogen activation assay, pNA is produced through a cascade of reactions, consisting of conversion of plasminogen to plasmin by the trophoblast plasminogen activator (PA) and plasmin-catalyzed hydrolysis of S-2251.



Assuming that both reactions above obey Michaelis-Menten kinetics, rate equations for

plasmin (1) and pNA (2) are described as follows:

$$(1) \quad \frac{d[Pln]}{dt} = \frac{k_{cat}[PA]}{1 + \frac{K_m}{[Plg]}}$$

$$(2) \quad \frac{d[pNA]}{dt} = \frac{k'_{cat}[Pln]}{1 + \frac{K'_m}{[S-2251]}}$$

If $[S-2251] \gg K'_m$, equation (2) can be written as

$$(3) \quad \frac{d[pNA]}{dt} = k'_{cat} [Pln]$$

Equation (3) demonstrates that plasmin concentration is directly proportional to the initial rate of hydrolysis of S-2251, i.e. plasmin activity. The rate of plasmin generation (plasminogen activation rate), in turn, is proportional to the acceleration of the appearance of pNA as shown by

$$(4) \quad \frac{d^2[pNA]}{dt^2} = k'_{cat} \frac{d[Pln]}{dt}$$

Integration of equation (1) yields

$$(5) \quad [Pln] = \frac{k_{cat}[PA]}{1 + \frac{K_m}{[Plg]}} t + [Pln_0]$$

where $[Pln_0]$ is the concentration of plasmin at time zero. Substituting $[Pln]$ from equation

(5) into equation (3) gives

$$(6) \quad \frac{d[pNA]}{dt} = \frac{k'_{cat}k_{cat}[PA]}{1 + \frac{K_m}{[Plg]}} t + k'_{cat} [Pln_0]$$

On integrating equation (6), concentration of pNA is obtained at any time as shown by

$$(7) \quad [pNA] = \frac{k'_{cat}k_{cat}[PA]}{2(1 + \frac{K_m}{[Plg]})} t^2 + k'_{cat}[Pln_0]t + pNA_0$$

where $[pNA_0]$ is the concentration of pNA at time zero.

When analyzing the experimental data, progress curves (time-absorbance curves) of pNA accumulation were constructed by fitting polynomials (degrees 1 to 6) to sets of experimental data representing the mean and S.E. of quadruplicate cultures, using a non-linear curve fitting computer program (Sigmaplot, Jandel). Curve fitting was performed only on early datapoints obtained before depletion of the substrates became significant. Plasmin concentrations (plasmin activities) were given as the first time derivative of the absorbance (dA/dt). The rates of plasmin generation (equivalent to PA activity) were calculated as the second time derivative of the absorbance (d²A/dt²) and expressed as percentage of the rate in control cultures. In all cases, the results shown were from one placenta and are representative of similar experiments performed in cultures from two or three placentas.

CHAPTER 3

ISOLATION, CHARACTERIZATION AND CULTURE OF VILLOUS

CYTOTROPHOBLASTS FROM HUMAN TERM PLACENTAS

3.1 INTRODUCTION

Trophoblast cell cultures obtained by disaggregation of placental tissue provide the potential of manipulating homogenous trophoblast populations. Although a variety of techniques have claimed success in isolation of human cytotrophoblasts (reviewed later in this chapter), the purification procedures employed often suggest that a heterogenous cell population was isolated, which contained not only cytotrophoblasts but also non-trophoblastic contaminating cells. An additional problem is that the isolated cytotrophoblasts themselves are also likely to be heterogenous in nature. Cellular contamination and heterogeneity of purified cytotrophoblast populations may lead to erroneous assignments of specific trophoblast functions.

The objective was to develop a procedure for purification and culture of a pure population of human villous cytotrophoblasts. Non-trophoblastic contaminants, such as macrophages, fibroblasts and endothelial cells, are known to produce constituents of the plasminogen activation system as well as various biologically important molecules, including ECM components, cytokines and growth factors, which may potentially obscure the evaluation of plasminogen activation by trophoblasts. It was thus felt imperative to eliminate contaminating cells as much as possible. Human villous cytotrophoblasts were obtained using a modification of the method of Kliman et al. (1986) and Douglas and King (1989), respectively. As described in Chapter 2, cytotrophoblasts were isolated from

human term placentas by mechanical and enzymatic dissociation of the placental tissue, followed by Percoll density gradient centrifugation and immunomagnetic separation employing monoclonal antibodies to monomorphic determinants of HLA class I and class II antigens.

3.2 RESULTS

3.2.1 Immunocytochemical identification of various cell types in the placenta

A variety of antigens were localized in the chorionic villous tissue. Table 3.1 summarizes the immunocytochemical characteristics of various cell types in human term placental villi. A monoclonal antibody to low molecular weight cytokeratins labelled both the syncytium and cytotrophoblasts of placental villi. The staining intensity was significantly increased by protease XIV pretreatment. In addition to the trophoblast epithelium in the placental villi, numerous cytokeratin positive extravillous cytotrophoblasts were observed in cell islands connected to the villous tree. Furthermore, the wall of larger blood vessels and some stromal elements in stem villi were also stained (Fig. 3.1a, b, c).

Distribution of vimentin immunoreactivity was in sharp contrast to that of cytokeratin positive cells. Virtually all cells in the central mesenchymal core of the placental villi were immunoreactive, including vascular endothelial and smooth muscle cells, and placental macrophages (Hofbauer cells). The trophoblastic epithelium, however, remained completely devoid of staining (Fig. 3.2a, b).

Immunocytochemical staining for HLA antigens demonstrated that all villous

Table 3.1. Immunocytochemical characterization of various cell types in placental villi

	Syncytium	Cyto- trophoblast	Endothelial cell	Smooth muscle cell	Macrophage
Cytokeratin	+	+	-	±	-
Vimentin	-	-	+	+	+
HLA-ABC	-	-	+	+	+
β-2-micro- globulin	-	-	+	+	+
HLA-DR	-	-	-	-	+
hCG	+	-	-	-	-
hPL	+	-	-	-	-
ACT	±	-	-	-	+
CD68	-	-	-	-	+

+ positive, - negative, ± inconsistently positive

stromal cells expressed HLA-ABC and beta-2-microglobulin, whereas HLA-DR immunoreactivity was localized only in Hofbauer cells. The trophoblast epithelium did not display any staining (Fig. 3.3).

Most of the syncytium displayed hCG immunoreactivity, but the staining intensity varied to a great extent (Fig. 3.4a, b). A strong uniform syncytial staining was obtained using a polyclonal antibody to hPL (Fig. 3.5a). No staining was observed for either hCG or hPL in cytotrophoblasts and the villous stroma.

A polyclonal antibody to α -1-antichymotrypsin (ACT) reacted strongly with Hofbauer cells (Fig. 3.6a, b). The apical surface of the syncytium was usually positive as well. Another macrophage associated antigen (CD68) was localized exclusively in Hofbauer cells (Fig. 3.7a, b), using two different monoclonal antibodies to CD68. Both antibody KP1 (Pulford et al., 1989) and EBM/11 (Kelly et al., 1988) reacted weakly and inconsistently with Hofbauer cells. However, the number and staining intensity of KP1 labelled cells increased dramatically after protease XIV treatment of paraffin sections. Protease pretreatment did not appreciably improve staining results obtained by antibody EBM/11. CD68 immunoreactivity in Hofbauer cells was also studied at the electron microscopic level, using immunogold technique. These studies revealed that CD68 is associated with lysosomal-like intracytoplasmic granules in these cells (Fig. 3.8). Finally, double immunocytochemistry demonstrated that CD68 and ACT are colocalized in the same macrophage population (Fig. 3.9). Some cells, however, stained positively only for ACT.

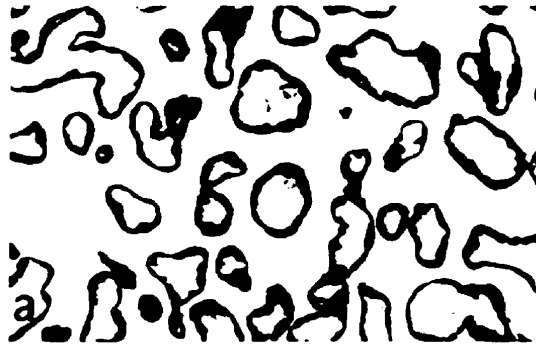
3.2.2 Characterization of Percoll purified cells

Immunocytochemical hPL staining of the finely chopped chorionic villous tissue revealed that the trophoblast epithelium of the placental villi almost completely dissociated 30 min after trypsin-DNase treatment (Fig. 3.5b). The denuded appearance of the villi suggested that both cytotrophoblasts and mesenchymal elements were released.

Following enzymatic disaggregation, the resultant placental cell suspension was subjected to discontinuous Percoll density gradient centrifugation. Three major bands were obtained in the gradients (Fig. 3.10). A wide band containing cellular debris but not viable cells was located at the top of the gradients. Another band containing red blood cells was invariably found at the bottom. In the middle of the gradient (between densities 1.051 and 1.063 g/mL), there was a band containing 5×10^7 - 2×10^8 cells/80 g villous tissue. Cells obtained from this band were 90-95% viable as assessed by trypan blue exclusion.

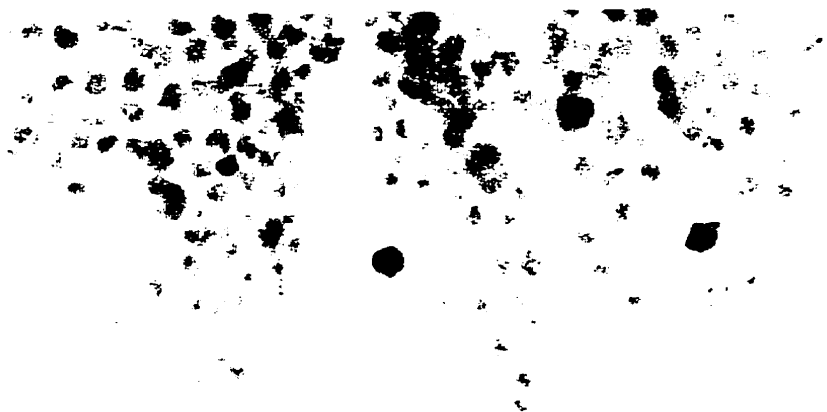
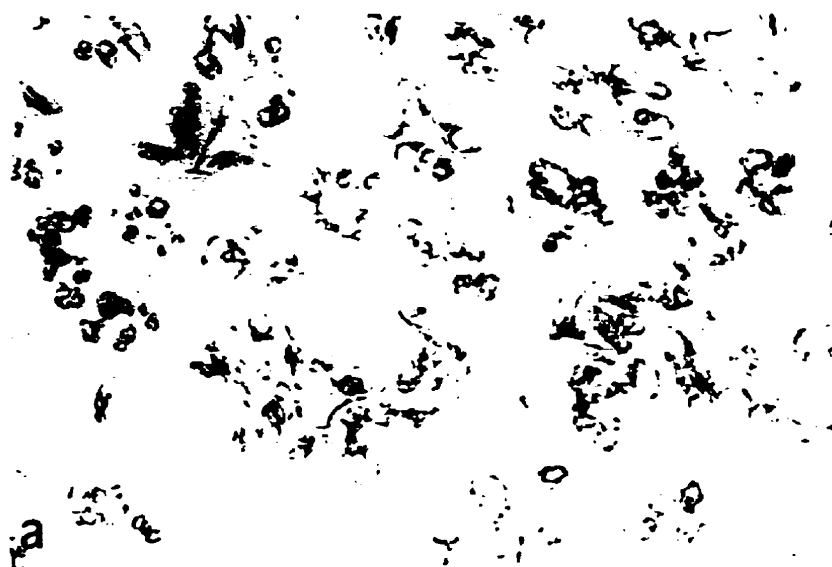
Immunocytochemical analysis of the cells collected from the middle band was performed on sections prepared from paraffin embedded cell pellets. Approximately 80-90% of these cells stained positively with an antibody to low molecular weight cytokeratins (Fig. 3.1d) and were considered as being mostly cytotrophoblasts. No specific staining was observed in pellets incubated with normal isotype control IgG (Fig 3.1e). A small percentage (2-4%) of Percoll purified cells were positively stained for hCG (Fig. 3.4c) or hPL (Fig. 3.5c). These cells were thought to be remnants of the syncytium which lysed during trypsin/DNase digestion. Vimentin (Fig. 3.2c), ACT (Fig. 3.6c) and

Fig. 3.1. Immunostaining for cytokeratin in placenta and Percoll purified cells. A monoclonal antibody to low molecular weight cytokeratins strongly labelled the trophoblastic epithelium of all chorionic villi (a, b, and c), blood vessels and some stromal cells in stem villi (b), and extravillous cytotrophoblasts in cell islands (c). The majority of Percoll purified cells were mononuclear and displayed cytokeratin immunoreactivity. Occasional syncytial remnants (arrow) were also cytokeratin positive (d). Percoll purified cells did not exhibit staining when the antibody to cytokeratin was replaced with normal isotype control IgG (e). (All sections were lightly counterstained with methyl green. Magnification: a, x 100; b, x 200; c, x 100; d, x 410; e, x 410)



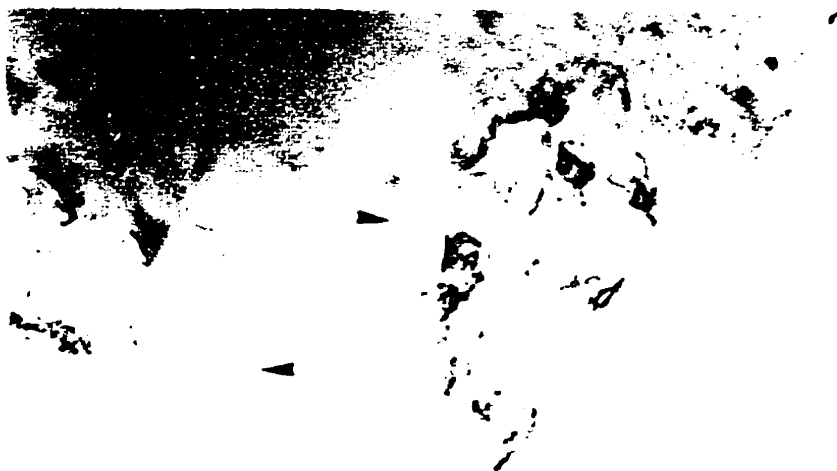
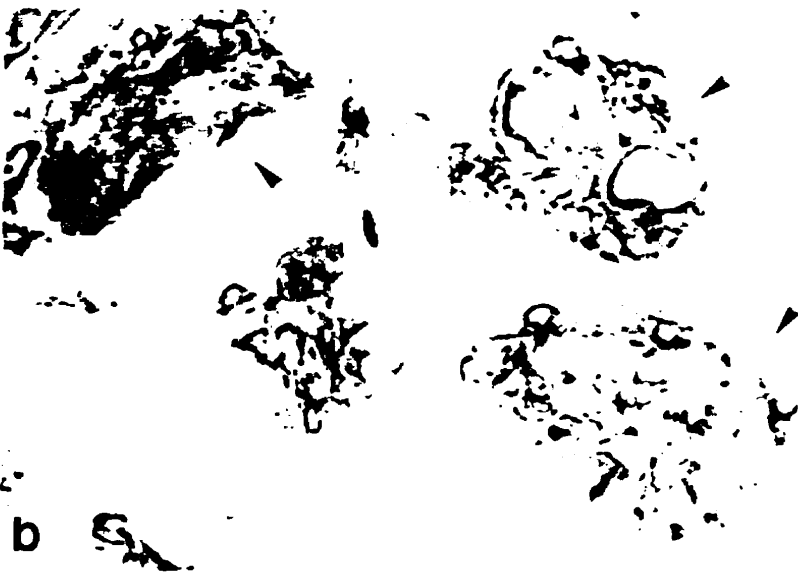
e

Fig. 3.2. Immunostaining for vimentin in placenta and Percoll purified cells. Cytokeratin immunoreactivity was distributed all over the central mesenchymal core of chorionic villi, whereas the trophoblastic epithelium remained completely devoid of staining (arrowheads, b) (a and b). Note intense labelling of vascular endothelial cells (thin arrow) and placental macrophages (thick arrow) (b). A few Percoll purified cells stained positively for vimentin (c). (Magnification: a, x 200; b, x 400; c, x 400.)



c

Fig. 3.3. Localization of HLA antigens in the placenta. Acetone-fixed cryostat sections were stained with a polyclonal antibody to beta-2-microglobulin (a), the monoclonal antibody W6/32 that recognizes a monomorphic determinant common to HLA-A, B, C, and G antigens (b), and a monoclonal antibody to a monomorphic determinant on HLA-DR/α (c). Both beta-2-microglobulin and HLA-ABC were widely distributed in the stroma of placental villi, whereas HLA-DR was found only in some sparsely scattered stromal cells (most likely macrophages). None of the above antibodies stained the trophoblastic epithelium (arrowheads). (Magnification: x 410).



c

Fig. 3.4. Immunostaining for hCG in placenta and Percoll purified cells. The syncytium displayed hCG immunoreactivity of variable intensity in the majority of chorionic villi. Some villi, however, remained strikingly unreactive (a and b). A few Percoll purified cells stained positively for hCG (c). (All sections were counterstained with methyl green. Magnification: a, x 100; b, x 200; c, 410.)

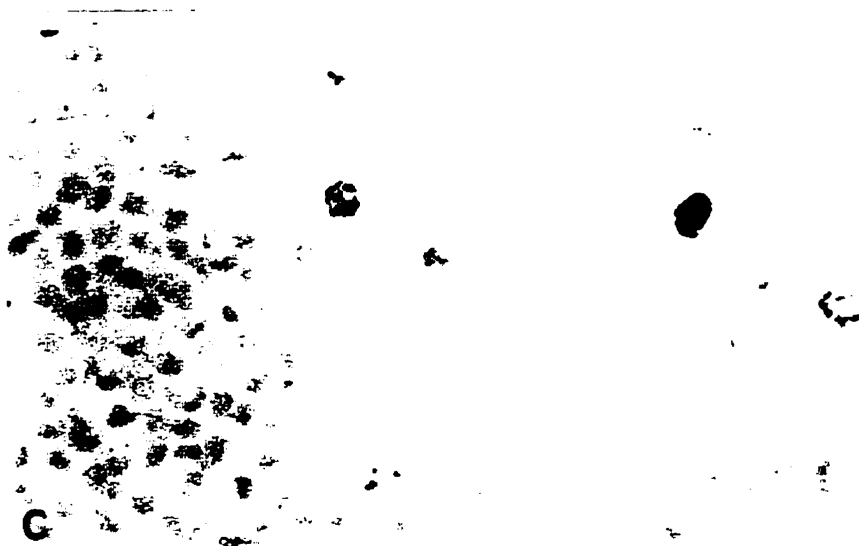


Fig. 3.5. Immunostaining for hPL in placenta and Percoll purified cells. The syncytium of chorionic villi was positively stained for hPL, whereas the villous core remained unreactive (a). Immunostaining of the finely minced and trypsin-DNase digested chorionic villous tissue demonstrated that the syncytium was almost completely dissociated after 30 min treatment (b). Only occasional Percoll purified cells were found to be hPL immunoreactive (c). (All sections are lightly counterstained with methyl green. Magnification: a, x 200; b, x 100; c, 410.)

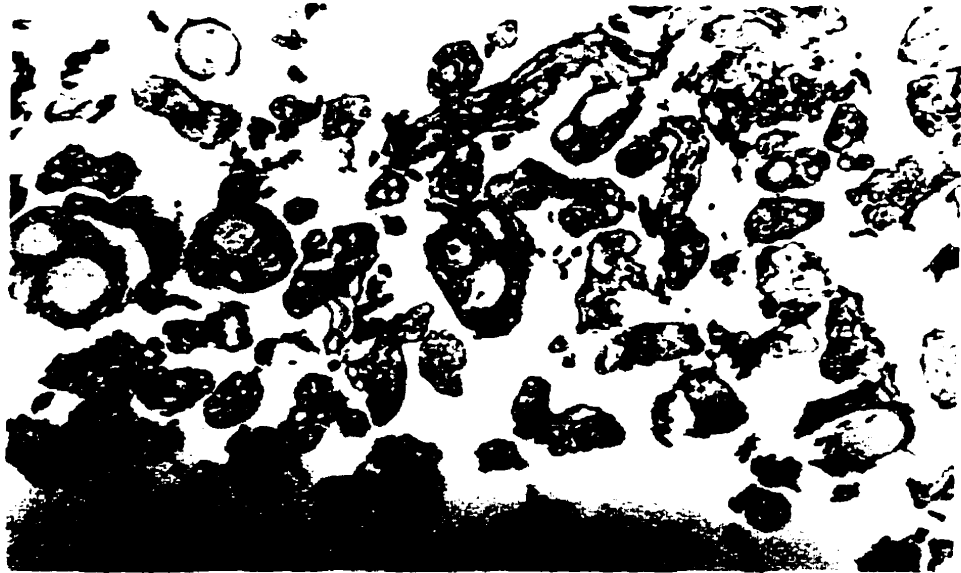


Fig. 3.6. Immunostaining for ACT in placenta and Percoll purified cells. Numerous macrophages were labelled in the core of placental villi. The apical surface of villi were also stained to a variable degree (a and b). A significant number of Percoll purified cells displayed ACT-like immunoreactivity (c). (Magnification: a, x 200; b, x 410; c, x 410.)

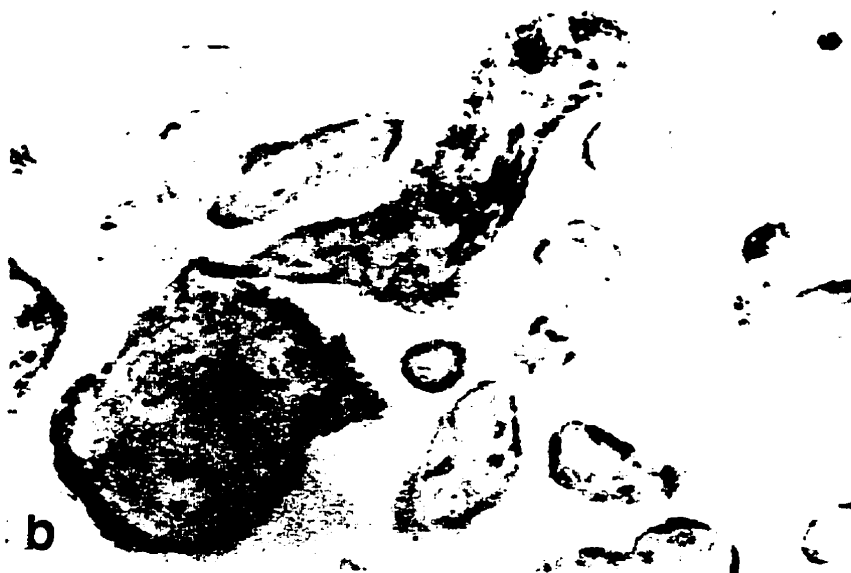


Fig. 3.7. Immunostaining for CD68 in placenta and Percoll purified cells. Placental macrophages (Hofbauer cells) were strongly labelled with a monoclonal antibody to CD68 (KP1) in the villous core of chorionic villi. Note the lack of staining on the surface of syncytium (a and b). A few Percoll purified cells were CD68 immunoreactive (c). (All sections were lightly counterstained with methyl green. Magnification: a, x 200; b, x 410; c, 410.)

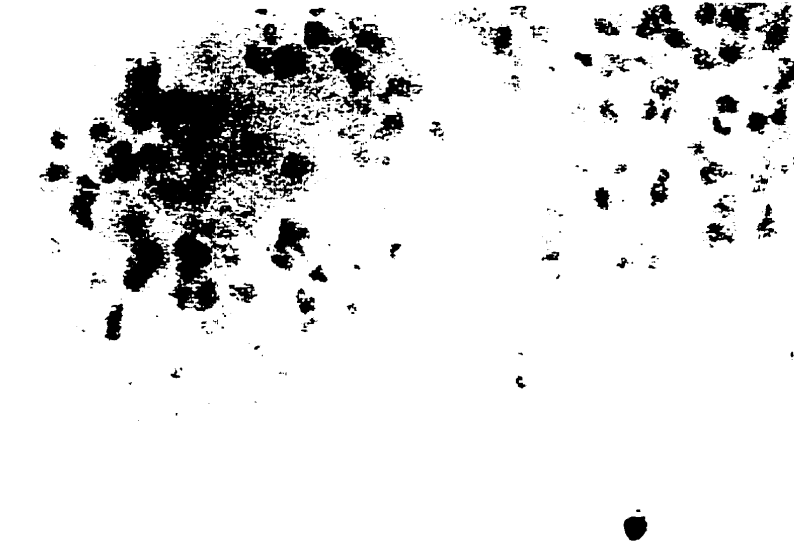


Fig. 3.8. Ultrastructural localization of CD68 immunoreactivity in Hofbauer cells.

Ultrathin sections of term placentae were incubated with a monoclonal antibody to CD68 (KP1). The sites of CD68 immunoreactivity were then visualized using goat anti-mouse IgG labelled with colloidal gold particles. Labelling was predominantly seen in lysosomal-like granules of Hofbauer cells. Magnification: x 60,000.

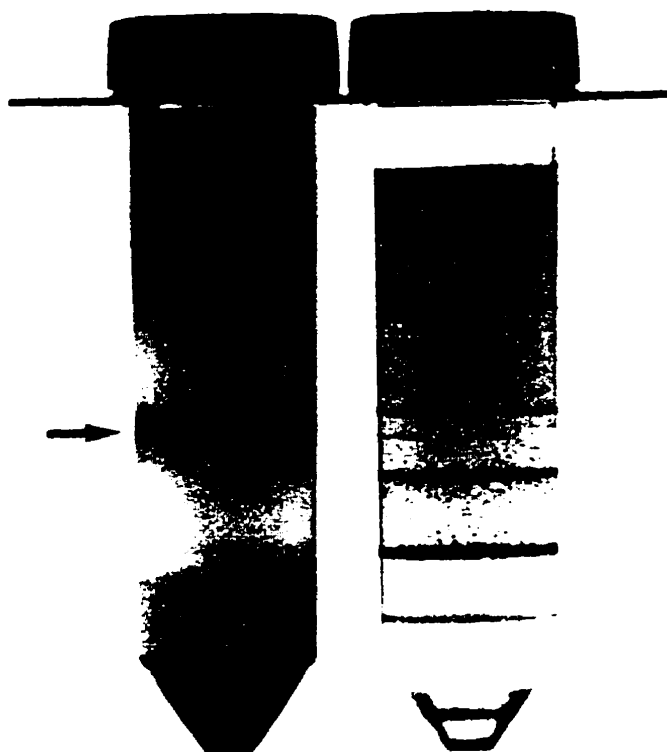


Fig. 3.9. Double immunocytochemical staining of placental sections for CD68 and ACT.

First, CD68 was localized by means of the ABC immunoperoxidase method, using the antibody KP1. Subsequently, ACT was detected with the PAP method. In these micrographs the dark black areas represent the intense bluish-black DAB-Ni precipitate of the CD68 staining, while the lighter black ones show the localization of the less intensive brown DAB reaction product produced by the ACT immunostaining. The two antigens were found to be codistributed in the cytoplasm of most placental macrophages (Hofbauer cells) (a and b). Some cells, however, exhibited only ACT staining (arrow). Note the strong granular appearance of the cytoplasmic staining for CD68 as distinct from the diffuse ACT immunoreactivity. (Section a was lightly counterstained with methyl green. Magnification: a, x 410; b, x 1020.)



Fig. 3.10. Separation of cytotrophoblasts by density gradient centrifugation. A 4 mL aliquot of placental cell suspension obtained by enzymatic disaggregation was layered onto a preformed discontinuous Percoll gradient in a 50 mL plastic tube (on the left). The gradient was centrifuged at 1200 x g for 20 min. Densities were determined by running an identical gradient loaded with color-coded density marker beads (right tube). Cytotrophoblasts were banded in the middle of the tube (arrow) between densities 1.051 and 1.063 g/mL.



CD68 (Fig. 3.7c) immunoreactive cells were identified as nontrophoblastic contaminants, such as endothelial cells, smooth muscle cells, fibroblasts or macrophages. They each accounted for about 10-20% of all Percoll purified cells. Since many of the vimentin positive cells were ACT and CD68 positive *in vivo*, these numbers are also an approximate estimate for the proportion of all nontrophoblastic contaminating cells in the Percoll purified fraction, the majority of which appear to be macrophage.

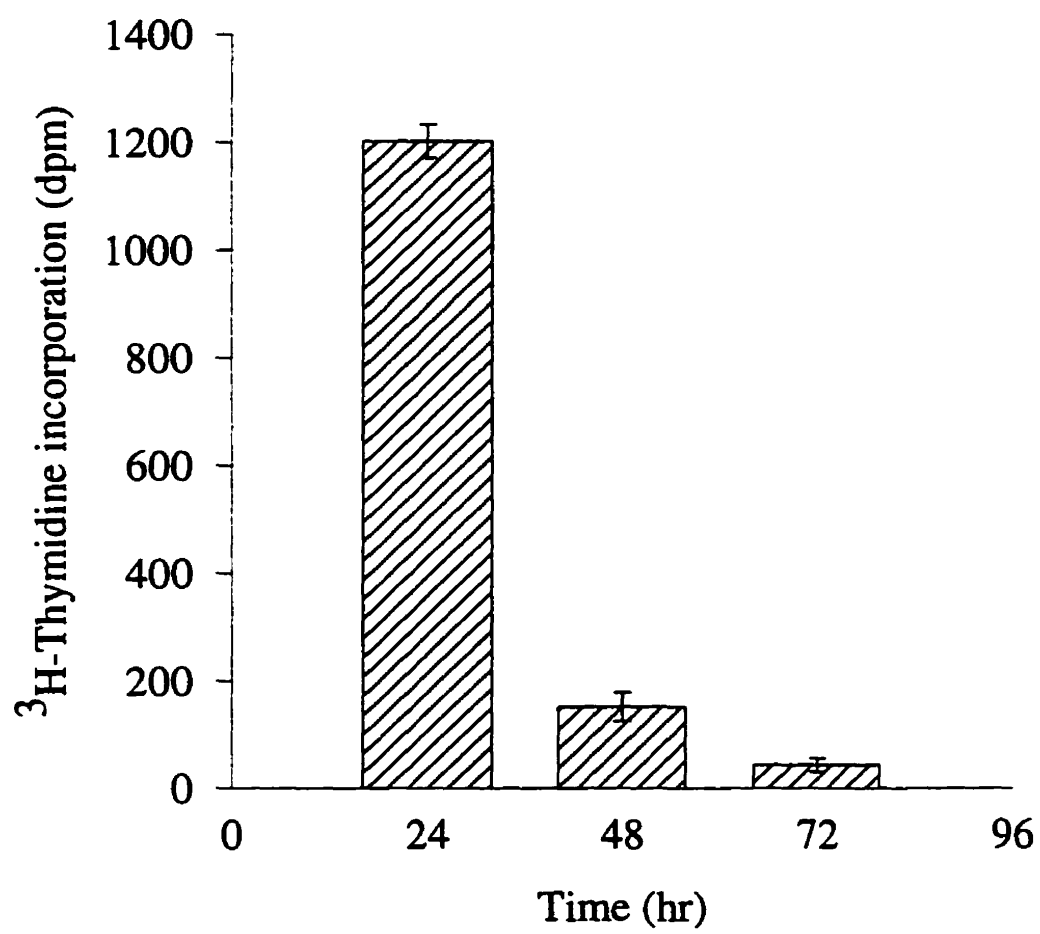
Percoll purified cytotrophoblasts demonstrated little replicative activity. No significant ^3H thymidine uptake was revealed when these cells were cultured in the presence of 20% fetal calf serum (Fig. 3.11).

3.2.3 Characteristics and culture of purified villous cytotrophoblasts

When Percoll purified cytotrophoblasts were cultured in the presence of fetal calf serum, three main cell types, i.e. single mononuclear cells, multinucleated aggregates and syncytial units could be observed in 24-36 hours (Fig. 3.12a, c). In the multinucleated aggregates, the outlines of aggregated cells were still visible, whereas the syncytial units did not possess recognizable cell borders. Occasionally, bi- and trinucleated cells were also seen. The multinucleated aggregates and syncytial structures were trophoblastic in nature and thus stained positively for cytokeratin (Fig. 3.12a, c) but were not labelled with monoclonal antibodies recognizing vimentin (Fig. 3.13a, c), CD68 (Fig. 3.14c) or monomorphic determinants on HLA class I (HLA-ABC) (Fig. 3.15a, c) and class II (HLA-DR) (Fig. 3.14a) antigens. Some of the single mononuclear cells were also cytokeratin immunoreactive (Fig. 3.12a, c) suggesting that they are trophoblastic in origin. Variable

Fig. 3.11. Time course of ³H-methyl-thymidine uptake by trophoblast cultures.

Cytotrophoblasts were plated at 1.6×10^5 /well into 96-well flat-bottom Nunclon culture plates and cultured in DMEM supplemented with 100 units/mL penicillin, 100 µg/mL streptomycin, 100 µg/mL kanamycin, 2.5 µg/mL Fungizone and 20% FCS. The culture medium was replaced daily with 200 µL of fresh medium. ³H-methyl-thymidine (1 µCi) was added to each well 6 h before concluding the assays. At the designated times the cultures were washed twice with Ca⁺⁺-Mg⁺⁺ free HBSS, treated with 0.25% trypsin, 1 mM EDTA for 30 min at 37 °C and harvested on microglass filters using a Skatron cell harvester. Radioactivity of the filters was measured by liquid scintillation spectrophotometry. Data shown are the mean and S.E. from quadruplicate cultures.



numbers of mononuclear, bi- and trinucleated cells were immunoreactive for vimentin (Fig. 3.13a, c), CD68 (Fig. 3.14c), HLA class I (Fig. 3.15a, c) or class II (Fig. 3.14a) antigens. On the basis of their immunoreactivity, these cells were classified as non-trophoblastic contaminating cells such as fibroblasts, endothelial cells and macrophages. The relative proportion of contaminating cells was progressively increased as the cultures grew older.

To obtain pure villous cytotrophoblasts, it was necessary to eliminate contaminating cells. This was accomplished using a mixture of the same anti-HLA-ABC and anti-HLA-DR antibodies that had been previously shown in immunocytochemistry to react with nontrophoblastic stromal elements but not villous trophoblasts (Fig. 3.3). Preformed complexes of these antibodies with goat anti-mouse IgG coupled to magnetic microspheres were incubated with Percoll purified cells, and the cells rosetted with microspheres were removed by a magnet. The proportion of rosette forming contaminating cells varied between 10-40% of the total number of purified cells. The specificity of rosette formation was tested by replacing the anti-HLA antibodies with a mixture of negative isotype controls of the same antibody concentrations. In this case, the number of rosetted cells was less than 0.5%. Cell viability with trypan blue exclusion was found to be about 85-90%, and the overall number of viable villous cytotrophoblasts obtained from 80 g of chorionic villous tissue ranged between 2×10^7 and 1.3×10^8 .

When placed in culture, villous cytotrophoblasts, obtained using Percoll density gradient centrifugation followed by immunomagnetic purification, formed cytokeratin

immunoreactive multinucleated trophoblast aggregates and syncytiotrophoblasts. Occasional round mononuclear cells were invariably cytokeratin positive (Fig. 3.12b, d). No vimentin (Fig. 3.13b, d) or CD68 (Fig. 3.14d) positive cells were found in these cultures. All cultures were HLA-ABC (Fig. 3.15b, d) and HLA-DR (Fig. 3.14b) negative at 24 h but some syncytiotrophoblasts became HLA-ABC positive after several days of incubation (not shown). The cultures were also shown to secrete immunoreactive hCG into the culture medium, and the hCG secretion could be stimulated by 8-Br-cAMP (Figs. 3.16 and 3.17), suggesting that the cultures were functionally viable. Viability was also confirmed by demonstrating the ability of cultured trophoblasts to cleave MTT (Fig. 3.18).

3.3 DISCUSSION

Human placental functions have been extensively studied in organ, explant and cell cultures (Loke, 1983; Ringler and Strauss, 1990; Contractor and Sooranna, 1993). Organ culture is a method whereby a small piece of tissue is maintained in its characteristic morphological and functional state. An essential difference between organ and explant cultures is that intensive cellular proliferation, migration and differentiation can only occur in explant culture (Freshney, 1987). Both placental organ and explant cultures have proved valuable in elucidating placental functions but suffered from the problem of cellular heterogeneity. By contrast, placental cell cultures offer the potential of manipulating defined cell populations and thereby the possibility of a less ambiguous assignment of cellular functions (Ringler and Strauss, 1990; Contractor and Sooranna, 1993).

Fig. 3.12. Micrographs of trophoblast cultures immunostained for cytokeratin. Cells isolated by Percoll density gradient centrifugation (a and c) or Percoll density gradient centrifugation followed by immunomagnetic separation (b and d) were cultured for 24 hours (a and b), 96 hours (c) or 120 hours (d). All forms of trophoblast (single mononuclear trophoblasts, multinucleated aggregates, and syncytial units) stained for cytokeratin (a, b, c, and d), while contaminating nontrophoblastic cells remained unstained (arrowheads, a and c). No cytokeratin negative cells were observed in cultures obtained using Percoll as well as immunomagnetic separation (b and d). Mononuclear trophoblasts and multinucleated aggregates (a, c, and d) exhibited stronger immunoreactivity than the flattened syncytial structures. The outlines of aggregated cells were invariably discernible in multinucleated aggregates (a, c, and d) as distinct from syncytial units in which cell borders could not be recognized any longer. In syncytial units numerous nuclei surrounded by vacuoles were concentrated at distinct sites of the cytoplasm. At the periphery of these units cytokeratin positive mononuclear cells were usually seen as possibly being incorporated into the syncytium (arrows, b and c). (All cultures were lightly counterstained with hematoxylin. Magnification: x 200.)

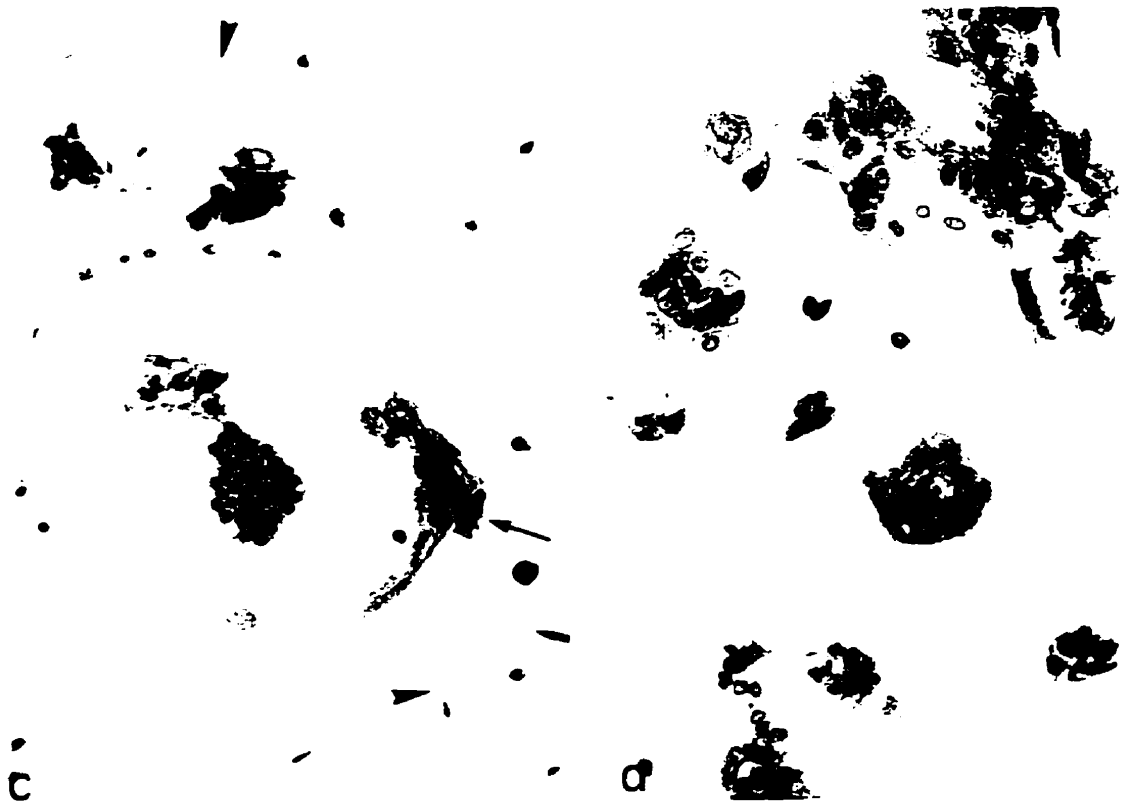
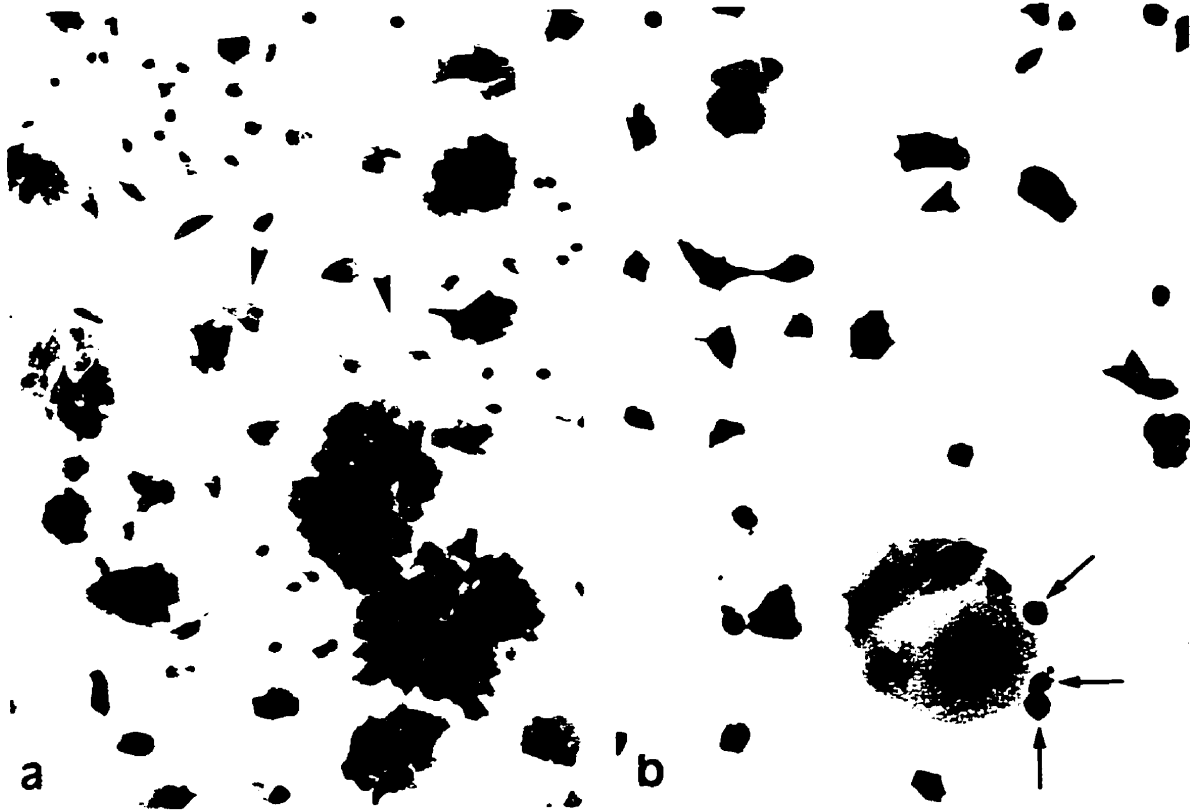


Fig. 3.13. Micrographs of trophoblast cultures immunostained for vimentin. Cells isolated by Percoll density gradient centrifugation (a and c) or Percoll density gradient centrifugation followed by immunomagnetic separation (b and d) were cultured for 24 hours (a and b) and 120 hours (c and d), respectively. Numerous round or elongated fibroblast-like vimentin immunoreactive cells were observed in cultures obtained using Percoll purification only (a and c), whereas no vimentin positive cells were present in cultures after combined Percoll and immunomagnetic purification (b and d). Note that multinucleated trophoblast aggregates (a, b, c, and d), syncytial structures (c and d), and mono- and binucleated trophoblast cells were vimentin negative. (All cultures were counterstained with hematoxylin. Magnification: a, x 100; b, x 100; c, x 200; d, x 200.)

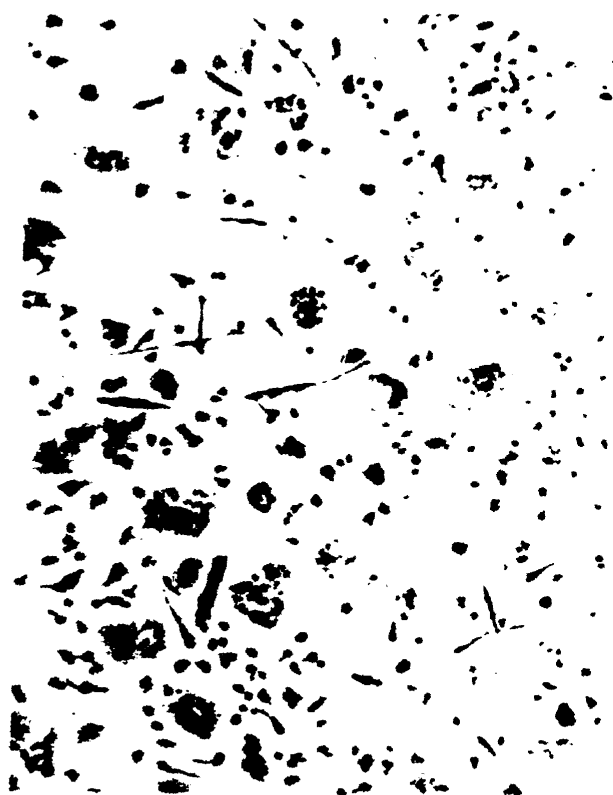


Fig. 3.14. Immunostaining of trophoblast cultures for HLA-DR and CD68. Cells isolated by Percoll density gradient centrifugation (a and c) or Percoll density gradient centrifugation followed by immunomagnetic separation (b and d) were cultured for 24 hours (a and b) and 120 hours (c and d), respectively. A monoclonal antibody to a monomorphic determinant on HLA-DR/ α stained some nontrophoblastic cells (most likely macrophages) (a), while trophoblasts remained unstained (a and b). Similarly, macrophages (c) but not trophoblasts displayed CD68 immunoreactivity (c and d). Note that trophoblast cultures established from villous cytotrophoblasts isolated by combined Percoll and immunomagnetic separation are completely devoid of HLA-DR/ α (b) and CD68 positive cells (d). (All cultures were counterstained with hematoxylin. Magnification: x 200.)



Fig. 3.15. Immunocytochemical staining of trophoblast cultures for class I HLA antigens.

Cells isolated by Percoll density gradient centrifugation (a and c) or Percoll density gradient centrifugation followed by immunomagnetic separation (b and d) were cultured for 24 hours (a and b) and 120 hours (c and d), respectively. The monoclonal antibody W6/32 that recognizes a monomorphic determinant common to HLA-A, B, C, and G antigens stained nontrophoblastic contaminating cells (a and c), but did not label mononuclear trophoblasts, multinucleated trophoblast aggregates or syncytia (a, b, c, and d). Note the complete absence of contaminating cells from cultures obtained after combined Percoll and immunomagnetic purification (b and d). (All cultures were counterstained with hematoxylin. Magnification: x 200.)

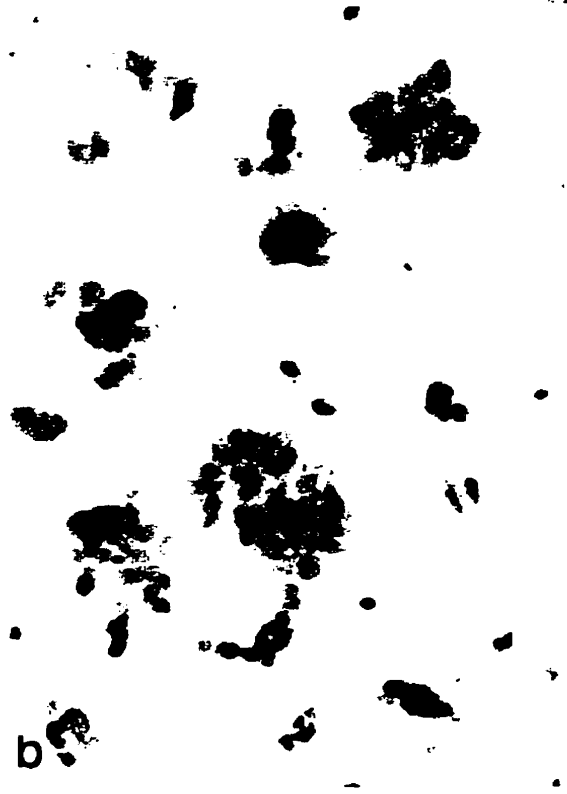
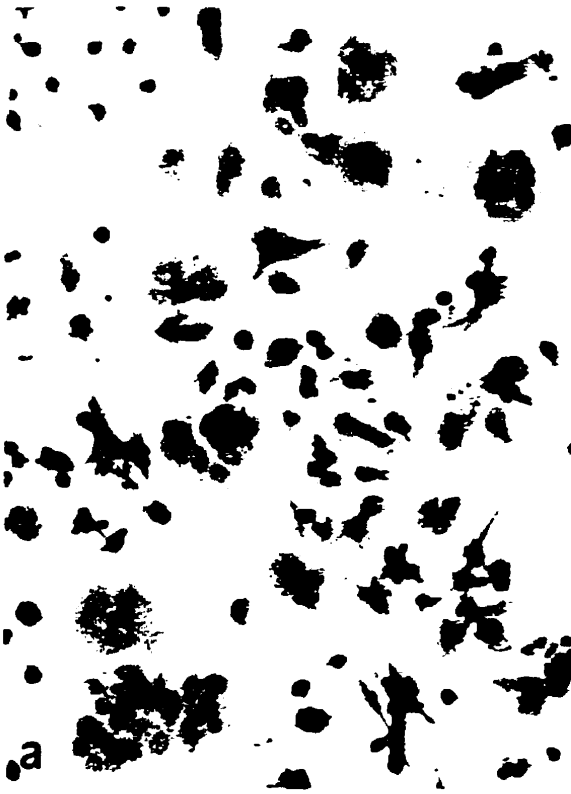


Fig. 3.16. Stimulation of hCG secretion by 8-bromo-cAMP in trophoblast culture.

Cytotrophoblasts were cultured at $8 \times 10^5/\text{mL}$ in DMEM supplemented with 100 units/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, 100 $\mu\text{g}/\text{mL}$ kanamycin, 2.5 $\mu\text{g}/\text{mL}$ Fungizone and 20% FCS in 24-well Nunclon culture plates in the presence or absence of 1.5 mM 8-bromo-cAMP. Culture medium was collected every 24 h and replaced with fresh medium. After centrifugation, the supernatants were stored at $-20\text{ }^{\circ}\text{C}$ until assayed for hCG using Serono hCG MAIAclone kits. The data shown are the mean and S.E. from quadruplicate cultures.

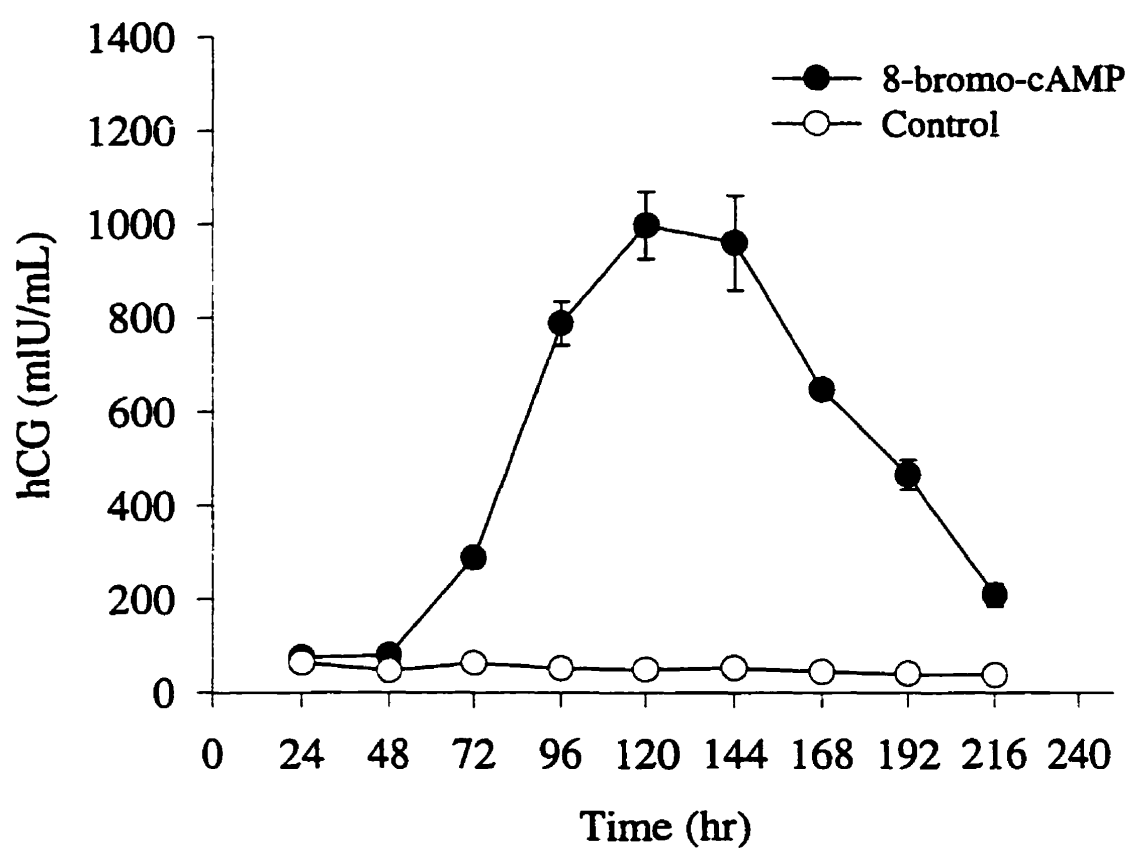


Fig. 3.17. Dose-dependent stimulation of hCG secretion by 8-bromo-cAMP in trophoblast culture. Villous trophoblasts cultured in the presence of 20% FCS were washed and incubated in serum-free medium alone (control) or serum-free medium containing increasing concentrations of 8-bromo-cAMP (0.001-3.2 mM). After 24 h conditioned media were collected, centrifuged and the supernatants were assayed for hCG. The data shown represent the mean \pm S.E. from quadruplicate cultures.

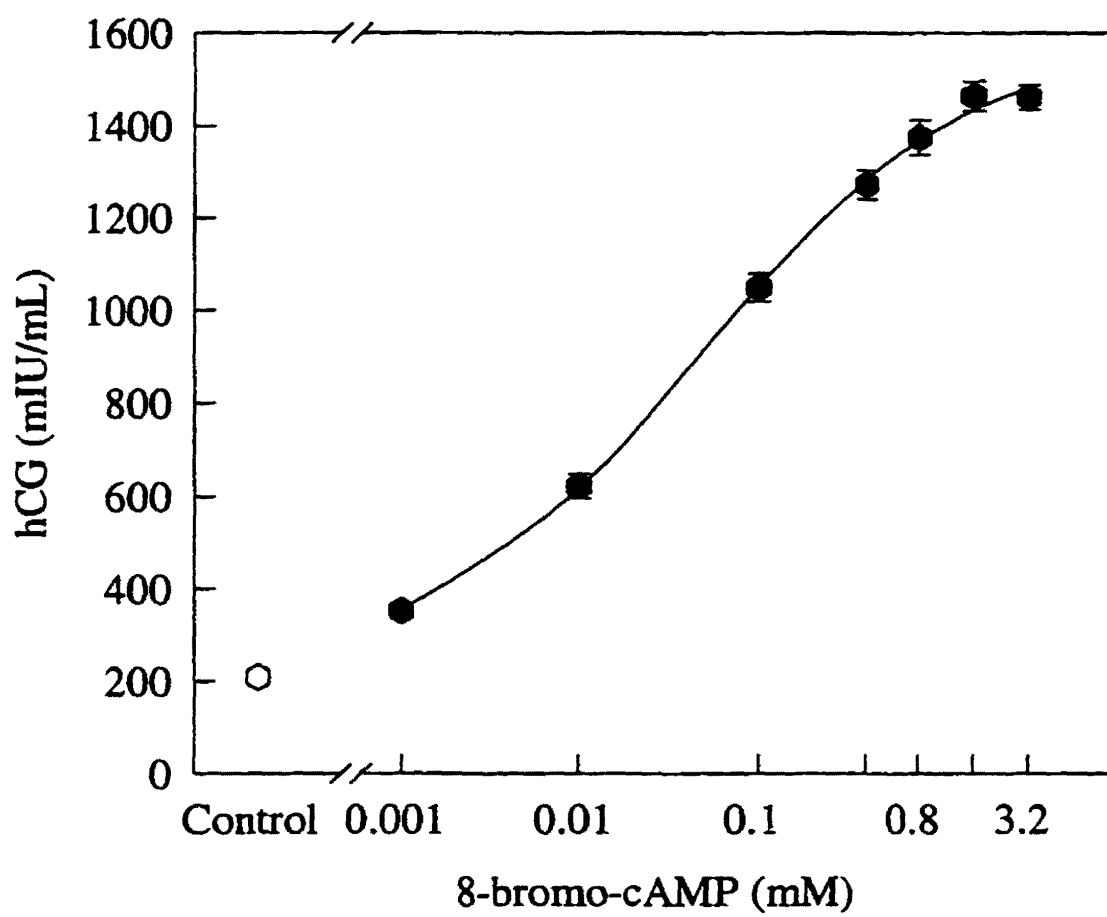
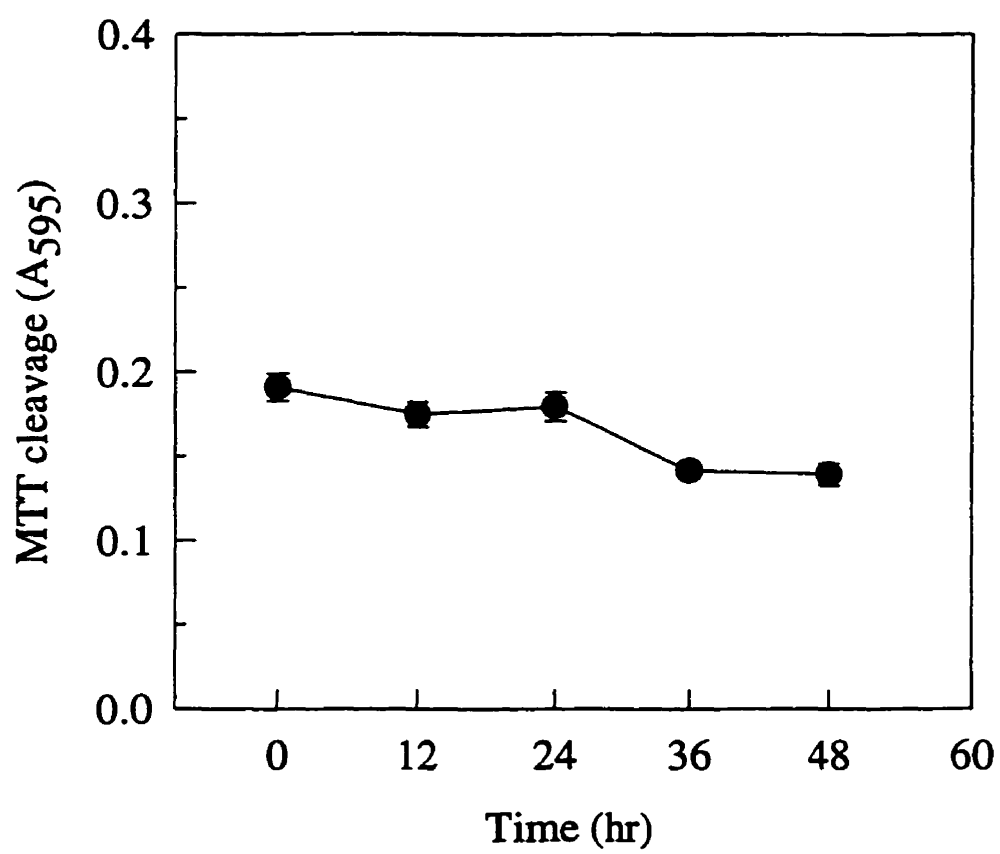


Fig. 3.18. MTT cleavage by cultured trophoblasts. Cytotrophoblasts were cultured at $2 \times 10^5/100 \mu\text{L}/\text{well}$ in 96-well culture plates in the presence of 20% FCS for 72 h. The cultures were then washed twice and further incubated in indicator-free medium. At the indicated times, the cultures were incubated with MTT for 2 h. After solubilization the absorbance of the blue formazan was measured at 595 nm. The data shown are the mean and S.E. from 8 wells.



Isolation and culture of cytotrophoblasts have been reported from many laboratories (for reviews see Loke, 1983; Ringler and Strauss, 1990; Contractor and Sooranna, 1993). A variety of methods involve mechanical and enzymatic (usually trypsin/DNase) disaggregation of the chorionic villous tissue, resulting in trophoblast preparations more or less contaminated with non-trophoblastic placental cells. The majority of cytotrophoblasts are mononuclear cytotrophoblasts, because the syncytium being very sensitive to enzymatic treatment is only recovered as occasional remnants. Currently, the most widely used method for purification of cytotrophoblasts is the one reported by Kliman et al. (1986). In this method, contaminating cells are removed through Percoll density gradient centrifugation. This technique is known to yield trophoblast preparations containing at least 90-95% cytotrophoblasts (Douglas and King, 1989; Zini et al., 1992), although considerably less purity (80%) has been also reported (Contractor and Sooranna, 1993).

Further purification of Percoll-purified cytotrophoblasts has been achieved by positive or negative immunoselection using monoclonal antibodies that bind to cytotrophoblasts or nontrophoblastic cells, respectively. Contractor and Sooranna (1988) used a panning technique to separate cytotrophoblasts from a heterogeneous mixture of placental cells obtained by enzymatic treatment. Their procedure involved incubation of placental cells with a trophoblast-specific antibody followed by absorption of labelled cells in plates coated with anti-mouse IgG. Kawata et al. (1984) developed a method to isolate cytotrophoblasts from human placental cell suspensions using trophoblast- and HLA-

specific antibodies, and fluorescence-activated cell sorting. Douglas and King (1989) employed immunomagnetic beads coated with anti-mouse IgG to separate villous cytotrophoblasts from nontrophoblastic cells labelled with mouse monoclonal antibodies against class I and class II HLA antigens. Expression of CD9 antigen by placental stromal cells but not by villous and extravillous trophoblasts has also been utilized to obtain pure cytotrophoblast preparations. CD9 expressing cells were removed by either immunomagnetic separation (Morrish et al., 1991) or second antibody-conjugated glass bead columns (Yui et al., 1994). Isolation of first-trimester extravillous trophoblasts has been reported by Loke et al. (1989). Their approach is based on selective binding of extravillous trophoblasts to laminin. Extravillous cytotrophoblasts isolated by attachment to laminin-coated magnetic beads are W6/32 and cytokeratin positive, and some but not all display hCG, hPL or SP1 immunoreactivity. This phenotype corresponds to that of intermediate trophoblasts described by Kurman et al. (1984). A monoclonal antibody specific for a framework determinant of HLA-B was used by Shorter et al. (1990). This antibody labelled all cell types present in the amniochorion and decidua, but did not bind to chorionic cytotrophoblasts. Hence, sorting the negative population by flow cytometry yielded extravillous cytotrophoblasts.

We have been successful in isolating and culturing pure villous cytotrophoblasts from human term placentas, using a modification of the methods of Kliman et al. (1986) and Douglas and King (1989), respectively. Our procedure consists of sequential trypsin-DNase digestions of the carefully selected chorionic villous tissue, discontinuous Percoll

density gradient centrifugation, and immunomagnetic removal of contaminating cells bearing HLA class I and class II antigens. Characterization and identification of the cells obtained after Percoll centrifugation and immunomagnetic separation, respectively, relied on their immunocytochemical staining characteristics in vivo. Differential expression of cytoskeletal proteins, HLA antigens and hormones by various cellular constituents of the chorionic villous tissue was utilized to determine the identity of the isolated cells.

Intermediate filaments are fibrous structures that form part of the cytoskeleton. Intermediate filament proteins display cell-type specific expression, which helps distinguish cells of various origin. In particular, expression of cytokeratins is characteristic of epithelial cells, whereas vimentin is a cytoskeletal component of cells of mesenchymal origin. Although a rare occurrence, coexpression of cytokeratin and vimentin has been also reported (Moll et al., 1982; Osborn and Weber, 1983). We found that both the syncytium and cytotrophoblasts of placental villi and extravillous cytotrophoblasts in cell islands are cytokeratin positive. Moreover, some mesenchymal elements in the vessel walls of larger villi were also stained. Our findings are in agreement with those reported earlier (Loke and Butterworth, 1987; Beham et al., 1988; Daya and Sabet, 1991) and confirm that cytokeratin is a reliable trophoblast marker. However, the fact that some mesenchymal elements in larger villi may also express cytokeratin suggests that cytokeratin positivity is a necessary, but not a sufficient condition for the identification of trophoblasts. Approximately 80-90% cells were cytokeratin positive in our Percoll purified preparations. We estimate that the actual

proportion of villous cytotrophoblasts is somewhat less than that, considering the possible contamination with cytokeratin positive extravillous cytotrophoblasts and mesenchymal elements.

Vimentin immunoreactivity was observed in practically all cellular components of the central core of placental villi, including vascular endothelial cells, smooth muscle cells of blood vessels, stromal mesenchymal cells and macrophages. The trophoblast covering of the placental layer, however, remained unstained. It should be mentioned, however, that extravillous cytotrophoblasts may stain for vimentin (Loke and Butterworth, 1987). Therefore, the estimated 10-20% vimentin positive cells detected in our Percoll purified preparations represent villous mesenchymal elements and some extravillous trophoblasts.

Our immunocytochemical investigations also confirmed previous results that villous cytotrophoblasts and syncytium express neither class I nor class II major histocompatibility complex (MHC) antigens (Faulk and Temple, 1976; Sunderland et al., 1981; Loke and Butterworth, 1987; Johnson, 1993). By contrast, all cellular components of the villous stroma were stained with the monoclonal antibody W6/32 that reacts with a monomorphic determinant common to HLA-A, B and C antigens (Barnstable et al., 1978), and HLA-DR was detected on Hofbauer cells. Although villous trophoblasts do not express MHC molecules, various forms of extravillous trophoblasts have been shown to react with the monoclonal antibody W6/32 (Loke and Butterworth, 1987). However, they failed to bind to antibodies directed at HLA-A or HLA-B polymorphic determinants of the fetal phenotype (Redman et al., 1984). Subsequent investigations revealed that the

trophoblast HLA antigen immunoprecipitated by W6/32 is identical with HLA-G, a nonclassical, nonpolymorphic class I MHC molecule (Ellis et al., 1986, 1990; Kovats et al., 1990).

Our immunocytochemical investigations performed on smears of Percoll purified cells revealed 20-30% each of HLA-ABC and HLA-DR immunoreactive cells. These numbers are close to those obtained for the percentage of rosette forming cells (10-40%) that reacted with the same anti-HLA-ABC and anti-HLA-DR antibodies during the immunomagnetic purification of villous cytotrophoblasts. The high proportion of HLA-ABC and HLA-DR positive cells suggest that our Percoll purified preparations were significantly contaminated with stromal cells, especially macrophages.

Placental macrophages were stained with antibodies to α -1-antichymotrypsin (ACT) and CD68. It appears that CD68 is a more specific marker for placental macrophages than ACT, because ACT staining was also seen on the luminal surface of the syncytium, whereas similar staining has never been observed using antibodies to CD68. Our electron microscopic findings corroborated that CD68 is exclusively localized in placental macrophages, and the predominant sites of CD68 immunoreactivity are intracytoplasmic lysosomal-like bodies. By double immunocytochemistry, we found that ACT and CD68 were codistributed in the villous stroma. Our finding provides evidence that both antigens are present in the same macrophage population, and CD68 is not expressed in stromal cells negative for ACT. Although the expression of CD68 is macrophage specific in the placental villi, other cell types may express this antigen in other organs (Pulford et al.,

1990).

In our Percoll purified preparations about 10-20% of cells were stained with the antibody to KP1. This is consistent with the proportion obtained for the HLA-DR positive cells and again points to considerable macrophage contamination.

Our immunocytochemical studies demonstrated that hCG and hPL are localized exclusively in the syncytium of placental villi. Percoll purified cytotrophoblasts were negative for both hCG and hPL. Only occasional (2-4%) isolated syncytial fragments were immunoreactive. This confirms that syncytial contamination of our Percoll purified preparations is not significant. These results support the findings reported earlier by several investigators (Kliman et al., 1986).

It is implicitly accepted by investigators that Percoll fractionation of placental cell suspensions as described by Kliman et al. (1986) yields pure cytotrophoblasts of the villous variety. However, in view of the relatively high number of vimentin positive extravillous cytotrophoblasts observed in cell islands, we believe that some extravillous cytotrophoblasts are also present in these preparations, because these cells cannot be completely avoided during selection of the villous tissue. Furthermore, we are not aware of any evidence that villous cytotrophoblasts separate at buoyant densities different from those of extravillous cytotrophoblasts.

In our hands, the method of Kliman et al. (1986) did not produce consistently pure cytotrophoblasts. Variations in the architecture and digestibility of the placental tissue as well as small unavoidable inconsistencies in the selection and mincing of the villous

material are likely to contribute significantly to this problem. In addition, there is always some subjectivity in determining the borders of the band in which the cytotrophoblasts are sedimented. Taking wider bands usually results in higher proportions of cellular contamination. In contrast to methods relying exclusively on a single Percoll purification step, the method used in this thesis yields villous cytotrophoblasts not contaminated by mesenchymal cells or extravillous cytotrophoblasts. Separation of villous cytotrophoblasts has been achieved by a one-step incubation employing complexes of anti-HLA antibodies and anti-mouse IgG coupled with magnetic microspheres. This procedure requires less time, is more convenient and less damaging to cytotrophoblasts than the indirect technique of Douglas and King (1989) that involves two incubations and repeated centrifugations.

CHAPTER 4

ACTIVATION OF PLASMINOGEN BY HUMAN TERM TROPHOBLASTS

4.1 INTRODUCTION

It is now well-known that the plasminogen activation system undergoes extensive alterations in pregnancy (see Chapter 1). The placental trophoblast appears to be critically involved in developing the suppressed fibrinolytic activity seen in both the maternal plasma and placenta during normal and preeclamptic pregnancies in particular. In addition, several studies suggest that the plasminogen activation system of trophoblasts is essential for mediating trophoblast invasiveness. Both trophoblast underinvasion and overinvasion of the maternal uterus in preeclampsia and choriocarcinoma, respectively, have been suspected of being associated with abnormal regulation of the trophoblast plasminogen activation cascade (Estellés et al., 1994; Graham et al. 1994). At present, however, little information is available on the factors that regulate the activation of plasminogen by trophoblasts with either normal or disturbed invasiveness. In this study, an attempt has been made to extend our understanding of the trophoblast plasminogen activation system. The emphasis was placed on testing various compounds, known or suspected to be important in trophoblast development and function, for their ability to modulate plasmin generation by trophoblasts.

4.2 RESULTS

4.2.1 Expression of plasminogen activators and their inhibitors by trophoblasts *in vivo* and *in vitro*

Immunohistochemical investigation of tissue sections from term placentas could

not demonstrate any staining for either u-PA or t-PA in the trophoblast lining of chorionic villi. Only occasional placental macrophages were found to be u-PA immunoreactive (Fig. 4.1d). In contrast, most trophoblasts expressed u-PA (Fig. 4.2a) but not t-PA (not shown) during the first 24 hours of culture. The most intense staining was observed in mononuclear trophoblasts and multinuclear aggregates. The syncytium showed patchy staining, the intensity of which diminished with advancing time in culture. Light to moderate PAI-1 staining was observed in the syncytium of the villi (Fig. 4.1a). Some placental macrophages were also labeled (Fig. 4.1b). All forms of trophoblasts showed moderate to strong diffuse as well as punctate PAI-1 staining in culture (Fig. 4.2b), and PAI-1 was also detected in trophoblast conditioned medium by ELISA (Fig. 4.3). The syncytium was strongly PAI-2 immunoreactive in vivo, and occasional placental macrophages were also immunostained (Fig. 4.1c). By contrast, only occasional syncytial units demonstrated light PAI-2 staining in culture whilst the majority of cultured trophoblasts remained unstained (Fig. 4.2c).

4.2.2 Kinetics of plasminogen activation by cultured trophoblasts

Villous cytotrophoblasts obtained from human term placentas were cultured at various densities for 36-72 hours in the presence of fetal calf serum. During this period, cytotrophoblasts formed multinuclear aggregates and fused into syncytial units. Trophoblasts were then washed and cultured in serum-free medium containing S-2251 in the presence or absence of Glu-plasminogen. Hydrolysis of S-2251 was measured by recording the appearance of pNA. Fig. 4.4A shows parabolic progress curves of pNA

Fig. 4.1. Immunostaining for PAIs and u-PA in the placenta. The syncytium of chorionic villi showed light to moderate PAI-1 staining (a). Some blood vessels and macrophage-like cells in the stroma were also stained (b). PAI-2 immunoreactivity was found in the syncytium (c). Occasional stromal cells (probably macrophages) displayed immunoreactivity for u-PA, but the trophoblastic epithelium were not labelled (d). (Sections a, b, and d were lightly counterstained with methyl green. Magnification: a, b, and d x 40; c, 20 x.)



Fig. 4.2. Immunocytochemical staining of trophoblast cultures for u-PA and PAIs.

Cultured trophoblasts were positively stained for u-PA (a) and PAI-1 (b). Note the diffuse u-PA staining as distinct from the mainly punctate distribution of PAI-1 immunoreactivity. Weak PAI-2 staining was observed in a few syncytial units, whereas the majority of trophoblasts remained unstained (c). (In culture a hematoxylin counterstaining was used. Magnification: x 410.)

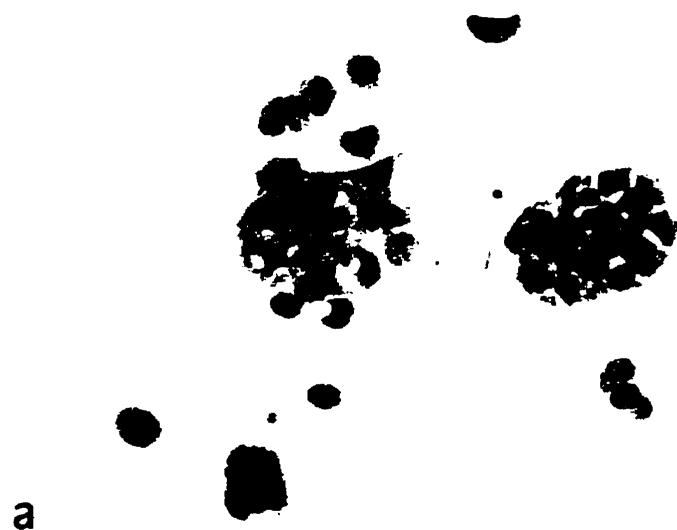
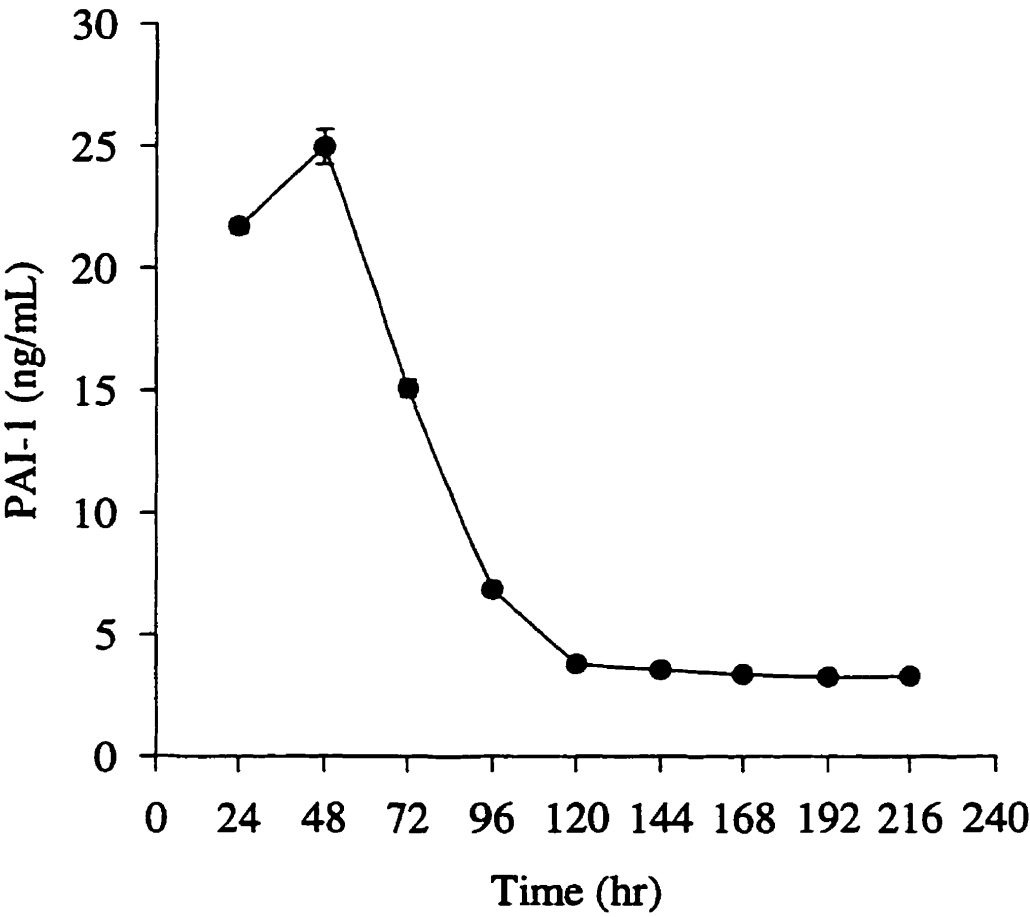


Fig. 4.3. Secretion of PAI-1 by cultured trophoblasts. Cytotrophoblasts were cultured at $8 \times 10^5/\text{mL}$ in DMEM supplemented with 100 units/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin, 100 $\mu\text{g/mL}$ kanamycin, 2.5 $\mu\text{g/mL}$ Fungizone and 20% FCS in 24-well Nunclon culture plates. Culture medium was collected and replaced with fresh medium every 24 h. After centrifugation, the supernatants were stored at -20°C until assayed for PAI-1 using Imubind PAI-1 ELISA kits (American Diagnostica). Results shown are the mean and S.E. from quadruplicate cultures.



formation, resulting from plasminogen-dependent hydrolysis of S-2251. These curves were obtained by non-linear curve-fitting and represent the best fit to the experimental data. In each case, the progress curve for pNA formation could be described by a quadratic polynomial ($y=a+bx+cx^2$), which had been predicted from the expected kinetics of plasmin generation and S-2251 hydrolysis (see Chapter 2). Plasmin activity, which is proportional to plasmin concentration, was calculated as the first derivative of these quadratics ($y'=b+2cx$). Thus, a linear increase in plasmin activity with time was obtained (Fig. 4.4B). Computation of the rates of plasmin generation as the second derivative of the quadratic equations describing the progress of plasminogen-dependent pNA formation yielded a constant ($y''=2c$) at each seeding density. The constant rate of plasmin generation implies that cultured villous trophoblasts maintain their PA activity at a constant level over the time investigated. Fig. 4.4C demonstrates that at a fixed plasminogen concentration ($0.28 \mu\text{M}$), the rate of plasmin formation plotted as a function of seeding density is approximately linear in the range between 1.5 and 3.0×10^5 cytotrophoblasts/well.

Fig. 4.5 shows the progress curve of S-2251 hydrolysis in the absence of plasminogen. A non-linear increase in pNA accumulation with time was obtained. The results obtained indicate the presence of trophoblast secreted proteases that are capable of hydrolyzing S-2251 in the absence of plasminogen. However, the magnitude of absorbance values arising from plasminogen independent cleavage of S-2251 was always much smaller than that observed in the presence of plasminogen.

Fig. 4.4. Effect of seed cell density on plasminogen activation by trophoblasts. Villous cytotrophoblasts were plated at varying densities and cultured in the presence of 20% FCS for 48 h. Trophoblast cultures were then washed and incubated in serum-free medium containing 0.4 mM S-2251 in the presence or absence of 0.28 μ M Glu-plasminogen. Hydrolysis of S-2251 was measured by recording the appearance of pNA at 405 nm. Plasminogen-dependent hydrolysis of S-2251 was calculated as the difference between absorbances measured in the presence and absence of plasminogen. Progress curves of plasminogen-dependent pNA generation (A) were drawn by fitting quadratics to the experimental points representing the mean and S.E. of quadruplicate cultures. Plasmin activities (B) were obtained by taking the first derivative of the quadratic equations. The rates of plasmin generation (C) were obtained from the second derivative of these equations.

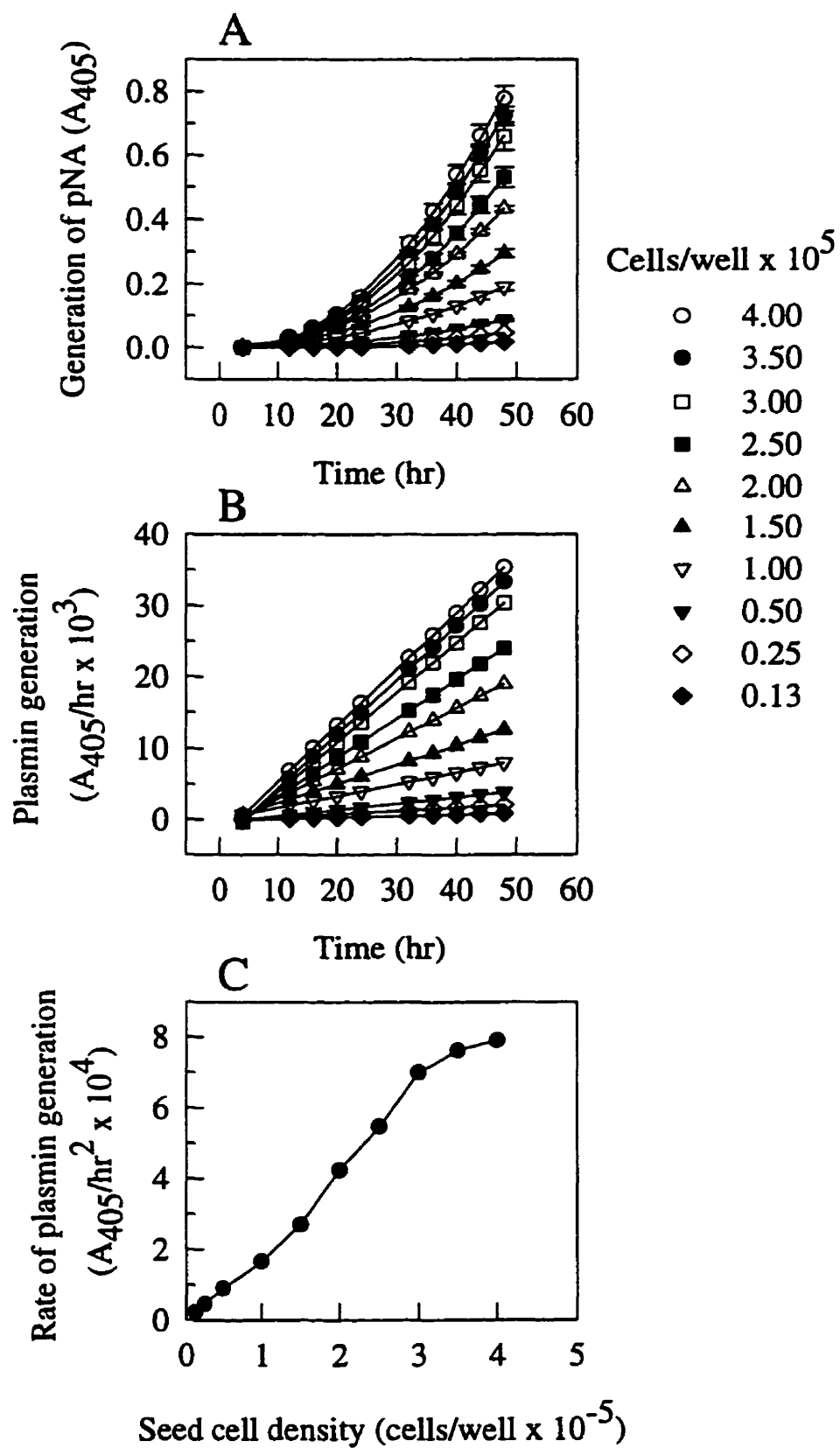
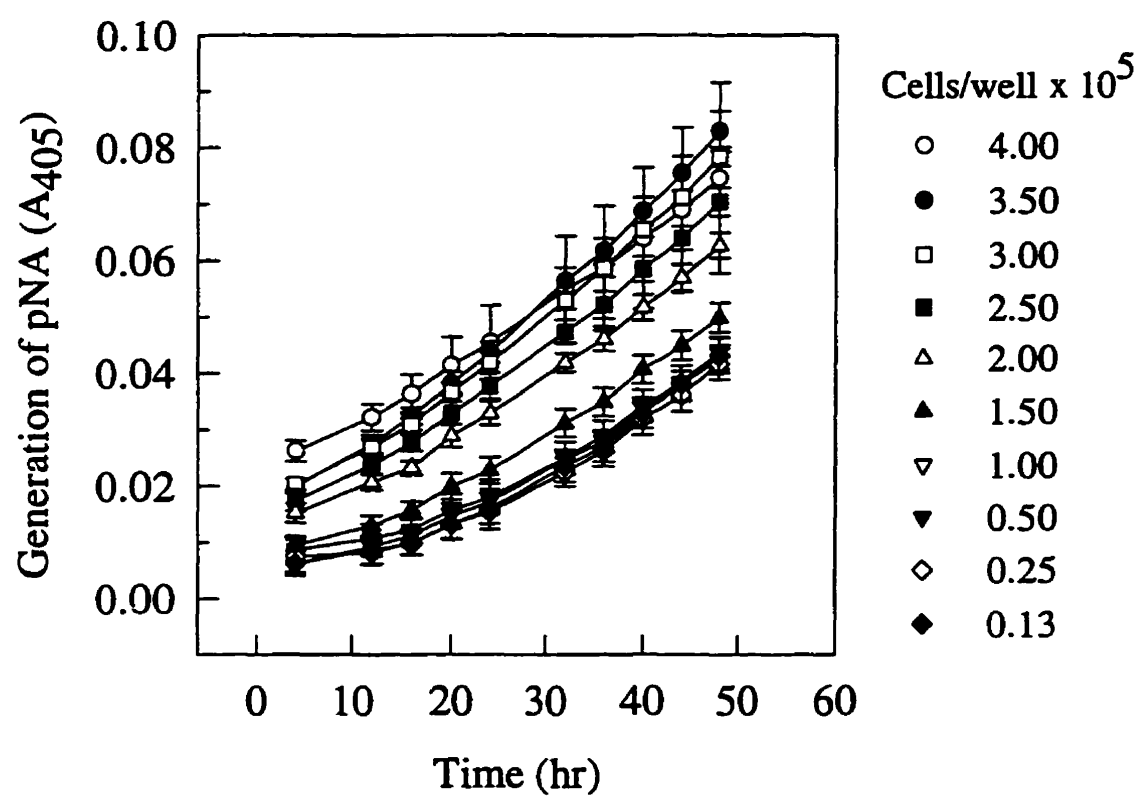


Fig. 4.5. Plasminogen-independent hydrolysis of S-2251 by trophoblasts. Villous cytotrophoblasts were plated at varying densities and cultured in the presence of 20% FCS for 48 h. Trophoblast cultures were then washed and incubated in serum-free medium containing 0.4 mM S-2251. Hydrolysis of S-2251 was measured by recording the appearance of pNA at 405 nm. The data shown are the mean and S.E. from quadruplicate cultures.



Plasmin generation by trophoblasts was also dependent on exogenously added plasminogen. Progress curves of pNA accumulation in the presence of varying concentration of Glu-plasminogen are shown in Fig. 4.6A. A plot of the initial rate of plasmin generation against Glu-plasminogen concentration resulted in a rectangular hyperbola (Fig. 4.6B). The hyperbola was transformed to a linear relationship when the variables were plotted in a double reciprocal manner (Lineweaver-Burk plot) (Fig. 4.6B, inset). This indicates that activation of plasminogen by cultured trophoblasts obeys Michaelis-Menten kinetics.

Plasminogen-dependent accumulation of pNA was completely abolished by the addition of the plasmin inhibitor aprotinin (Fig. 4.7), confirming that it entirely results from plasmin-catalyzed hydrolysis of S-2251.

4.2.3 Characterization of trophoblast PA activity

To determine which of the two known types of physiological plasminogen activators are responsible for the PA activity measured in villous trophoblast cultures, we examined the effect of neutralizing anticatalytic antibodies to u-PA and t-PA respectively on trophoblast plasminogen activation. The basal rate of plasmin generation was dose-dependently reduced by the antibody to u-PA, and the highest antibody concentration resulted in nearly complete inhibition (Fig. 4.8). By contrast, the antibody to t-PA did not inhibit the activation of plasminogen (Fig. 4.9). Likewise, normal goat IgG that served as negative control for both the anti-u-PA and anti-t-PA IgGs had no effect (Fig. 4.10). The results therefore indicate that PA activity of human villous trophoblasts results from

Fig. 4.6. Kinetics of Glu-plasminogen activation by trophoblasts. Cultured trophoblasts (2×10^5 /well) were washed twice and incubated in serum-free medium containing 0.4 mM S-2251 and Glu-plasminogen at varying concentrations. Hydrolysis of S-2251 was measured by recording the appearance of pNA at 405 nm. Plasminogen-dependent hydrolysis of S-2251 was calculated as the difference between absorbances measured in the presence and absence of plasminogen. Progress curves of plasminogen-dependent pNA generation (A) were constructed by fitting quadratics to the experimental points representing the mean and S.E. of quadruplicate cultures. The rates of plasmin generation were obtained from the second derivative of these curves and plotted against the plasminogen concentration (B). The inset shows the data replotted in a double reciprocal manner. The value of K_m was found to be 0.42 μ M.

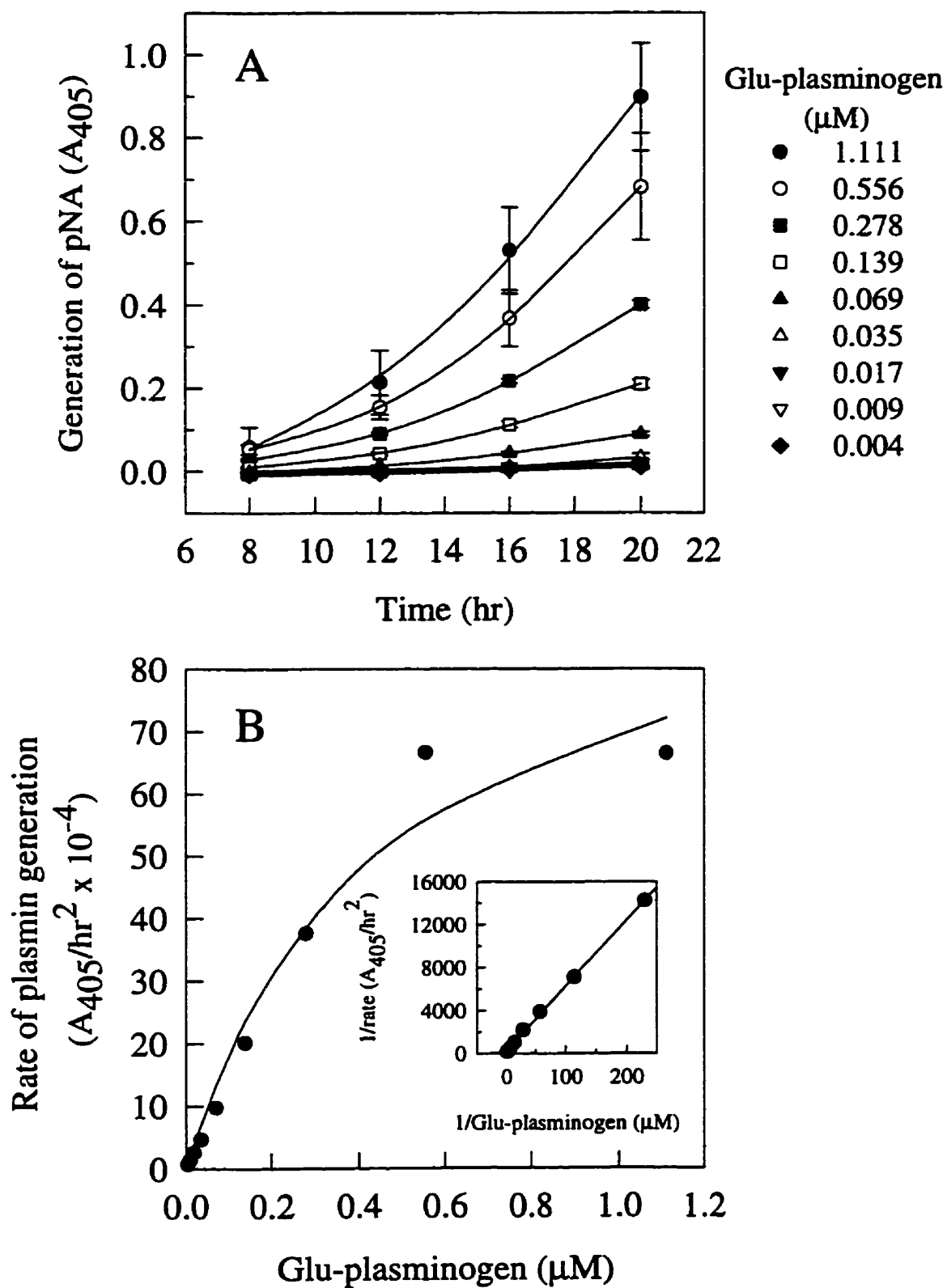


Fig. 4.7. Inhibition of trophoblast-associated plasminogen activation by aprotinin.

Cultured villous trophoblasts (2×10^5 per well) were washed and incubated in serum-free medium containing 0.4 mM S-2251 and aprotinin (Trasylol) at varying concentrations, in the presence or absence of 0.28 μ M Glu-plasminogen. Hydrolysis of S-2251 was measured by recording the appearance of pNA at 405 nm. Plasminogen-dependent hydrolysis of S-2251 was calculated as the difference between absorbances measured in the presence and absence of plasminogen. Progress curves of plasminogen-dependent pNA generation were constructed by fitting quadratics to the experimental points representing the mean and S.E. of quadruplicate cultures. The rates of plasmin generation (inset) were obtained from the second derivative of these curves and expressed as percentage of the rate in control cultures with no aprotinin added.

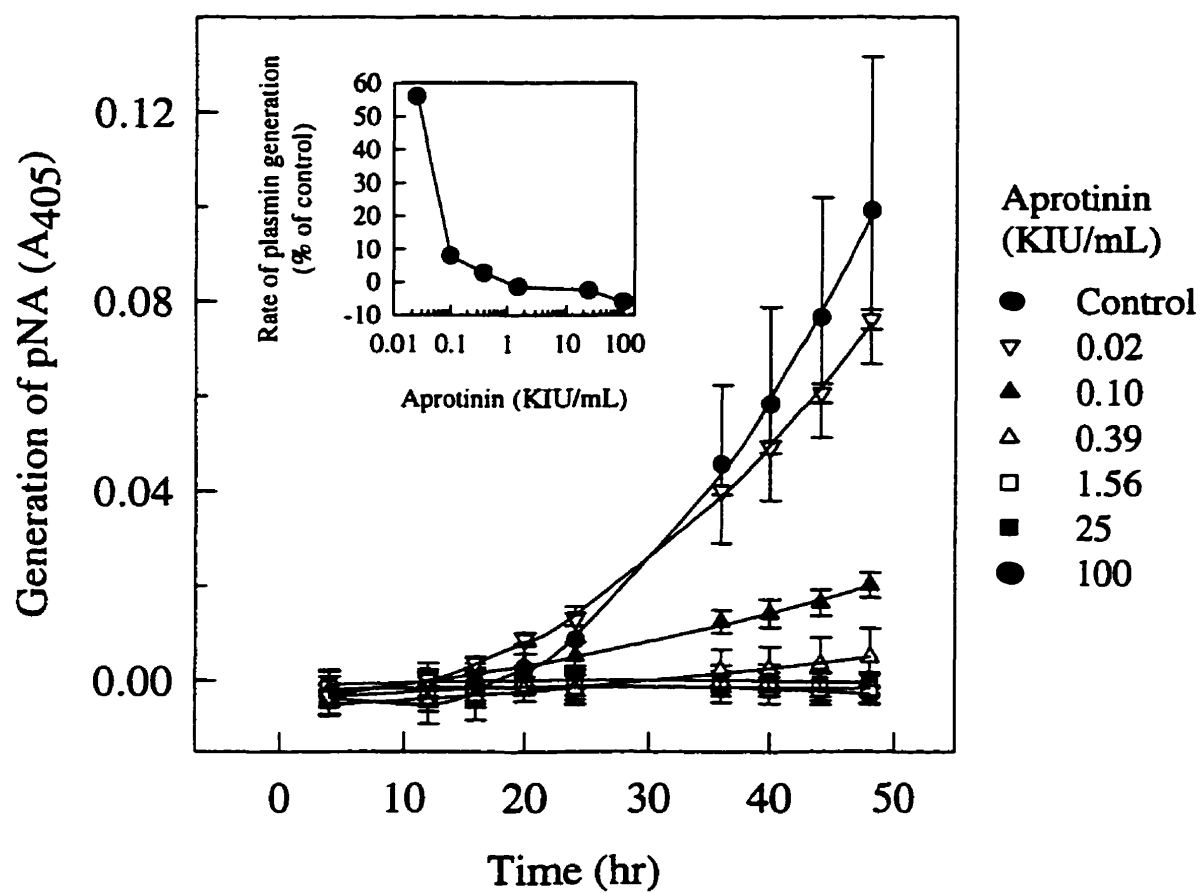


Fig. 4.8. Inhibition of trophoblast plasminogen activation by a neutralizing antibody to u-PA. Cultured villous trophoblasts (2×10^5 per well) were washed and incubated in serum-free medium containing 0.4 mM S-2251 and anti-u-PA at varying concentrations, in the presence or absence of 0.28 μ M Glu-plasminogen. Hydrolysis of S-2251 was measured by recording the appearance of pNA at 405 nm. Plasminogen-dependent hydrolysis of S-2251 was calculated as the difference between absorbances measured in the presence and absence of plasminogen. Progress curves of plasminogen-dependent pNA generation were constructed by fitting quadratics to the experimental points representing the mean and S.E. of quadruplicate cultures. The rates of plasmin generation (inset) were obtained from the second derivative of these curves and expressed as percentage of the rate in control cultures with no antibody addition.

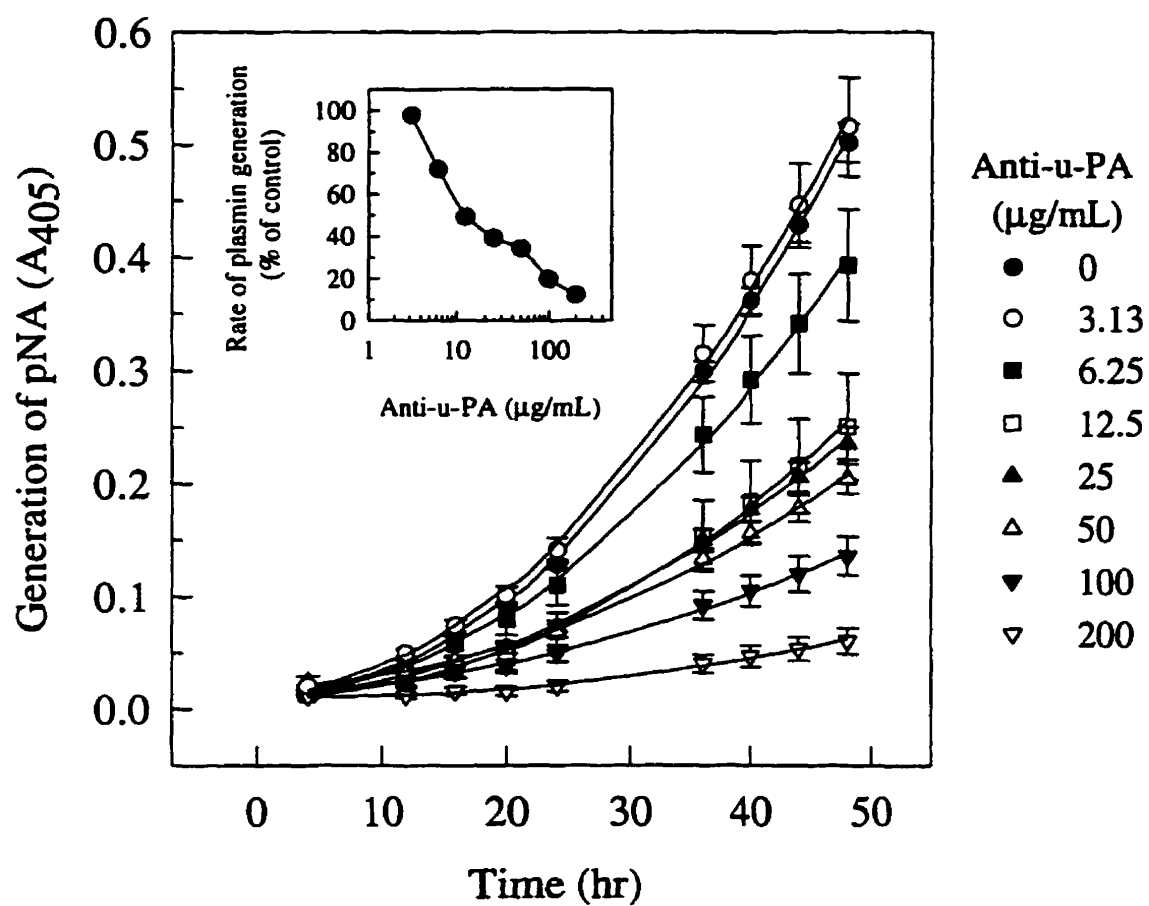


Fig. 4.9. Effect of a neutralizing antibody to t-PA on plasminogen activation by trophoblasts. Cultured villous trophoblasts (2×10^5 per well) were washed and incubated in serum-free medium containing 0.4 mM S-2251 and anti-t-PA at varying concentrations, in the presence or absence of 0.28 μ M Glu-plasminogen. Hydrolysis of S-2251 was measured by recording the appearance of pNA at 405 nm. Plasminogen-dependent hydrolysis of S-2251 was calculated as the difference between absorbances measured in the presence and absence of plasminogen. Progress curves of plasminogen-dependent pNA generation were constructed by fitting quadratics to the experimental points representing the mean and S.E. of quadruplicate cultures. The rates of plasmin generation (inset) were obtained from the second derivative of these curves and expressed as percentage of the rate in control cultures with no antibody addition.

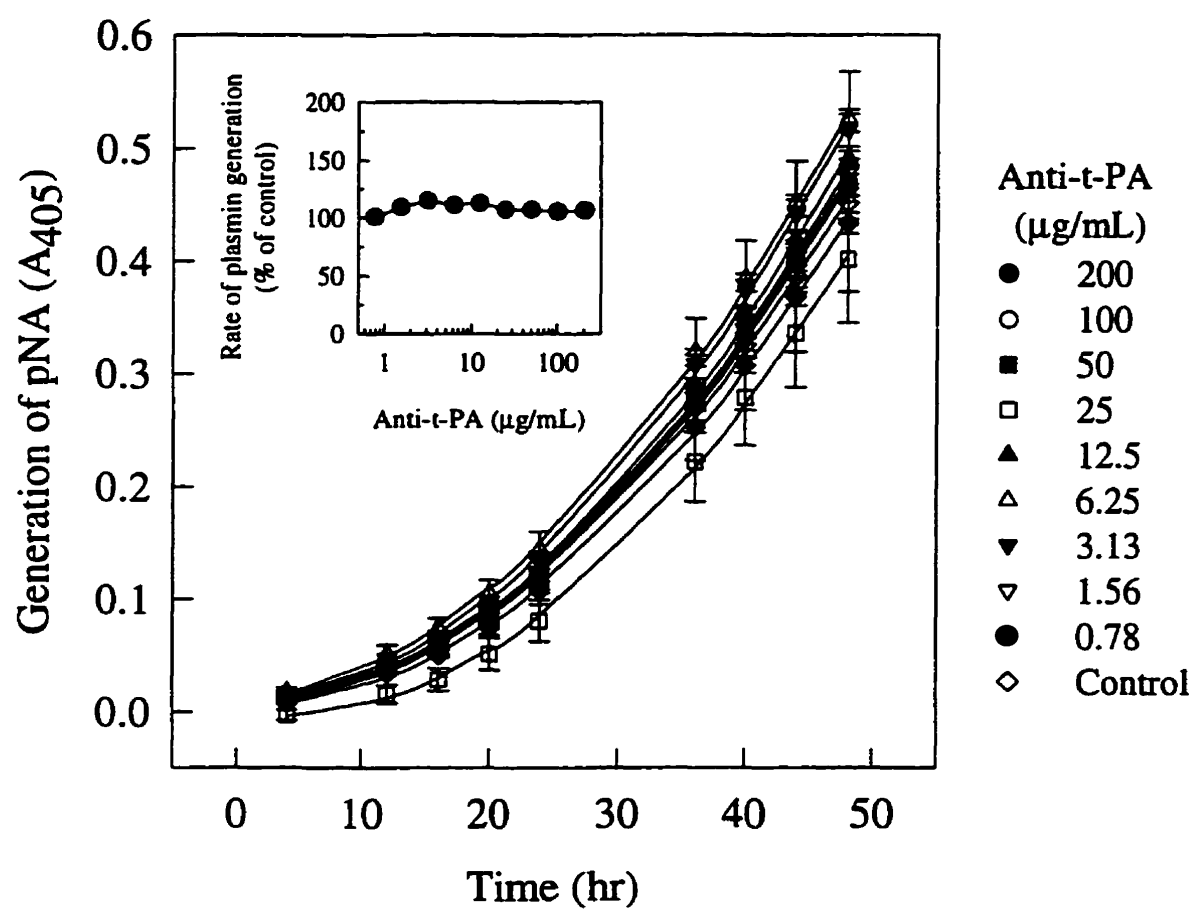
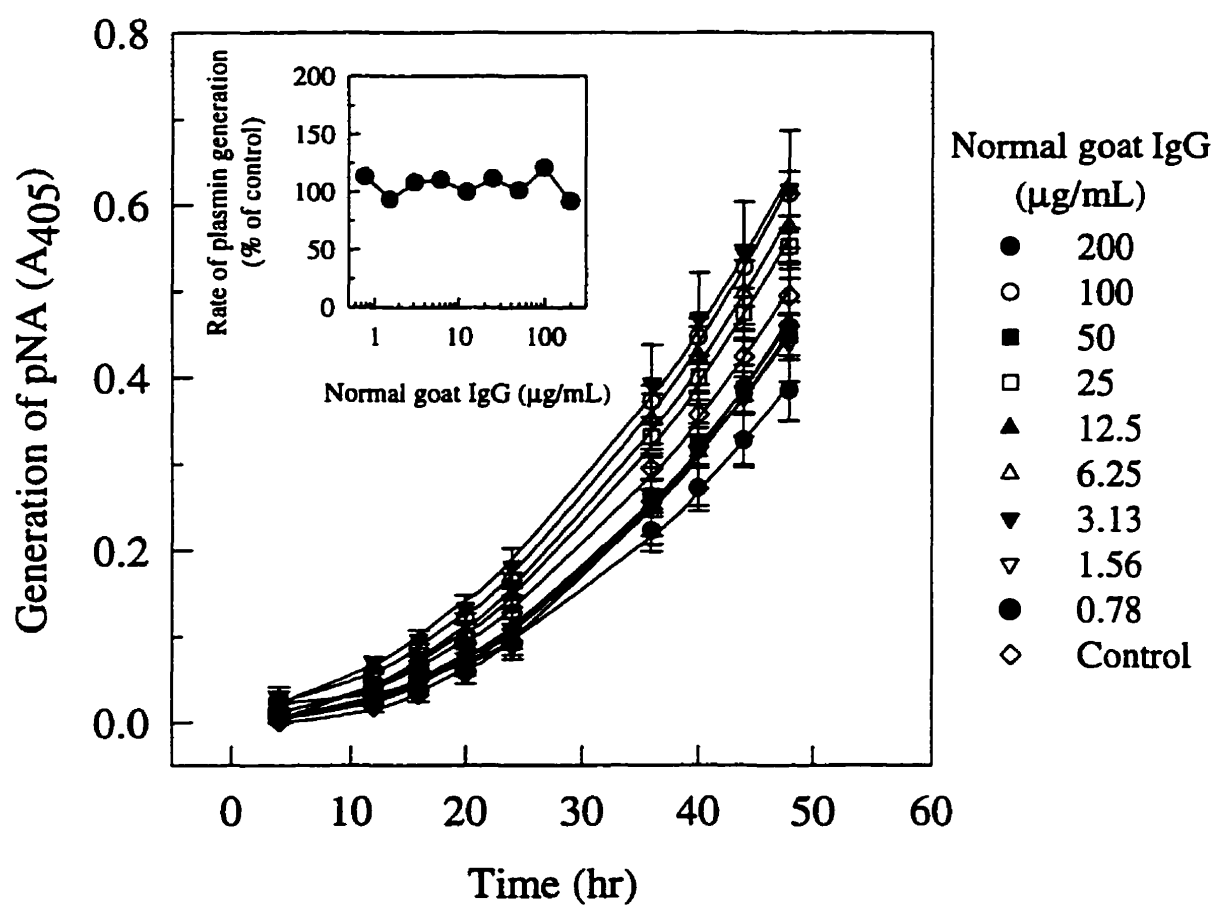


Fig. 4.10. Effect of normal goat IgG on plasminogen activation by trophoblasts. Cultured villous trophoblasts (2×10^5 per well) were washed and incubated in serum-free medium containing 0.4 mM S-2251 and normal goat IgG at varying concentrations, in the presence or absence of 0.28 μ M Glu-plasminogen. Hydrolysis of S-2251 was measured by recording the appearance of pNA at 405 nm. Plasminogen-dependent hydrolysis of S-2251 was calculated as the difference between absorbances measured in the presence and absence of plasminogen. Progress curves of plasminogen-dependent pNA generation were constructed by fitting quadratics to the experimental points representing the mean and S.E. of quadruplicate cultures. The rates of plasmin generation (inset) were obtained from the second derivative of these curves and expressed as percentage of the rate in control cultures with no antibody addition.



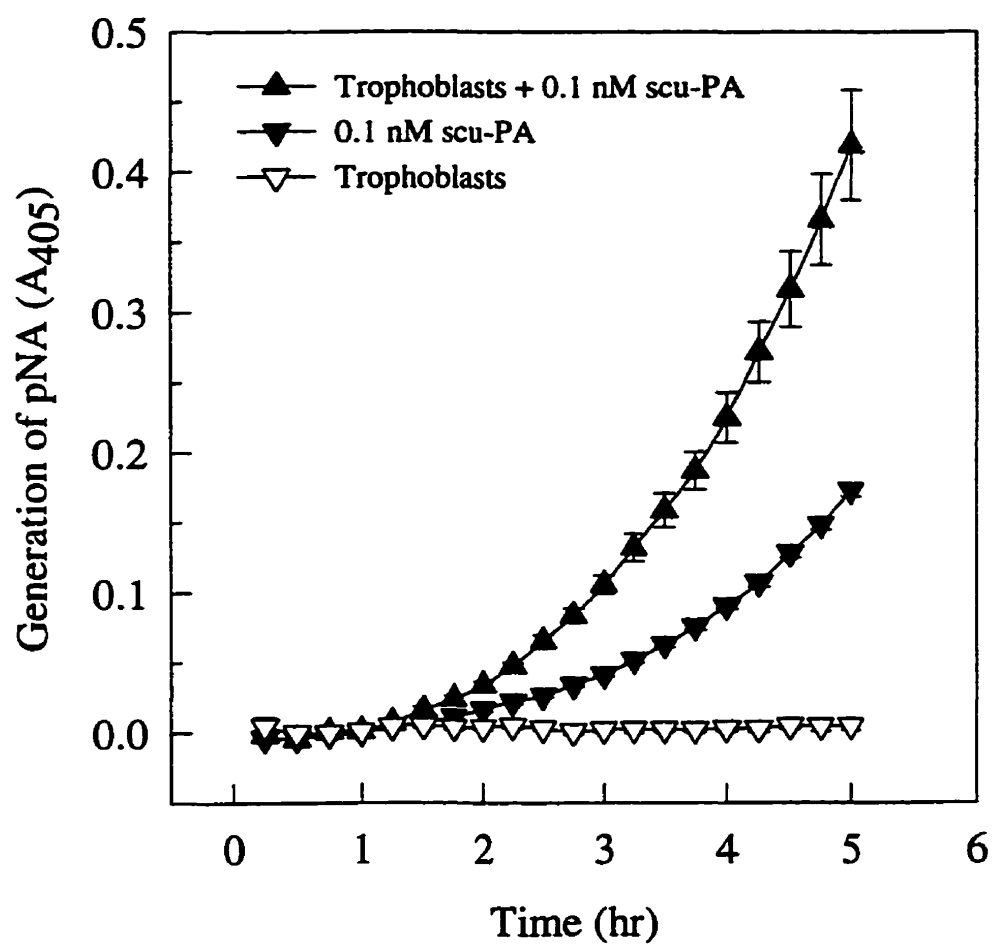
u-PA but not t-PA.

4.2.4 Trophoblasts enhance plasminogen activation initiated by single-chain u-PA (pro-u-PA)

Cell surface binding of pro-u-PA and plasminogen is thought to enhance plasminogen activation through an increased plasmin mediated feedback activation of pro-u-PA (Ellis et al., 1989; Duval-Jobe and Parmely, 1994). Human term trophoblasts possess u-PA receptors, and the generation of plasmin initiated by trophoblast-bound pro-u-PA is accompanied by an accelerated conversion of pro-u-PA to u-PA (Zini et al., 1992). We therefore sought to investigate whether activation of plasminogen is increased in the presence of trophoblasts. In these experiments, cultured trophoblasts were treated with aprotinin followed by acid treatment to inhibit trophoblast associated serine proteinases and remove cell-bound u-PA, respectively. After neutralizing the acidic buffer, the cultures were washed and cultured in serum-free medium containing plasminogen, S-2251, and scu-PA. Plasminogen activation was simultaneously measured in the absence of trophoblasts, using the same concentrations of plasminogen, S-2251 and scu-PA. As shown in Fig. 4.11, scu-PA (0.1 nM) induced plasminogen activation is enhanced in the presence of trophoblasts as compared to fluid phase activation in the absence of trophoblasts. High (presumably saturating) concentrations of scu-PA did not accelerate plasminogen activation (data are not shown). No significant plasmin generation was detected in cultures treated with aprotinin and acidic buffer, indicating that endogenous u-PA or plasmin entrapped on the trophoblast surface during serum culture

Fig. 4.11. Effect of trophoblasts on exogenous pro-u-PA induced plasminogen activation.

Cultured villous trophoblasts (2×10^5 per well) were washed twice in indicator-free medium, and the cultures were treated with 1000 K.I.U./mL aprotinin (Trasylol) for 15 min to inhibit trophoblast associated serine proteinases. Subsequently, the cells were incubated with 50 mM glycine-HCl buffer containing 0.1 M NaCl (pH 3.0) for 5 min to remove u-PA from the trophoblast surface. After neutralization with 0.5 M HEPES, 0.1 M NaCl (pH 7.3) for 1 min followed by two washes with indicator-free medium, pro-u-PA (0.1 nM) and S-2251 (0.4 mM) were added to the cultures. Hydrolysis of S-2251 was measured in the presence or absence of 0.28 μ M Glu-plasminogen. Pro-u-PA induced hydrolysis of S-2251 was simultaneously measured in the absence of trophoblasts as well. Plasminogen-dependent hydrolysis of S-2251 was calculated as the difference between absorbances measured in the presence and absence of plasminogen. The data shown are the mean and S.E. from quadruplicate wells.



are not implicated in trophoblast-mediated enhancement of plasminogen activation.

4.2.5 Inhibition of trophoblast PA activity by PAIs

We demonstrated earlier in this chapter that cultured villous trophoblasts express both PAI-1 and PAI-2. Here we show that exogenous PAIs are efficient inhibitors of trophoblast-associated u-PA activity. Fig. 4.12 demonstrates the inhibition of trophoblast plasminogen activation by varying concentrations of recombinant PAI-1. The dose-dependent inhibition of plasminogen activation by recombinant PAI-2 is shown in Fig. 4.13.

4.2.6 Regulation of trophoblast PA activity by endogenous PAIs

Next, we examined whether trophoblast u-PA activity is regulated by endogenously secreted PAIs. Fig. 4.14 demonstrates that a neutralizing polyclonal antibody to PAI-1 increases trophoblast PA activity in a dose dependent manner. An approximately sixfold enhancement was reached at 200 $\mu\text{g/mL}$ of antibody concentration. Normal goat IgG that served as negative control had no effect on PA activity of trophoblasts at the same concentrations (Fig. 4.10). The results again point to the capacity of human villous trophoblasts to produce PAI-1 and indicate that the basal level of trophoblast PA activity is under autocrine control of PAI-1.

We were unable to obtain evidence for the regulation of trophoblast PA activity by PAI-2. Neither of the two different antibodies to PAI-2 tested increased trophoblast PA activity (Fig. 4.15), suggesting that these cultures may not secrete significant amount of PAI-2.

Fig. 4.12. Inhibition of trophoblast plasminogen activation by PAI-1. Cultured villous trophoblasts (2×10^5 per well) were washed and incubated in serum-free medium containing 0.4 mM S-2251 and PAI-1 at varying concentrations, in the presence or absence of 0.28 μ M Glu-plasminogen. Hydrolysis of S-2251 was measured by recording the appearance of pNA at 405 nm. Plasminogen-dependent hydrolysis of S-2251 was calculated as the difference between absorbances measured in the presence and absence of plasminogen. Progress curves of plasminogen-dependent pNA generation were constructed by fitting quadratics to the experimental points representing the mean and S.E. of quadruplicate cultures. The rates of plasmin generation (inset) were obtained from the second derivative of these curves and expressed as percentage of the rate in control cultures with no PAI-1 addition.

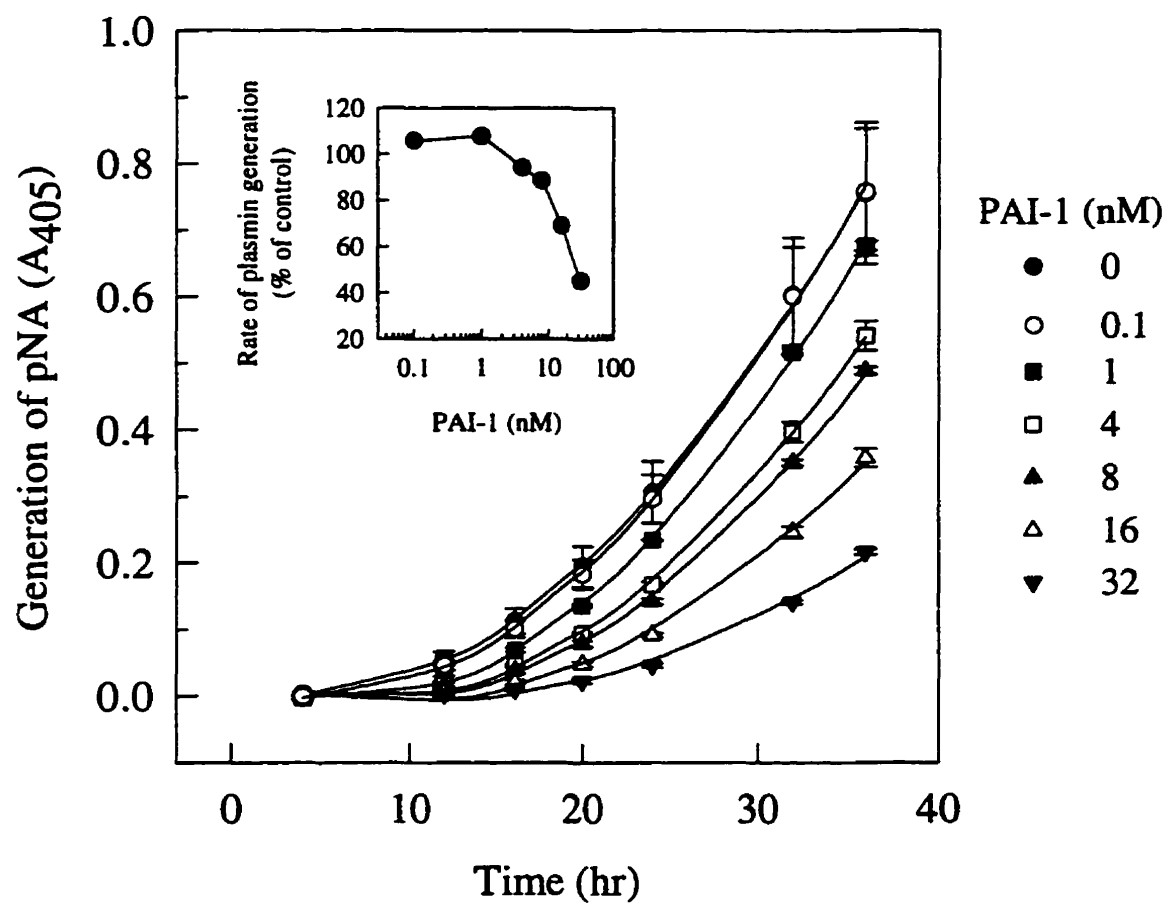


Fig. 4.13. Inhibition of trophoblast plasminogen activation by PAI-2. Cultured villous trophoblasts (2×10^5 per well) were washed and incubated in serum-free medium containing 0.4 mM S-2251 and PAI-2 at varying concentrations, in the presence or absence of 0.28 μ M Glu-plasminogen. Hydrolysis of S-2251 was measured by recording the appearance of pNA at 405 nm. Plasminogen-dependent hydrolysis of S-2251 was calculated as the difference between absorbances measured in the presence and absence of plasminogen. Progress curves of plasminogen-dependent pNA generation were constructed by fitting quadratics to the experimental points representing the mean and S.E. of quadruplicate cultures. The rates of plasmin generation (inset) were obtained from the second derivative of these curves and expressed as percentage of the rate in control cultures with no PAI-2 addition.

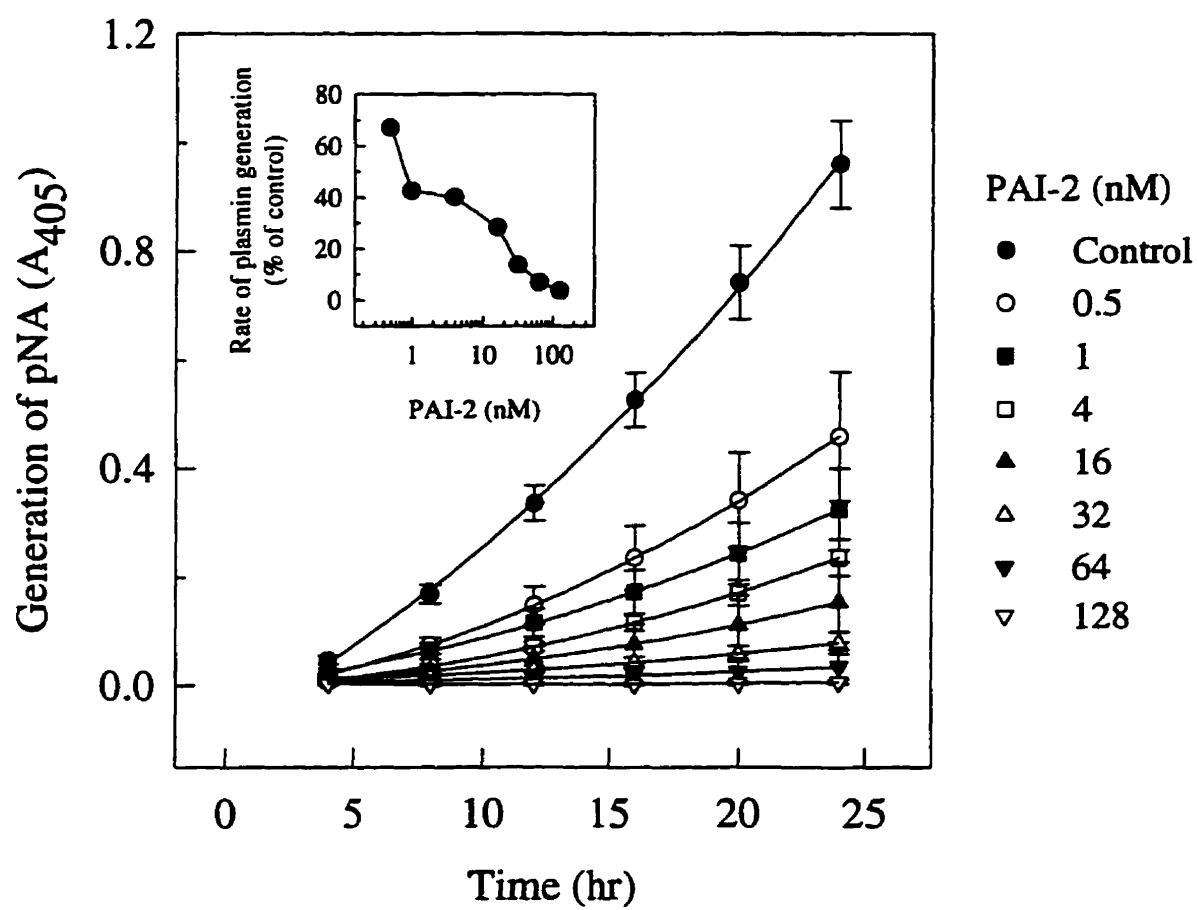


Fig. 4.14. Enhancement of trophoblast plasminogen activation by anti-PAI-1. Cultured villous trophoblasts (2×10^5 per well) were washed and incubated in serum-free medium containing 0.4 mM S-2251 and anti-PAI-1 at varying concentrations, in the presence or absence of 0.28 μ M Glu-plasminogen. Hydrolysis of S-2251 was measured by recording the appearance of pNA at 405 nm. Plasminogen-dependent hydrolysis of S-2251 was calculated as the difference between absorbances measured in the presence and absence of plasminogen. Progress curves of plasminogen-dependent pNA generation were constructed by fitting quadratics to the experimental points representing the mean and S.E. of quadruplicate cultures. The rates of plasmin generation (inset) were obtained from the second derivative of these curves and expressed as percentage of the rate in control cultures with no antibody addition.

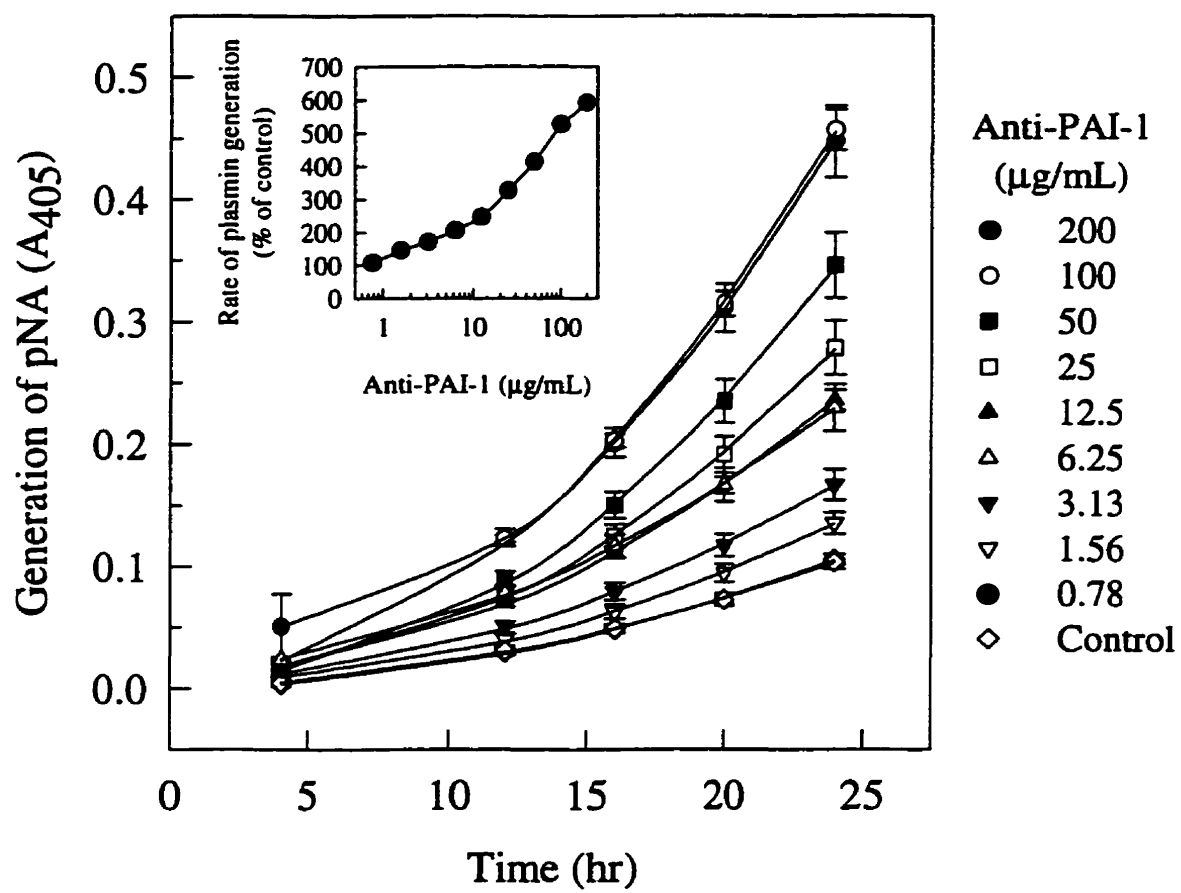
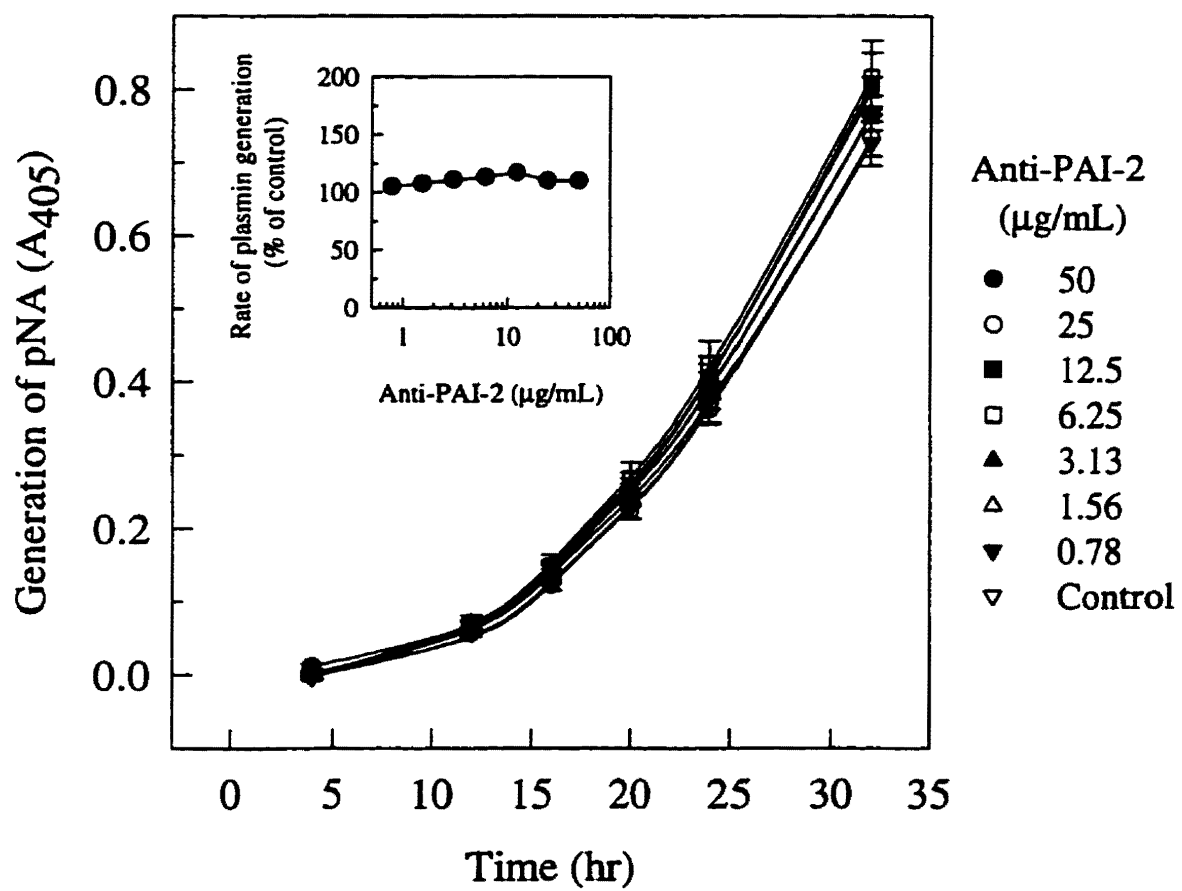


Fig. 4.15. Effect of anti-PAI-2 on plasminogen activation by trophoblasts. Cultured villous trophoblasts (2×10^5 per well) were washed and incubated in serum-free medium containing 0.4 mM S-2251 and anti-PAI-2 at varying concentrations, in the presence or absence of 0.28 μ M Glu-plasminogen. Hydrolysis of S-2251 was measured by recording the appearance of pNA at 405 nm. Plasminogen-dependent hydrolysis of S-2251 was calculated as the difference between absorbances measured in the presence and absence of plasminogen. Progress curves of plasminogen-dependent pNA generation were constructed by fitting quadratics to the experimental points representing the mean and S.E. of quadruplicate cultures. The rates of plasmin generation (inset) were obtained from the second derivative of these curves and expressed as percentage of the rate in control cultures with no antibody addition.



4.2.7 Modulation of trophoblast PA activity by various compounds

To define modulators of trophoblast plasminogen activation, various substances known to be important in trophoblast development and invasiveness have been tested for their ability to alter the rate of plasmin generation in trophoblast cultures.

4.2.7.1 Effects of components of the blood coagulation system

Thrombin and activated protein C (APC) have been shown to modulate PA activity in endothelial cell cultures (Loskutoff, 1979; Sakata et al., 1985). As it has been suggested that these proteases may be involved in the pathogenesis of preeclampsia (Aznar et al., 1986; de Boer et al., 1989; Espana et al., 1991) where disturbed plasminogen activation occurs, we have examined whether they also modulate PA activity in trophoblasts. Thrombin caused a dose dependent inhibition of trophoblast plasminogen activation, which was complete at very low concentrations (Fig. 4.16). APC increased the plasminogen-dependent hydrolysis of S-2251 (Fig. 4.17). However, when the rates of plasmin generation were calculated, no increased rates were obtained. In fact, at the highest concentration tested (16 nM) the time course of pNA generation was approximately linear, thus resulting in a zero rate of plasmin generation. This suggests a mechanism involving an initial stimulation of plasminogen activation (during the initial 4-hour time interval when pNA accumulation is too low to be detected), accompanied by later inhibition.

4.2.7.2 Effects of growth factors/cytokines

As shown in Fig. 4.18, EGF stimulated plasminogen activation in a dose dependent

Fig. 4.16. Inhibition of trophoblast plasminogen activation by thrombin. Cultured villous trophoblasts (2×10^5 per well) were washed and incubated in serum-free medium containing 0.4 mM S-2251 and thrombin at varying concentrations, in the presence or absence of 0.28 μ M Glu-plasminogen. Hydrolysis of S-2251 was measured by recording the appearance of pNA at 405 nm. Plasminogen-dependent hydrolysis of S-2251 was calculated as the difference between absorbances measured in the presence and absence of plasminogen. Progress curves of plasminogen-dependent pNA generation were constructed by fitting quadratics to the experimental points representing the mean and S.E. of quadruplicate cultures. The rates of plasmin generation (inset) were obtained from the second derivative of these curves and expressed as percentage of the rate in control cultures with no thrombin addition.

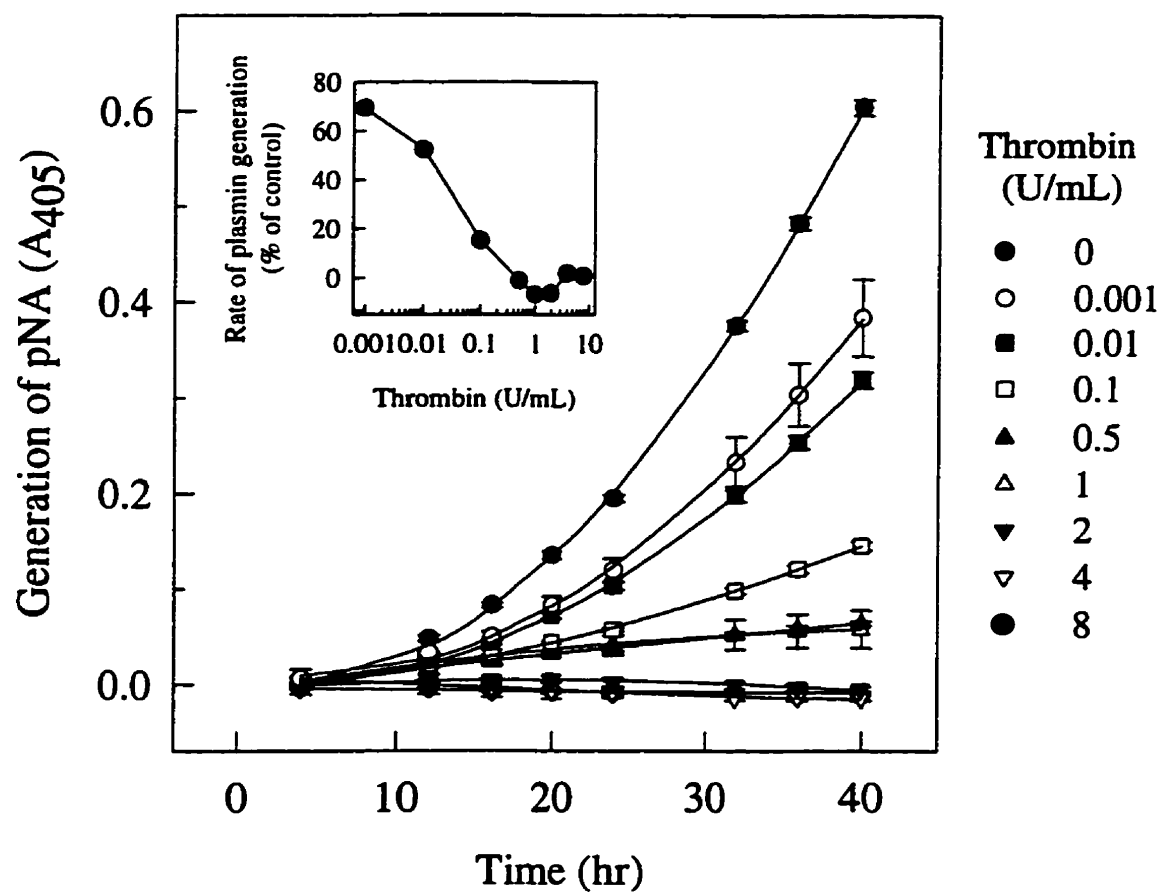
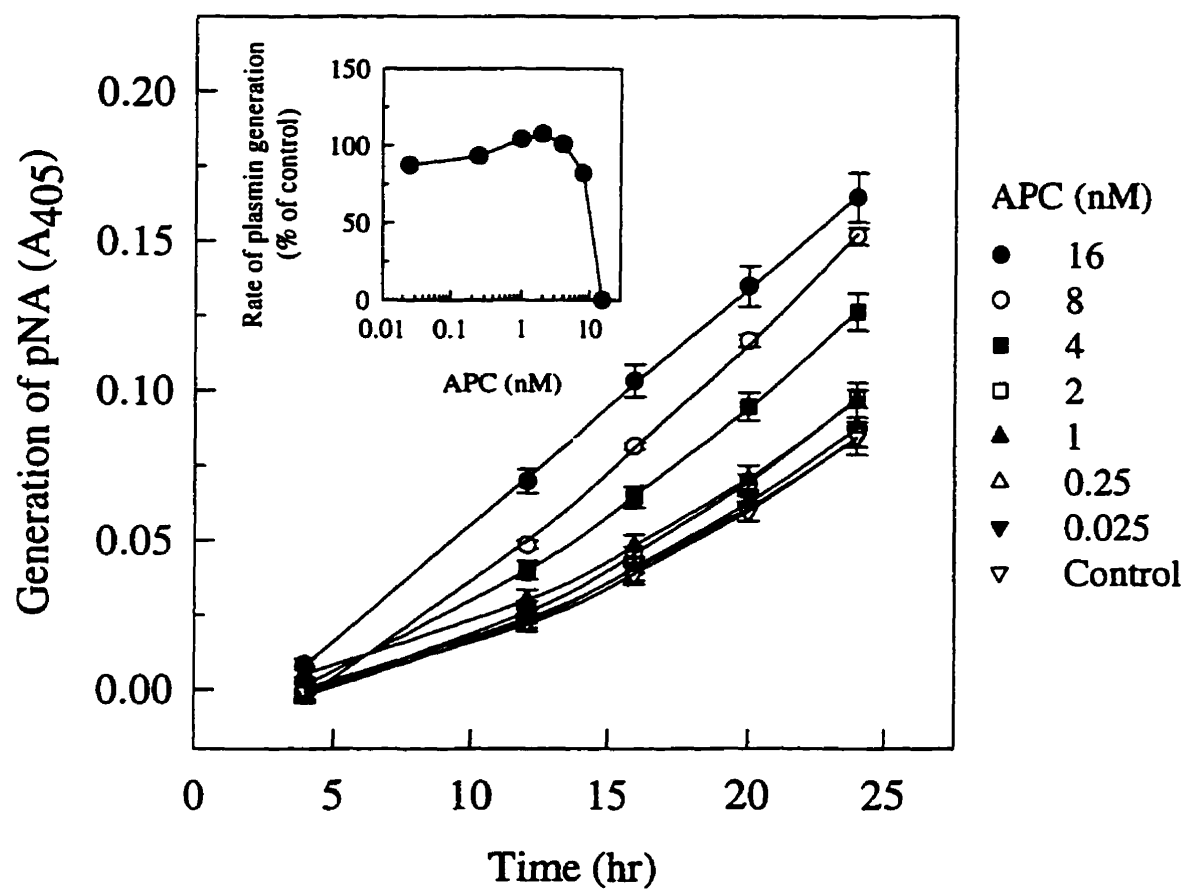


Fig. 4.17. Effect of APC on plasminogen activation by trophoblasts. Cultured villous trophoblasts (2×10^5 per well) were washed and incubated in serum-free medium containing 0.4 mM S-2251 and APC at varying concentrations, in the presence or absence of 0.28 μ M Glu-plasminogen. Hydrolysis of S-2251 was measured by recording the appearance of pNA at 405 nm. Plasminogen-dependent hydrolysis of S-2251 was calculated as the difference between absorbances measured in the presence and absence of plasminogen. Progress curves of plasminogen-dependent pNA generation were constructed by fitting quadratics to the experimental points representing the mean and S.E. of quadruplicate cultures. The rates of plasmin generation (inset) were obtained from the second derivative of these curves and expressed as percentage of the rate in control cultures with no APC addition.



manner. As little as 1 ng/mL of EGF induced an increase in the rate of plasmin generation, and maximal stimulation was reached at 10 ng/mL. Likewise, TGF- α , a structural analog of EGF caused a dose dependent stimulation of PA activity (Fig. 4.19).

We also sought to determine whether the EGF induced stimulation of trophoblast PA activity is accompanied by cellular proliferation and/or increased metabolic activity. To this end, the MTT reductive cleavage of trophoblast cultures was determined following the measurement of PA activity in the presence of EGF. Fig. 4.20 shows that EGF does not appreciably change the capacity of trophoblasts to cleave MTT, indicating that EGF stimulation of trophoblast PA activity is not connected to proliferation/increased metabolic capacity.

To determine whether endogenous secretion of EGF has a role in regulating the basal level of PA activity, trophoblasts were cultured in serum-free medium alone or with increasing doses of a neutralizing antibody to EGF. Fig. 4.21 shows that the antibody did not cause a decrease in plasminogen activation. This result suggests that term villous trophoblast cultures do not produce sufficient amount of EGF to modulate PA activity. Alternatively, EGF may be produced in a precursor form not recognized by the antibody.

TGF- β has been reported to inhibit trophoblast PA activity in first trimester trophoblasts (Graham et al., 1993, 1994). Our experiments suggest, however, that similar inhibition does not occur in primary cultures of term villous trophoblasts. As demonstrated in Fig. 4.22, TGF- β_1 does not affect plasminogen activation in our cultures.

Our results suggest that IGF-II (Fig. 4.23) and IL-1 β (Fig. 4.24) have no

Fig. 4.18. Stimulation of trophoblast plasminogen activation by EGF. Cultured villous trophoblasts (2×10^5 per well) were washed and incubated in serum-free medium containing 0.4 mM S-2251 and EGF at varying concentrations, in the presence or absence of 0.28 μ M Glu-plasminogen. Hydrolysis of S-2251 was measured by recording the appearance of pNA at 405 nm. Plasminogen-dependent hydrolysis of S-2251 was calculated as the difference between absorbances measured in the presence and absence of plasminogen. Progress curves of plasminogen-dependent pNA generation were constructed by fitting quadratics to the experimental points representing the mean and S.E. of quadruplicate cultures. The rates of plasmin generation (inset) were obtained from the second derivative of these curves and expressed as percentage of the rate in control cultures with no EGF addition.

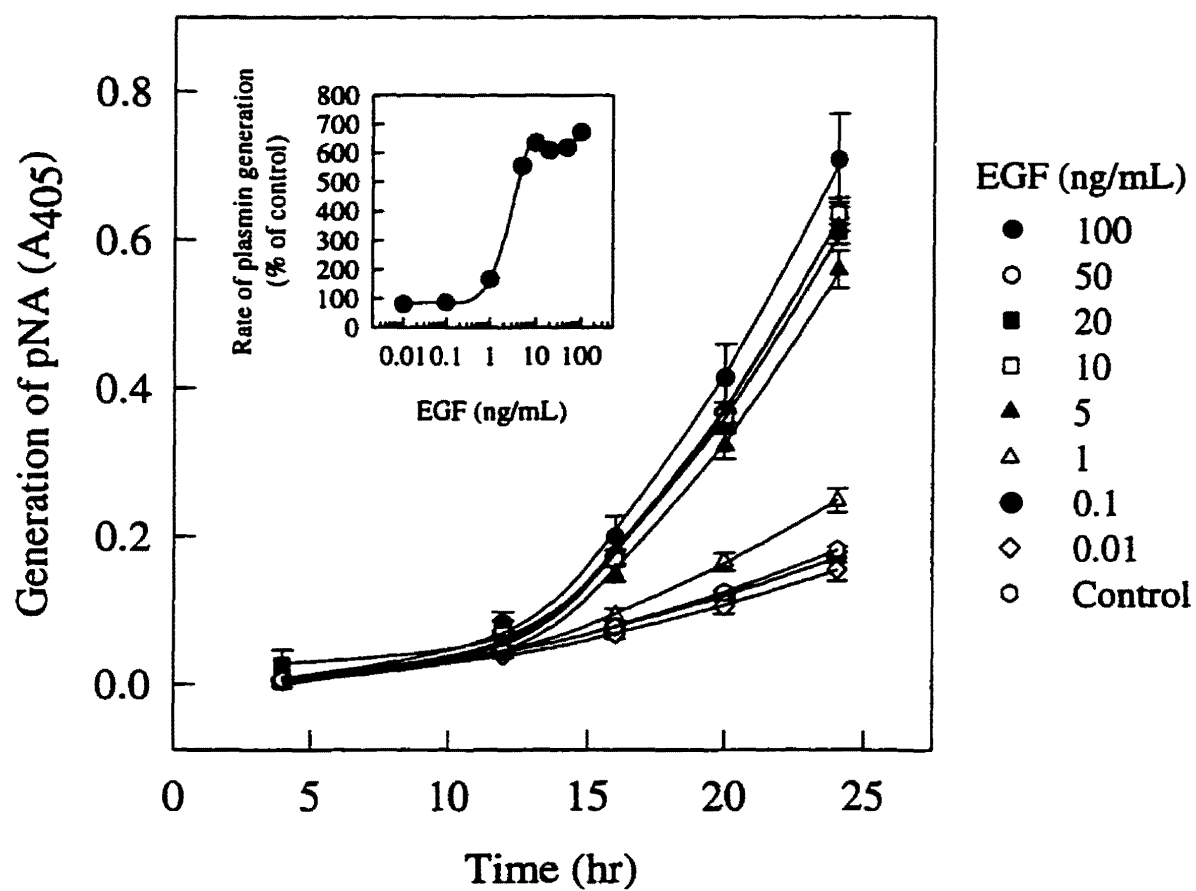


Fig. 4.19. Stimulation of trophoblast plasminogen activation by TGF- α . Cultured villous trophoblasts (2×10^5 per well) were washed and incubated in serum-free medium containing 0.4 mM S-2251 and TGF- α at varying concentrations, in the presence or absence of 0.28 μ M Glu-plasminogen. Hydrolysis of S-2251 was measured by recording the appearance of pNA at 405 nm. Plasminogen-dependent hydrolysis of S-2251 was calculated as the difference between absorbances measured in the presence and absence of plasminogen. Progress curves of plasminogen-dependent pNA generation were constructed by fitting quadratics to the experimental points representing the mean and S.E. of quadruplicate cultures. The rates of plasmin generation (inset) were obtained from the second derivative of these curves and expressed as percentage of the rate in control cultures with no TGF- α addition.

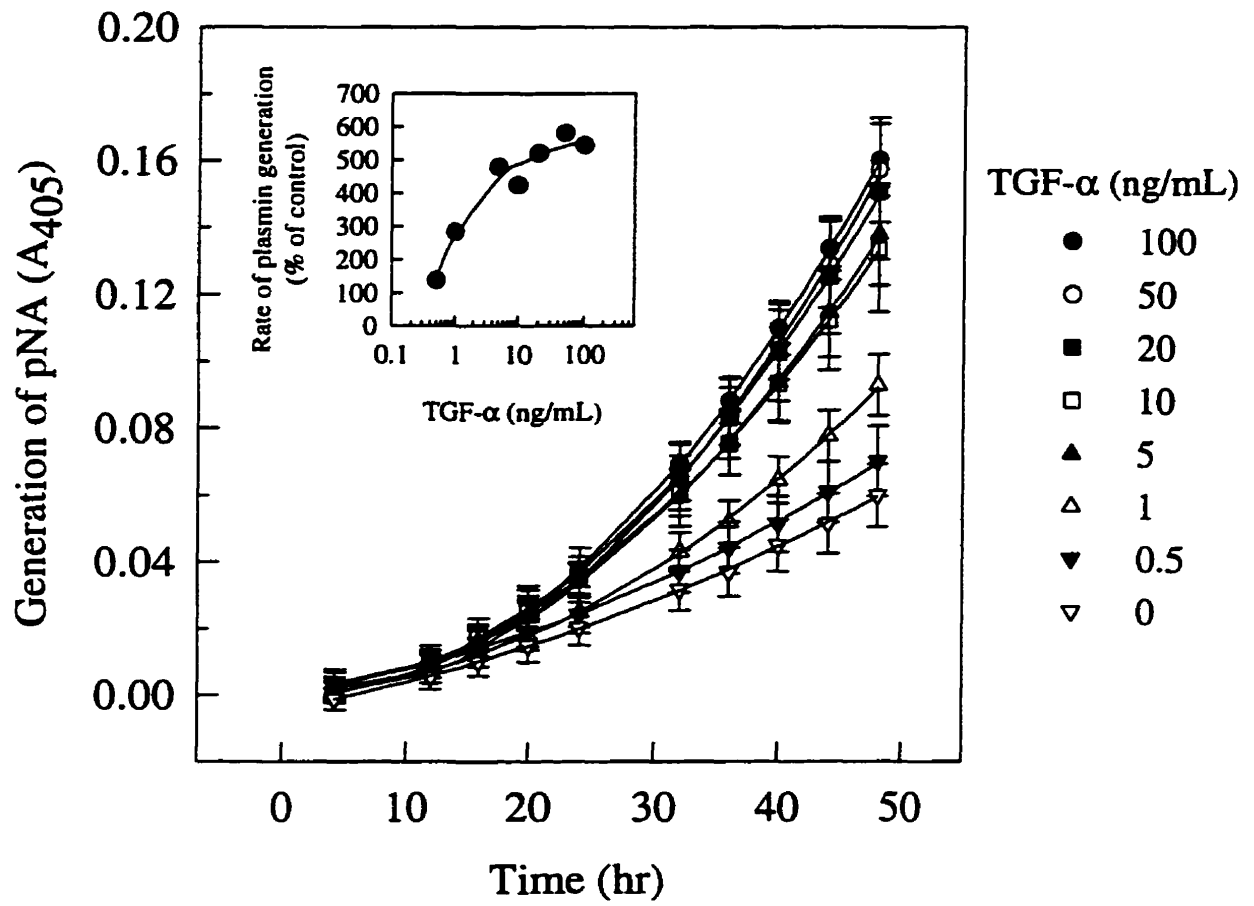


Fig. 4.20. MTT reductive cleavage by cultured trophoblasts following EGF stimulation of plasminogen activation. PA activity of cultured villous trophoblasts was determined as described in Chapter 2. Subsequently, the cultures were washed twice with indicator-free medium and incubated with MTT for 2 h. After solubilization the absorbance of the blue formazan was measured at 595 nm. The data shown are the mean and S.E. from 4 wells.

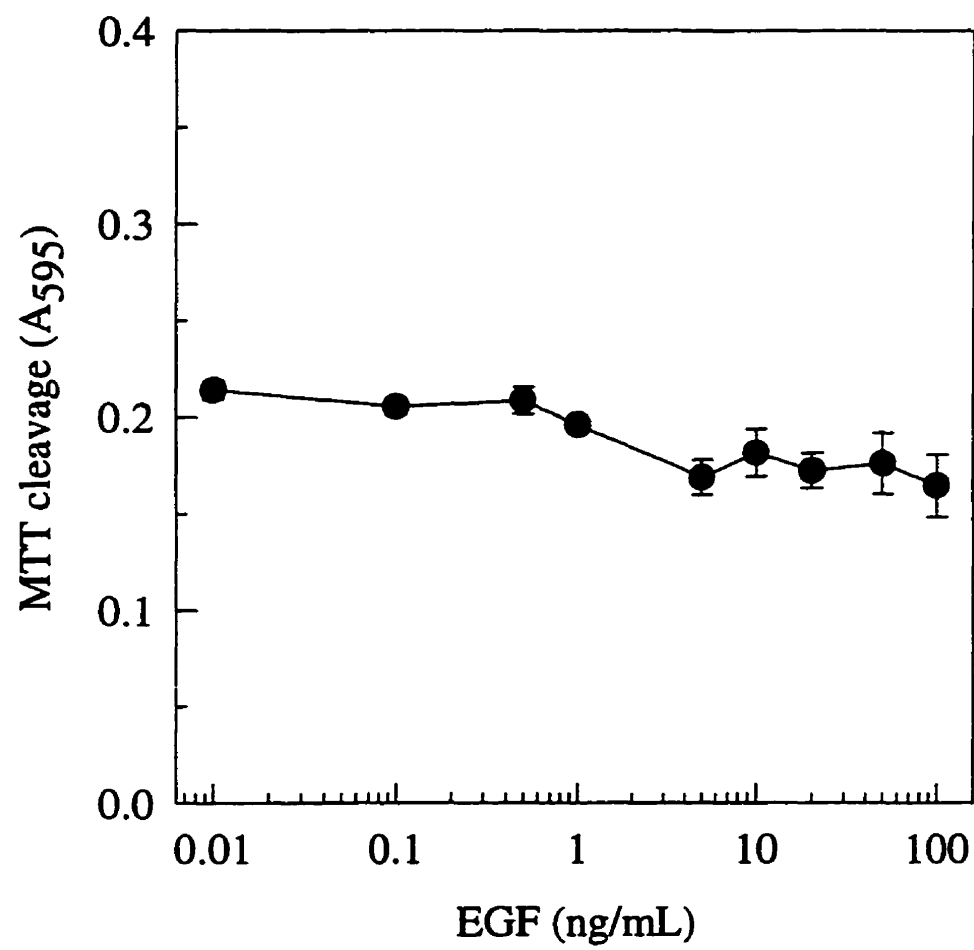


Fig. 4.21. Effect of anti-EGF on plasminogen activation by trophoblasts. Cultured villous trophoblasts (2×10^5 per well) were washed and incubated in serum-free medium containing 0.4 mM S-2251 and anti-EGF at varying concentrations, in the presence or absence of 0.28 μ M Glu-plasminogen. Hydrolysis of S-2251 was measured by recording the appearance of pNA at 405 nm. Plasminogen-dependent hydrolysis of S-2251 was calculated as the difference between absorbances measured in the presence and absence of plasminogen. Progress curves of plasminogen-dependent pNA generation were constructed by fitting quadratics to the experimental points representing the mean and S.E. of quadruplicate cultures. The rates of plasmin generation (inset) were obtained from the second derivative of these curves and expressed as percentage of the rate in control cultures with no antibody addition.

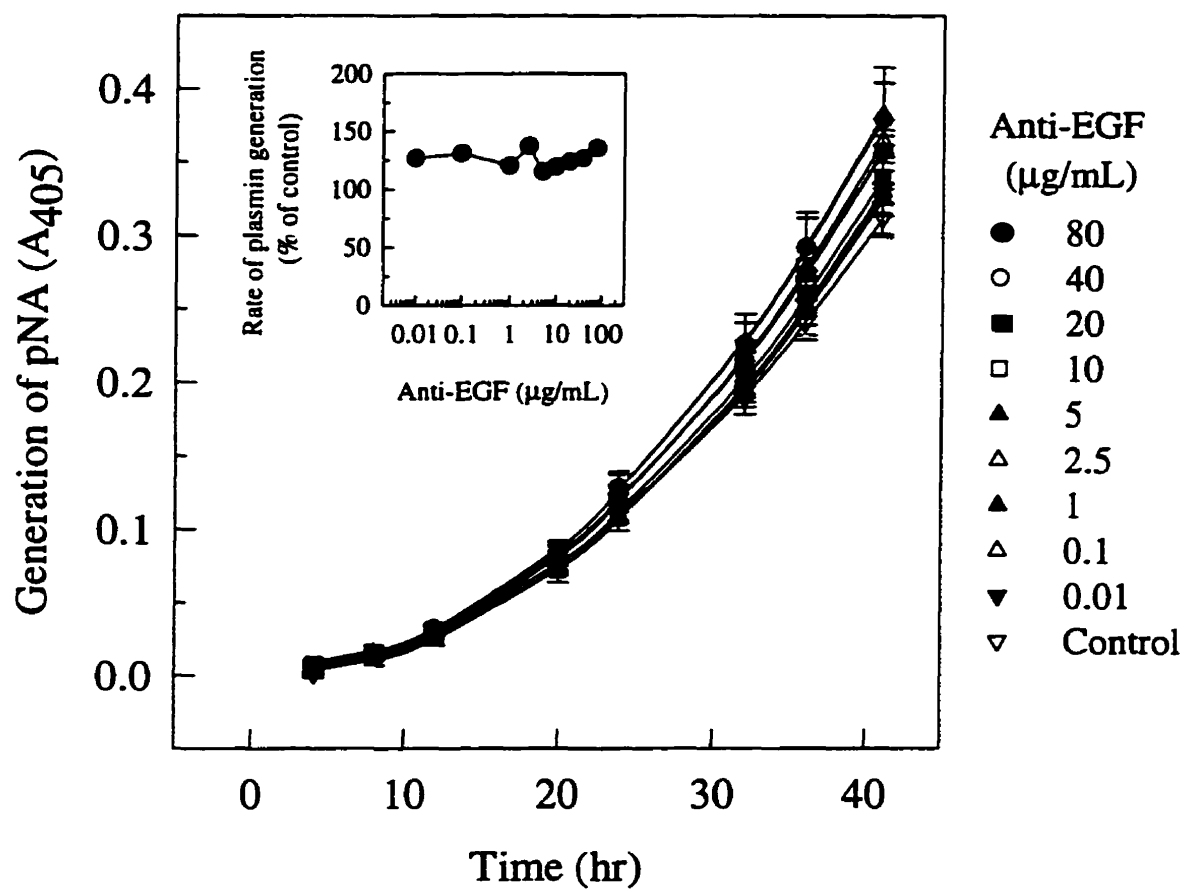


Fig. 4.22. Effect of TGF- β 1 on plasminogen activation by trophoblasts. Cultured villous trophoblasts (2×10^5 per well) were washed and incubated in serum-free medium containing 0.4 mM S-2251 and TGF- β 1 at varying concentrations, in the presence or absence of 0.28 μ M Glu-plasminogen. Hydrolysis of S-2251 was measured by recording the appearance of pNA at 405 nm. Plasminogen-dependent hydrolysis of S-2251 was calculated as the difference between absorbances measured in the presence and absence of plasminogen. Progress curves of plasminogen-dependent pNA generation were constructed by fitting quadratics to the experimental points representing the mean and S.E. of quadruplicate cultures. The rates of plasmin generation (inset) were obtained from the second derivative of these curves and expressed as percentage of the rate in control cultures with no TGF- β 1 addition.

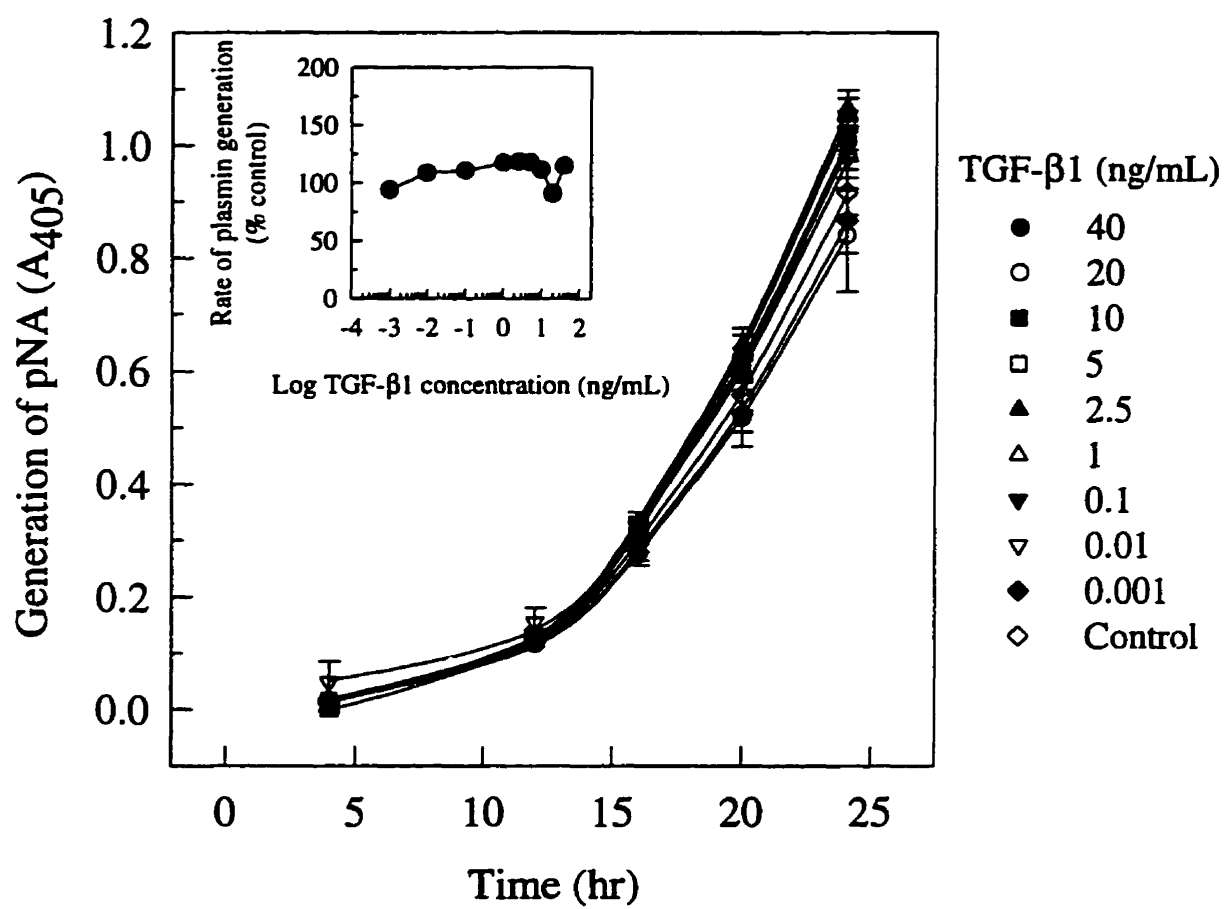


Fig. 4.23. Effect of IGF-II on plasminogen activation by trophoblasts. Cultured villous trophoblasts (2×10^5 per well) were washed and incubated in serum-free medium containing 0.4 mM S-2251 and IGF-II at varying concentrations, in the presence or absence of 0.28 μ M Glu-plasminogen. Hydrolysis of S-2251 was measured by recording the appearance of pNA at 405 nm. Plasminogen-dependent hydrolysis of S-2251 was calculated as the difference between absorbances measured in the presence and absence of plasminogen. Progress curves of plasminogen-dependent pNA generation were constructed by fitting quadratics to the experimental points representing the mean and S.E. of quadruplicate cultures. The rates of plasmin generation (inset) were obtained from the second derivative of these curves and expressed as percentage of the rate in control cultures with no IGF-II addition.

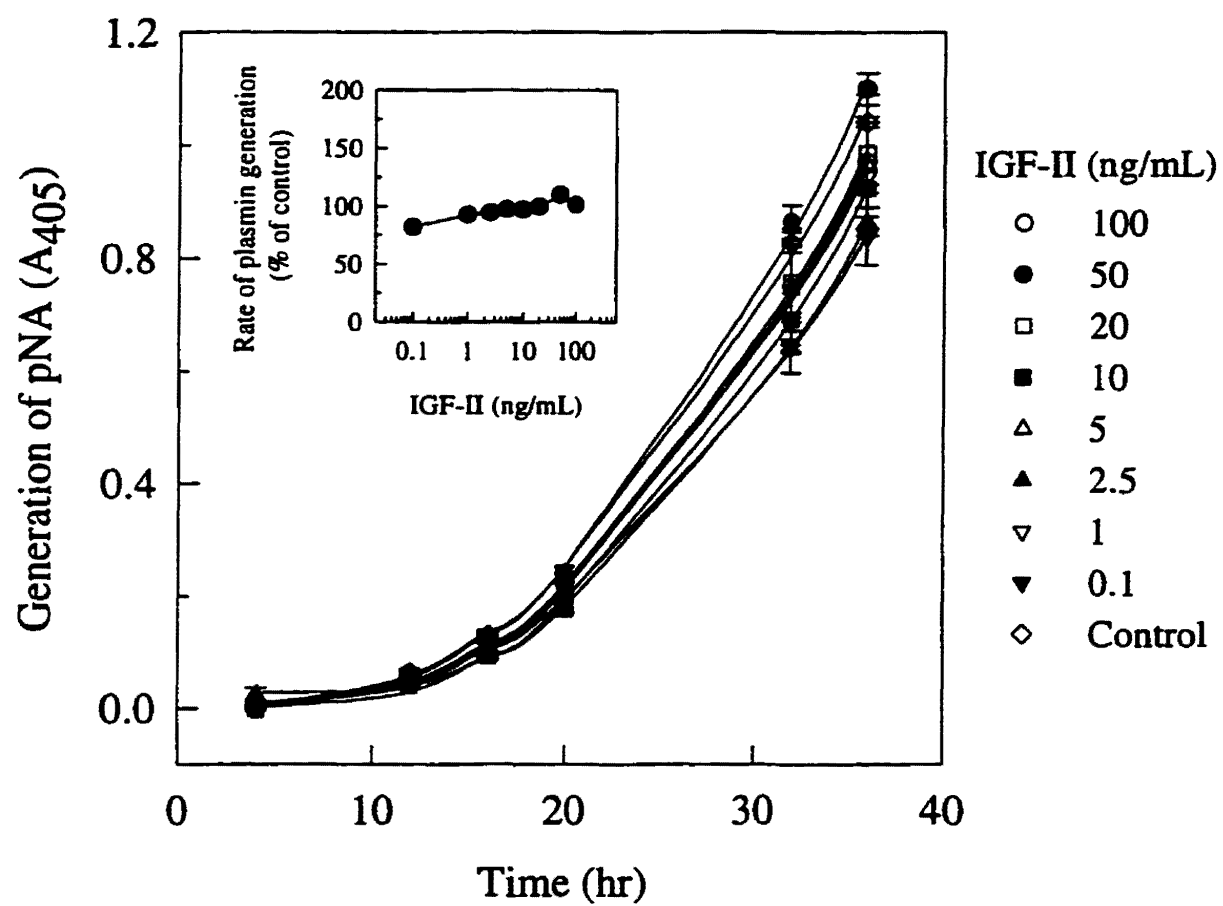
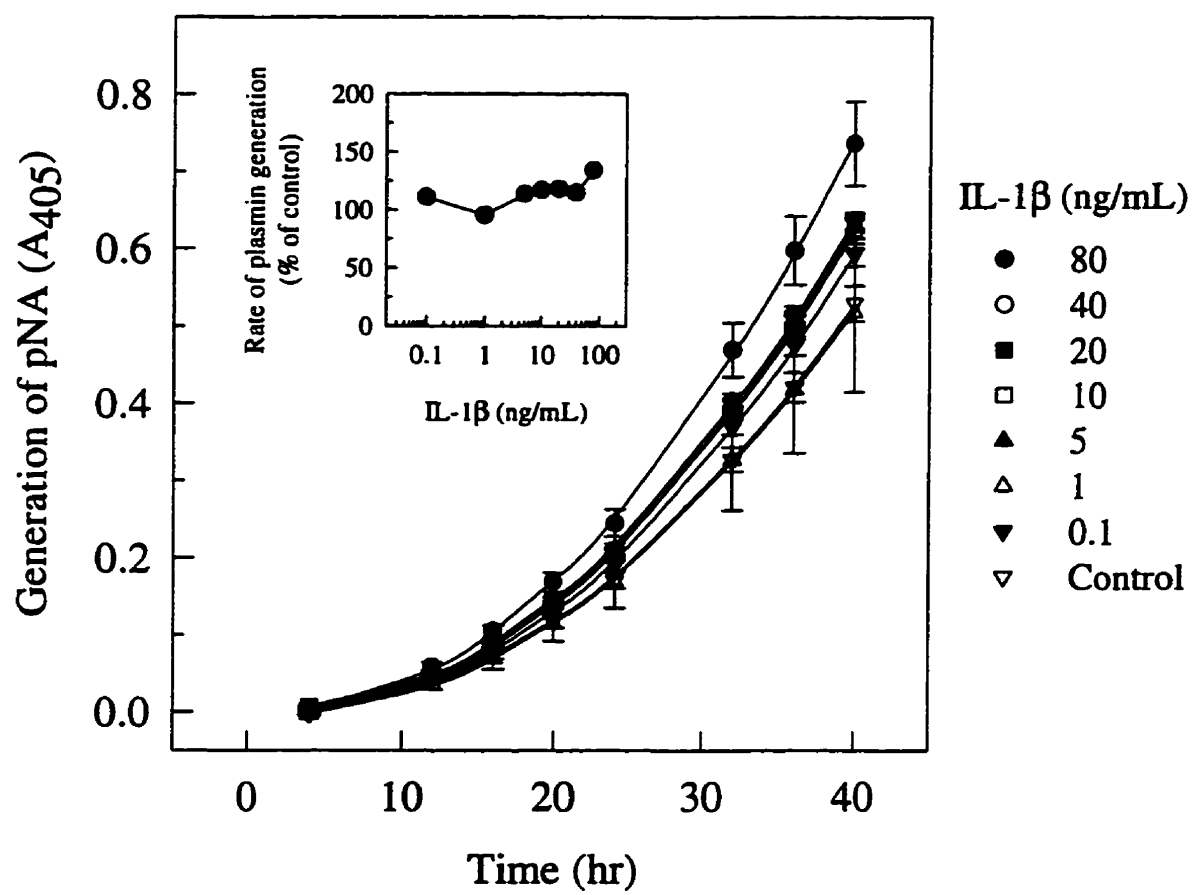


Fig. 4.24. Effect of IL-1 β on plasminogen activation by trophoblasts. Cultured villous trophoblasts (2×10^5 per well) were washed and incubated in serum-free medium containing 0.4 mM S-2251 and IL-1 β at varying concentrations, in the presence or absence of 0.28 μ M Glu-plasminogen. Hydrolysis of S-2251 was measured by recording the appearance of pNA at 405 nm. Plasminogen-dependent hydrolysis of S-2251 was calculated as the difference between absorbances measured in the presence and absence of plasminogen. Progress curves of plasminogen-dependent pNA generation were constructed by fitting quadratics to the experimental points representing the mean and S.E. of quadruplicate cultures. The rates of plasmin generation (inset) were obtained from the second derivative of these curves and expressed as percentage of the rate in control cultures with no IL-1 β addition.



appreciable effect on trophoblast plasminogen activation. LPS, which is known to increase secretion of IL-1 β in trophoblasts (Librach et al., 1994), did not alter the activation of plasminogen either (Fig. 4.25).

4.2.7.3 Effects of hormones

Treatment of trophoblast cultures with hCG caused an inhibition of plasminogen activation (Fig. 4.26).

Dexamethasone elicited a dose-dependent decrease in the rate of plasminogen activation (Fig. 4.27). A significant decrease was noticed at 10^{-8} M. Maximal inhibition was reached at 10^{-6} M.

4.2.7.4 Effect of 8-bromo-cAMP, forskolin and PMA

Finally, the involvement of the protein kinase-A and -C pathways in mediating regulatory effects on trophoblast plasminogen activation was examined. We found that 8-bromo-cAMP (Fig. 4.28) and forskolin (Fig. 4.29) that increases intracellular cAMP levels inhibited plasminogen activation. PMA, however, which is known to exert effects through the protein kinase-C pathway had no effect (Fig. 4.30).

4.3 DISCUSSION

Implantation and subsequent placentation are critically dependent on the controlled invasion of the maternal uterus by trophoblasts. This process is accomplished through coordinated interactions of several classes of molecules expressed at the materno-fetal interface. Among these molecules, trophoblast-secreted proteases appear to play a central role in mediating trophoblast invasion, during which extensive ECM breakdown, tissue

Fig. 4.25. Effect of LPS on plasminogen activation by trophoblasts. Cultured villous trophoblasts (2×10^5 per well) were washed and incubated in serum-free medium containing 0.4 mM S-2251 and LPS at varying concentrations, in the presence or absence of 0.28 μ M Glu-plasminogen. Hydrolysis of S-2251 was measured by recording the appearance of pNA at 405 nm. Plasminogen-dependent hydrolysis of S-2251 was calculated as the difference between absorbances measured in the presence and absence of plasminogen. Progress curves of plasminogen-dependent pNA generation were constructed by fitting quadratics to the experimental points representing the mean and S.E. of quadruplicate cultures. The rates of plasmin generation (inset) were obtained from the second derivative of these curves and expressed as percentage of the rate in control cultures with no LPS addition.

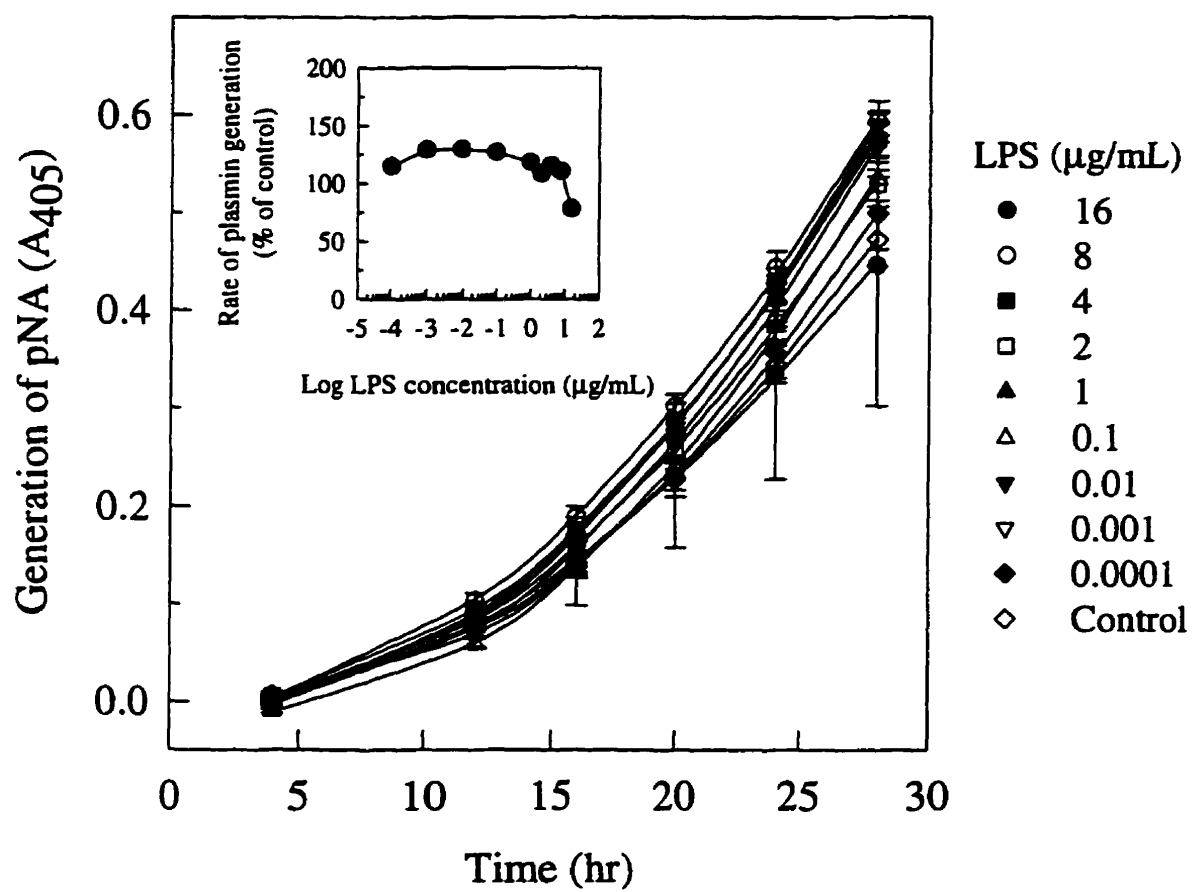


Fig. 4.26. Inhibition of trophoblast plasminogen activation by hCG. Cultured villous trophoblasts (2×10^5 per well) were washed and incubated in serum-free medium containing 0.4 mM S-2251 and hCG at varying concentrations, in the presence or absence of 0.28 μ M Glu-plasminogen. Hydrolysis of S-2251 was measured by recording the appearance of pNA at 405 nm. Plasminogen-dependent hydrolysis of S-2251 was calculated as the difference between absorbances measured in the presence and absence of plasminogen. Progress curves of plasminogen-dependent pNA generation were constructed by fitting quadratics to the experimental points representing the mean and S.E. of quadruplicate cultures. The rates of plasmin generation (inset) were obtained from the second derivative of these curves and expressed as percentage of the rate in control cultures with no hCG addition.

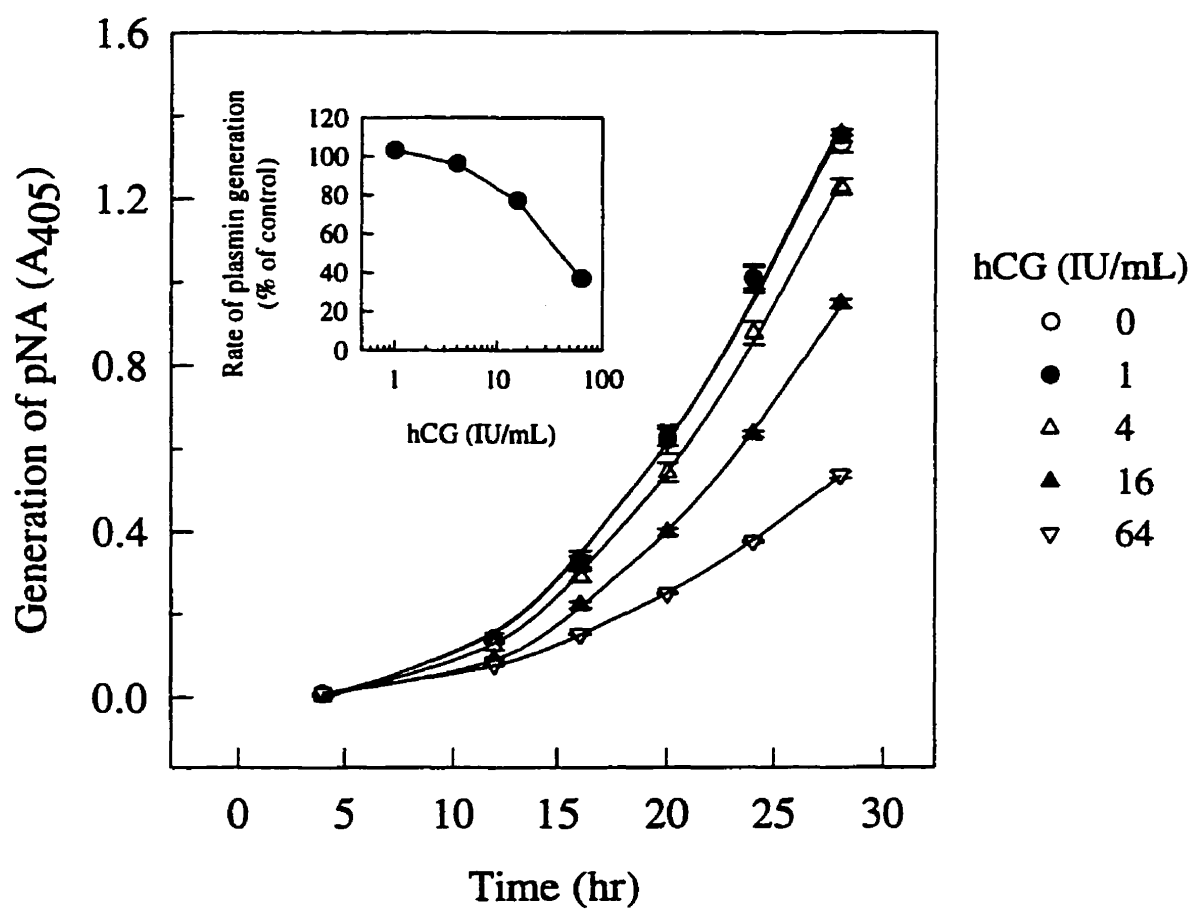


Fig. 4.27. Inhibition of trophoblast plasminogen activation by dexamethasone. Cultured villous trophoblasts (2×10^5 per well) were washed and incubated in serum-free medium containing 0.4 mM S-2251 and dexamethasone at varying concentrations, in the presence or absence of 0.28 μ M Glu-plasminogen. Hydrolysis of S-2251 was measured by recording the appearance of pNA at 405 nm. Plasminogen-dependent hydrolysis of S-2251 was calculated as the difference between absorbances measured in the presence and absence of plasminogen. Progress curves of plasminogen-dependent pNA generation were constructed by fitting quadratics to the experimental points representing the mean and S.E. of quadruplicate cultures. The rates of plasmin generation (inset) were obtained from the second derivative of these curves and expressed as percentage of the rate in control cultures with no dexamethasone addition.

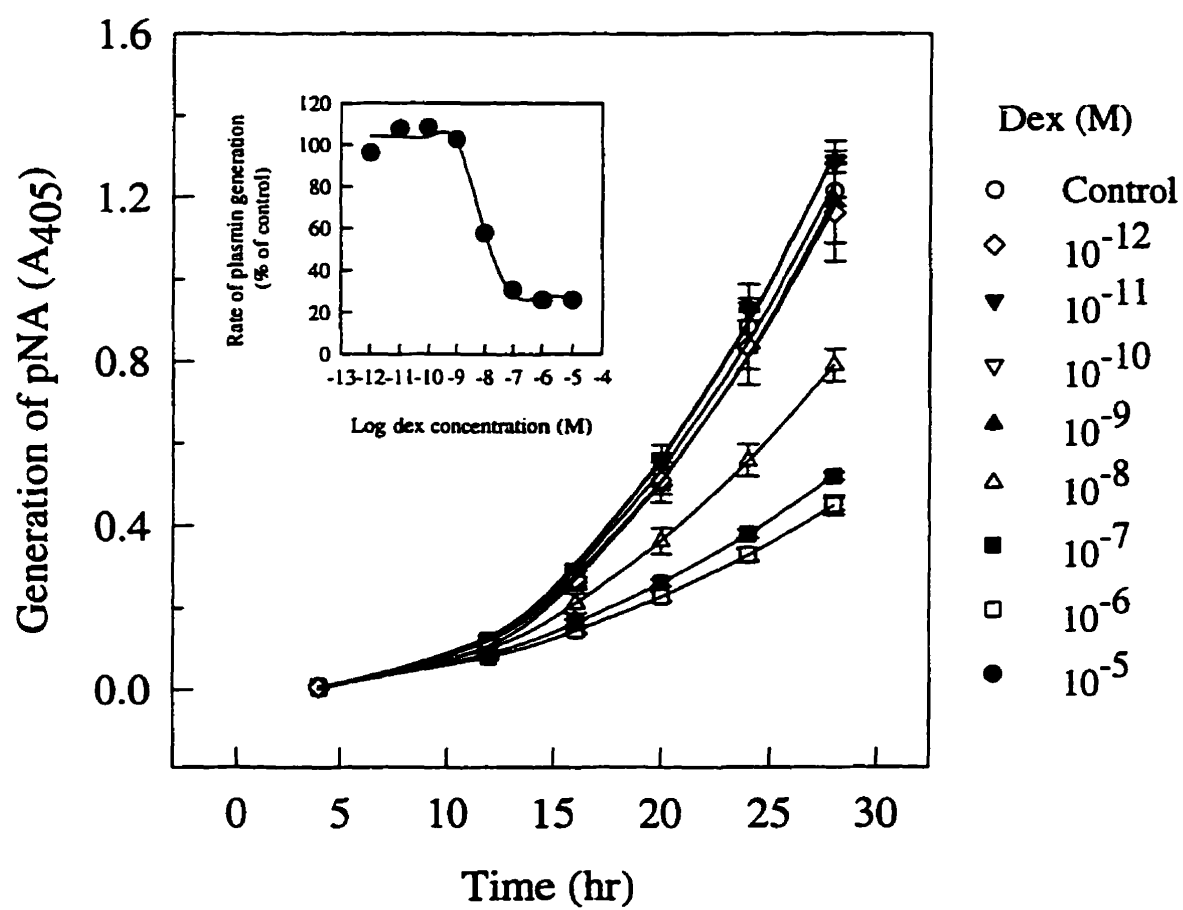


Fig. 4.28. Inhibition of trophoblast plasminogen activation by 8-bromo-cAMP. Cultured villous trophoblasts (2×10^5 per well) were washed and incubated in serum-free medium containing 0.4 mM S-2251 and forskolin at varying concentrations, in the presence or absence of 0.28 μ M Glu-plasminogen. Hydrolysis of S-2251 was measured by recording the appearance of pNA at 405 nm. Plasminogen-dependent hydrolysis of S-2251 was calculated as the difference between absorbances measured in the presence and absence of plasminogen. Progress curves of plasminogen-dependent pNA generation were constructed by fitting quadratics to the experimental points representing the mean and S.E. of quadruplicate cultures. The rates of plasmin generation (inset) were obtained from the second derivative of these curves and expressed as percentage of the rate in control cultures with no 8-bromo-cAMP addition.

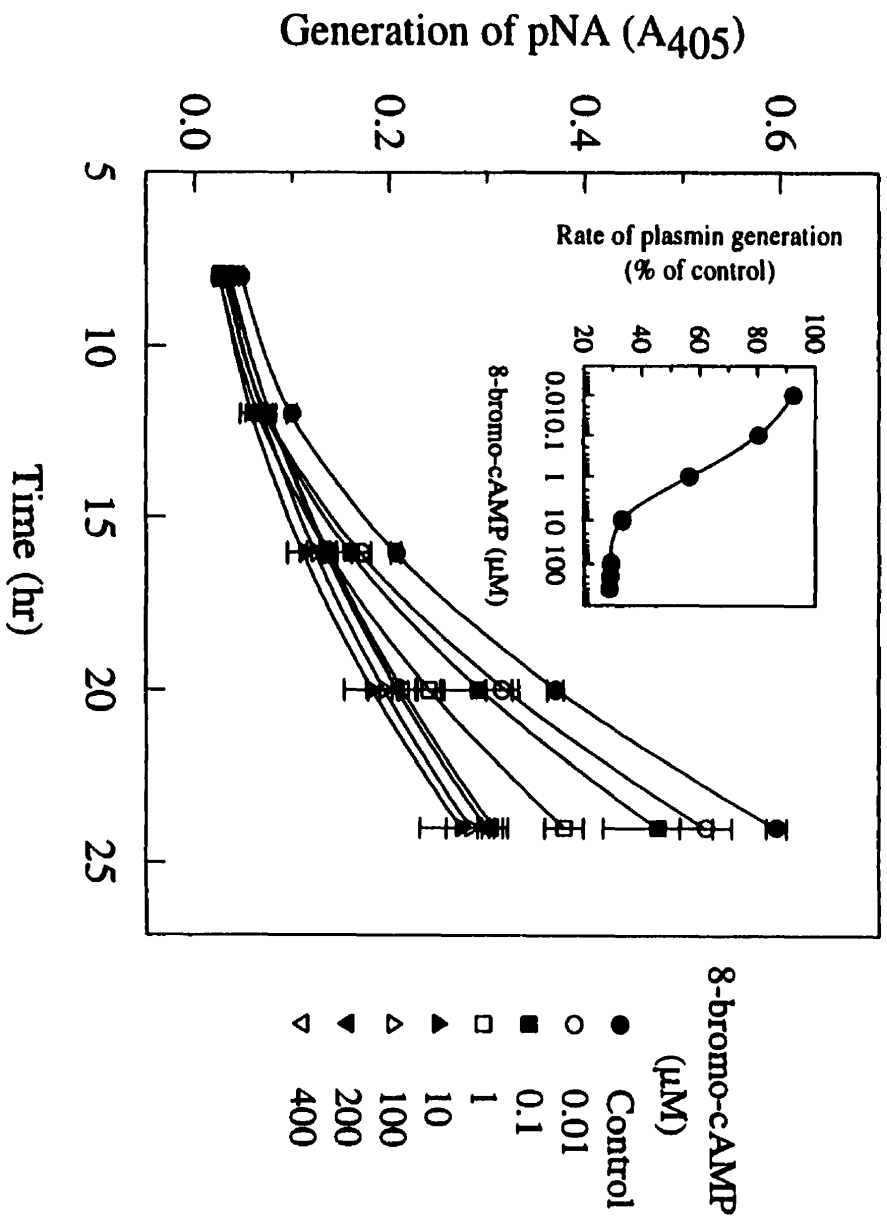


Fig. 4.29. Inhibition of trophoblast plasminogen activation by forskolin. Cultured villous trophoblasts (2×10^5 per well) were washed and incubated in serum-free medium containing 0.4 mM S-2251 and forskolin at varying concentrations, in the presence or absence of 0.28 μ M Glu-plasminogen. Hydrolysis of S-2251 was measured by recording the appearance of pNA at 405 nm. Plasminogen-dependent hydrolysis of S-2251 was calculated as the difference between absorbances measured in the presence and absence of plasminogen. Progress curves of plasminogen-dependent pNA generation were constructed by fitting quadratics to the experimental points representing the mean and S.E. of quadruplicate cultures. The rates of plasmin generation (inset) were obtained from the second derivative of these curves and expressed as percentage of the rate in control cultures with no forskolin addition.

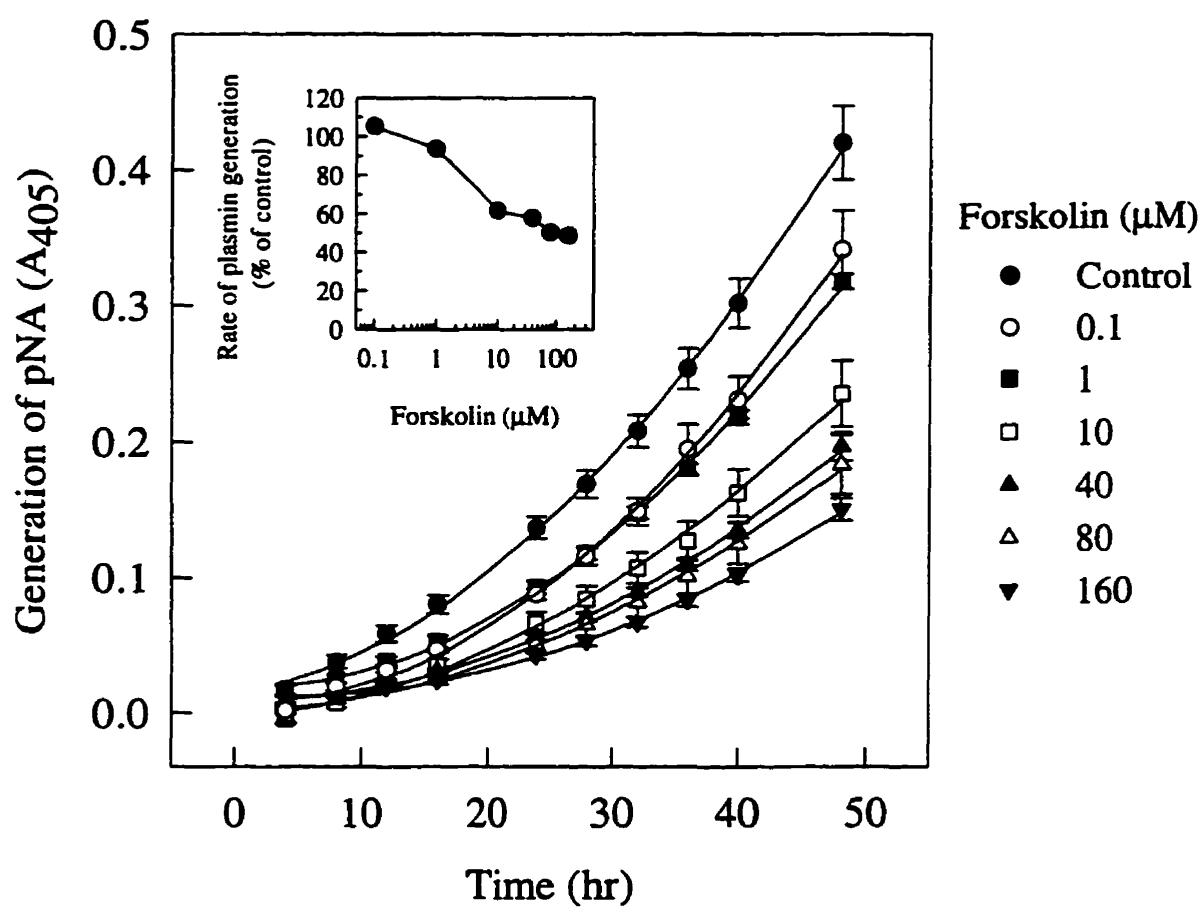
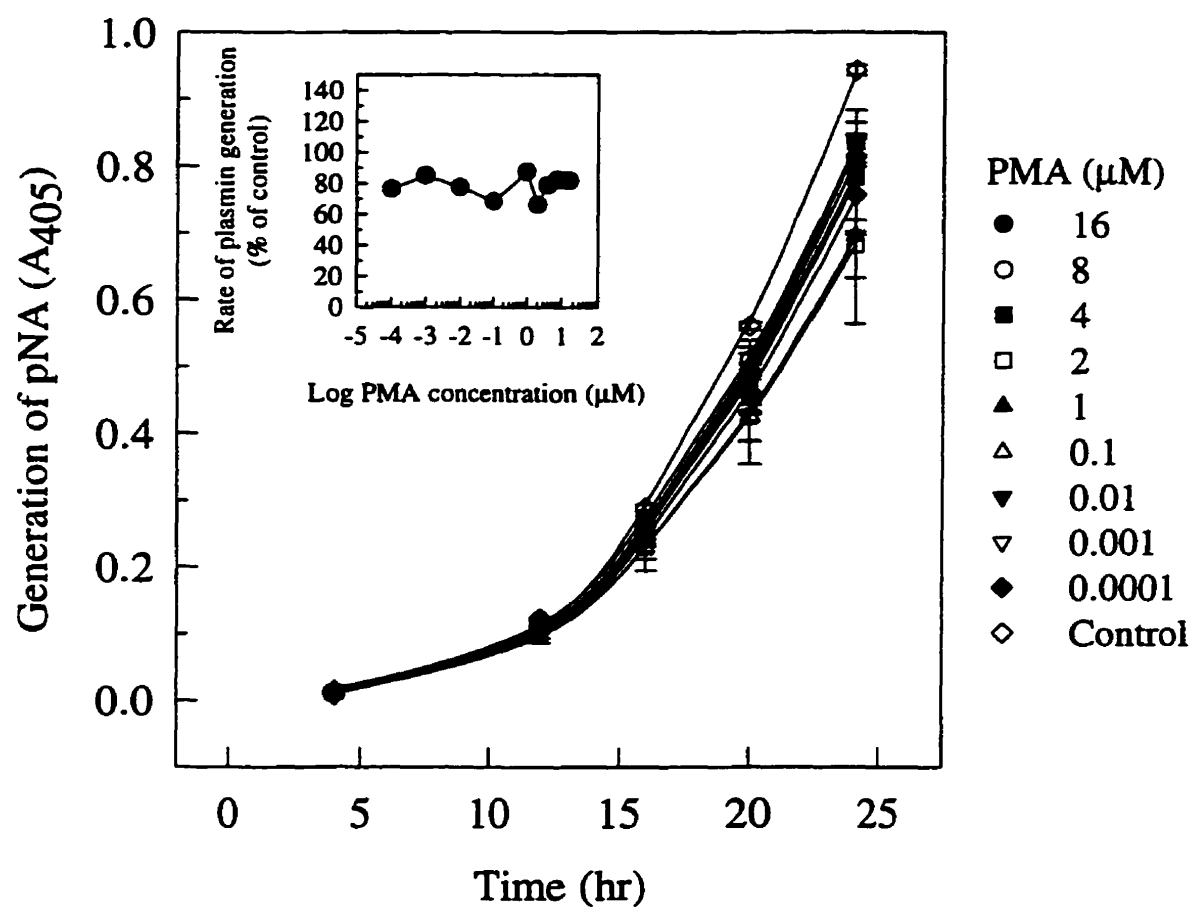


Fig. 4.30. Effect of PMA on plasminogen activation by trophoblasts. Cultured villous trophoblasts (2×10^5 per well) were washed and incubated in serum-free medium containing 0.4 mM S-2251 and PMA at varying concentrations, in the presence or absence of 0.28 μ M Glu-plasminogen. Hydrolysis of S-2251 was measured by recording the appearance of pNA at 405 nm. Plasminogen-dependent hydrolysis of S-2251 was calculated as the difference between absorbances measured in the presence and absence of plasminogen. Progress curves of plasminogen-dependent pNA generation were constructed by fitting quadratics to the experimental points representing the mean and S.E. of quadruplicate cultures. The rates of plasmin generation (inset) were obtained from the second derivative of these curves and expressed as percentage of the rate in control cultures with no PMA addition.



remodelling and fibrin deposition occur.

We investigated the mechanisms that control PA activity in human villous trophoblasts. The method employed for the measurement of trophoblast-mediated activation of plasminogen was adapted from Zini et al. (1992) who demonstrated the conversion of exogenous plasminogen to plasmin in the presence of trophoblasts, using the synthetic plasmin substrate, S-2251. In the present study, human villous cytotrophoblasts purified from term placentas were cultured in the presence of fetal calf serum for 36 to 72 hours. During this period, the great majority of mononuclear cytotrophoblasts differentiated to multinucleated trophoblast aggregates and syncytial units. At this stage the cultures were washed and maintained in serum-free medium during the plasminogen activation assay to ensure that serum proteases and protease inhibitors did not interfere with plasminogen activation by trophoblasts. Plasminogen activation was measured through plasminogen-dependent hydrolysis of S-2251 in the presence of trophoblasts. Trophoblasts remained viable during serum-free culture and were not adversely affected by increasing concentrations of plasmin generated during the assay. This implies that the assay measures only secreted plasminogen activator, and the possibility of measuring some plasminogen activator activity resulting from cell death is minimal. An advantage of this assay is that it allows continuous monitoring of plasminogen activator activity in the presence of added modulator(s) without the necessity of collecting and assaying conditioned media. The assay, however, was unable to detect early changes in trophoblast plasminogen activator activity due to low sensitivity of the

plasmin substrate, S-2251. Although the use of more sensitive plasmin-specific fluorogenic substrates could possibly overcome this problem, the relative unavailability of an expensive microplate fluorimeter would still hamper the extensive application of assays of this type.

Activation of plasminogen by cultured trophoblasts produced parabolic progress curves of pNA accumulation. These curves could be transformed to linear progress curves of plasmin formation, the slopes of which yielded constant rates of plasmin formation. Thus, term villous trophoblasts in culture maintain steady PA activity at their syncytial stage. This finding is consistent with the steady metabolic and non-proliferating state of the syncytium (Yui et al., 1994; see also Fig. 3.18). Apparently contrasting results have been reported by Zini et al. (1992). These authors found that human term trophoblasts in culture expressed gradually declining numbers of urokinase receptors accompanied with a parallel reduction in antigenic u-PA concentration of trophoblast conditioned medium. This suggests that PA activity may decrease during in vitro differentiation of cytotrophoblasts into syncytiotrophoblasts. However, PA activity is determined by complex interactions between several elements of the plasminogen activation system, including urokinase receptor affinity, PAI activity and the number and affinity of plasminogen receptors expressed on the cell surface, the balance of which may result in a steady PA activity.

PA activity of villous trophoblast cultures was found to result from u-PA but not t-PA. Furthermore, cultured trophoblasts demonstrated only u-PA immunoreactivity by

immunocytochemistry. These findings confirm earlier observations that human term trophoblasts synthesize and secrete u-PA (Queenan et al., 1987; Zini et al., 1992). Production of u-PA by our cultures is entirely due to villous trophoblasts, because HLA-positive extravillous cytotrophoblasts, macrophages, endothelial cells and fibroblasts are completely eliminated through the immunomagnetic purification step employed. Interestingly, we were unable to detect any u-PA immunoreactivity in trophoblasts *in situ*. By contrast, occasional macrophages in the placental villi expressed u-PA. One explanation for the lack of immunostaining in trophoblasts *in situ* may be that paraffin embedment of the placental tissue reduced the number of u-PA epitopes on trophoblasts below detection level. To our knowledge, no report has been so far published on successful localization of u-PA in term trophoblasts, although u-PA was previously detected in villous and non-villous cytotrophoblasts, intermediate trophoblasts and syncytiotrophoblasts of early human implantation sites (Hofmann et al., 1994).

In contrast to term trophoblasts, cultures established from first trimester placentas appear to secrete both u-PA and t-PA (Graham et al., 1993, 1994). The reason why these invasive extravillous trophoblasts secrete t-PA in addition to u-PA is not clear. However, it seems beyond doubt that human trophoblasts produce u-PA at all gestational ages (Queenan et al., 1987; Zini et al., 1992; our results). Although we have no indication on which of the molecular forms of urokinase have been produced by our cultures, Percoll-purified human term cytotrophoblasts, which are largely of the villous variety, are known to synthesize urokinase in the single-chain form (prourokinase) (Queenan et al., 1987).

Trophoblast-associated pro-u-PA is converted to two-chain u-PA through a primarily plasmin(ogen)-dependent mechanism (Zini et al., 1992). This suggests that during plasminogen activation under serum-free conditions, such as in our plasminogen activation assay, plasmin contamination of the plasminogen preparation and/or trace amounts of plasmin associated with the trophoblast surface in serum culture may have an important role in initiating the activation of plasminogen by trophoblasts.

Plasminogen activation in U937 cells has been shown to be more efficient on the cell surface than in solution. The enhancement of plasmin generation on the cell surface is due primarily to an enhanced plasmin-mediated feedback activation of receptor-bound pro-u-PA (Ellis et al., 1989, 1991; Duval-Jobe and Parmely, 1994). Although accelerated conversion of cell-bound pro-u-PA to u-PA had been also demonstrated in trophoblasts (Zini et al., 1992), direct evidence for trophoblast-mediated potentiation of plasminogen activation was lacking. In this study, we have shown that plasminogen activation initiated by exogenous pro-u-PA progressed more rapidly in the presence of villous trophoblasts than in solution in the absence of trophoblasts. This finding is consistent with an enhanced plasmin-mediated feed-back activation of pro-u-PA on the trophoblast surface.

Our immunocytochemical results showed that villous trophoblasts express PAI-1 and PAI-2 both in vivo and in vitro. In addition, PAI-1 production has been verified in culture, using ELISA. In agreement with previous investigations (Feinberg et al., 1989; Estellés et al., 1994), weak PAI-1 immunoreactivity was observed in the syncytium of term placentas in situ. In culture, however, nearly all trophoblasts showed moderate to

strong diffuse as well as punctate PAI-1 staining. The staining pattern observed may suggest that some PAI-1 is associated with focal adhesions, although the extracellular location of PAI-1 could not be unequivocally determined because of permeabilization of the cells prior to immunocytochemistry. By contrast, Feinberg et al. (1989) claimed both intra- and extracellular location for PAI-1 in cultured trophoblasts.

PA activity of our trophoblast cultures was specifically augmented by a neutralizing antibody to PAI-1 and inhibited by exogenous PAI-1. This finding suggests that the basal u-PA activity is under autocrine/paracrine control by trophoblast-secreted PAI-1. The general model of PAI-1 modulation of cell-surface proteolytic activity proposed by Blasi et al. (1993) may apply to trophoblast plasminogen activation, too. Accordingly, receptor-bound u-PA at focal contact sites is inhibited by PAI-1. In this process, covalent u-PA:PAI-1 complexes are formed, internalized and degraded. The u-PA receptor itself is also internalized but it recycles back to a different position on the cell surface. The relocated u-PAR binds another u-PA molecule and the cycle starts again. This mechanism creates continuously changing proteolytically active and adhesion promoting areas required for cell movement, adhesion and invasion. Some evidence that trophoblast PA activity may also be regulated through u-PA:PAI-1 complex formation has come from the work of Zini et al. (1992). After trophoblasts cultured in serum-free medium had been incubated with ^{125}I -prourokinase and plasminogen, the authors demonstrated radioactivity that upon SDS-PAGE migrated with sizes corresponding to those of u-PA:PAI-1 complexes.

In accord with previous investigations (Åstedt et al., 1986; Feinberg et al., 1989; Estellés et al., 1994) we demonstrated strong PAI-2 immunostaining in the syncytium of term placentas in vivo. Interestingly, cultured trophoblasts stained weakly and very inconsistently for PAI-2. Thus, our results are similar to those reported by Feinberg et al. (1989) who observed a mosaic pattern of PAI-2 immunoreactivity with variable intensity. The role of PAI-2 in the regulation of trophoblast PA activity is not clear. Although exogenous recombinant PAI-2 completely inhibited PA activity of cultured trophoblasts, we were unable to obtain evidence for the involvement of secreted PAI-2 in modulating trophoblast PA activity. One possible explanation for this is that PAI-2 is not secreted by our cultures in sufficient amount to compete with PAI-1 which inhibits u-PA with a second order rate constant 10 times higher than that for the interaction of PAI-2 with u-PA (Andreasen et al., 1990). In fact, PAI-2 appears to be secreted at much lower concentrations in trophoblast culture, and its secretion starts considerably later than that of PAI-1 (Zini et al., 1992).

Regulation of PA activity has not been studied extensively in human trophoblasts. Expression of the components of the trophoblast plasminogen activation system is a developmentally tightly regulated process, linked to trophoblast differentiation and invasion (Feinberg et al., 1989; Zini et al., 1992; Mulhaupt et al., 1994). Despite the obvious importance of the area, comparatively few studies have been conducted to determine the factors that regulate the activation of plasminogen by human trophoblasts (Martin and Arias, 1982; Queenan et al., 1987; Milwidsky et al., 1993; Yagel et al., 1993;

Graham et al., 1993, 1994). In this thesis, various compounds, including proteases, growth factors/cytokines, and hormones, have been tested for their ability to modulate plasmin generation in primary villous trophoblast cultures established from term human placentas.

This study provides evidence that components of the blood coagulation system have the potential to modulate trophoblast plasminogen activation. The addition of thrombin to trophoblast cultures caused a dose dependent loss of PA activity. A similar thrombin-induced reduction in PA activity had been reported earlier in bovine endothelial (Loskutoff, 1979) and human fibrosarcoma HT-1080 cell cultures (Osada et al., 1991). Thrombin has profound effects on cell-associated fibrinolysis. Most of our knowledge in the area has come from investigations conducted in endothelial cell cultures. Exposure of cultured human endothelial cells to thrombin stimulates secretion of t-PA (Levin et al., 1984; Gelehrter and Sznycer-Laszuk, 1986; Hanss and Collen, 1987; Van Hinsbergh et al., 1987; Dichek and Quertermous, 1989) and u-PA (Van Hinsbergh et al., 1987; Shatos et al., 1995) antigens. The enhanced PA secretion is active site dependent and appears to result from increased synthesis (Dichek and Quertermous, 1989; Shatos et al., 1995). Thrombin has also been reported to stimulate acute release (completed in minutes) of t-PA and u-PA from human endothelial cells (Booyse et al., 1986; Schrauwen et al., 1995). Thrombin enhances PAI-1 secretion and synthesis in cultured endothelial (Gelehrter and Sznycer-Laszuk, 1986; Hanss and Collen; Van Hinsbergh et al., 1987; Dichek and Quertermous, 1989; Heaton et al., 1992) and vascular smooth muscle cells (Noda-Heiny

et al., 1993; Cockell et al., 1995). In addition, experiments carried out with endothelial cells have led to the realization that thrombin can directly inactivate and degrade PAI-1 even in the absence of cells (Hanss and Collen, 1987; de Fouw et al., 1987). Thrombin has been found to modulate u-PA binding at the cell surface. This effect appears to involve both new u-PAR synthesis and an increased cleavage of u-PAR by possible involvement of PI-PLC (Miles et al., 1988; Reuning and Bang, 1992; Reuning et al., 1994; Li et al., 1995; Noda-Heiny and Sobel, 1995). Thrombin induced stimulation of plasminogen binding has also been reported (Miles et al., 1988). Finally, thrombin is known to catalyze conversion of prourokinase to an inactive two-chain form that cannot be activated by plasmin, plasma kallikrein and factor XIIa (Ichinose et al., 1986). The thrombin mediated inactivation of prourokinase is accelerated in the presence of soluble thrombomodulin (de Munk et al., 1991; Wilhelm et al., 1994). From the foregoing it is clear that thrombin has the ability to modulate the plasminogen activation cascade at numerous levels. At present, nothing is known on the mechanisms that brought about the loss of PA activity in trophoblasts. The fibrinolytic system of the trophoblast lining at the intervillous surface of placental villi and in spiral arteries probably functions to maintain proper nonthrombogenicity, similar to that on the endothelial surface. As thrombomodulin, a binding protein for thrombin at the endothelial surface (Esmon, 1995) has been also localized in the microvilli of syncytiotrophoblasts (Matsumoto et al., 1987), thrombin may directly interact with various components of the fibrinolytic system while actually bound to thrombomodulin on the trophoblast surface. The thrombin induced

reduction in trophoblast PA activity may serve as a mechanism that regulates fibrin deposition in spiral arteries and intervillous spaces. The significance of such a mechanism in the pathogenesis of preeclampsia, where excessive fibrin deposition (Kanfer et al., 1996), coagulation activation (de Boer et al., 1989; Espana et al., 1991) and depressed fibrinolysis occur, also remains to be determined.

Activated protein C (APC), another component of the coagulation system, stimulated PA activity of cultured trophoblasts. Protein C is a vitamin K-dependent glycoprotein that circulates in the plasma as the zymogen for the serine proteinase APC. Activation of protein C is carried out by the thrombin-thrombomodulin complex. The resultant APC possesses both anticoagulant and profibrinolytic properties (Halkier, 1991; Comp and Esmon, 1981; Esmon, 1995). APC increased the fibrinolytic activity in cellular extracts and conditioned media of cultured endothelial cells. This effect was also observed when APC was added to conditioned media collected from untreated cultures (Sakata et al., 1985). Moreover, APC treatment of endothelial cell cultures or of conditioned medium in the absence of cells caused a rapid decrease in PAI-1 activity (Sakata et al., 1985; Van Hinsbergh et al., 1985; de Fouw et al., 1987). These results suggested a partially or entirely cell-independent decrease in PAI-1 activity and a consequent increase in the fibrinolytic activity. The APC induced inactivation of PAI-1 is associated with the formation of APC-PAI-1 complexes (Sakata et al., 1986; de Fouw et al., 1987) and with the appearance of a 42 kD irreversibly inactivated form of PAI-1 (de Fouw et al., 1987). APC does not react with inactive (latent) PAI-1 and the active

site of APC is also required for the formation of enzyme-inhibitor complexes and for the degradation of PAI-1 to occur (de Fouw et al., 1987).

At present, it is difficult to assess the significance of the finding that APC stimulates plasminogen activation by trophoblasts. As the second order rate constant for the inhibition of APC by PAI-1 is about three orders of magnitude lower than that for the inhibition of plasminogen activators, APC can only compete with plasminogen activators for complex formation with PAI-1 if its concentration is very much higher than those of plasminogen activators, or the interaction of APC with PAI-1 is accelerated with some mechanism (Fay and Owen, 1989). Although activation of protein C can occur on the surface of the endothelium (Esmon, 1995), cellular potentiation of APC induced PAI-1 inactivation has not been reported. Recently, APC was found to facilitate basement membrane invasion by tumor cells, including a choriocarcinoma cell line (SMT-ccl), most probably via a mechanism that involves direct inactivation of PAI-1 (Kobayashi et al., 1994). Hence, it is possible that APC, formed as a result of coagulation activation, promotes invasion and/or lysis of fibrin by normal trophoblasts in vivo. These proposed roles for APC would be consistent with the reduced protein C level observed in preeclampsia (Aznar et al., 1986) where decreased fibrinolysis and impaired trophoblast invasion occurs.

Epidermal growth factor (EGF) and its structural analog, transforming growth factor- α (TGF- α) were found to increase trophoblast PA activity. These findings are in line with the recently reported stimulatory effect of EGF on u-PA activity of mouse

blastocyst outgrowths (Harvey et al., 1995). EGF may affect plasminogen activation at various levels of the plasminogen activation cascade. Stimulation of PA synthesis has been reported in a variety of cell types (Lee and Weinstein, 1978; Grimaldi et al., 1986; Stoppelli et al., 1986; Galway et al., 1989; Kessler and Markus, 1991; Mawatari et al., 1991; Jarrard et al., 1994). Several examples of EGF induced modulation of PAI-1 (Thalacker and Nilsen-Hamilton, 1987; Lucore et al., 1988) or u-PAR (Boyd, 1989; Estreicher et al., 1989; Lund et al., 1995) expression are also known. EGF may even be implicated in the process of initiating the activation of plasminogen, because its binding protein (EGF-BP), a serine proteinase, becomes activated upon releasing from the $(\text{EGF})_2(\text{EGF-BP})_2$ complex (Taylor et al., 1970; Server et al., 1976), and the active proteinase is capable of converting pro-u-PA to active u-PA both in solution and on the cell surface (Wolf and Brown, 1995). Thus, it is conceivable that the stimulatory effect of EGF on trophoblast PA activity results from the regulation of more than one component of the plasminogen activation system. Stimulation of PA activity occurs in the absence of cellular proliferation (see Fig. 4.20), which suggest that trophoblast plasminogen activation and proliferation are not necessarily interconnected. Lack of proliferation in our trophoblast cultures upon EGF stimulation confirms the generally accepted view that syncytiotrophoblasts do not possess any proliferative potential (Benirschke and Kaufmann, 1990). Furthermore, our observations are consistent with those of Morrish et al. (1987) who reported EGF induced differentiation of term cytotrophoblasts into syncytiotrophoblasts without cell proliferation. Whether induction of PA activity by EGF

has relevance to syncytium formation remains to be elucidated.

EGF affects trophoblast function in various ways. It stimulates proliferation of cytotrophoblasts in very early (4-5 week) placentas (Maruo et al., 1992), inhibits cytokine induced apoptosis of term cytotrophoblasts and syncytium (Garcia-Lloret et al., 1996), and is also involved in trophoblast differentiation (Morrish et al., 1987; Maruo et al., 1987, 1992). Of particular interest are the observations suggesting that EGF is implicated in implantation and trophoblast invasion. These include: (1) Maternal estrogen induces the synthesis of EGF by the murine uterus at the time of implantation. However, EGF is not detected in the uterus if implantation has been delayed by ovariectomy (Huet-Hudson et al., 1990), (2) EGF receptor protein is downregulated in the blastocyst trophoectoderm during delayed implantation caused by ovariectomy, and subsequent estrogen administration restores both EGF receptor expression and implantation (Paria et al., 1993), (3) Production of trophoblast invasion-promoting proteases such as u-PA and MMP-9 is enhanced by trophoblast outgrowths of cultured blastocysts exposed to EGF, at the time which coincides with trophoblast invasion in vivo (Harvey et al., 1995), (4) EGF and its analog, TGF- α have been also shown to stimulate mouse trophoblast outgrowths in vitro (Machida et al., 1995), (5) mouse blastocysts having no EGF receptors die at various stages of gestation or shortly after birth (Threadgill et al., 1995; Sibilla and Wagner, 1995), (6) EGF upregulates invasion of both first trimester and term cytotrophoblasts purified from human placentas (Bass et al., 1994). It should be added, however, that lack of effect of EGF and TGF- α on trophoblast invasiveness has also been reported (Lysiak

et al., 1994) so that this question remains to be further investigated. Our observation that EGF stimulates plasminogen activation by syncytial trophoblasts suggests that this may also be the case in invasive trophoblasts. One can also speculate that EGF stimulation of plasminogen activation by villous trophoblasts may be involved in controlling intervillous fibrin deposition or fusion of cytotrophoblasts into syncytiotrophoblast.

We have found that TGF- α also stimulates plasminogen activation in human villous trophoblasts. This is not surprising, because the biological functions of TGF- α are linked to its ability to activate the EGF receptor (Burgess, 1989). Other EGF receptor ligands so far described include heparin-binding EGF-like growth factor (HB-EGF), amphiregulin and betacellulin (Willey et al., 1995). One can hypothesize that these ligands also have the ability to stimulate trophoblast plasminogen activation. Furthermore, it is also conceivable that EGF, HB-EGF, TGF- α and amphiregulin, which are all produced in the uterus during the peri-implantation period (Das et al., 1994; Cross et al., 1994; Willey et al., 1995), act in concert to upregulate trophoblast PA activity in a paracrine manner.

Whether upregulation of trophoblast PA activity by EGF and TGF- α is mediated via autocrine and/or paracrine mechanisms remains to be determined. Our finding that a neutralizing monoclonal antibody to EGF did not affect trophoblast plasminogen activation argues against an autocrine mechanism. EGF receptors have been detected in both villous and extravillous (invasive) trophoblasts at all gestational ages (Maruo et al., 1987; Hofmann et al., 1992; Mühlhauser et al., 1993). EGF and TGF- α appear to be

synthesized by the mouse endometrium (Huet-Hudson et al., 1990; Tamada et al., 1991 Biol Reprod 45:365) and are also produced in the human decidua (Haining et al., 1991). However, the synthesis of these growth factors by trophoblasts is uncertain. TGF- α but not EGF mRNA transcripts were detected in whole mouse blastocysts (Rappolee et al., 1988). In humans, EGF immunostaining has been detected in trophoblasts (Hoffman et al., 1992; Chia et al., 1995), but there is no consensus as to whether EGF is synthesized by trophoblasts themselves (Amemiya et al., 1994; Bass et al., 1994; Chia et al., 1995). Similarly, TGF- α is present in trophoblasts (Lysiak et al., 1993), but no mRNA transcripts could be demonstrated (Bass et al., 1994). Taken together, the available information suggests that trophoblast PA activity is likely to be controlled through EGF/TGF- α mediated paracrine (maternal) mechanisms, although autocrine mechanisms may also exist.

Other growth factors such as TGF- β , IL-1 β , IGF-II and known to be relevant to trophoblast development, had no appreciable effect on trophoblast PA activity. TGF- β is a major regulator of cell proliferation and differentiation, extracellular matrix composition, and pericellular proteolytic activity (Hsuan et al., 1989; Keski-Oja et al., 1991). It usually inhibits both cell growth and PA activity, although growth inhibition in association with enhanced PA activity has been also reported (Keski-Oja and Koli, 1992). TGF- β often simultaneously regulates the secretion of plasminogen activators and their inhibitors, and thus the net PA activity reflects a new balance between their altered levels. A major mechanism whereby the human decidua limits trophoblast invasiveness involves the decidual secretion of TGF- β (Graham and Lala, 1991; Graham et al., 1993). As first

trimester trophoblasts themselves secrete TGF- β , an autocrine invasion regulating loop may also function. TGF- β appears to inhibit invasiveness of normal first trimester trophoblasts in several ways (see Chapter 1), including downregulation of u-PA activity. However, it does not affect PA secretion by malignant trophoblasts cells. In this thesis, I have pointed out that TGF- β does not have an effect on PA activity by cultured term villous syncytiotrophoblasts. One possible explanation for this finding is that PA activity of syncytiotrophoblasts may be already inhibited by endogenously secreted TGF- β . The fact that in vivo, villous syncytiotrophoblasts, as opposed to villous and extravillous cytotrophoblasts, express TGF- β (Lysiak et al., 1995) gives some support for the idea.

IL-1, a known regulator of plasminogen activation in endothelial cells (Bevilacqua et al., 1986; Michel and Quertermous, 1989; Loskutoff, 1991), has been shown to stimulate invasion and MMP-9 activity of first trimester cytotrophoblasts (Librach et al., 1994). As cytotrophoblasts produce IL-1 β and express IL-1 receptor (Librach et al., 1994), and the human decidua is also a source of IL-1 (Romero et al., 1989) it is likely that both autocrine and paracrine mechanisms have roles in regulating invasion and protease secretion by trophoblasts. At present, no information is available on whether first trimester trophoblasts can regulate the activation of plasminogen. However, as our results show, IL-1 does not have a significant effect on PA activity in term trophoblasts. We also found that bacterial endotoxin (lipopolysaccharide, LPS) did not alter PA activity of term trophoblasts either. IL-1 secretion of cultured trophoblasts has been found to be stimulated by LPS (Taniguchi et al., 1991; Librach et al., 1994). Hence, the inability of

LPS to alter PA activity gives further support to our conclusion that IL-1 may not regulate PA activity in term trophoblasts.

Our results indicate that IGF II has no appreciable effect on plasminogen activation in term trophoblasts. The insulin-like growth factors (IGF I and IGF II) are single chain polypeptides that are structurally homologous to proinsulin (Cohick and Clemmons, 1993). They regulate proliferation, differentiation (Cohick and Clemmons, 1993), and migration (Manske and Bade, 1994) of a variety of cell types. IGF-I induced stimulation of PA activity has been also reported (Tranque et al., 1994). The IGFs exert most of their biological actions through the IGF-I receptor but also bind to the IGF-II and insulin receptors as well as their specific binding proteins (IGFBPs). The latter inhibit or enhance the biological actions of IGFs at the cellular level (Cohick and Clemmons, 1993). IGF-I is expressed in syncytiotrophoblasts (Wang et al., 1988), while the expression of IGF-II is cytotrophoblast specific (Ohlsson et al., 1989). Furthermore, cytotrophoblasts have been shown to express both IGF-I and IGF-II receptors (Ohlsson et al., 1989). The expression of IGF-II have been also described in the early pregnancy decidua (Giudice et al., 1993), and both trophoblasts and the decidua are known to express IGFBPs (Waites et al., 1989; Petraglia et al., 1996). Thus, the localization of the expression of IGFs, their receptors and binding proteins suggest autocrine/paracrine regulation by IGFs of trophoblast functions. IGF-II and IGFBP-1 have been reported to stimulate trophoblast invasion (Lysiak et al., 1993, 1994) and migration (Irving and Lala, 1995). In the light of our results, the IGF-II induced stimulation of trophoblast invasiveness/migration may not be

mediated by a mechanism involving plasminogen.

In this study, I have shown that hCG inhibits PA activity in term trophoblasts. Inhibition of trophoblast urokinase activity has been first described in human first trimester cultures (Milwidsky et al., 1993; Yagel et al., 1993), using a methodology different from that employed in this thesis. Specifically, urokinase-like activity was determined by measuring aprotinin-resistant amidolytic activity with substrate S-2444, in the absence of plasminogen. This method is based on the observation that aprotinin, a serine protease inhibitor, does not inhibit urokinase activity (Danø et al., 1985; Mayer et al., 1986). In addition to its inhibitory effect on urokinase activity, hCG inhibits collagenase production by first trimester trophoblasts (Lala et al., 1989), and this inhibition appears to result from decreased urokinase mediated activation of collagenase (Yagel et al., 1993). hCG has also been found to retard trophoblast invasion (Yagel et al., 1993), consonant with its inhibitory effect on the invasion promoting enzymes, u-PA and collagenase. As trophoblasts synthesize large amounts of hCG (Hoshina et al., 1982) and express hCG receptors (Licht et al., 1993), they may have the ability to regulate their own invasiveness by an autocrine mechanism involving hCG induced inhibition of trophoblast u-PA activity. It is also possible that modulation of trophoblast urokinase activity by hCG has also some role in differentiation of cytotrophoblasts into syncytiotrophoblasts, considering that hCG is known to promote this process (Shi et al., 1993).

At present, little is known on the role of hCG in the pathology of trophoblasts. Elevated hCG levels (Said et al., 1984; Hsu et al., 1994) and excessive suppression of

fibrinolytic activity (Bonnar et al., 1971; Yoshimura et al., 1985; Bonnar et al., 1990; Kanfer et al., 1996) have been reported in women with preeclampsia. These observations together with the facts that hCG inhibits trophoblast u-PA activity and trophoblast invasion is impaired in preeclampsia (Brosens et al., 1972; Gerretsen et al., 1981; Khong et al., 1986; Meekins et al., 1994) suggest that one way in which hCG may be implicated in the pathogenesis of preeclampsia is by inhibition of trophoblast and perhaps systemic plasminogen activation.

Addition of the synthetic glucocorticoid dexamethasone to human term trophoblast cultures suppressed PA activity in a dose-dependent manner. Glucocorticoids have been found to inhibit u-PA activity in nearly all cell types studied. The response of t-PA activity to glucocorticoids is more variable: suppression, enhancement and lack of effect have all been reported (Danø et al., 1985). Glucocorticoids may decrease PA activity in three major ways: (1) by inhibiting PA synthesis (Busso et al., 1986, 1987; Medcalf et al., 1986), (2) by an enhancement of PAI production (Busso et al., 1987; Gelehrter et al., 1987; Grant and Medcalf, 1990), and by decreasing plasminogen binding on the cell surface (Pöllänen, 1989).

Dexamethasone has also been found to decrease PA activity of trophoblast cultures established from first trimester human placentas (Martin and Arias, 1982). In addition, Librach et al. (1994) reported that dexamethasone inhibited human cytotrophoblast metalloproteinase (MMP-9) activity and invasion. The fact that downregulation of MMP-9 activity was observed in serum-free cultures suggests that dexamethasone exerts its

effect via a mechanism independent of plasminogen activation. Nevertheless, it is conceivable that under conditions when plasminogen is present, a reduction in metalloproteinase activity is linked, at least in part, to dexamethasone induced suppression of plasminogen activation.

We have shown that 8-Br-cAMP and forskolin decrease PA activity in human trophoblasts. Elevation of intracellular cAMP level may regulate plasminogen activation at several levels. Activation of adenylate cyclase by forskolin or increasing cAMP content with 8-Br-cAMP induced t-PA mRNA in rat granulosa cells (Ohlsson et al., 1988). By contrast, forskolin decreased t-PA mRNA levels in HT-1080 human fibrosarcoma cells (Georg et al., 1990). Elevation of cAMP level has been reported to downregulate PA production in macrophages (Vassali et al., 1976, 1992). Forskolin decreased the synthesis of PAI-1 in HT-1080 cells (Georg et al., 1990). Treatment of endothelial cells with forskolin, 3-isobutyl-1-methylxanthine (IBMX) or dibutyryl cAMP reduced PAI-1 secretion (Santell and Levin, 1988). Evidence for the involvement of cyclic AMP dependent pathways in the regulation of u-PA expression has been also presented (Langer et al., 1993).

Agents that enhance the intracellular cAMP level have profound effects on the expression of various endocrine functions in human trophoblasts (Feinman et al., 1986; Zhou et al., 1987; Benoit et al., 1988; Nulsen et al., 1988; Ringler et al., 1989). In addition, cultured cytotrophoblasts have been reported to raise their u-PA mRNA levels and u-PA secretion upon exposure to 8-bromo-cAMP. However, this stimulation is

limited to the first 24 h of culture, and u-PA mRNA and u-PA activity declined during the subsequent day (Queenan et al., 1987). It has been also shown that 8-bromo-cAMP treatment leads to a marked inhibition of ECM degradation by human term trophoblasts (Kliman and Feinberg, 1990). Our observation that both 8-bromo-cAMP and forskolin inhibit PA activity in trophoblasts is consistent with the above findings and suggests that inhibition of plasminogen activation is implicated in the 8-bromo-cAMP induced inhibition of trophoblast mediated ECM degradation. Furthermore, our data suggest that cAMP dependent pathways are involved in mediating inhibitory effects on trophoblast plasminogen activation.

PMA did not alter PA activity in our trophoblast cultures. Phorbol esters such as PMA have been reported to increase the synthesis of several components of the plasminogen activation system in various cell types (Danø et al., 1985; Loskutoff et al., 1989; Picone et al., 1989; Niiya et al., 1991). Thus, it is possible that PMA affects the expression of some components of the trophoblast plasminogen activation system, yet the net balance of these components (i.e. the PA activity) remains unaltered. At present, the effects of phorbol esters on the plasminogen activation system in trophoblasts is totally unknown.

CHAPTER 5

CONCLUSION AND PERSPECTIVES

This study was undertaken to reveal some characteristics of the plasminogen activation system in human trophoblasts both *in vivo* and in culture. We have been successful in isolating and culturing pure villous cytotrophoblasts from human term placentas, using a modification of the procedures originally described by Kliman et al. (1986) and Douglas and King (1989), respectively. Villus cytotrophoblasts, characterized as being cytokeratin positive as well as vimentin, HLA-ABC, HLA-DR and CD68 negative, were isolated using sequential trypsin/DNase digestions of the selected placental villous tissue, Percoll density gradient centrifugation and immunomagnetic removal of contaminating cells. The purity of our trophoblast cultures ensured that plasminogen activation by villous trophoblasts could be studied in the absence of contaminating cells.

We carried out immunocytochemical experiments to investigate the expression of the components of the plasminogen activation system in trophoblasts. The results, in accordance with previous findings (Feinberg et al., 1989; Hofmann et al., 1994), demonstrate that trophoblasts express PAI-1 and PAI-2 both *in vivo* and *in vitro*. As also shown earlier (Zini et al., 1992), u-PA has been expressed in culture, but could not be demonstrated *in vivo*. No immunoreactive t-PA has been found in trophoblasts.

Our results also support and extend previous observations (Queenan et al., 1987; Zini et al., 1992) that human term trophoblasts produce PA activity that results from u-PA but not t-PA secretion. In addition, we demonstrated that plasminogen activation by cultured trophoblasts followed Michaelis-Menten type kinetics. We also found that

trophoblasts enhanced plasminogen activation initiated by pro-u-PA, which is consistent with previous findings that trophoblasts accelerate plasmin mediated conversion of pro-u-PA to u-PA (Zini et al., 1992).

Our data show that both PAI-1 and PAI-2 are efficient inhibitors of trophoblast secreted u-PA, and u-PA activity is regulated by PAI-1 through an autocrine mechanism. We have also shown that plasminogen activation by villous trophoblasts is modulated by a variety of agents. Components of the coagulation system appear to have roles in modulating trophoblast plasminogen activation, because thrombin inhibited, whereas activated protein C stimulated trophoblast PA activity. Of the growth factors/cytokines studied, EGF and its analog, TGF- α were found to stimulate plasminogen activation. Experiments with a neutralizing antibody to EGF suggest that EGF may not exert an autocrine regulatory influence on trophoblast PA activity. Our results are consistent with the recently published finding that u-PA activity secreted by cultured mouse blastocysts is enhanced by EGF (Harvey et al., 1995). Another important cytokine, TGF- β , which is known to inhibit PA activity in first trimester trophoblasts (Graham et al., 1993, 1994), has no effect in term trophoblasts. Hormones such as dexamethasone, a synthetic glucocorticoid or hCG, an important secretory product of syncytiotrophoblasts inhibit activation of plasminogen by trophoblasts. The inhibitory effect of dexamethasone and hCG in term villous trophoblasts is consistent with their inhibition of PA activity in first trimester trophoblasts (Martin and Arias, 1982; Milwidsky et al., 1993; Yagel et al., 1993). Finally, agents that increase intracellular cAMP levels such as 8-bromo-cAMP and

forskolin inhibit trophoblast plasminogen activation, suggesting that cAMP dependent mechanisms are involved in mediating negative regulatory effects on trophoblast plasminogen activation.

Our findings may have relevance to several trophoblast functions. As plasminogen activation is implicated in mediating trophoblast invasiveness (Graham and Lala, 1992), modulation of trophoblast PA activity by growth factors, hormones and coagulation products suggests that these molecules may have significant roles in controlling trophoblast invasion during implantation and placentation. These compounds may also act as regulators of fibrin deposition at the sites of trophoblast invasion and on the luminal surface of syncytium covering the chorionic villi. In addition, we suggest that fusion of cytotrophoblasts into syncytium may involve the activation of plasminogen. This is an intriguing possibility that could be easily tested *in vitro* using neutralizing antibodies to u-PA and PAIs, respectively. The above modulators of trophoblast activation may also be implicated in trophoblast pathology, especially in preeclampsia where disturbed invasion is associated with suppressed plasminogen activation.

The process of plasminogen activation in trophoblasts is far from being characterized. A lot of work has to be done to clarify various aspects of the regulatory mechanism of plasminogen activation. Currently, very little is known about the regulation of u-PA synthesis and secretion (Queenan et al., 1987), and virtually no information is available regarding the compounds that regulate PAI-1 and PAI-2 activity/production. Although the u-PA receptor has been characterized on trophoblasts (Zini et al., 1992;

Multhaupt et al., 1994), regulation of the receptor is not known. Mechanisms of plasminogen binding by trophoblasts have never been addressed and the details of trophoblast plasminogen activation kinetics also remain to be investigated. Determination of the kinetic parameters, characterizing plasmin generation and pro-u-PA activation, could help understand how the trophoblast surface may become a preferential site of plasminogen activation. Finally, the underlying mechanisms of the disordered plasminogen activation in trophoblast-related diseases such as preeclampsia need to be understood. Identification of such mechanisms may have potential pathogenetic and therapeutic implications.

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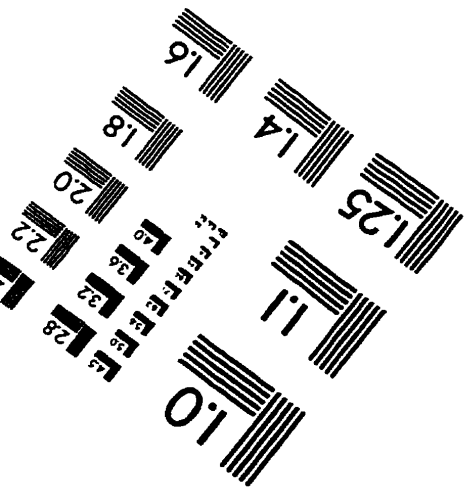
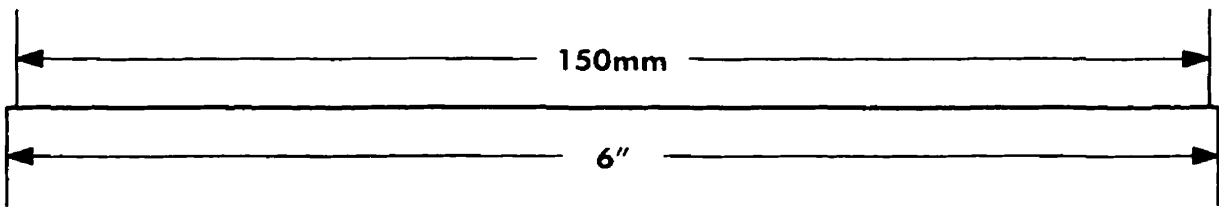
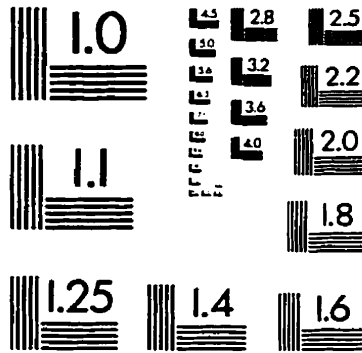
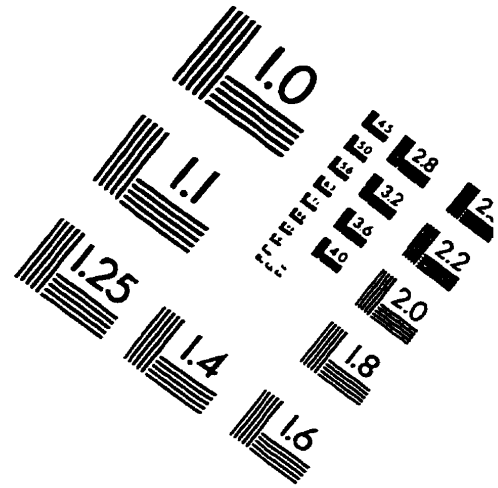
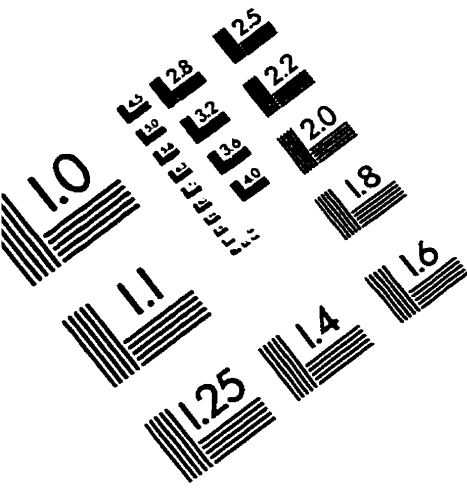
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IMAGE EVALUATION TEST TARGET (QA-3)



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