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The interaction of plasminogen with the cell surface: involvement of plasminogen binding and activation during cell death

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The interaction of plasminogen with the cell surface: Involvement of plasminogen binding and activation during cell death

A thesis submitted in fulfilment of the requirements for the award of the degree

Doctor of Philosophy

from

The University of Wollongong

by

Matthew J O’Mullane B. Sc. (Hons) (UW)

Dept of Biological Sciences

1999
DECLARATION

This thesis is submitted in accordance with the regulations of the University of Wollongong in fulfilment of the degree of Doctor of Philosophy. It does not include any material previously published by another person except where due reference is made in the text. The experimental work described in this thesis is original work and had not been submitted for a degree in any University or Institution.

Matthew J O'Mullane

July 1999.
ABSTRACT

Cell-surface plasminogen activation (PA) occurs by virtue of the presence of specific receptors for plasminogen (plg) and urokinase or tissue plasminogen activator (uPA and tPA respectively). Receptor binding of plg induces a conformational change in the molecule, which makes it more susceptible to activation by uPA/tPA. Plasmin (plm) generated on the cell-surface in this manner is protected from fast acting inhibitors and can subsequently participate in various extracellular proteolytic events such as cell migration (e.g. angiogenesis, tumour cell invasion, inflammation, wound healing, bacterial invasion), ovulation, tissue remodelling and the activation of other protease classes and growth factors. Evidence is also emerging that PA plays as yet undefined roles in cell death either as a cytoprotective or cytotoxic agent.

$^{125}$I-plg binding studies on the human monocytic leukemic cell line U937, the breast carcinoma cell lines MCF-7 and MDA-MB-231, and the colon carcinoma cell line HCT116 indicated that the presence of plg-Rs (i.e. specific plg binding) was highly variable on both U937 and MDA-MB-231 cells. This suggested that $^{125}$I-plg binding assays might not be suitable for measuring plg binding on all cell types and/or that plg-R expression is a variable phenomenon. Plg-binding proteins of 40-43kDa and 48-50kDa were consistently isolated from the plasma membranes of HCT116 cells using plg-affinity chromatography. The low concentrations of these proteins and the apparent loss of their plg-binding ability following isolation made it difficult to proceed with any further studies. Collectively these observations
indicated that it was essential to develop alternative techniques for measuring plg binding.

A flow cytometric plg binding assay was developed, using fluorescein isothiocyanate-labelled plg (FITC-plg), to enable the measurement of plg-Rs (i.e. specific plg binding) on various cell types (e.g. adherent and non-adherent cells) and on specific subpopulations of cells (e.g. viable and non-viable). A direct relationship was found to exist between cell viability [propidium iodide (PI) uptake] and the magnitude of lysine-dependent plg binding with non-viable subpopulations of cells binding up to 100-fold more plg than viable cells. This relationship was observed on numerous cell lines and indicated that the presence of non-viable cells could artifactually elevate the measurement of plg binding using non-cytometric assays, and more importantly, that plg-Rs (i.e. specific plg binding) might play a role during cell death.

U937 cells that had been induced to undergo apoptosis with the protein synthesis inhibitor cycloheximide (CHX) were also shown to have highly elevated levels of plg-Rs (i.e. specific plg binding) in addition to a large transient increase in cell-surface uPA. This increase in plg-Rs was a late apoptotic event, coincident with PI uptake and internucleosomal DNA fragmentation but occurring after elevations in phosphatidylserine (PS) exposure. Plg was also observed to dramatically increase the rate of CHX-induced apoptosis. These observations suggested that plg-Rs play a role during the degradative phase of apoptosis.
In order to ascertain whether apoptotic cells could activate plg, a novel flow cytometric PA assay was developed utilising fluorescein isothiocyanate-labelled aprotinin (FITC-aprotinin), to detect cell-surface plm. PA was found to be highly increased on apoptotic cells, which supports the observations that both plg-Rs and uPA are increased during apoptosis.

In summary, evidence is provided that plg-Rs (i.e-specific plg binding) are dramatically increased during cell death. The presence of both plg-Rs (i.e cell-surface plg) and uPA is critical for cell surface PA to proceed and this was predominantly restricted to apoptotic U937 cells which displayed highly elevated levels of PA. These novel observations support the important role of flow cytometry in cellular plg binding and activation studies and strongly implicate the PA cascade in apoptosis, particularly on those cells that express uPA.
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ABBREVIATIONS

\( \alpha_2\text{-AP} = \) alpha-2-antiplasmin

\( \alpha_2\text{-MG} = \) alpha-2-macroglobulin

\( \alpha_2\text{-MGR} = \) alpha-2-macroglobulin receptor

apo(a) = apolipoprotein A

bFGF = basic fibroblast growth factor

CHX = cycloheximide

DMSO = dimethyl sulphoxide

ECM = extracellular matrix

EGF = epidermal growth factor

\( \varepsilon\text{-ACA} = \) epsalon aminocaproic acid

EDTA = ethylenediamine tetraacetic acid

FACS = fluorescence-activated cell sorting

FCS = fetal calf serum

FITC = fluorescein isothyocyanate

FPLC = fast protein liquid chromatography

FSC = forward scatter

FSH = follicular stimulating hormone

G3PDH = glyceraldehyde-3-phosphate dehydrogenase

HBSS = Hank's balanced salt solution

HGF = hepatocyte growth factor

HPRG = histidine-proline-rich glycoprotein

ICE = interleukin -1\( \beta \)-converting enzyme

IFN\( \gamma \) = interferon gamma
K1, K2 \ldots = \text{kringle 1, kringle 2} \\
K_d = \text{dissociation constant} \\
LBS = \text{lysine binding site} \\
LRP = \text{low density lipoprotein receptor-related protein} \\
M_r = \text{molecular weight} \\
NTP = \text{N-terminal peptide} \\
PA = \text{plasminogen activation} \\
PAI-1 = \text{plasminogen activator inhibitor type 1} \\
PAI-2 = \text{plasminogen activator inhibitor type 2} \\
PBS = \text{phosphate buffered saline} \\
PEG = \text{polyethylene glycol} \\
PI = \text{propidium iodide} \\
Plg = \text{plasminogen} \\
Plm = \text{plasmin} \\
PMA = \text{phorbol 12-myristate 13 acetate} \\
PMSF = \text{phenylmethylsulfonyl fluoride} \\
PS = \text{phosphatidylserine} \\
PVDF = \text{polyvinylidene difluoride} \\
rpm = \text{revolutions per minute} \\
RT = \text{room temperature} \\
SDS = \text{sodium dodecyl sulphate} \\
SDS-PAGE = \text{sodium dodecyl sulphate polyacrylamide gel electrophoresis} \\
serpin = \text{serine protease inhibitor} \\
7-AAD = 7\text{-aminoactinomycin D} \\
SSC = \text{side scatter}
STA = staphylokinase
SK = streptokinase
TA = tranexamic acid
TGFβ = transforming growth factor beta
TNFα = tumour necrosis factor alpha
TSP = thrombospondin
tPA = tissue plasminogen activator
sctPA = single chain tissue plasminogen activator
tctPA = twin chain tissue plasminogen activator
tPAR = tissue plasminogen activator receptor
uPA = urokinase plasminogen activator
scuPA = single chain urokinase plasminogen activator
tcuPA = twin chain urokinase plasminogen activator
uPAR = urokinase plasminogen activator receptor
suPAR = soluble urokinase activator receptor
VEGF = vascular endothelial growth factor
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Chapter 1

The Biology of Plasminogen Activation - Literature Review
1.1 Plasminogen activation - an overview

Plasminogen activation (PA) is the proteolytic conversion of plasminogen (plg) to the serine protease plasmin (plm). The predominant function of PA is the removal of fibrin blood clots from the circulature (fibrinolysis) but it is also involved in numerous other processes which involve the degradation of extracellular matrix (ECM) or basement membrane (BM) components such as cell migration (tumour cell invasion and metastasis, inflammation, angiogenesis, bacterial invasion, wound healing), tissue remodelling (embryogenesis) and ovulation. Additionally PA is important for activating certain hormones, growth factors and other protease classes.

Although plm is considered to be a broad range serine protease, a number of key regulatory mechanisms ensure that PA is directed to specific sites within the body. It is the fine interplay between these mechanisms that determines the specific role that PA plays at any one time. PA occurs by the action of either of two plasminogen activators, urokinase-type plasminogen activator (uPA) or tissue-type plasminogen activator (tPA) which are both secreted as zymogons that need to be converted to their proteolytically active forms. The activity of uPA and tPA is regulated by the presence of plasminogen activator inhibitors such as plasminogen activator inhibitor type 1 (PAI-1) and plasminogen activator inhibitor type 2 (PAI-2). The proteolytic activity of plm is modulated by plm inhibitors such as \( \alpha_2 \)-antiplasmin (\( \alpha_2 \)AP) and \( \alpha_2 \)-macroglobulin (\( \alpha_2 \)MG). The presence of specific binding sites for plg and plg activators on cells, fibrin and ECM also regulate PA by localising plg/plg activators to specific extracellular sites, and, inducing conformational
changes to plg which make its activation kinetically more favourable. The nature of the binding event also protects cell- and fibrin-bound plm from inactivation by α₂-AP and α₂-MG. It is thus the levels of bound plg, receptor-associated uPA/tPA, serpin expression, cellular receptors and substrate binding sites that determines the location and ultimately the function of PA at any given time. Figure 1.1 illustrates the key features of PA including its various components, regulatory mechanisms and physiological outcomes.

Another important feature of PA is that it operates as a positive feedback loop whereby an initial small amount of plm, that was generated by the action of uPA/tPA, can activate more plg. In this way a proteolytic cascade is spawned which significantly and quickly amplifies the generation of plm. PA is also functionally linked to other proteolytic systems as plm can activate other protease classes (e.g metalloproteases). In this manner the role of PA can be either direct (i.e via plm) or indirect (i.e via the activation of other protease classes) and indicates that plm can work in unison with other proteases during extracellular proteolytic events.

In summary, the key features of PA include: (1) the generation of the serine protease plm; (2) a number of regulatory mechanisms which can quickly switch PA on and off; (3) a positive feedback loop which amplifies PA; (4) linking with other proteolytic pathways; (5) is involved in numerous biological and pathobiological processes.
Fig 1.1: Overview of plasminogen activation.
1.2 Components of the PA system

1.2.1 Plasminogen

Plg is a single chain glycoprotein of 791 amino acid residues and has a molecular weight of 92kDa. Native plg has a glutamic acid residue at its amino terminus and is consequently referred to as glu-plg. Although plg can be activated by uPA or tPA, plm can also activate plg by hydrolysing the peptide bond at Lys\(^{76}\)-Lys\(^{77}\) causing the release of a 76 amino acid (~8kDa) preactivation peptide and the formation of lys-plg (Castellino, 1984). Lys-plg is not found in the circulation and is considered to be a short-lived intermediate in the activation pathway (Hajjar & Nachman, 1988). There are two different glycoforms of plg that possess identical amino acid sequences but differ in their glycosylation sites (Lijnen et al 1981): type I plg is glycosylated at Asn\(^{289}\) and Thr\(^{346}\); type II plg is glycosylated only at Thr\(^{346}\). The kinetics of activation of each glycoform is slightly different (Collen et al 1975; Hall et al 1990) in addition to their binding to cells (Gonzalez-Gronow et al 1989; Hall et al 1990), \(\alpha_2\)-AP, fibrin and lysine analogues (Lijnen et al 1981).

A schematic representation of native glu-plg is given in Fig 1.2. The plg molecule consists a short N-terminal peptide (NTP), 5 kringle domains consisting of approximately 80 amino acids (Sottrup-Jensen et al, 1978) and a serine protease domain. The N-terminal peptide is thought to be important for regulating the conformation and activation of glu-plg (Ponting et al 1992). Kringles are a common structural motif in serum proteins associated with blood coagulation and fibrinolysis and are found in proteins such as
uPA, tPA, coagulation factor XII, prothrombin and hepatocyte growth factor (Donate et al 1994).

Of the five kringle, kringle 1, 4 and 5 (K1, 4 and 5 respectively) contain preformed lysine binding sites (LBS) (Wu et al, 1991) which enable plg to interact with a variety of lysine-containing molecules (Wiman et al 1979; Thorsen et al 1981; Knudson et al 1986; Silverstein et al 1985; Plow et al 1991). Strong LBS are located in K1 and K4, however the LBS in K4 is thought to be

**Fig 1.2:** Schematic representation of plg (adapted from Castellino 1984). Cleavage at residues 77/78 releases the N-terminal peptide that liberates lys-plg. Subsequent cleavage at residues 561/562 liberates lys-plm. An N-linked polysaccharide is linked to residue 289 with an O-linked glycan found at position 346.
hidden in the intact molecule (Vali & Patthy 1981). The primary role of the strong LBS in K1 is to mediate the interaction with $\alpha_2$-AP (Wiman et al 1981; Christensen 1984) however other evidence suggests it is important for plg binding to cells (Plow et al 1986), ECM (Knudsen et al 1986) and fibrin (Thorsen et al 1981). K5 contains the weak LBS which is also termed the AH-site as it binds to the aminohexyl group of lysine (Christensen 1984; Thewes et al 1990). K5 also contains a weak binding site for benzamidine (Thewes et al 1987). K2 and 3 have been reported to possess weak LBS (Matsuka et al 1990).

Native glu-plg possesses a closed, right-handed spiral conformation (Ponting et al 1992) due to the lysine-dependent interaction between the NTP and K5 (Christensen 1984). Upon binding to lysine residues, such as $\varepsilon$-ACA or TA, plg undergoes a large conformational change (Markus et al 1978; Mangel et al 1990; Christensen 1991) which appears to involve two weak LBS in K4 and K5 (Christensen & Molgaard 1992). This open conformation can also be induced via plm-hydrolysis of the NTP that yields lys-plg (Violand et al 1975). The difference between the closed and open conformation is apparently due to changes in the interactions of the various plg domains (Vuk-Pavlovic et al 1979).

Plg is synthesised in the liver and is present in serum and extracellular tissue spaces at concentrations of approximately 2$\mu$M (Castellino 1984). Recent evidence has shown that retinoic acid enhances the secretion of plg from rat microglia (Nakajima et al 1992). The gene for human plg has
recently been characterised and found to be 52.5kb (Petersen et al 1990) and mapped to chromosome 6q26-6q27 (Murray et al 1987). The plg gene probably evolved from a common ancestral gene consisting of an N-terminal preactivation domain, three copies of the kringle domain and a serine proteinase domain (Donate et al 1994). This gene also gave rise to hepatocyte growth factor/scatter factor, hepatocyte growth factor-like/macrophage stimulating protein and lipoprotein (a) (Donate et al 1994) (Fig 1.3).

![Phylogenetic relationship of pig to other kringle-containing and serine proteinase domain-containing proteins (adapted from Donate et al 1994).](image)

**Fig 1.3:** Phylogenetic relationship of plg to other kringle-containing and serine proteinase domain-containing proteins (adapted from Donate et al 1994).

1.2.2 Plasmin

Hydrolysis of the Arg$^{560}$-Val$^{561}$ bond in plg by uPA or tPA yields the disulphide-linked heterodimer of plm (Castellino 1984). The heavy chain of plm contains the five plg kringles that are important for binding to fibrin, ECM components or cellular receptors. The light chain contains the active
site which consists of the catalytic triad of His$^{603}$, Asp$^{646}$ and Ser$^{741}$ which is characteristic of the serine protease family which also includes trypsin and elastase (Castellino 1995). The light and heavy plm chains are covalently linked by two disulphide bonds between Cys$^{548}$ of the heavy chain and Cys$^{666}$ of the light chain, and Cys$^{558}$ of the heavy chain and Cys$^{566}$ of the light chain (Castellino 1995). The secondary structure of the light chain consists of a $\beta$-barrel (Ponting et al 1992).

Plm hydrolyses peptide bonds on the C-terminal side of lysine and arginine residues (Ponting et al 1992) and for this reason it is generally considered to have broad substrate specificity. However the physiological targets of plm are very specific due to regulatory mechanisms that ensures PA occurs at specific sites within the body. The main target of plm is fibrin but it can also hydrolyse several ECM components such as laminin (Moser et al, 1993), fibronectin (Moser et al, 1993; Wang et al, 1994), type IV collagen (Mackay et al 1990) in addition to the protein core of proteoglycans (Richardson et al, 1988). This targeting of plm to the ECM implicates it in processes requiring ECM degradation such as cell migration (e.g. angiogenesis, inflammation, tumor cell invasion) and tissue remodelling. The role of plm in ECM dissolution is augmented by the fact that plm can activate both ECM metalloproteinases (He et al 1989; Murphy et al, 1992) and cell-surface gelatinases (Mazzieri et al 1997).

An interesting by-product of plm-catalysed ECM degradation is the release of bound growth factors, such as TGF-β (Taipale et al, 1992). Plm also causes
the release of basic fibroblasts growth factor (bFGF) and TGF-β from macrophages and endothelial cells respectively (Falcone et al, 1993 & 1994). Plm has several other substrates including van Willebrand factor (Hamilton et al 1985), glycoprotein IIIa (Pasche et al 1994), insulin-like growth factor (Campbell et al 1992), interferon γ (Gonias et al 1989) and the thrombin receptor (Turner et al 1994). Plm can also cleave adhesion proteins such as vitronectin (Kost et al 1996).

1.2.3 Plasminogen activators

There are two classes of plasminogen activators. Firstly there are serine proteases such as tPA and uPA that proteolytically activate plg to plm via the cleavage of a single peptide bond. Secondly there are bacterial activators such as streptokinase (SK) and staphylokinase (STA) which induce conformational activation.

1.2.3.1 uPA

Single chain uPA (scuPA) is a ~55kDa proenzyme which is converted to twin chain uPA (tcuPA) via the cleavage of Lys^{158}-Ile^{159} by plm. Conversion of scuPA to tcuPA can also occur via plasma kallikrein (Ichinose et al 1986), thrombin (Lijnen et al 1987), cathepsin B (Kobayashi et al 1991), cathepsin L (Goretzki 1992) and nerve growth factor-γ (Wolf et al 1993). Plm can also generate an active low molecular weight 33kDa form of uPA by hydrolysing Lys^{135}-Lys^{136} (Stump et al 1986). Thrombin cleavage of the Arg^{156}-Phe^{157} yields inactive tcuPA (Duffy 1993). The two polypeptide chains of tcuPA (M_r = 20 and 34kDa) are held together by a single disulphide bridge.
Phosphorylated and non-phosphorylated variants of uPA exist which are functionally identical but differ in their sensitivity to PAI-1 with phosphorylated uPA more than a magnitude less sensitive to PAI-1 than non-phosphorylated uPA (Franco et al 1992). Although scuPA is a zymogen it has been found to possess some plg activating ability (Ellis et al 1987) and can interact with glu and lys-plg via a high affinity site (Longstaff et al 1992). In some circumstances uPA can be activated indirectly via the activation of cathepsin C after thrombin stimulation (Lenich et al 1997). A single copy gene for uPA is found on chromosome 10 (Rajput et al 1985).

uPA consists of a growth factor domain that contains the uPAR binding site (Appella et al 1987), a single kringle and a protease domain that contains the catalytic triad of His$^{204}$, Asp$^{255}$ and Ser$^{356}$ (Pennica et al 1983; Verde et al 1984). The kringle domain contains a high-affinity PAI-1 binding site that facilitates the reversible interaction with PAI-1 (Mimuro et al 1992). The protease domain may also contain a second reversible PAI-1 binding site (Mimuro et al 1992). The predominant substrate for uPA is plg however there is evidence that uPA can directly cleave fibronectin at high concentrations of uPA (Gold et al 1989). uPA has also been found to activate collagenase IV (Reith et al 1992). uPA-induced PA occurs on a variety of cell types including thrombin stimulated platelets (Lenich et al 1997). uPA is also found associated with strands of vinculin on the undersurface of human fibroblasts (Hebert & Baker 1988). This suggests a role for uPA-catalysed PA during cellular detachment and adhesion.
uPA is synthesised by a variety of cells including endothelial cells (Levin et al 1982), monocytes/macrophages (Vassalli et al 1984); keratinocytes (Hashimoto et al 1988) and leukemic cells (Wilson et al 1983). uPA synthesis and/or secretion is increased by various growth factors such as estradiol (Ryan et al 1984), FSH (Lacroix & Fritz 1982), EGF (Laiho et al 1986; Boyd & Brattain 1989), TGFα (Laiho et al 1986), TGF-β (Hamilton et al 1991; Falcone et al 1995), hepatocyte growth factor (HGF) (Pepper et al 1992), basic fibroblast growth factor (bFGF) (Montesano et al 1986), IL-1 (Hamilton et al 1991), TNFα (Niedbala & Stein 1990) and LPS (Schwartz & Bradshaw 1992; Chapman & Stone 1985). The significance of this mode of uPA regulation is that growth factor-mediated processes (such as angiogenesis, cell migration and ovulation) can seconder the input of uPA.

1.2.3.2 tPA

tPA is a ~68kDa glycoprotein that is predominantly synthesised by vascular endothelial cells as a single-chain proteolytically active form (Levin & Loskutoff 1982). tPA is also produced by cerebellar cells (Verrall and Seeds), leukemic cells (Wilson et al 1983) and monocytes (Hart et al 1989). Plm can cleave single-chain tPA (sctPA) to twin-chain tPA (tctPA) with only a minor increase in activity (Madison et al 1993). There are two glycoforms of tPA: type I has N-linked oligosaccharides at sites 117, 184 and 448 while type II is glycosylated only at sites 117 and 448. Both glycoforms have an O-linked fucose residues at Thr61 (Harris et al 1991). Type II tPA has a 2-fold higher activity than type I (Mori et al 1995).
tPA consists of a finger domain, a growth factor-like domain, two kringles and a serine protease domain that contains the catalytic triad of His$^{322}$, Asp$^{371}$ and Ser$^{478}$ (Pennica et al 1983; Verde et al 1984). PAI-1 binding sites are located in the finger domain (Wittwer & Sanzo 1990; Kaneko et al 1992), kringle 2 domain (Knaeko et al 1991) and the catalytic domain (Madison et al 1990, Bennett et al 1991) and mediate the reversible interaction between tPA and PAI-1.

A single copy gene for tPA is located on chromosome 8 (Rajput et al 1985). Dexamethasone causes an increase in tPA production in mammary epithelial cells due to a direct effect on tPA gene expression (Bussi et al 1986). tPA production by monocytes is increased by LPS and IL-4 (Hart et al 1989). tPA is responsible for activating plg on fibrin blood clots and is consequently important for fibrinolysis. The predominant substrate for tPA is plg however there is evidence that tPA can directly cleave fibrinogen at high tPA concentrations (Weitz et al 1988).

1.2.3.3 Bacterial activators
Streptokinase (SK) is a 414 amino acid, 48kDa protein that is secreted by group A, C and G streptococci and is able to bind and activate human plg (Bennsar et al 1994). Unlike uPA and tPA that are serine proteases, SK has no intrinsic proteolytic activity. The process of SK-induced PA begins by the initial formation of a series of stoichiometric plg:SK complexes. SK binds rapidly and with high affinity to the plg light chain (Summaria & Robbins 1976; Cederholm-Williams et al 1979). This binding event is mediated by a
17kDa region spanning Val43-Lys293 in SK (Rodriguez 1994). Lysine residues in SK are important for the binding of plg (Lin et al, 1996). The formation of the 'activator complex' induces a conformational change in plg which exposes the plg active site (McClintock & Bell 1971; Reddy & Markus 1972; Summaria et al 1982). The resulting complex is then capable of activating plg - that is the plg:SK complex is a plg activator. Plg:SK has been shown to bind to hepatocytes and endothelial cells which may help to localise PA near the cell surface (Humphries et al 1993). This process is mediated by the LBS in the plg kringles which become exposed upon formation of plg:SK and also enable binding to fibrin (Fears 1989). Recent evidence suggests SK can bind to receptor-bound plg on the bacterial surface (Rigdahl et al 1998).

Staphylokinase (STA) is a plg activator produced by various strains of Staphylococcus aureus. It is a single chain protein of 136 amino acids (Sako & Tsuchida 1983) and binds to glu and lys-plg with similar affinity and via similar sites to SK (Lijnen et al 1994) to form a 1:1 stoichiometric complex (Lijnen et al 1993). Plm generation is essential for exposure of the active site in plg:STA complexes (Collen et al 1993). Neither the protease domain nor kringles 1-4 of plg are involved in STA binding (Lijnen et al 1994). The LBS in plg kringles 1-4 have been proposed to play a role in the interaction with STA (Arai et al 1998). α2-AP is able to rapidly inhibit plm:STA complexes which causes dissociation of active STA which can subsequently bind to other plg molecules (Silence et al 1993).
1.2.4 Plasminogen activator inhibitors

The proteolytic activity of both uPA and tPA is modulated predominantly by PAI-1 and PAI-2 that belong to the serpin family of protease inhibitors (serine protein inhibitors). Serpins undergo a large conformational change upon complex formation with proteases which is essential for their inhibitory properties (Gettins et al 1993). PAI-/PAI-2 form 1:1 molar complexes with uPA/tPA via a reactive centre loop that interacts with the catalytic site of the serine protease. Complexes are then cleared from the circulation or from the cell surface via receptor mediated endocytosis.

1.2.4.1 PAI-1

PAI-1 is a single chain glycoprotein of ~54kDa and is the major extracellular inhibitor of both uPA and tPA. For this reason PAI-1 is a regulator of both fibrinolysis and cell-surface proteolysis. PAI-1 has also been found to inactivate α-thrombin (Naski et al 1993). PAI-1 is synthesised by a variety of cells including endothelial cells (Kruithof et al 1984) and monocytes/macrophages (Wohlwend et al 1987a & b) and is found in plasma at about 0.4nm (Booth et al 1988; Declerck et al 1988) and on the surface of platelets (Booth et al 1988). Although platelet-bound PAI-1 accounts for the majority of circulating PAI-1, it possesses a lower specific activity than free PAI-1 (Booth et al 1988; Declerck et al 1988).

The interaction between PAI-1 and tPA is a two step mechanism involving an initial rapid, reversible interaction, followed by a slow irreversible reaction (Heckman & Loskutoff 1988). In contrast the interaction between
uPA and PAI-1 is a single step interaction between the active site of uPA and the reactive centre of PAI-1 (Heckman & Loskutoff 1988). PAI-1 can also bind to uPAR-bound uPA (Cubellis et al 1989; Ellis et al 1990) and the resulting complex binds to the $\alpha_2$-MG/lipoprotein receptor protein which causes the endocytic clearance of PAI-1/uPA/uPAR from the cell surface (Cubellis et al 1990; Conease et al 1995). gp330 is also responsible for clearing uPA:PAI-1 complexes from renal proximal tubules (Moestrup et al 1993). Evidence suggests that in addition to their involvement in extracellular proteolysis PAI-1/uPA/uPAR complexes may be involved in cell fusion by acting as a bridge between cells and ECM (Bonavaud et al 1997). Free tPA and tPA:PAI-1 complexes are also cleared and degraded from the surface of endothelial cells (Grobmyer et al 1993), fibroblasts (Willnow et al 1992) and hepatoma cells (Owensby et al 1988; Owensby et al 1989; Morton et al 1990; Owensby et al 1991; Bu et al 1993) via $\alpha_2$-MGR.

Vitronectin serves as a co-factor for PAI-1 and acts as a PAI-1 binding protein, allowing the accumulation of PAI-1 at sites of tissue damage (Declerck et al 1988; Podor et al 1992). This association of PAI-1 with vitronectin apparently stabilises PAI-1 in its active conformation without interfering with its inhibitory activity (Seiffert et al 1990). Vitronectin:PAI-1 complexes are able to inhibit both uPA and tPA (Salonen et al 1989). This tight complex between PAI-1 and vitronectin has been found to accelerate the inactivation of human $\alpha$-thrombin (Naski et al 1993). PAI-1 can also interact with heparin which enhances its reactivity towards thrombin
(Ehrlich et al 1991). PAI-1 is a major component of the ECM of cultured human fibroblasts and sarcoma cells (Pollanen et al 1987). The incorporation of PAI-1 into the ECM has been proposed to protect ECM components from the proteolytic activity on the surface of migrating cells (Seiffert et al 1990). PAI-1 released from endothelial cells is also incorporated into developing fibrin clots which similarly may be a mechanism of regulating tPA-catalysed fibrinolysis (Handt et al 1994).

A variety of growth factors have been shown to increase the synthesis and secretion of PAI-1 such as IL-1 (Bevilacqua et al 1986), thrombin (van Hinsbergh et al 1987), bFGF (Gospodarowicz et al 1987), TNFα (Schleef et al 1987; Handt et al 1994), TGF-β1 (Lund et al 1987; Albo et al 1994; Pepper et al 1990; Handt et al 1994), TSP (Albo et al 1994) and LPS (Chapman & Stone 1985; Schwartz & Bradshaw 1992). These observations suggest that growth factor driven processes, such as cell migration, angiogenesis and inflammation, can utilise PAI-1.

1.2.4.2 PAI-2

PAI-2 is a protein which exists as a 46kDa non-glycosylated intracellular form and a 60kDa glycosylated extracellular form (Astedt et al 1987; Wohlwend et al 1987; Wun et al 1987.). Glycosylated tPA has three N-linked glycosylation sites at Asn75, Asn115 and Asn339 (Ye et al 1988). Both forms of PAI-2 are encoded by a single mRNA (Belin et al 1989). The gene for PAI-2 is located on chromosome 18q21-23 and is the closest mammalian homologue to ovalbumin (Ye et al 1989). The reactive centre of PAI-2 is located at Arg380.
Thr$^{381}$ (Kiso et al 1988). PAI-2 can inactivate both uPA and tctPA but less effectively than PAI-1 (Kruithof 1988). uPA:PAI-2 complexes are rapidly cleared from the surface of monocytes (Estreicher et al 1990).

PAI-2 was originally isolated from human placenta and is sharply increased during the third trimester of pregnancy (Kruithof et al 1987). PAI-2 is found intracellularly in a variety of cells including monocytes/macrophages (Wohlwend et al 1987a & b), fibroblasts (Hamilton et al 1992; Pytel et al 1990), syncytiotrophoblasts (Astedt et al 1986), keratinocytes (Hashimoto et al 1989), vascular smooth muscle cells (Laug et al 1989), endothelial cells (Webb et al 1987), granulosa cells (Piquette et al 1993) and various cells of the brain including mesothelial cells (van Hinsbergh et al 1990; Idell et al 1992) and microglia (Akiyama et al 1993).

IL-1 increases PAI-2 expression in monocytes (Gyetko et al 1993), endothelial cells (Zoellner et al 1993), synovial fibroblasts (Hamilton et al 1992; Hamilton et al 1993), bone marrow stromal cells (Hannocks et al 1992) and granulosa cells (Piquette et al 1994). PAI-2 expression in monocytes/macrophages is also increased by IL-2 (Gyetko et al 1993), IFNγ (Gyetko et al 1992), macrophage and granulocyte-macrophage colony stimulating factors (Wohlwend et al 1987; Hamilton et al 1993). EGF also increases PAI-2 expression in epidermoid carcinoma (George et al 1990) and granulosa cells (Piquette et al 1993).

PAI-2 expression in macrophages/monocytes is decreased by TGFβ (Hamilton et al 1993) and glucocorticoids such as dexamethasone (Wohlwend et al 1987; Medcalf et al 1988). Non-glycosylated PAI-2 is released by U937 cells after prolonged exposure to PMA (Kruithof et al 1986). Cell death has been proposed to be another way in which non-glycosylated PAI-2 might be released from cells (Kruithof et al 1995).

1.2.5 Plasmin inhibitors

There are a number of serpins which function to inactivate plm by forming irreversible enzyme-inhibitor complexes. Some of these inhibitors are specific for plm, for example α2-antiplasmin (α2-AP) while others are able to inhibit a variety of serine proteases, for example α2-macroglobulin and protease nexin 1. Plm can also complex with other serpins such as
antithrombin III, α₁-antitrypsin and C1 inhibitor (Levi et al 1993), however the main physiological plm inhibitor is α₂-AP followed by α₂-MG.

1.2.5.1 α₂-antiplasmin

α₂-AP is a single chain glycoprotein of 65-70kDa (452 amino acids) and is the main physiological plasmin inhibitor. Under certain in vitro conditions α₂-AP can also inhibit factor XII, plasma kallikrein, activated factor XI and thrombin (Saito et al 1979). It is found in mammalian plasma at a concentration of 1μM (Wiman & Collen 1977) and a small amount (~0.5%) is found on the surface of platelets (Plow & Collen 1981). α₂-AP exists as either a plg binding or non-plg binding form. The plg binding form inhibits plm, reversibly interacts with plg and covalently attaches to fibrin during coagulation. The non-plg binding form inhibits plm more slowly and does not bind to plg or fibrin (Tamaki & Aoki 1982; Kluft et al 1984). The plg binding form of α₂-AP is produced by the liver while the non-plg binding form is formed in the circulation (Kluft et al 1986).

α₂-AP consists of a plg/plm binding site, a reactive site (which binds to the active site of plm) at Arg₃₆₄-Met₃₆₅ and a fibrin binding site (which binds to the α-chain of fibrin) (Wiman et al 1989). The binding of plm to α₂-AP is rapid with the rate limiting step being the interaction between the LBS in K1 and a C-terminal lysine residue in α₂-AP (Wiman & Collen 1978; Wiman 1981; Sasaki et al 1986). This facilitates the interaction between the active site
of plm and the reactive site peptide in $\alpha_2$-AP to form a stable enzyme inhibitor complex (Wiman & Collen 1979; Nilsson & Wiman 1982). The major sites of $\alpha_2$-AP production are the liver and the kidney (Menoud et al 1996) however cultured monocytes (Kloczko et al 1990) and glioblastoma organ cultures (Keohane et al 1990) can release $\alpha_2$-AP. Numerous studies have shown that $\alpha_2$-AP can not inhibit cell- (Ellis et al 1991) or fibrin-bound plm (Anonick & Gonias 1991).

1.2.5.2 $\alpha_2$-macroglobulin

$\alpha_2$-MG is a 726kDa protein consisting of four identical 185kDa subunits that are non-covalently associated via two disulphide bonded pairs of subunits. In addition to plm, $\alpha_2$-MG is capable of inhibiting trypsin, chymotrypsin, neutrophil elastase and fibroblast collagenase. Only when the inhibitory capacity of $\alpha_2$-AP is exceeded by high plasma concentrations of plm does $\alpha_2$-MG come into play (Saito 1988). $\alpha_2$-MG is produced by monocytes and macrophages (Ganter et al 1989) and tumour cells (Bizik et al 1986). $\alpha_2$-MG is found at relatively high concentrations in plasma and lymph, pleural and amniotic fluids (Starkey & Barrett 1977). The interaction of plm with $\alpha_2$-MG does not depend on the LBS in K1 (Steiner et al 1987). Studies have demonstrated that fibrin-bound plm is protected from inhibition by $\alpha_2$-MG (Anonick & Gonias 1991).
α₂-MG:plm complexes bind to the α₂-MG/LRP receptor (α₂-MGR) which is present on a range of cell types including hepatocytes, macrophages, fibroblasts and neurons (Moestrup et al 1992). PAI-1:uPA/tPA complexes can also bind to the α₂-MGR but bind to a site distinct from α₂-MG:plm (Nykjaer et al 1992). Once bound to the cell surface α₂-MG:plm is cleared via receptor mediated endocytosis.

1.2.6 Cellular Receptors

Another critical component of the PA cascade is binding sites for uPA, tPA and plg. There are two general classes of binding sites: (1) cellular receptors and (2) receptors/binding determinates on fibrin/ECM components. Binding of plg, tPA or uPA serves to localise PA to the cell surface, ECM or fibrin clot thereby focussing proteolytic activity at these regions. Additionally, immobilised plm is protected from fast-acting plasma inhibitors such as α₂-AP and α₂-MG (Lottenberg et al 1987; Hall et al 1991; Fuchs et al 1994). In this manner cellular receptors are an important regulatory element of the PA system.

1.2.6.1 uPAR (CD87)

uPA (both sc and tc-uPA) is able to specifically interact with a variety of cells (Table 1.1) via a ~55kDa single glycosylphosphoinositol (GPI)-anchored membrane glycoprotein which consists of three homologous domains (Cubellis et al 1986; Ploug et al 1991). uPA can also interact with gangliosides on U937 cells (Miles et al 1989). uPAR possesses an N-linked carbohydrate
which appears to be important for its cellular transport and maturation (Moller et al 1993) and also contributes to the affinity for uPA (Behrendt 1991; Moller et al 1993). There is outstanding evidence that the N-terminal growth factor domain of uPA is responsible for mediating the high affinity binding to uPAR (Stopelli et al 1985; Appella et al 1987; Goretzki et al 1999; Quax et al 1999). Recent evidence indicates that intracellular stores of uPAR occur in some cells that do not express cell-surface uPAR suggesting that translocation may occur if necessary (Jardi et al 1996). A truncated form of uPAR, lacking the uPA binding domain, has been found on normal and tumour thyroid cells (Ragno et al 1998). Studies have also revealed that unoccupied uPAR is relatively mobile (Myohanen et al 1993).

**TABLE 1.1: Characteristics of cellular uPA binding**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>No receptors/cell</th>
<th>Kd</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>U937 (monocytoid)</td>
<td>5.0 ± 2.9 x 10⁴/cell</td>
<td>0.5nm</td>
<td>Cubellis et al 1986</td>
</tr>
<tr>
<td>GM1380 (fibroblast)</td>
<td>1.8 ± 0.3 x 10⁴/cell</td>
<td>2.0 ± 1.6nm</td>
<td>Plow et al 1986</td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>2.6 ± 0.8 x 10⁵/cell</td>
<td>0.8 ± 0.5nm</td>
<td>Felez et al 1990</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>2.9 ± 2.9 x 10⁵/cell</td>
<td>2.1 ± 1.5nM</td>
<td>Plow et al 1986</td>
</tr>
<tr>
<td>Monocytes</td>
<td>5.6 ± 3.1 x 10³/cell</td>
<td>1.5nM</td>
<td>Miles et al 1987</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>5.7 ± 1.6 x 10³/cell</td>
<td>0.5nm</td>
<td>Miles et al 1987</td>
</tr>
<tr>
<td>Platelets</td>
<td>1 ± 0.2 x 10³/cell</td>
<td>1 ± 0.2x10³/cell</td>
<td>Miles et al 1987</td>
</tr>
<tr>
<td>RBC</td>
<td>&lt; 70/cell</td>
<td>1 ± 0.2x10³/cell</td>
<td>Miles et al 1987</td>
</tr>
<tr>
<td>THP-1 (monocytoid)</td>
<td>2.1 ± 0.1 x 10⁴/cell</td>
<td>1 ± 0.2x10³/cell</td>
<td>Felez et al 1990</td>
</tr>
<tr>
<td>HL-60 (monocytoid)</td>
<td>1.3 ± 0.3 x 10⁴/cell</td>
<td>2.1 ± 0.1x10⁴/cell</td>
<td>Felez et al 1990</td>
</tr>
</tbody>
</table>
Vitronectin can also bind with high affinity to uPAR (Hoyer-Hansen et al 1992) at a site different from uPA. This co-localisation of vitronectin and uPA on uPAR contributes to cell adhesion and migration (Kanse et al 1996; Chapman 1997). Recent evidence indicates the existence of a soluble uPAR (suPAR) which is secreted by human endothelial and monocytic cells and is increased by PMA stimulation (Chavakis et al 1998). Vitronectin mediates the binding of uPA/suPAR to cell surfaces and ECM (Chavakis et al 1998) presumably via binding to suPAR. uPAR is found in close association with β₂-integrins on leukocytes where it is required for leukocyte-endothelial cell interactions and migration to inflamed areas (May et al 1998).

uPAR is expressed on a variety of cell types including leukemic cells and peripheral blood cells (Jardi et al 1996), keratinocytes (Reinartz et al 1994), human thyroid cells (Ragno et al 1998), and mouse spermatozoa (Zhou & Vassalli 1997). uPAR expression on leukemic cells appears to be confined to more differentiated cells along the myelo-monocytic pathway (Jardi et al 1996). This is supported by a previous observation that uPAR expression increases during the differentiation of mouse erythroleukemic cells (Del Rosso et al 1987). As with other components of the PA system, uPAR expression is increased by a variety of growth factors such as phorbol 12-myristate 13 acetate (PMA) (Stopelli et al 1985; Estreicher et al 1989; Picone et
al 1989; Lund et al 1991; Wang et al 1994; Jardi et al 1996), HGF (Pepper et al 1992), IFNγ (Kirchheimer et al 1988; Lu et al 1988), TNFα (Kirchheimer et al 1988; Lu et al 1988; Wang et al 1994), TGF-β1 (Falcone et al 1995) and thrombin (Yoshida et al 1997). These results suggest that the increase in uPAR expression during processes such as cell migration and inflammation can be attributed to the effect of growth factors.

The obvious role of uPA binding to uPAR is to localise PA to the cell surface. The enzymic activity of scuPA is increased 100-fold upon binding to uPAR on monocytes (Manchandra & Schwartz 1991). Unlike cell-surface plm which is relatively resistant to inactivation by its circulating inhibitors, receptor-bound uPA is efficiently inactivated by both PAI-1 and PAI-2 (Ellis et al 1990; Pollanen et al 1990). Another outcome of uPA binding is the initiation of signal transduction pathways that can cause phosphorylation of protein kinases (Dumler 1993; Busso 1994; Bohuslav 1995; Resnati 1996; Dumler et al 1998) and intracellular calcium mobilisation (Cao et al 1995). Recent evidence (Dumler et al 1998) has shown that uPA/uPAR signalling utilises a direct Jak/Stat cascade and a second pathway utilising Src-like protein-tyrosine kinases. Although the Jak/Stat pathway appears to be involved in cell migration, the role of the Src-like protein-tyrosine kinase pathway remains to be elucidated. Evidence also suggests that uPA binding can actually transactivate the PAI-2 gene promoter in HT-1080 cells (Dear et al 1997). The mechanism of uPA/uPAR signal transduction is thought to be via related transmembrane integrins (Petty & Todd 1996) as uPAR does not contain any transmembrane or cytoplasmic domains (May et al 1998).
Infact uPAR is found associated with various integrins in focal contacts (Pollanen et al 1991) where it appears to be involved in cell migration (Busso et al 1994; Gyetko et al 1994).

Additionally uPA can bind to several ECM components such as heparin, laminin and nidogen (Stephens et al 1992a & b). uPA binds weakly to fibronectin (Salonen et al 1985).

### 1.2.6.2 tPAR

tPA can bind to a variety of cells (Table 1.2) via two mechanisms. One mechanism is mediated by PAI-1 which binds (and hence inactivates) functionally active tPA (Cheng et al 1992). The resulting tPA:PAI-1 complexes bind to α₂-MGR, low density lipoprotein receptor-related protein (LRP) or the mannose receptor on the surface of liver parenchymal cells (Bu et al 1993; Smedsrod & Einarsson 1990) and endothelial cells (Einarsson et al 1988; Smedsrod & Einarsson 1990) and are then cleared by receptor mediated endocytosis. The second mechanism involves tPA binding directly to specific membrane receptors and does not involve the protease (Cheng et al 1992) or EGF domain of tPA (Verrall & Seeds 1989). This second mechanism also results in the internalisation and degradation of tPA (Nguyen et al 1992).

tPA has a unique receptor on some bacteria (Ullberg et al 1994) and vascular smooth muscle cells (Ellis & Whawell 1997), however both tPA and plg share a common set of binding sites on nucleated cells (Felez et al, 1996).
tPA can bind to several plg-R candidates such as α-enolase, gangliosides (Felez et al 1993) annexin II (Hajjar et al 1994), a 45kDa protein on endothelial cells (Dudani et al 1994) and E. Coli Fimbriae (Parkkinen et al 1991). tPA can also bind to the mannose receptor via its high mannose-type

**TABLE 1.2: Characteristics of cellular tPA binding**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>No receptors/cell</th>
<th>Kd</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>U937</td>
<td>1.6 ± 0.1 x 10⁶/cell</td>
<td></td>
<td>Felez et al 1993</td>
</tr>
<tr>
<td>THP-1</td>
<td>2.3 ± 1.2 x 10⁶/cell</td>
<td></td>
<td>Felez et al 1993</td>
</tr>
<tr>
<td>K562 (leukemic)</td>
<td>3.2 ± 0.2 x 10⁶/cell</td>
<td></td>
<td>Felez et al 1993</td>
</tr>
<tr>
<td>Raji (lymphoma)</td>
<td>4.0 ± 0.3 x 10⁶/cell</td>
<td></td>
<td>Felez et al 1993</td>
</tr>
<tr>
<td>Nalm-6 (pre-B leukemic)</td>
<td>1.3 ± 0.1 x 10⁶/cell</td>
<td></td>
<td>Felez et al 1993</td>
</tr>
<tr>
<td>Rat Novikoff (hepatoma cells)</td>
<td>5.4 x 10³/cell</td>
<td>12mM</td>
<td>Nguyen et al 1992</td>
</tr>
<tr>
<td>HepG2 (liver)</td>
<td>1.4 ± 0.1 x 10⁶/cell</td>
<td></td>
<td>Bu et al 1993</td>
</tr>
<tr>
<td>A431 (epidermoid)</td>
<td>1.4 ± 0.1 x 10⁶/cell</td>
<td>50pM</td>
<td>Felez et al 1993</td>
</tr>
<tr>
<td>mouse cerebellar cells</td>
<td>3.2 ± 1.0 x 10⁶/cell</td>
<td>70nM</td>
<td>Verral &amp; Seeds 1989</td>
</tr>
<tr>
<td>Neisseria (Mo 52)</td>
<td>8.5 x 10³/cell</td>
<td></td>
<td>Ullberg et al 1994</td>
</tr>
<tr>
<td></td>
<td>1 x 10⁴/cell</td>
<td>20nM</td>
<td>Ullberg et al 1994</td>
</tr>
<tr>
<td>----------------</td>
<td>--------------</td>
<td>------</td>
<td>------------------</td>
</tr>
<tr>
<td><strong>Haemophilus influenzae (HI23354)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vascular smooth muscle cells</td>
<td></td>
<td>25nM</td>
<td>Ellis &amp; Whawell 1997</td>
</tr>
<tr>
<td>muscle cells</td>
<td></td>
<td>300nM</td>
<td></td>
</tr>
</tbody>
</table>

oligosaccharide (Otter et al 1991) however other studies have shown that the carbohydrate moiety of tPA is not important for cellular binding (Verrall & Seeds 1989). The clearance of tPA from the surface of Novikoff hepatoma cells does not involve the mannose receptor (Nguyen et al 1992).

tPA has been shown to bind to ECM produced by both smooth muscle and endothelial cells (Stack et al 1994). tPA can interact with laminin (Salonen et al 1984) and to heparan sulphate- and chondroitin sulphate-containing proteoglycans produced by human endothelial cells (Bohm et al 1996). tPA can also bind to heparin which stimulates fibrin-dependent PA (Andrade-Gordon & Strickland 1986; Paques et al 1986; Stein et al 1989). tPA can also bind to fibronectin via a 55kDa amino terminal fragment (Salonen et al 1985; Moser et al 1993). Binding of tPA (and plg) to intact fibronectin does not enhance PA, however, proteolytic fragments of fibronectin are potent stimulators of PA (Stack and Pizzo 1993). tPA (and plg) binds with high affinity to thrombospondin which results in a 40-fold increase in the efficiency of PA (Silverstein et al 1985). tPA binds with high affinity to both fibrin and fibrin fragments which enhances tPA-catalysed PA (Hoylaerts et al 1982) but has a lower affinity for fibrin than plg (Fleury et al 1993). Fibrin-bound tPA appears to be protected from inhibition by PAI-2 (Leung et al 1987). Antimicrobial peptides called defensins have been shown to inhibit
tPA binding to endothelial cells and fibrin and consequently inhibit PA (Higazi et al 1996).

Although the main physiological outcome of tPA binding to its receptor/s is an enhancement of tPA-dependent PA (Parkkinen et al 1991; Hajjar et al 1994) there is some evidence that tPA binding may initiate a signal transduction pathway. The binding of tPA to its receptor/s appears to induce endothelial cell proliferation which is independent of plm activity but apparently dependent on protein kinase A activity (Welling et al 1996).

1.2.6.3 Plg-Rs

Plg-Rs are a heterogenous group of molecules that are characterised by the presence of terminal lysine residues that serve as a binding site for plg via the LBSs (Winram & Lottemberg 1998). C-terminal lysine residues appear to be the defining feature of plg-Rs however, recent evidence indicates that N-terminal lysine residues are also important in plg binding to α-enolase on pathogenic streptococci (Pancholi & Fischetti 1988). Although cellular plg binding is a widespread phenomena most plg-R candidates are restricted to specific cell types (Table 1.3) and are generally proteins, however some non-proteinaceous molecules, such as gangliosides, function as plg-Rs on U937 cells (Miles et al, 1989). The general consensus is that several different plg-Rs are responsible for the total plg binding potential of cells. For example, although glyceraldehyde-3-phosphate functions as a bacterial plg-R it does not account for all the plg-binding ability of cells as bacterial strains with
mutated plg-R genes can still bind the same level of plg as wild type strains (Winram & Lottenberg, 1998).

Plg has also been found to share some cellular binding sites with tPA (Parkkinen & Rauvala 1991; Felez et al 1993; Cesarman et al 1994). Lipoprotein(a) can compete with plg for cellular binding sites due to the ability of Lp(a) to bind to plg-Rs on U937 cells and endothelial cells (Miles et, 1988)

TABLE 1.3: Candidate plg-Rs

<table>
<thead>
<tr>
<th>Receptor Candidate</th>
<th>Cell Type/s</th>
<th>MWt</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPIIb/IIIa</td>
<td>platelets</td>
<td></td>
<td>Miles et al 1986; Adelman et al 1988</td>
</tr>
<tr>
<td>Type I Fimbrae</td>
<td>Salmonella enterica</td>
<td></td>
<td>Kukkonen et al 1998</td>
</tr>
<tr>
<td>G Fimbrae</td>
<td>Escherichia coli</td>
<td></td>
<td>Parkkinen &amp; Korhonen 1989</td>
</tr>
<tr>
<td>S Fimbrae</td>
<td>Escherichia coli</td>
<td></td>
<td>Miles et al 1989</td>
</tr>
<tr>
<td>Gangliosides</td>
<td>Endothelial cells, granulaocytes, platelets, U937s</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Annexin II</td>
<td>Endothelial cells</td>
<td>40kDa</td>
<td>Hajjar et al 1994</td>
</tr>
<tr>
<td>45kDa protein</td>
<td>Rat neocortical neurons</td>
<td>45kDa</td>
<td>Nakajima et al 1993</td>
</tr>
<tr>
<td>Thrombospondin/</td>
<td>platelets</td>
<td>450kDa</td>
<td>Silverstein et al 1985</td>
</tr>
<tr>
<td>Histidine-rich glycoprotein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-enolase / α-</td>
<td>U937s</td>
<td>54kDa</td>
<td>Miles et al 1991; Redlitz et al 1995; Arza et al 1997</td>
</tr>
<tr>
<td>Protein/Pathogen</td>
<td>Cells/Cells Type</td>
<td>Molecular Weight</td>
<td>References</td>
</tr>
<tr>
<td>------------------</td>
<td>------------------</td>
<td>------------------</td>
<td>------------</td>
</tr>
<tr>
<td>Cytokeratin 8</td>
<td>55-60kDa</td>
<td>MCF-7 cells</td>
<td>Lopez-alemany et al 1994</td>
</tr>
<tr>
<td>Actin</td>
<td>45kDa</td>
<td>endothelial cells</td>
<td>Pancholi &amp; Fischetti 1998</td>
</tr>
<tr>
<td>50-60kDa protein</td>
<td>45kDa</td>
<td>endothelial cells</td>
<td>Hembrough et al 1995</td>
</tr>
<tr>
<td>PAM</td>
<td>45kDa</td>
<td>Streptococcus pyogenes</td>
<td>Durliat et al 1992</td>
</tr>
<tr>
<td>Flagella</td>
<td>43kDa</td>
<td>E. Coli LE393</td>
<td>Berge &amp; Sjobring 1993; Wistedt et al, 1995</td>
</tr>
<tr>
<td>97kDa protein</td>
<td>97kDa</td>
<td>Rheumatoid arthritis, human synovial fibroblasts</td>
<td>Gonzalez-Gronow et al 1994</td>
</tr>
<tr>
<td>amphoterin</td>
<td>30kDa</td>
<td>rat kidney cells</td>
<td>Parkkinen &amp; Rauvala 1991</td>
</tr>
<tr>
<td>Rat heymann</td>
<td>76kDa</td>
<td>rat kidney cells</td>
<td>Kanalas &amp; Makker 1991</td>
</tr>
<tr>
<td>nephritis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>autoantigen gp330</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cell-surface</td>
<td></td>
<td>Borrelia Burgdorferi</td>
<td>Fuchs et al 1994</td>
</tr>
<tr>
<td>lipoprotein A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>55kDa protein</td>
<td></td>
<td>corneal fibroblasts</td>
<td>Lopez-Alemany et al 1995</td>
</tr>
<tr>
<td>Tetranectin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>glyceraldehyde-3-phosphate</td>
<td></td>
<td>Streptococcus equisimilis</td>
<td>Broder et al 1991; Gase et al 1996</td>
</tr>
<tr>
<td>dehydrogenase</td>
<td></td>
<td>H46A</td>
<td></td>
</tr>
</tbody>
</table>
surface protein A  Borrelia Burgdorferi  Hu et al 1997
70kDa protein  Borrelia Burgdorferi  70kDa  Hu et al 1997
Tissue factor  CHO (transfected)  Fan et al 1998
Complement protein  endothelial cells
C9
Aspartase  Haemophilus influenzae  55kDa  Sjostrom et al 1997

al 1995). The basis for this competition is that Lp(a) contains kringle (analogous to plg kringle 4) which have lysine binding ability.

The regulation of plg-R expression is another important mechanism whereby cellular PA can be modulated. A variety of plg binding experiments have been performed (without identification of an actual plg-R) which indicate that plg-R expression can be modulated by a variety of growth factors. Interferon γ and vitamin D₃ have been shown to increase plg-R expression on U937 cells (Lu et al 1988 & 89). Plg-Rs are decreased by glucocorticoids on HT-1080 fibrosarcoma cells (Pollanen, 1989) and thrombin on endothelial cells (Miles et al 1988) which contrasts with thrombin stimulated platelets which express more plg-Rs (Miles et al, 1986). Plg-Rs are also upregulated following β1-intergrin-dependent cellular adherence (Kim et al 1996). PMA has also been shown to increase plg-R expression on monocytoid cells (Felez et al 1990). Plg-R expression is not modulated by TGF-β₁ on THP-1 cells (Falcone et al 1995). Some candidate plg-Rs have also been suggested to be important for cell viability (Winram & Lottenberg 1998).
1.2.6.4 Characteristics of cellular plg binding

Plasminogen binding has been reported on a variety of both prokaryotic (Table 1.4) and eukaryotic cells (Table 1.5). The main features of cellular plg binding are: (1) it is specific - it can be inhibited with an excess of unlabelled plg, and lysine analogues such as L-lysine, ε-amino caproic acid (ε-ACA) and tranexamic acid (TA) and partially by the arginine analogue benzamidine; (2) it is saturable - there are a finite number of cellular binding sites ranging from $10^2$ on some bacteria (Kuusela & Saksela 1990) up to $10^7$ on some eukaryotic cells (Miles et al 1988) and (3) it is a relatively low affinity interaction with dissociation constants ($K_d$) on eukaryotes of up to 2.6μM. Generally, plg binding to prokaryotes is a much higher affinity interaction than binding to eukaryotes.

### TABLE 1.4: Characteristics of plg binding to prokaryotic cells

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>No of Receptors</th>
<th>Affinity ($K_d$)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Helicobacter pylori</td>
<td>3-6 x 10^3/cell</td>
<td>50nm</td>
<td>Ringner et al 1994; Khin et al 1996; Pantzar et al 1998</td>
</tr>
<tr>
<td>Neisseria meningitidis</td>
<td>1-2 x 10^4/cell</td>
<td>200nm</td>
<td>Ullberg et al 1992</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Eberhard et al 1995</td>
</tr>
<tr>
<td>Neisseria gonorrhoeae</td>
<td></td>
<td></td>
<td>Ullberg et al 1992</td>
</tr>
<tr>
<td>Group A streptococcus</td>
<td>780/cell</td>
<td>1.7nm</td>
<td>Lottenberg et al 1987; Wang et al 1994; Eberhard et al 1995</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td></td>
<td></td>
<td>Kuusela &amp; Saksela 1990</td>
</tr>
</tbody>
</table>
Table 1.5: Characteristics of plg binding to eukaryotic cells

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>No of Receptors</th>
<th>Affinity (Kd)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>THP-1 (monocytoid)</td>
<td>0.3 ±0.1 x 10⁶/cell</td>
<td>15.8 ±6.2nM</td>
<td>Felez et al 1990</td>
</tr>
<tr>
<td></td>
<td>1.6 ±0.2 x 10⁶/cell</td>
<td></td>
<td>Felez et al 1993</td>
</tr>
<tr>
<td>THP-1 macrophage</td>
<td>1.4 ± 0.3 x 10⁶/cell</td>
<td>1.9-2.6μM</td>
<td>Falcone et al 1994</td>
</tr>
<tr>
<td>platelets (resting)</td>
<td>3.7 ± 1 x 10⁴/cell</td>
<td>1.9-2.6μM</td>
<td>Miles &amp; Plow 1985;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Miles et al 1986; Adelman et al 1988</td>
</tr>
<tr>
<td>platelets (thrombin-stimulated)</td>
<td>1.9 ± 0.45 x 10⁵/cell</td>
<td>1.9-2.6μM</td>
<td>Miles &amp; Plow 1985; Miles et al 1986;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Edelberg et al 1990</td>
</tr>
<tr>
<td>U937 (monocytoid)</td>
<td>1.6 ± 1.4 x 10⁷/cell</td>
<td>0.8 ±0.5μM</td>
<td>Plow et al 1986</td>
</tr>
<tr>
<td></td>
<td>0.4 ±0.1 x 10⁶/cell</td>
<td></td>
<td>Felez et al 1990</td>
</tr>
<tr>
<td></td>
<td>2.0 ± 0.7 x 10⁶/cell</td>
<td></td>
<td>Edelberg et al 1990</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Gonzalez-Gronow et al 1991</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Felez et al 1993</td>
</tr>
<tr>
<td>Cell Type</td>
<td>Value</td>
<td>Source</td>
<td></td>
</tr>
<tr>
<td>-------------------------------</td>
<td>--------------------------------</td>
<td>----------------------</td>
<td></td>
</tr>
<tr>
<td>GM1380 (fibroblast)</td>
<td>$3.1 \pm 0.4 \times 10^7$/cell</td>
<td>Plow et al 1986</td>
<td></td>
</tr>
<tr>
<td>SW1116 (fibroblast)</td>
<td>$1.5 \times 10^7$/cell</td>
<td>Comacho et al 1989</td>
<td></td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>$1.4 \pm 1 \times 10^6$/cell</td>
<td>Hajjar et al 1986</td>
<td></td>
</tr>
<tr>
<td>SW1116 (colonic adenocarcinoma)</td>
<td>$1.8 \pm 1.3 \times 10^7$/cell</td>
<td>Miles et al 1988</td>
<td></td>
</tr>
<tr>
<td>PROb (rat colon carcinoma)</td>
<td>$0.8 \pm 0.5 \times 10^6$/cell</td>
<td>Felez et al 1993</td>
<td></td>
</tr>
<tr>
<td>REGb (rat colon carcinoma)</td>
<td>$5 \times 10^4$ M</td>
<td>Burtin &amp; Fondaneche 1988</td>
<td></td>
</tr>
<tr>
<td>HT-1080 (fibrosarcoma cells)</td>
<td>$6 \times 10^9$/cell (plm)</td>
<td>Pollanen 1989</td>
<td></td>
</tr>
<tr>
<td>rat hepatocytes</td>
<td>$9.4 \pm 0.8 \times 10^7$/cell</td>
<td>Hall et al 1990</td>
<td></td>
</tr>
<tr>
<td>rat C6 glioma cells</td>
<td>$9.8 \pm 1.3 \times 10^7$/cell</td>
<td>Hall et al 1990</td>
<td></td>
</tr>
<tr>
<td>MCF-7 cells (breast carcinoma cells)</td>
<td>$0.5 \pm 0.2 \times 10^6$/cell</td>
<td>Felez et al 1990</td>
<td></td>
</tr>
<tr>
<td>HL-60 (monocytoid)</td>
<td>$-7.5 \times 10^6$/cell</td>
<td>Campbell et al 1994</td>
<td></td>
</tr>
<tr>
<td>K562 (leukemic)</td>
<td>$3.1 \pm 0.2 \times 10^6$/cell</td>
<td>Felez et al 1993</td>
<td></td>
</tr>
<tr>
<td>Cell Type</td>
<td>Pig Binding (x 10^6/cell)</td>
<td>Reference</td>
<td></td>
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<tr>
<td>---------------------------</td>
<td>---------------------------</td>
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<td></td>
</tr>
<tr>
<td>Raji (lymphoma)</td>
<td>3.4 ± 0.1</td>
<td>Felez et al 1993</td>
<td></td>
</tr>
<tr>
<td>Nalm-6 (pre-B-leukemic)</td>
<td>1.2 ± 0.2</td>
<td>Felez et al 1993</td>
<td></td>
</tr>
<tr>
<td>Chang (liver)</td>
<td>0.5 ± 0.1</td>
<td>Felez et al 1993</td>
<td></td>
</tr>
<tr>
<td>A431 (epidermoid)</td>
<td>1.6 ± 0.1</td>
<td>Felez et al 1993</td>
<td></td>
</tr>
<tr>
<td>HT1080 (fibrosarcoma)</td>
<td>2.4 ± 1.9</td>
<td>Felez et al 1993</td>
<td></td>
</tr>
<tr>
<td>corneal fibroblasts</td>
<td>4 x 10^5</td>
<td>Lopez-Alemany 1995</td>
<td></td>
</tr>
<tr>
<td>WEHI-38 murine Leukemic cells</td>
<td>0.38μM</td>
<td>Martinez &amp; Santibanez 1994</td>
<td></td>
</tr>
<tr>
<td>rat hepatocytes</td>
<td>1.41 ± 1.1</td>
<td>Gonias et al 1989</td>
<td></td>
</tr>
<tr>
<td>human keratinocytes</td>
<td>1.3 x 10^6</td>
<td>Reinartz et al 1993</td>
<td></td>
</tr>
</tbody>
</table>

Various proteins can mediate the binding of plg to cells including complement protein C5B-9 which has been found to increase plg binding and activation on human endothelial cells (Christiansen et al 1997). Plg binding to U937 cells is also enhanced by complestatin which is a small peptide-like metabolite from streptomycyes (Tachikawa et al 1997). Additionally, antimicrobial peptides called defensins have been shown to modulate the binding of both plg and tPA to endothelial cells (Higazi et al 1996). Plg binding to endothelial cells has been inhibited with hypertriglyceridemic VLDL (Li et al 1996).

Proteolytic treatment of cells with plm has been shown to increase the number of plg binding sites on platelets (Ouimet et al 1994) tumor cells (Comacho et al 1989) and bacteria (Gase et al 1996). Limited plm treatment of the candidate plg-R G3PDH, has been shown to enhance its binding capacity...
for plg (Gase et al 1996). The mechanism of this observation is that plm enhances plg binding by exposing new C-terminal lysine residues.

LBS in the plg molecule have been repeatedly shown to mediate cellular plg binding, however there is some evidence that the carbohydrates of plg also contribute to cellular binding. Edelberg et al (1990) determined that neonatal plg, that differs in carbohydrate composition to adult plg, interacts differently with cells. Hall et al (1990) also found that plg type 2 had a slightly higher affinity for rat C6 glioma cells than plg type 1. A recent study on the real-time binding of plg to immobilised l-lysine (mimicking fibrin, ECM or plg-Rs) also indicated that plg binding is very complex and may not be entirely mediated by plg kringles (Warkentin et al 1998).

The main physiological outcome of plg binding to cells is the generation of cell-surface plm which then participates in various extracellular proteolytic events. There is some evidence that plg binding followed by PA may be involved in other cellular processes. uPA-catalysed PA on human rheumatoid arthritis synovial fibroblasts stimulates an increase in intracellular calcium which is not evident in normal cells (Gonzalez-Gronow et al 1993). K4 and the Thr245-linked carbohydrate are involved in mediating this increase in calcium (Gonzalez-Gronow et al 1993). On platelets, plm can indirectly induce calcium mobilisation by proteolytically modifying adhesive receptors (Winters et al 1990). Plm has been shown to induce receptor-mediated arachidonate release which is coupled with G proteins in endothelial cells (Chang et al 1993). The early phase of this
release involved the LBS and active site of plm, and was mediated by the calcium-dependent activation of phospholipase A2 via a GTP-binding protein (Chang et al 1993). The late phase of this release was calcium independent and involved the active site of plm (Chang et al 1993).

1.2.7 Plg binding to extracellular proteins
C-terminal lysine residues also serve as binding sites for plg on ECM and fibrin. Just as with cells, plg binding to ECM/fibrin serves to direct the proteolytic activity of plm against ECM and fibrin which is crucial for processes like cell migration, tissue remodelling and fibrinolysis. Plg binding to ECM and fibrin also induces suitable conformational changes in plg which make it more susceptible to activation by uPA/tPA.

1.2.7.1 Plg binding to ECM
Plg has been shown to bind to intact ECM (Knudson et al 1986; Stack et al 1994; Lopez-Alemany 1995; Pekelharing et al 1996) in addition to individual ECM components such as type 1 collagen (Martinez & Santibanez 1994), type IV collagen (Stack et al 1992), heparin (Stack & Pizzo 1993), laminin (Salonen et al 1984; Moser et al 1993) and fibronectin (Moser et al 1993). Plg has a relatively high affinity for ECM components such as type 1 collagen (Martinez & Santibanez 1994), thrombospondin (35nM) (Silverstein et al 1985), fibronectin (91nM) (Salonen et al 1985) and type IV collagen (12nM) (Stack et al 1992) which suggests that plg would preferentially bind to ECM rather than cells in vivo.
The binding of plg to ECM, like binding to fibrin or cells, results in more efficient activation and serves to direct PA to the extracellular tissue environment. For example, plg binding to type IV collagen enhances the rate of tPA-catalysed PA (Stack et al 1990). The binding of plg to ECM components also establishes a reservoir of plg, which when activated, degrades ECM (Martinez and Santibanez 199) either directly with plm or via the activation of matrix metalloproteases. Additionally, the degradation of ECM by plm also causes the release of matrix-bound growth factors such as TGF-β (Taipale et al, 1992). Lp (A) and LDL have both been shown to compete with plg for binding sites on ECM thereby inhibiting PA (Pekelharing et al 1996).

1.2.7.2 Plg binding to Fibrin

Both glu- and lys-plg can bind to fibrin with lys-plg having a higher affinity ($K_d = 0.32\mu M$) for both noncross-linked and crosslinked fibrin than glu-plg ($K_d = 38\mu M$) (Lucas et al 1983). These binding sites for plg pre-exist in intact fibrin and are not modified by the presence of tPA (Fleury et al 1993). More glu- and lys-plg can bind to non-crosslinked fibrin compared to crosslinked fibrin (Sakata et al 1984). This difference is apparently due to structural changes in the fibrin α-chain which reduces either the affinity or number of binding sites (Sakata et al 1984).

Initial plm digestion of fibrin generates new high affinity binding sites (C-terminal lysine residues) for glu-plg ($K_d = 0.3\mu M$) (Trang-Thang et al (1986) which accelerates PA and hence fibrinolysis. (Bok & Mangel 1985; Harpel et
Plg binding to fibrin is mediated by two fibrin-binding domains: a high affinity site in K5 and a lower affinity site in K1-3 (Wu et al 1990). It is however the binding via K5 which is thought to be essential for tPA-catalysed PA to proceed (Wu et al 1990).

Plg binding to fibrin can be both enhanced and dampened by several proteins. The mechanism by which plg-Rs and fibrin fragments enhance plg binding and activation is similar but not identical (Felez et al 1996). Plg binding and subsequent activation on fibrin is enhanced by staplabin due to a conformational change in plg which is different from that induced by lysine analogues (Takayasu et al 1997). Plg binding to fibrin and subsequent fibrinolysis is enhanced by chloropeptin I (Tachikawa et al, 1997a) and complestatin (Tachikawa et al, 1997b).

Plg binding to plm-modified fibrin is inhibited by apolipoprotein(a) [apo (a)] by the formation of solution phase plg:apo(a) complexes (Sangrar et al 1997). Apo(a) can also bind to C-terminal lysine residues on the surface of fibrin and this binding increases following treatment with plm which blocks plg binding (Angles-Cano et al 1994). This suggests that apo(a) binding may impair fibrinolysis (Angles-Cano et al 1994). The mechanism for this inhibition is due to the strong homology between plg and apo(a) (Angles-Cano 1994). Additionally, small antimicrobial peptides called defensins can compete with plg for binding to fibrin due to defensin having close structural similarity to plg kringles (Higazi et al, 1995). This caused the inhibition of tPA-catalysed fibrinolysis.
1.2.7.3 Plg binding to other proteins

Plg can specifically bind to several other proteins including histidine-proline-rich glycoprotein (Leung, 1986) which is has been suggested to play a physiological role in fibrinolysis considering that approximately 50% of serum plg is bound to HPRG (Lijnen et al 1980). Like binding to other proteins, plg binding to HPRG stimulates tPA-induced activation (Borza & Morgan 1997). Plg can bind, in a lysine-dependent, manner to apolipoprotein (A) in a 1:1 stoichiometry (Sangrar et al 1997). Complement component C7 is a plasminogen-binding protein and is mediated by the LBS of plg (Reinartz et al 1995). Plg can bind, with relatively high affinity to the multifunctional platelet α-granule thrombospondin (TSP) via the LBS (Silverstein et al 1984). uPA and tPA have been shown to increase plg binding to TSP via the generation of cryptic C-terminal lysine residues by small amounts of plm (Silverstein et al 1986). Once again this is an example of the positive feedback loop in the PA system. Plm generated on TSP is also protected from α2-AP (Silverstein et al 1985). Plg can bind to tissue factor which is mediated by the LBS in K1 and 3 (Fan et al 1998). Plg has been shown to bind to vitronectin following plm modification due to the liberation of C-terminal lysine residues (Kost et al 1996).

1.3 The mechanism of plasminogen activation

The first step in the activation pathway is the binding of plg to a C-terminal lysine residue on either a plg-R, fibrin or ECM component. This binding event induces a reversible structural transition from the closed to the open conformation, which exposes the Arg561-Val562 activation bond thus
stimulating activation by uPA (Peltz et al 1982) or tPA Hoylaerts et al 1982). The overall mechanism for the conversion of glu-plg to lys-plm is illustrated in Fig 1.4.

![Overall pathway of activation of glu-plg to lys-plm](image)

**Fig 1.4:** Overall pathway of activation of glu-plg to lys-plm (Adapted from Castellino 1995).

Initially tPA/uPA cleaves glu-plg at the Arg^{561}.Val^{562} peptide bond which generates a small amount of glu-plm. This glu-plm then cleaves a 77 amino acid peptide from the amino terminus of glu-plm to generate lys-plm, or from the amino terminus of glu-plg to yield lys-plg. Lys-plg is then converted to lys-plm via the activity of uPA/tPA. The key feature of this pathway is that the end product (i.e plm) is able to feed back and activate more plg. In this manner PA can be accelerated quickly to generate large quantities of plm.
As PA proceeds plm degrades fibrin or ECM components which generates additional lysine residues thus facilitating the binding and activation of more glu-plg. Cellular plg binding has been shown to increase when cells are pretreated with plm (Comacho et al 1989; Ouimet et al 1994; Gase et al 1996). Plm bound to the surface of macrophages is also capable of autoproteolysis which has been proposed to be another mechanism for regulating membrane-bound plm activity (Falcone et al 1994). Plg binding is also increased on fibrin that has been partially degraded with plm (Bok & Mangel 1985).

1.4 Roles of plasminogen activation

The main physiological role of PA is fibrinolysis but it also contributes to processes of cell migration (e.g angiogenesis, metastasis, inflammation, wound healing, bacterial invasion), tissue remodelling and development, ovulation and the activation of growth factors and other protease classes. Although a myriad of in vitro studies have supported these diverse roles, the development of plg 'knockout' mice have proven a vital model to ascertain the in vivo role/s of PA.

Plg-deficient mice exhibit impaired thrombolysis which leads to spontaneous fibrin deposition in various organs including the liver, lungs and stomach (Ploplis et al 1995). The thrombolytic activity of these mice can be restored by the bolus administration of plg (Lijnen et al 1996). Plg-deficient mice also suffer from reduced fertility and retarded growth (Ploplis et al 1995), and are protected from tPA-induced neuronal degeneration
(Tsirka et al 1997a & b). The role of PA in metastasis is supported by ‘knockout’ mouse studies showing that PA contributes to the morbidity and mortality of lewis lung carcinoma (Bugge et al 1997) and metastasis of polyoma virus middle T antigen-induced mammary cancer (Bugge et al 1998). A role for PA in wound healing is supported by studies showing that plg-deficient mice exhibit defects in corneal epithelial healing (Kao et al 1998). Future knockout mouse studies will confirm/refute the other proposed roles of PA in addition to the potential discovery of other roles.

1.4.1 Fibrinolysis

The predominant role of PA (i.e plm) is the proteolytic degradation of fibrin clots (fibrinolysis). The formation of a fibrin clot is a complex process involving the cleavage of fibrinogen (340kDa) initially by thrombin, and then by Factor XIII and plm. The process of fibrin formation and clot dissolution is summarised in Figure 1.5. Plm is responsible for degrading fibrin I and cross-linked fibrin II polymers that yields fragment X polymers which are inturn degraded to yield fibrin fragments D and E.

Fibrin and fibrinogen possess binding sites (C-terminal lysine residues) for plg and tPA which facilitate the binding of both to the clot and the progression of tPA-induced PA. As plm degrades the clot, new C-terminal lysine residues are exposed which allow more plg to bind and be activated, thereby accelerating PA. In this way polymerised fibrin acts as both a catalyst and substrate for PA (Fluery et al 1993). Plm is also able to ‘feedback’ and activate both tPA and plg. $\alpha_2$-AP is also incorporated into the fibrin clot by
cross-linking with the α-chain of fibrin (Aoki et al 1983). The rate of fibrinolysis appears to be determined by the initial amount of bound plg (Aoki et al 1983). PAI-1 secreted by endothelial cells is also incorporated into the developing fibrin clot (Handt et al 1994). This ensures that when the clot
is degraded there is a ready supply of α₂-AP and PAI-1 to inhibit the free plm and tPA. Additionally serum α₂-AP or α₂-MG can also switch off released plm.

1.4.2 Cell migration

Considerable evidence implicates the involvement of PA in various types of cell migration such as tumour metastasis (Ossowski & Reich 1983; Carroll & Binder 1999), bacterial invasion (Korhonen et al 1993; Coleman et al 1995; Virkola et al 1996; Pantzar et al 1998; Li et al 1999), inflammation (Unkeless et al 1974; Wohlwend et al 1987), trophoblast invasion (Sappino et al 1989; Salamonsen 1999; Floridin et al 1999), keratinocyte migration during wound healing (Morioka et al 1991; Reinartz et al 1994) and angiogenesis (Gross et al 1983; Pepper et al 1993). In all cases PA causes the degradation of BM/ECM which enables cells to either penetrate a tissue barrier or to detach from other cells or ECM/BM.

1.4.2.1 Cancer invasion and metastasis

The complex process of tumour cell invasion and metastasis involves a cascade of events including the detachment of neoplastic cells from a primary tumour mass, dissolution of the BM/ECM, migration of the neoplastic cell into (intravasation) and then out (extravasation) of the capillary endothelial lumen and finally the establishment of a metastatic foci at a distant site (Blood and Zetter 1990). Angiogenesis also plays a key role in this process by providing new blood vessels to the primary tumour mass. These newly generated blood vessels provide nourishment to the expanding
metastatic foci and also serve as pathways for tumour cell entry into the circulation (Liotta and Stetler-Stevenson 1991). The ECM/BM is the major barrier to tumour cell invasion and is thus the target for any cell-surface proteolytic activity. This proteolytic activity breaks cell:cell and cell:ECM/BM attachments and facilitates cell migration not unlike other normal processes of cell migration such as angiogenesis and monocyte migration.

Strong evidence supports the involvement of various proteases in metastasis including metalloproteases, plasminogen activators, heparitinases, cathepsins and elastases (Blood and Zetter 1990). These proteases either directly attack the ECM or activate other proteases which in turn degrade ECM. For example, a proteolytic cascade involving both uPA/plm and collagenase IV is necessary for the invasion of basement membranes by metastatic cells (Reich et al 1988). Most proteases operate at very localised sites near the cell:ECM/BM boundary where the amount of active protease outbalances its inhibitor/s (Brown et al 1990). Although the ability of cancer cells to metastasise may be related to an increase in protease production/secretion it may also be related to a decrease in inhibitor concentration (Mignatti & Rifkin 1993).

Numerous studies have shown that various components of the PA system are increased during certain types of cancer. There is an increased presence of components of the PA system in lymph node metastasis (Burtin et al 1987) and on a range of large lung cancer cell lines (Pappot & Brunner 1995).
uPA and uPAR production is elevated in ovarian carcinoma cells (Moser et al 1994) and malignant ovarian tumours (Pujade-Lauraine et al 1993). uPA is produced by malignant squamous cell carcinoma but not by basal cell carcinoma (Sappino et al 1991). High levels of tPA are produced by melanoma cells (Quax et al 1991). Several studies have shown that uPA and uPAR are expressed at invading foci in colon (Pyke et al 1991) and lung cancer (Brodt et al 1992). uPA and tPA are also secreted by leukemic cells (Wilson et al 1983). Invasive prostate cancer cell lines have been shown to express 10-fold more uPA than their benign counterparts (Hollas et al 1992).

Various studies have indicated that uPA is important for ECM/BM degradation and invasion. Antibodies against uPA have been shown to inhibit or prevent metastasis of human carcinoma Hep3 in a chick embryo model (Ossowski & Reich 1983). Indirect evidence (Ossowski 1988) implicates uPA as playing an essential role in tumour cell intravasation. The metastatic potential of melanoma cells has been correlated with uPA secretion (Yu & Schultz 1990). uPA expression on the surface of melanoma cells is crucial for their ability to degrade fibronectin (Hearing et al 1988). uPA and tPA-catalysed PA are essential for in vitro invasion of human melanoma cells (Meissauer et al 1991). The presence of active uPA is essential for in vitro invasion of tumour cells through BM (Kobayashi et al 1993).

Experimental evidence also suggests an important role for uPAR and subsequently uPA binding to uPAR in cancer invasion and metastasis.
uPAR expression on osteosarcoma cells has been shown to facilitate matrix degradation and invasion (Kariko et al, 1991) via the binding and activation of uPA. Overexpression of uPAR in a human osteosarcoma cell line has been shown to increase ECM invasion (Kariko et al 1993). uPA binding to uPAR has also been shown to be vital for the in vitro metastasis of lewis lung carcinoma (Brodt et al 1992; Kobayashi et al 1994). Malignancy of human fibroblasts is also correlated with increased receptor-bound uPA activity (Jankun et al 1991).

Experimental evidence also points to a role for PAI-1 in tumour cell invasion and metastasis. PAI-1 apparently inhibits uPA-induced tissue degradation in murine Lewis Lung carcinoma while it is absent from areas where tissue destruction is evident (Kristensen et al 1990). This has generated the hypothesis that PAI-1 may protect the ECM in tumour tissue against uPA-induced dissolution (Pyke et al 1991). Several lines of evidence also indicate that cancer cells secrete and thus may utilise PAI-1 to protect themselves from uPA-mediated tissue degradation (Skriver et al 1984; Kristensen et al 1990; Sappino et al 1991). Additionally lung carcinomas with the worst clinical prognosis no longer express PAI-1 (Gris et al 1993) which may permit cancer spread.

Convincing clinical evidence for the involvement of PA in cancer is provided by studies showing that uPA and PAI-1 levels are independent and significant prognostic indicators of survival in breast cancer patients (Duffy et al 1990; Janicke et al 1991; Foekens et al 1992; Grondahl-Hansen et al 1993).
PAI-2 has also been shown to have some prognostic value in breast cancer (Bouchet et al 1994). PAI-1 and uPA have also been demonstrated to have prognostic significance in gastric (Nekarda et al 1994), colorectal (Ganesh et al 1994; Mulcahy et al 1994) and bladder cancer (Hasui et al 1994). Evidence also suggests that PAI-2 may be a marker for certain types of myelomocytic leukemia (Scherrer et al 1991; Wada et al 1993).

The main contribution of PA to metastasis is ECM degradation which enables cells to move, however, evidence is emerging that PA is important for releasing certain growth/angiogenic factors (e.g. bFGF and TGF-β1) from the ECM that contribute to the metastatic process. Thrombospondin (TSP) and TGFβ promote PAI-1 production in human lung carcinoma cells which facilitates tumor cell attachment (Albo et al 1994). Retraction of endothelial cells which is an important step in the extravasation of blood and metastatic cells has been shown to be due to PA (Conforti et al 1994). Oncogenes and growth factors associated with cancer have been shown to increase uPA/uPAR production or secretion. For example, ras-oncogene transformed malignant fibroblasts exhibit significantly higher levels of receptor-bound tPA than their untransformed counterparts (Jankun et al 1991). uPA gene expression has also been shown to be induced by the src oncogene product and tumour promoters (Bell et al 1990).

The logical mechanism of uPA-mediated metastasis is via the activation of plm which in turn degrades ECM/BM components or activates other protease classes. An alternate mechanism is via the direct action of uPA
which has been shown to directly hydrolyse fibronectin (Gold et al 1989) and activate collagenase IV (Reith et al 1992). Vitronectin-dependent carcinoma cell migration, mediated by integrin αvβ5, is also dependent on uPAR-bound uPA (Yebra et al 1996).

1.4.2.2 Angiogenesis

Angiogenesis is the formation of new blood vessels by the endothelium and is important in embryo and organ development, wound healing and tumour growth. It is a complex process which involves the degradation of the BM by endothelial cells; the migration of endothelial cells towards an angiogenic stimulus; infiltration of endothelial cells into the perivascular stroma; and proliferation of endothelial cells to form the new lumen (Ausprunk & Folkman 1977). Several protease systems have been implicated in angiogenesis particularly the PA and metalloproteases systems which play key roles in BM/ECM degradation and the invasive process of angiogenesis (Mignatti et al 1989).

Numerous studies have shown that endothelial cells can bind (Hajjar et al 1986; Miles et al 1988; Felez et al 1993) and activate plg via the presence of specific plg-Rs such as actin (Dudani et al 1996) and Annexin II (Hajjar et al 1994). Both uPA (Miles et al 1988) and tPA (Beebe 1987; Hajjar et al 1987; Cheng et al 1992; Felez et al 1993) can bind to endothelial cells. Migrating endothelial cells have been shown to upregulate both uPAR expression and uPA production via bFGF (Pepper et al 1993). This results in an increase in receptor-bound uPA which in turn increases PA.
Angiogenesis seems to be regulated by various cell:ECM/BM interactions in addition to anti/angiogenic factors. bFGF is one such angiogenic factor which has been shown to increase the production of uPA and uPAR by endothelial cells (Moscatelli et al 1986; Rifkin et al 1990; Mignatti et al 1991). Plm can also cause the release of bound bFGF from ECM via the proteolytic cleavage of proteoglycans (Rifkin et al 1990). Another potent angiogenic factor is vascular endothelial growth factor (VEGF) which increases uPAR-expression on endothelial cells (Mandriota et al 1995). TGFβ is an anti-angiogenic factor which has been shown to be a potent inhibitor of endothelial cell uPA production (Rifkin et al 1990). The activation of latent TGFβ to TGFβ requires plm (Lyons et al 1990; Rifkin et al 1990). The TGFβ thus formed causes an increase in PAI-1 in endothelial and smooth muscle cells which acts to dampen any further TGFβ activation via plm (Rifkin et al 1990; Sato et al 1990).

Recently, a 32kDa form of plg consisting of the internal 342 amino acid fragment (i.e. K1-3), has been found to act as an anti-angiogenic factor (O'Reilly et al 1994). This protein, called angiostatin, has been found to induce and sustain dormancy of human primary tumors in mice via apoptotic mechanisms (O'Reilly et al 1996).

1.4.2.3 Inflammation

Cells that are involved in the inflammatory response such as monocytes, macrophages and lymphocytes produce, secrete and/or utilise various components of the PA system in order to migrate to inflammatory sites. For
example, uPAR is polarised to the leading edge of migrating monocytes (Estreicher et al 1990) and is also present on lymphocytes (Miles et al 1987). Macrophages have been shown to secrete both uPA (Unkeless et al 1974) and PAI-2 (Wohlwend et al 1987) with uPA production being a marker of the activation state of inflammatory macrophages (Vassalli et al 1992). Additionally, uPAR in association with β2-integrins is required for leukocyte migration to inflamed areas (May et al 1998).

Various inflammatory mediators have been shown to modulate components of the PA system. PA is increased in the presence of IFNγ but decreased in the presence of M-CSF (Vassalli et al 1992). Peripheral blood monocytes produce uPA and tPA in response to LPS and IL-4 (Hart et al 1989). PMA, LPS, IL-1 and TNF induce PAI-2 in monocytic cells while anti-inflammatory agents such as dexamethasone and IL-4 downregulate PAI-2 (Kruithof et al 1995). Plg/plm receptors are decreased in response to dexamethasone (Pollanen et al 1989). IL-1 increases uPA synthesis in synovial fibroblasts (Hamilton et al 1991).

1.4.2.4 Bacterial Invasion

Numerous bacteria can bind and activate plg (refer Table 1.4) by virtue of the presence of plg-Rs, such as G3PDH, and it is the plm generated on the surface of these cells which has been hypothesised to promote penetration into host tissue (Sako & Tsuchida 1983; Parkkinen & Korhonen 1989; Korhonen et al 1993). Infact, plm generation on the surface of Borrelia burgdoferi enhances the penetration of endothelial monolayers (Coleman et al 1995). PA occurs
on the surface of Helicobacter pylori which has been proposed to be a mechanism for gastric tissue penetration (Pantzar et al 1998). Cell surface uPA has been found to be critical for the spread of Yersinia pestis from the original site of infection (Sodeinde et al 1992). The ability of both Streptococcus enterica (Lahteenmaki et al 1995) and Haemophilus influenzae (Virkola et al 1996) to penetrate a reconstituted BM is due to cell-surface plm. Pathogenic strains of group A streptococci, not only possess plg-Rs but can also secrete SK which generates plm (Wang et al 1994). A similar story holds for certain strains of Staphylococcus aureus which produce STA (Sako & Tsuchida 1983). Thus certain bacteria are equipped with both plg-Rs and plg activators which would appear to contribute to their virulence.

1.4.3 Activation of prohormones and growth factors

Evidence is emerging that plm can activate a variety of proteases and growth factors that are important for processes like tissue remodelling, inflammation and angiogenesis. Plm has been shown to directly or indirectly activate connective tissue metalloproteinases such as stromelysin, collagenase and gelatinase (Murphy et al 1992). Specifically, plm is responsible for activating proMMP-1, 3 and 9 (Nagase & Salvesen 1993). Several studies have shown that plm-dependent ECM degradation can actually release bound growth factors such as bFGF (Rifkin et al 1990; Falcone et al 1993 & 1994) and TGF-β (Taipale et al 1992; Falcone et al 1993 & 1994).
1.4.4 Tissue remodelling and development

The development, differentiation and remodelling of tissues consists of three steps that involve the direct input of the PA system - detachment of cells from the substratum (ECM/BM) and surrounding cells, cell migration and cell adhesion. From the earliest moments of life, plm-catalysed ECM degradation and cell adhesion occur. For example, uPA is temporally expressed in trophoblast cells during both the implantation and early growth of the embryo (Sappino et al 1989).

Cell detachment utilises the direct proteolytic input of plm which degrades various ECM components such as laminin (Moser et al 1993), fibronectin (Wang et al 1994), type IV collagen (Mackay et al 1990) and proteoglycans (Richardson et al 1988). Plm can also activate metalloproteases (He et al 1989; Mazzieri et al 1997) which in turn can promote ECM degradation. One of the key regulatory mechanisms of cell detachment is the localisation of various components of the PA system to sites where detachment is initiated: uPA is located at discrete cell:substratum and cell:cell contacts (Pollanen et al 1987); scuPA, tcuPA and uPAR are confined to focal adhesions and cell-cell contacts (Myohanen et al 1993); cell surface uPA co-localises with vinculin (Myohanen et al 1993; Hebert & Baker 1988) and is found in close proximity to αvβ3 integrin at focal adhesion sites (Myohanen et al 1993). In contrast is the broad distribution of plg throughout the ECM by virtue of its specific interaction with various ECM components (refer section 1.2.6.7.1). Additionally PAI-1 is distributed evenly over the cellular substratum (Pollanen et al 1987).
Evidence is accumulating that uPA/uPAR contributes to cell adhesion independent of plm generation. The adhesion and migration of monocytic cells requires both uPAR and inactive uPA (Nusrat & Chapman 1991; Gyetko et al 1994) which led some to hypothesise (Chapman 1997) that uPAR could couple to the cytoskeleton and mediate adhesion and migration. Vitronectin can bind with high affinity to uPAR (Hoyer-Hansen et al 1992) which is a logical explanation for the ability of uPAR to promote cell adhesion. Additionally the association of uPAR with leukocyte β2-integrins is required for leukocyte endothelial cell interactions and migration to inflamed areas (May et al 1998). Plg itself has been shown to be essential for the adhesion of leukemic cells to type I collagen (Martinez & Santibanez 1994).

There is evidence that PA also plays a role in normal epidermal differentiation. Studies have shown that PAI-2 is distributed throughout the epidermis (Kruithof et al 1995). Additionally, uPA and PAI-1 are associated with the least differentiated keratinocytes while tPA is found associated with more differentiated keratinocytes (Chen et al 1993).

1.4.5 Ovulation

Ovulation is the process whereby oocytes are released from a mature ovarian follicle via the proteolytic degradation of the follicular wall. This is a particularly complex and dynamic process that is preceded by the development and differentiation of the follicle. The collective process of follicular development, differentiation and ovulation involves numerous
extracellular proteolytic events including the generation of plm. Early studies indicated a positive correlation between ovulation and PA (Beers 1975; Beers et al 1975; Strickland & Beers 1976). Additionally, ovulation can be suppressed with $\alpha_2$-AP or $\alpha$-tPA antibodies (Tsafriri et al 1989).

Plg is found in follicular fluid at levels equivalent to that in serum (Beers 1975), with uPA, tPA and PAI-1 all found to be present in ovaries (Beers 1975; Liu et al 1991). tPA and PAI-1 appear to be the main regulators of ovarian PA with the levels of both being regulated by the same set of hormones that control ovulation (Ny et al 1993). tPA levels in granulosa cells and follicular fluid are increased sharply just prior to ovulation (Lui et al 1991; Peng et al 1993; Reich et al 1986) while a corresponding decrease in PAI-1 levels occur (Reich et al 1986). The secretion of tPA by granulosa cells is stimulated by the major ovulatory hormones follicle stimulating hormone and luteinizing hormone (Ny et al 1993).

Oocytes themselves are able to synthesize tPA (Huarte et al 1985; Liu et al 1986; Sappino et al 1989) with levels increasing dramatically during ovulation (Liu et al 1985; Sappino et al 1989; Bicsak et al 1989). The role of oocyte tPA is unknown but it has been speculated that plm could facilitate follicular disruption and prevent premature clot formation in the ovarian stigma (Huarte et al 1985). Additionally plm could prevent premature arrest of the preimplantation embryo by blocking egg adhesion to fibrin deposits (Liedholm & Astedt 1975; Huarte et al 1985; Sappino et al 1989). uPA synthesized by epithelial cells in the male genital binds to spermatozoa and
may then participate in the proteolytic events involved in capacitation and fertilisation (Huarthe et al 1987).

1.4.6 Cell death

Necrosis and apoptosis are the two mechanisms whereby cells die. Necrosis is a non-specific form of cell death characterised by a loss in plasma membrane integrity, cytomplasmic swelling and progressive damage to organelles (Wyllie et al 1980). In contrast, apoptosis is a genetically controlled form of cell death which contributes to development and tissue homeostasis (Wilson 1998) in addition to a variety of diseases including AIDS, cancer, alzheimer's disease, stroke (Whyte 1996) and rheumatoid arthritis (Hasunuma et al 1997). Apoptosis is a complex process involving a myriad of structural and biochemical changes ultimately leading to the dissolution and resorption of cells by the body. Apoptosis consists of three distinct stages: (1) induction which involves a cell receiving a death signal from a specific apoptotic inducer (e.g chemical, inflammatory mediator, uv light etc); (2) execution where a series of biochemical changes induced by the death signal progress to a point where the cell is committed to die - the 'point of no return'; (3) degradation where the cells are essentially autodigested and packaged ready for engulfment by macrophages.

Apoptosis is characterised by an ordered progression of morphological changes yet a heterogenous series of biochemical changes involving several pathways that utilise different genes and enzymes (Sen & D'Incalti 1992; Williams & Smith 1993). The biochemical events of the initiation phase are
determined by the type of apoptotic inducer and vary from one cell type to another. During the execution phase the heterogenous pathways of initiation converge into a homogenous death pathway that ultimately leads to the degradative phase. The major changes occurring during apoptosis include chromatin condensation (Wyllie et al 1980), decreased membrane phospholipid packing (Mower et al 1994), cell shrinkage (Mower et al 1994), plasma membrane blebbing, internucleosomal DNA fragmentation, and phosphatidylerine (PS) exposure, the latter being important for stimulating phagocytosis of apoptotic cells by macrophages thereby preventing inflammation (Savill et al 1993). Each of these characteristics can be measured using a variety of techniques, for example cell shrinkage and membrane blebbing can be measured microscopically or by flow cytometry, DNA fragmentation can be measured using agarose gel electrophoresis and PS exposure can be measured via the flow cytometric analysis of Annexin-V binding.

1.4.6.1 The role of proteases in apoptosis

Numerous proteases are involved in both the initiation and progression of apoptosis including caspases (e.g ICE, CPP32, Nedd-2/Ich-1L, TX ICErel II/Ich-2, ICErel III, Mch2), perforin, serine proteases (e.g granzymes, fragmentin-2, elastase) and calpains (Patel at al 1996). The targets of apoptotic proteases include both structural (e.g β-actin, laminin, histones, fodrin, non-laminin nuclear matrix proteins) and functional proteins [e.g poly (ADP-ribose) polymerase, 70kDa polypeptide of the U1 small nuclear ribonuclear protein, endonucleases, nucleolin, PITSLRε1 protein kinase, topoisomerase 1] (Patel
et al 1996). Therefore apoptotic proteases are responsible for activating other proteases/factors or digesting the cellular architecture which leads to the characteristic morphological changes. Apoptosis then must be considered as being regulated, to some extent, by proteases.

1.4.6.2 The role of serine proteases in apoptosis

A group of serine proteases called granzymes (granule-associated enzymes) are critical for induction of apoptosis in virally infected and malignant cells by cytotoxic T lymphocytes (CTL) and natural killer (NK) cells. The recognition and subsequent interaction of a CTL with the target cell causes the exocytosis of cytoplasmic granules which contain a 66-70kDa pore-forming protein called perforin (Smyth et al 1995). Perforin monomers insert into the target cell membrane and aggregate to form pores which facilitate the entry of granzymes into the target cell (Liu et al 1995). Granzymes thus synergise with perforin to induce target cell DNA fragmentation and chromatin condensation (Jans et al 1988).

Granzymes have been divided into three categories: (1) tryptases such as Granzyme A (GrA) and Tryp-2; (2) aspases such as Granzyme B (GrB), and (3) metases (elastase-like) such as Met-1 (Smyth et al 1996). The division of granzymes is based on both genetic information and their substrate specificities, with each group able to preferentially cleave one or more specific amino acid residues. Granzymes contain the same catalytic triad that is characteristic of all serine proteases – His$^{57}$, Asp$^{102}$ and Ser$^{195}$ (Smyth et al 1996). GrA or fragmentin-1 is the most abundant granzyme and cleaves
target proteins such as nucleolin (Pasternack et al 1991), IL-1β (Irmler et al 1995) and the thrombin receptor (Suidan et al 1994) at basic amino acid residues (Poe et al 1991). GrB (or fragmentin-2) cleaves target proteins at aspartic acid and glutamic acid residues (Poe et al 1991) and has been shown to participate in apoptosis via the activation of caspase-10. GrB also participates in a secondary apoptotic mechanism involving the activation of other caspases such as caspase 7 and 3 (Talanian et al 1997). Both GrA and B have been shown to accumulate in the nucleus via a novel nuclear import pathway that requires perforin (Jans et al 1998). The specificity of several granzymes (such as granzymes C to H) remains to be defined (Smyth et al 1996).

Increasing evidence suggests a prominent role for the serine protease thrombin in apoptotic cell death. Apoptotic smooth muscle cells were shown to generate significant levels of thrombin following PS exposure (Flynn et al 1997). This process could be inhibited with Annexin V suggesting that pro/thrombin binds to PS during serum starvation-induced apoptosis. Additionally, thrombin has been shown to decrease neutrite length, neutrite branching and induce motoneuron death via an apoptotic mechanism (Turgeon et al 1998).

1.4.6.3 The role of PA in cell death

Evidence is accumulating that components of the PA system are somehow involved in cell death processes. Some confusion exists since it has been
suggested that PA may contribute to the death process or play as yet undefined cytoprotective roles.

Independent studies have shown that plg binding is increased on dead epithelial cells (Green 1978), damaged amniotic epithelial cells (Burgos et al 1982; Jenkins et al 1983) necrotic breast cancer epithelial cells (Burtin et al 1985; Ranson et al 1998) and apoptotic/necrotic U937 cells (O'Mullane & Baker 1998). Equally, "knockout" mouse studies have suggested that PA plays a role in tPA-mediated neuronal cell degeneration (Tsirka et al 1997a & b) where plg synthesis increases during kainic acid-induced neurodegeneration. Here PA may contribute to neuronal death by disrupting cell:ECM interactions (Matsuoka et al 1998). In contrast, other studies have shown that plg may act as a neuroprotectant against nitric oxide-induced neuronal apoptosis (Toku et al 1998). Additionally, uPA mRNA is increased in granulosa cells undergoing apoptosis (Johnson et al 1997). Apoptosis of large dysplastic hepatocytes is associated with an increase in uPA (Santonirugiu et al 1996). Also, tPA is increased 50-fold in X-ray irradiated melanoma cells (Boothman et al 1991) and may play a role in inducible repair systems of mammalian cells (Boothman et al 1994). In contrast, apoptotic endothelial cells were shown to have greatly reduced levels of tPA, PAI-1, PAI-2 and uPAR, however uPA levels remained unchanged compared to their non-apoptotic counterparts (Zoellner et al 1998). Inflammatory mediators such as TNFα, that are known to induce apoptosis in a range of cell types, can also increase uPA mRNA (Simonitsch & Krupitza 1998) and this has led some to suggest that uPA may facilitate
anoikis (cell detachment) during TNFα-induced apoptosis (Simonitsch & Krupitza 1998). Thrombin activity can be generated by apoptotic vascular smooth muscle cells after phosphatidylserine exposure (Flynn et al 1997) which in turn could activate uPA and subsequently plg. Finally, overexpression of plg has been shown to be cytotoxic to mammalian cells (Busby et al 1991).

PAIs have been recently implicated in protection against some apoptotic stimuli. For example, a cleaved intracellular PAI-2 isoform was found to be a biochemical marker of apoptosis in the human promyelocytic leukemic cell line NB4 (Jensen et al 1994) and overexpression of intracellular PAI-2 resulted in protection against TNFα-induced apoptosis (Dickinson et al 1995), the cytopathic effects of alphavirus infection (Antalis et al 1988) and infection of human macrophages with Mycobacterium avium (Gan et al 1995). As uPA and tPA are established cognate serine protease targets of PAI-2 it is logical to assume that the cytoprotective ability of PAI-2 might have been due to the inhibition of either uPA or tPA activity, however, this has not been shown. Equally the ability of PAI-2 to act as a competitive substrate in these models has not been examined. Recent evidence however, has shown that PAI-2 can induce IFN-α/β and ISGF3 genes, which subsequently stimulate antiviral genes (Antalis et al 1998) and that the distribution of PAI-2 in hair and nail is appropriately positioned to protect epithelial cells from apoptosis (Lavker et al 1998).
1.5 Conclusion

PA is vital for a range of extracellular biological processes due to the broad substrate specificity of plm. It is subtle changes in the cell-surface or ECM concentrations of plg, plg activators, serpins, plm inhibitors, receptors/binding determinates and substrates that determines the role of PA at a given time.

Although the regulatory input of uPAR has been extensively studied during such processes as tumor cell invasion and metastasis, only fundamental studies on plg-Rs have been performed. This has probably been due to the fact that plg-R candidates are a heterogenous group of molecules that seem to be restricted to specific cell types. It is imperative to determine the contribution of plg-Rs to biological processes such as cell migration since they are a critical regulatory component of PA.

The list of roles for PA is growing with recent evidence suggesting a role for PA in cell death playing either a cytodestructive or cytoprotective role. There is clearly a need to examine the contribution of plg-Rs and hence PA to cell death.

1.6 Thesis Objectives

This thesis examines the binding of native human plg to viable and non-viable subpopulations of cancer cells with an emphasis on the role of plg binding and activation during apoptosis. A major component of this thesis is the development of novel flow cytometric assays for measuring plg
binding (i.e plg-R expression) and PA (i.e plm generation) on specific subpopulations of cells (i.e viable, apoptotic and necrotic cells). The following chapter is a preliminary investigation into both plg-R expression on several cancer cell lines utilising $^{125}$I-plg binding assays, and the isolation of several plg-R candidates from HCT116 colon carcinoma cells. Chapter 3 describes the development of a flow cytometric plg binding assay and the characterisation of plg binding to viable and non-viable subpopulations of cells. Chapter 4 utilises this assay to investigate plg-R expression on apoptotic cells. Chapter 5 details the development of a flow cytometric PA assay and measures PA on viable, apoptotic and necrotic cells. Finally, Chapter 6 chronicles plg-R expression during apoptosis and the influence of plg on the entry of cells into apoptosis.
Chapter 2

Preliminary Plasminogen Binding and Receptor Isolation Studies
2.1 INTRODUCTION

Cellular plg binding is a dynamic process with numerous studies suggesting that it is specific, lysine-dependent, reversible, concentration dependent and saturable. Another important characteristic is its low affinity with reported $K_d$ ranging from 0.024-200nM on prokaryotes (refer Table 1.4) and 0.0158-5μM on eukaryotes (refer Table 1.5). Although a specific receptor for uPA has been identified (Cubellis et al 1986) that facilitates uPA binding to a variety of cell types (refer Table 1.1), plg-Rs appear to be a heterogenous group of molecules that are typically proteins with C-terminal lysine residues. Although plg binding is a widely observed phenomenon, most proteinacious plg-R candidates appear to be restricted to specific cell types (refer Table 1.3). The contribution of cell surface carbohydrates or lipids to plg binding has been poorly studied which is puzzling considering that some non-proteinacious molecules, such as gangliosides, have been reported to function both as plg and uPA binding sites on U937 cells (Miles et al 1989).

Plg binding has been historically studied using radioligand binding assays. Typically plg is labelled with $^{125}$I, incubated with cells and the amount of radioactivity then quantified using a scintillation counter. The ability of lysine analogues such as TA and $\varepsilon$-ACA to inhibit plg binding has formed an integral component of these assays as they enable the measurement of non-specific (i.e non lysine-independent) binding. Although non-specific binding can also be measured using a large (i.e 50-100-fold) molar excess of unlabelled plg all
researchers have appeared to favour the use of lysine analogues. Besides the handling and disposal of radioactive material, one of the major disadvantages of using $^{125}$I-plg binding assays is that they do not enable the measurement of plg binding on specific subpopulations of cells (e.g. cells in different stages of the cell cycle). Thus if plg binding varies between subpopulation of cells within the total population these differences will be undetectable. An additional problem is the need to wash away unbound $^{125}$I-plg in order to measure cell-associated radioactivity. This action affectively reduces the free concentration of ligand (i.e. plg) and since plg binding is a concentration-dependent and reversible interaction, cell-bound plg will dissociate in order to re-establish equilibrium binding (i.e. where the concentration of free plg is in equilibrium with bound plg).

The identification of the cell-surface molecules responsible for plg binding has predominantly focussed on proteins with little credence given to the input of cell-surface lipids or carbohydrates. All of the proteinacious plg-R candidates identified to date have been isolated using plg-affinity chromatography. Typically, plg is coupled to CNBr-activated Sepharose 4B and a sample likely to contain plg-Rs (e.g. solubilised whole cell lysates or plasma membrane preparations) is adsorbed. Plg-Rs are then eluted using high concentrations of lysine or ε-ACA, analysed by SDS-PAGE and then usually microsequenced. One criticism that can be levelled at most of the work on proteinacious plg-Rs
is that they have been isolated from whole cell lysates rather than plasma membrane preparations.

Although there is ample evidence to suggest that uPA/uPAR plays a role in tumour cell invasion and metastasis (refer section 1.4.2.1) no studies have examined the role of plg-Rs in the invasive phenotype. Several plg-R candidates have been identified on cancer cells including cytokeratin 8 on hepatocellular carcinoma and breast cancer cells (Hembrough et al 1995), α-enolase on breast cancer cells (Lopez-Alemany et al 1994) and a 50-60kDa protein on rat colon carcinoma cells (Durliat et al 1992). Additionally plg has been found to bind to leukemic cells (Plow et al 1986; Felez et al 1990; Edelberg et al 1990; Gonzalez-Gronow et al 1991; Felez et al 1993; Santibanez et al 1994), mammary carcinoma cells (Correc et 1990; Burtin et al 1993), fibrosarcoma cells (Pollanen 1989; Felez et al 1993), osteosarcoma cells (Campbell et al 1994) and colon adenocarcinoma cells (Burtin & Fondaneche 1988; Comacho et al 1989).

The aim of this chapter was to: (1) measure plg binding on several cancer cell lines using 125I-plg binding assays; and (2) isolate plg-Rs on a cell line selected from (1)
2.2 MATERIALS AND METHODS

2.2.1 Materials

All cell culture reagents and plasticware were purchased from Trace BioSciences (Sydney, Australia) except for FCS, which was purchased from CSL (Melbourne, Australia). Human glu-plg was purified according to method of Grant (1990) or was generously provided by American Diagnostica (ADI) (Greenwich CT, USA). $^{125}$I was purchased from Amersham (Sydney, Australia) and Iodo-beads were purchased from Pierce (Rockford, IL, USA). Prepacked NAP10 columns (Sephadex G25), Lysine Sepharose 4B, CNBR-activated Sepharose, prepacked Superdex™ 75 HR 10/30 column, prepacked NHS-activated Superose HR 10/2 and low molecular weight size exclusion standards were purchased from Amrad Pharmacia Biotech (Sydney, Australia). SDS-PAGE standards, precast polyacrylamide gels, silver staining kit and DC protein assay were purchased from Bio-Rad (Sydney, Australia). 0.2\(\mu\)M and 0.45\(\mu\)M syringe filters were purchased from Sartorius (Sydney, Australia). Unless indicated, all chemicals were purchased from Sigma (Sydney, Australia).

2.2.2 Cell lines and cell culture

The human breast cancer cell lines MCF-7 and MDA-MB-231 were obtained from Dr R. Sutherland (Garvan Institute, Sydney Australia). The human colon cancer cell line HCT116 was provided by Dr M Baker (Dept. Biological Sciences, University of Wollongong, Australia). The human monocytic leukemic cell
line U937 was obtained from the American Type Culture Collection. All cell lines were cultured in RPMI1640/10% FCS at 37°C in a humidified atmosphere of 5% CO₂. Adherent cells were passaged using 0.05% (w/v) trypsin/5mM EDTA/phosphate buffered saline (PBS) to lift them into suspension.

2.2.3 Radio-iodination of plasminogen

Plg was iodinated using Iodo-beads™ according to the manufacturer’s instructions (Pierce, Rockford IL, USA). Just prior to use, three beads were washed twice with 3ml 0.1M phosphate buffer (pH 7.2). 10μl ¹²⁵I (1mCi) was added to 0.5ml 0.1M phosphate buffer (pH 7.2) in an eppendorf tube and allowed to react for 5min/RT. Plg (800µg) in 500μl 0.1M phosphate buffer (pH 7.2) was added, mixed gently and allowed to react for 15min/RT. To separate ¹²⁵I-plg from free ¹²⁵I a prepacked column of Sephadex G-25 was equilibrated with phosphate buffered saline (PBS) (pH 7.4) and the 1ml reaction sample loaded. 12 x 0.5ml fractions were collected and the radioactivity in a 2μl sample from each fraction measured using a γ-counter (Cobra Auto Gamma, Canberra Packard, Canberra, Australia). Those fractions containing the first peak of radioactivity were pooled, aliquoted and stored at -20°C.

2.2.4 Preparation of Lysine Sepharose 4B

Lysine Sepharose 4B was prepared according to the manufacturer’s instructions (Amrad Pharmacia Biotech, Sydney, Australia) by swelling the powder in PBS
overnight at 4°C. Lysine Sepharose 4B was stored in PBS containing 0.1% (w/v) NaN₃ at 4°C until required.

2.2.5 Depletion of plg from FCS

FCS was heat inactivated at 56°C for 30min, cooled then filtered through a 0.45μM syringe filter to remove any particulate matter. FCS (500ml) was circulated overnight through a 50ml Lysine Sepharose column that had been equilibrated with PBS. All FCS was collected and filter sterilised through a 0.2μM syringe filter aliquoted and stored at -20°C.

2.2.6 ¹²⁵I-plg binding assays

Cells were cultured in RPMI1640/10% (v/v) plg-depleted FCS for 24h. Adherent cells were detached using a solution of 5mM EDTA in Hank's Balanced Salt Solution (HBSS) (pH 7.4) (Ca/Mg-free) and washed thrice with HBSS at 37°C. Cells were incubated for 1h on ice with ¹²⁵I-plg in HBSS containing 0.1% BSA, 1mM CaCl₂ and 1mM MgCl₂. Cells were washed thrice with HBSS at 4°C and cell-associated radioactivity measured using a γ-counter (Cobra Auto Gamma, Canberra Packard, Canberra, Australia). Lysine-dependent (specific) plg binding was calculated as the difference in the radioactivity of cells incubated solely with ¹²⁵I-plg (total binding) and cells incubated with ¹²⁵I-plg and 10mM TA (non-specific binding).
2.2.7 Viable Cell counts

A 100μl aliquot of cells that had been washed once with HBSS was mixed with 100μl 0.4% (w/v) Trypan blue and a small amount loaded onto a haemocytometer. Viable cells (those excluding Trypan blue) were counted in the four 1mm corner squares. The number of viable cells/ml = average count per four corner squares x 2 (dilution factor) x 10⁴.

2.2.8 Plasma membrane isolation

Plasma membranes were isolated, using an established two phase polymer system consisting of PEG and dextran, which was modified from the methods of Klockman and Deppert (1983), and Rana and Majumber (1987). The two-phase polymer system was prepared as follows: Dextran (Mₚ = 266,000) and PEG (Mₚ = 20,000) were dissolved in 0.1M phosphate buffer (pH 6.5) at concentrations of 5.5% (w/v) and 4.2% (w/v) respectively. Both phases were allowed to separate overnight at 4°C in a separating funnel, collected and stored at 4°C until required.

Adherent cells were scraped off flasks into HBSS using a cell scraper. All cells were washed thrice with HBSS and resuspended in ice-cold 5mM Tris-HCl (pH 7.4) containing 5mM EDTA and 1mM PMSF. Cells were allowed to swell for 15min on ice then disrupted using a dounce homogeniser. The degree of cell breakage was monitored by light microscopy every 5 strokes, until over 80% of the cells had lysed. Cells were centrifuged at 5000rpm for 10min in corex tubes.
using a Sorvall HB-4 swing-out rotor. The pellet was resuspended in 10ml top phase (PEG) and mixed thoroughly with 10ml bottom phase (dextran) then centrifuged at 8000rpm for 10min at 4°C using a Sorvall HB-4 swing-out rotor. Plasma membranes were aspirated from the interface of the two phases, transferred to a clean corex tube, and the remainder of the two phases added to the same tube. The sample was centrifuged at 8000rpm for 10min at 4°C, the plasma membranes aspirated off and diluted in 4 volumes of MilliQ H2O. Plasma membranes were pelleted at 8000rpm for 10min at 4°C and resuspended in 1ml of solubilisation buffer [PBS (pH 7.4), 1mM CaCl2 and 1mM MgCl2, 1% v/v Triton-X-100, 1% (w/v) sodium deoxycholate, 1mM PMSF, 5mM EDTA]. Plasma membranes were solubilised overnight at 4°C by end-over-end mixing. Unsolubilised material was removed by ultracentrifugation at 100,000g for 1h at 4°C. The supernatant was collected and stored at -80°C until required.

2.2.9 Coupling plg to CNBr'-activated Sepharose

CNBr'-activated Sepharose 4B was pretreated according to the manufacturer’s instructions (Amrad Pharmacia Biotech, Melbourne, Australia). Briefly, 2g dry gel was pre-swollen in 20ml 1mM HCl then washed on a glass sintered filter with 50ml ice-cold 1mM HCl. Glu-plg (10mg) was reconstituted in 10ml coupling buffer (0.1M NaHCO3/0.5M NaCl pH 8.3), the washed gel added and mixed end-over-end overnight at 4°C. The plg-Sepharose was washed with 5 volumes of coupling buffer, resuspended in 10ml blocking buffer (0.1M Tris-HCl pH 8) and mixed end-over-end overnight at 4°C. The gel was washed
alternatively thrice with 0.1M sodium acetate/0.5M NaCl (pH 4) and 0.1M Tris-HCl/0.5M NaCl (pH 8). Plg-Sepharose was stored in PBS/0.1% NaN₃ at 4°C.

2.2.10 Coupling plg to NHS-activated Superose HR 10/2

Plg was coupled to pre-packed NHS activated Superose HR 10/2 according to the manufacturer’s instructions by FPLC (Amrad Pharmacia Biotech, Melbourne, Australia). The column was washed with 10 column volumes of 1mM HCl at a flow rate of 1ml/min. Glu-plg (10mg) was reconstituted in 10ml coupling buffer (0.2M NaHCO₃/0.5M NaCl pH 8.3) and circulated through the column for 1h at a flow rate of 1ml/min. The column was washed alternatively with five column volumes of 0.1M Tris-HCl (pH 8)/0.5M NaCl followed by ten column volumes of 0.1M sodium acetate (pH 4). This was repeated once and the column allowed to stand for 1h in 0.1M Tris-HCl (pH 8)/0.5M NaCl. The wash procedure was then repeated twice and then the column equilibrated with Buffer A [PBS, 1mM CaCl₂, 1mM MgCl₂, 1mM PMSF, 0.2% Triton-X-100 (reduced)] overnight at a flow rate of 0.5ml/min. The column was stored in 0.1% NaN₃ (v/v) in Buffer A.

2.2.11 Plg affinity chromatography

Batch elution: Plg-Sepharose was washed extensively with PBS to remove the storage solution and equilibrated with Buffer A overnight at 4°C by end-over-end mixing. Plasma membrane preparations were incubated with plg-Sepharose overnight at 4°C with gentle agitation. Plg-Sepharose was washed
extensively with buffer A and plg-binding proteins eluted with Buffer B (Buffer A containing 0.2M TA) at 4°C with gentle agitation. Samples containing plg-Rs were concentrated and desalted using a microconcentrator with a 10K Mr cut-off.

**FPLC (fast protein liquid chromatography)** Plg-Sepharose was packed into an FPLC column (HR 5/2, Amrad Pharmacia Biotech, Melbourne, Australia) and equilibrated with Buffer A overnight at 4°C at a flow rate of 0.5ml/min. Alternatively plg-Superose was equilibrated with Buffer A overnight at a flow rate of 0.5ml/min. Plasma membrane preparations were loaded onto the column via a 15ml or 1ml sample loop, depending on the volume, at a flow rate of 0.5ml/min. Plg-Rs were eluted from plg-Sepharose or plg-Superose using a 0-0.2M TA gradient according to the following program:

<table>
<thead>
<tr>
<th>Time (s)</th>
<th>Concentration %B</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>0.00</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>0.00</td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td>0.00</td>
<td>1.2</td>
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</tr>
<tr>
<td>0.00</td>
<td>5.7</td>
<td></td>
</tr>
<tr>
<td>0.00</td>
<td>6.7</td>
<td></td>
</tr>
<tr>
<td>30.00</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>30.00</td>
<td>6.1</td>
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</tr>
<tr>
<td>30.00</td>
<td>1.1</td>
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</tr>
<tr>
<td>60.00</td>
<td>30.0</td>
<td></td>
</tr>
<tr>
<td>60.00</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>
0.5ml fractions were collected and those containing protein were concentrated using microconcentrator with a 10K Mr cut-off.

2.2.12 Size exclusion chromatography

Size exclusion chromatography was performed by FPLC using a pre-packed Superdex™ 75 HR 10/30 column which has a fractionation range of 3-70kDa. The column was equilibrated with PBS at a flow rate of 0.5ml/min and calibrated with low molecular weight standards consisting of ribonuclease A (13.7kDa), chymotrypsinogen A (25kDa), ovalbumin (42kDa), bovine serum albumin (67kDa) and blue dextran (2000kDa). The void volume of the column was determined using blue dextran 2000. The $K_v$ for each standard was calculated as follows: $K_v = (V_e - V_o)/(V_t - V_o)$ where $V_o$ = void volume of the column, $V_e$ = elution volume of the protein and $V_t$ = the total bed volume. The following standard curve was constructed and used to determine the molecular weight of any proteins:
2.2.13 SDS-PAGE

All reagents and gel preparation for SDS-PAGE mini-gels were based on the method of Laemmli (1970). Samples were mixed with an equal volume of 2x non-reducing sample buffer [Tris-HCl (pH 6.8), 20% (v/v) glycerol, 4% (w/v) SDS, and 0.005% (w/v) bromophenol blue] and electrophoresed on either 4-20% or 10% precast polyacrylamide gels (Bio-Rad, Sydney, Australia) using a Bio-Rad protein apparatus. Broad-range molecular weight standards consisting of myosin (200kDa), β-galactosidase (116.5kDa), phosphorylase B (97.4kDa), bovine serum albumin (66.2), ovalbumin (45kDa), carbonic anhydrase (31kDa), soybean trypsin inhibitor (21.5kDa), lysozyme (14.4) and aprotinin (6kDa) were included for comparison. Samples were electrophoresed for 3h at 100V (20-40mAmp).
2.2.14 Silver staining

Protein bands were visualised by silver staining using a commercially available kit according to the manufacturer's instructions (BioRad, Sydney, Australia).

2.2.15 Ligand blotting

Following SDS-PAGE, proteins were transferred to polyvinylidene difluoride (PVDF) membrane, at 15V for 20min, using a semi-dry transfer system (BioRad, Sydney, Australia). PVDF had been pre-equilibrated with transfer buffer [48mM Tris-base, 39mM glycine, 0.037% (w/v) SDS and 20% (v/v) methanol] for 15min prior to use. The semi-dry transfer cell was configured as follows:

```
-ve

5x whatman filter paper

gel

PVDF

5x whatman filter paper

+ve
```

PVDF was blocked overnight in blotto then incubated with 1.5µg/ml biotinylated-plg (kindly provided by Nicholas Andronicus, Dept of Biological Sciences, University of Wollongong) in PBS containing 0.1% tween-20 (PBS-T) for 90min/RT. PVDF was washed thrice for 10min with PBS-T then incubated with streptavidin:horseradish peroxidase (1µg/ml) in PBS-T for 1h/RT. PVDF was washed thrice with PBS-T then once with PBS and then developed with 0.06% (w/v) 4-chloro-1-naphthol in 0.1M Tris-HCl (pH 7.4), 20% (v/v)
methanol, 0.03% (v/v) H₂O₂ for 30min/RT. The blot was washed extensively with water then air dried.

2.2.16 Autoradiography
Following electrophoresis, gels were fixed in methanol:acetic acid:H₂O (4:1:5) for 1h/RT. Gels were dried using a gel drier (BioRad, Sydney, Australia) and exposed to X-ray film (Amersham, Sydney, Australia) overnight at -80°C. Film was developed with GBX developer (Kodak, Sydney, Australia) for 2min/RT, fixed with GBX fixer (Kodak, Sydney, Australia) for 2min/RT.

2.2.17 Molecular weight determination
Gels and autoradiograms were scanned using an imaging densitometer Model GS170 (Bio-Rad, Sydney, Australia). The molecular weights of any bands were determined by comparison with molecular weight standards using Molecular Analyst software (Bio-Rad, Sydney, Australia).

2.2.18 Protein assay
Protein concentrations were measured using a detergent compatible microplate protein assay according to the manufacturer's instructions (Bio-Rad, Sydney, Australia). This assay is based on the method of Lowry et al (1951).
2.2.19 Photography

Cells were photographed using a MPS45 photoautomat (Wild Heerbrugg, Switzerland) and 100ASA film (Kodak, Sydney Australia) attached to an inverted light microscope (Leitz, Sydney, Australia).

2.2.20 Data analysis

All experimental measurements were performed in triplicate. Unless indicated, all results are expressed as means ± 1 SEM.

2.3 RESULTS

2.3.1 Labelling of plg with $^{125}$I

In the current study, Iodobeads™ were used to iodinate glu-plg to a specific activity of $0.41 ± 0.0807 \mu \text{Ci/\mu g}$ (N=11) which is comparable with a previous study (Gonias et al, 1989). As shown in Fig 2.3.1A, $^{25}$I-plg was eluted as the first radioactive peak from a Sephadex G25 column and unconjugated $^{125}$I was eluted in the second peak. Autoradiography confirmed that the iodinated material had an apparent molecular weight consistent with that of plg (Fig 2.3.1B).

2.3.2 Morphological alterations to cells cultured in plg-depleted media

A previous study had reported that plg from different species could bind to the same cell types (Durliat et al 1991). It is logical to assume then that cells cultured in FCS would have a proportion of their plg-Rs occupied by bovine
plg. To eliminate the potential problem of bovine plg competing with human plg during binding assays, all cell lines were cultured in plg-free media. Preliminary observations indicated that the adhesion of the breast carcinoma cell line MCF-7 was altered when they were cultured in plg-free media however this phenomenon was not evident on other adherent cell lines such as HCT116 and MDA-MB-231 cells.

To investigate the hypothesis that plg is important for cell adhesion, MCF-7 cells were cultured in plg-free media ± 2μM human glu-plg, or standard media containing FCS. 18h after passaging control cells were obviously well-adhered with individual cobblestone-like cells clearly evident (Fig 2.3.2). In contrast, cells cultured in plg-free media were poorly adhered with clumps of cells appearing rounded with the perimeter of individual cells unclear. Cells cultured in the presence of human plg exhibited a similar morphology as control cells (Fig 2.3.2). At 36h after passaging, there was a marked increase in the adhesion of cells cultured in plg-free conditions (Fig 2.3.2), however some clumps of poorly adhering cells were still evident. By 72h after passaging the level of adhesion of all cells was virtually the same. Collectively these results suggest that plg plays a non-essential role in the initial adhesion of MCF-7 cells to plastic tissue culture flasks.
Fig 2.3.1: Iodination of human glu-plg. Human glu-plg was labelled with 125I using Iodobeads according to the manufacturers instructions (Pierce). A. Elution profile of 125I-plg from a Sephadex G25 column. B. Confirmation of the identity of 125I-plg by autoradiography.
Fig 2.3.2: Influence of plg on the adhesion of MCF-7 cells. MCF-7 cells were passaged into media containing either 10% (v/v) FCS (control), 10% (v/v) plg-free FCS or 10% (v/v) plg-free FCS + 2μM human glu-plg. At 18, 36 and 72h the relative adhesion of cells was microscopically examined and photographed.
2.3.3 $^{125}$I-plg binding studies

The presence of plg-Rs (i.e specific plg binding) was analysed on the colon carcinoma cell line HCT116, the monocytic leukemic cell line U937 and the breast carcinoma cell lines MCF-7 and MDA-MB-231. Specific $^{125}$I-plg binding was detected on HCT116, MDA-MB-231 and U937 cells, but was absent on MCF-7 cells (Fig 2.3.3A). HCT116 cells exhibited the highest level of specific plg binding relative to the other cell lines and was subsequently chosen as the candidate from which to isolate proteinacious plg-Rs. An obvious problem with the binding data was the high level of interassay variability (Fig 2.3.3B). For example, specific plg binding was detected on MDA-MB-231 in only 50% of experiments. This variability suggests that $^{125}$I-plg binding assays may not be the ideal method for analysing cellular plg binding on certain cell lines.

2.3.4 Isolation of plg-Rs from HCT116 cells

Plasma membranes from HCT116 cells were isolated, solubilised then batch adsorbed to plg-Sepharose or Superose. Plg binding proteins were eluted with 200mM TA and then analysed by SDS-PAGE. Following silver staining, three major proteins with molecular weights of >250, 50 and 42kDa (Fig 2.3.4A) were evident. When these ‘plg-Rs’ were transferred to PVDF they could not be detected by ligand blotting (Fig 2.3.4B, lane 3) indicating that they had lost their plg binding.
A.

Fig 2.33: Comparison of plg binding on a variety of cancer cell lines. HCT116, U937, MCF-7 and MDA-MB-231 cells were cultured in plg-free media for 48h then analysed for plg binding (i.e plg-R expression) using a 125I-plg binding assay. A. Comparison of 125I-plg binding to each cell line showing total binding (cells incubated solely with 125I-plg), non-specific binding (cells incubated with 125I-plg and 10mM TA) and specific binding (the difference between total and non-specific binding). Each bar represents the mean of triplicate determinations ± 1SEM. The absence of error bars indicates that the error was smaller than the graphical symbol. B. Interassay variability in the detection of specific plg binding to each cell line.

B.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>No of experiments</th>
<th>No. of times specific plg binding detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT116</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>MCF-7</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>U937</td>
<td>3</td>
<td>1</td>
</tr>
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</table>

Fig 2.33: Comparison of plg binding on a variety of cancer cell lines. HCT116, U937, MCF-7 and MDA-MB-231 cells were cultured in plg-free media for 48h then analysed for plg binding (i.e plg-R expression) using a 125I-plg binding assay. A. Comparison of 125I-plg binding to each cell line showing total binding (cells incubated solely with 125I-plg), non-specific binding (cells incubated with 125I-plg and 10mM TA) and specific binding (the difference between total and non-specific binding). Each bar represents the mean of triplicate determinations ± 1SEM. The absence of error bars indicates that the error was smaller than the graphical symbol. B. Interassay variability in the detection of specific plg binding to each cell line.
Fig 2.3.4: Isolation of plg-Rs by plg affinity chromatography and ligand blotting. Plasma membranes from HCT116 cells were isolated, solubilised then batch adsorbed to plg-Sepharose. Plg binding proteins were batch eluted with 200mM TA and then analysed by SDS-PAGE and silver staining. These proteins were subsequently analysed for plg binding ability using ligand blotting. A. Silver stained 4-20% polyacrylamide gel showing plg-Rs batch eluted from plg-Sepharose. (lane 1 = HCT116 plasma membrane preparation; lane 2 = batch eluted plg-Rs; lane 3 = broad range SDS-PAGE standards. B. Ligand blotting of HCT116 plasma membranes and batch eluted plg-Rs (lane 1 = biotinylated standards; lane 2 = HCT116 plasma membrane preparation; lane 3 = batch eluted plg-Rs).
ability. In contrast, ligand blotting of solubilised HCT116 plasma membranes revealed proteins of 250kDa and a doublet band at approximately 42kDa that possessed plg binding ability (Fig 2.3.4B, lane 2). These results indicate that several plg-binding species are present in the plasma membranes of HCT116s but they lose their plg-binding ability following isolation.

In a further attempt to separate the three plg binding proteins, HCT116 plasma membranes were adsorbed to plg-Superose or Sepharose then eluted with a 0-200mM TA gradient. A typical elution profile is shown in Fig 2.3.5A with proteins eluted at 2, 16 and 22mM TA. Several protein bands were evident in each of the three fractions following SDS-PAGE and silver staining (Fig 2.3.5B) suggesting that the three proteins could not be adequately separated by FPLC. Peak 1 contained a single major protein band of 42.8kDa, peak 2 contained two proteins of 48 and 43.7kDa and peak 3 contained a protein of 38.4kDa. In all experiments overstaining of gels was necessary to visualise protein bands indicating that these proteins were present in very low concentrations. The >250kDa protein that was detected by ligand blotting was never observed following FPLC. Plg-Rs isolated by FPLC, like batch elution, could not be visualised by ligand blotting which indicated that they had either lost their plg binding ability, or were present in extremely low concentrations. Several large scale purifications were attempted, however the yield of plg-Rs was not improved. Additionally, the efficiency of plg affinity media appeared to rapidly decline with use so generally it could be used but once.
Fig 2.3.5: Isolation of plg-Rs by FPLC. Plasma membranes from HCT116 cells were isolated, solubilised then adsorbed to plg-Sepharose. Plg-Rs were eluted with a 0-200mM TA gradient using FPLC then analysed by SDS-PAGE and silver staining. A typical elution profile is shown and displays the presence of three protein peaks. INSET: Silver stained 4-20% polyacrylamide gel showing the presence of plg-Rs in each of the three fractions (lane 1 = fraction 1; lane 2 = fraction 2; lane 3 = fraction 3).
In a further attempt to isolate individual plg binding proteins, plg-Rs were firstly isolated using the batch plg-affinity technique and then fractionated by size exclusion chromatography using a Sephadex G75 column. Two protein peaks were detected possessing molecular weights of >100kDa and 73.45kDa (Fig 2.3.6A). Because the amount of protein in each fraction was very low, each fraction was iodinated with $^{125}$I, electrophoresed and then visualised by autoradiography. Numerous iodinated protein bands were evident in both fractions including major proteins of >205kDa and 80kDa (Fig 2.3.6B) and minor ones of 50 and 40kDa. Collectively these results indicate that several plg binding proteins are present in plasma membrane preparations from HCT116 cells but they appear to be difficult to isolate possibly due to their similar affinities for plg. Additionally these plg-Rs appear to lose their plg binding ability once isolated.

2.4 DISCUSSION

These preliminary studies were targeted at analysing plg-R expression (i.e plg binding) on several different types of cancer cells. An unexpected observation was that the absence of plg appeared to reduce the rate of adhesion of the breast carcinoma cell line MCF-7 to tissue culture flasks (Fig 2.3.2). This suggested that plg plays a role during the adhesion of MCF-7 cells and is supported by a previous study showing that murine leukemic cells require plg for adhesion to collagen type I (Martinez & Santibanez 1994). Several other components of the PA system including uPA and uPAR are located at focal contacts and cell:cell
Fig 2.3.6: Isolation of plg-Rs by plg affinity and size exclusion chromatography. Plg-Rs were initially isolated from HCT116 plasma membranes using the batch affinity technique and then further fractionated by size exclusion chromatography using a Sephadex G75 column. Protein were labeled with 125I using Iodobeads (Pierce) and visualised by autoradiography. A. Elution profile of plg-Rs from a Sephadex G75 column. B. Autoradiogram of 125I-labeled plg-Rs eluted in fraction 1 and 2 from the G75 column [lane 1 = fraction 1, reducing conditions; lane 2 = fraction 1, non-reducing conditions; lane 3 = fraction 2, non-reducing conditions; lane 4 = fraction 2 reducing conditions].
contacts (Pollanen et al 1987; Hebert & Baker 1988; Mayohanen et al 1993; Kanse et al 1996; Chapman 1997) where they are likely to contribute to processes of cell adhesion and migration. Other evidence also indicates that plg-R expression is increased during β1-integrin-dependent cell adhesion (Kim et al 1996). Whatever the role of plg during cell adhesion it appears to be cell-specific as the attachment of HCT116 and MDA-MB-231 cells to tissue culture flasks was unaffected by the absence of plg (Fig 2.3.2). Additionally, the mechanism of plg-dependent adhesion does not appear to require specific plg binding sites as no plg-Rs were detected on MCF-7 cells (Fig 2.3.3).

Nearly all studies of cellular plg binding have utilised 125I-plg binding assays. In the current study, considerable variability was evident in the detection of specific (i.e lysine-dependent) plg-binding on U937, MDA-MB-231 and HCT116 cells (Fig 2.3.3B). For example, in only 50% of experiments specific plg binding was detected on MDA-MB-231 and U937 cells. Additionally specific plg binding could not be detected on MCF-7 cells (Fig 2.3.3A) which contrasts with previous studies on the same cell line (Correc et al 1990; Burtin et al 1993). Numerous 125I-plg binding studies have been performed on U937 cells (Plow et al 1986; Felez et al 1990; Edelberg et al 1990; Gonzalez-Gronow et al 1991; Felez et al 1993) however, no comment was made regarding any variability in the data. One possible explanation is that plg-R expression varies between different populations of cells due to factors linked to the cell cycle. An alternative scenario is that cells could sustain damage to their plasma membranes during
experimental manipulation which alters the number of plg-Rs (i.e. plg-R expression is linked to cell viability). Regardless of the cause it is clearly evident that in their current format $^{125}$I-plg binding assays are not a satisfactory means of measuring plg binding and alternative methods should be developed.

Despite the aforementioned limitation with $^{125}$I-plg binding assays, repeated attempts were made to isolate proteinaceous plg-Rs from HCT116 cells as they appeared to specifically bind the highest level of plg compared to U937, MDA-MB-231 and MCF-7 cells (Fig 2.3.3). Several plg-binding proteins were isolated from HCT116 plasma membrane preparations using plg-affinity chromatography, however the molecular weight species isolated varied depending on the purification technique used. For example, >250, 50 and 42kDa proteins were isolated using a batch elution technique where the plg binding proteins were eluted with a single concentration of TA (Fig 2.3.4); 38.4, 43 and 48kDa proteins were isolated using a FPLC TA gradient (Fig 2.3.5); >100, 80 50 and 40kDa proteins were isolated using a combination of affinity and size exclusion chromatography (Fig 2.3.6). Two proteins were repeatedly isolated using all techniques and had molecular weights between 40-43kDa and 48-50kDa. The molecular weights of these proteins is comparable with several eukaryotic plg-R candidates such as α-enolase (54kDa) (Miles et al 1991), Annexin II (40kDa) (Hajjar et al 1994), actin (45kDa) (Dudani et al 1996). Additionally a 50-60kDa plg-binding protein has been identified on the surface of rat colon carcinoma cells (Durliat et al 1992). Although >250kDa and 40kDa
proteins could be detected by ligand blotting of plasma membrane preparations (Fig 2.3.4B) no isolated plg-Rs were ever visualised by ligand blotting. This could be due to the low concentration of isolated plg-Rs or to the loss of plg-binding ability by isolated plg-Rs.

In summary, plg appears to play a non-essential role during in vitro cell adhesion on of some cell lines. The current study also throws doubt on the reliability of using $^{125}$I-plg binding assays to measure plg-Rs due to high interexperimental variability. This consistent observation indicates that more robust methods for measuring plg binding need to be developed. Several plg binding proteins were found associated with the plasma membranes of HCT116 cells. The identity of these 'plg-Rs' remains to be determined however they do have molecular weights similar to several reported plg-R candidates.
Chapter 3

Development of a Flow Cytometric Plasminogen Binding Assay
3.1 INTRODUCTION

Flow cytometry is a technique for measuring both the physical attributes and fluorescent emission from single, intact cells. Cells are passed in the path of a laser beam and any light that is scattered by or emitted from individual cells is detected using various sensors. Light which is scattered in the forward direction (forward scatter or FSC) is indicative of cell size while light scattered sideways (side scatter or SSC) indicates granularity or how differentiated/internally complex the cell is. If the cells are stained with a fluorescently-labelled probe, such as an antibody, then cell-associated fluorescence is detected with a sensor set to the wavelength emission of the fluorophore. Flow cytometry can be used to detect extracellular and intracellular proteins, in ligand binding studies, measurement of DNA and RNA content, cell cycle analysis, measurement of intracellular calcium, various metabolites, membrane potential, membrane lipids and enzyme activity.

One of the key advantages of using flow cytometry is that specific subpopulations of cells can be analysed separately from other cells in the sample. This is extremely useful, particularly if the parameter being measured is linked to another parameter or process. For example, if the presence of a certain surface protein is linked to the cell cycle, then flow cytometry enables the measurement of that protein on cells in different stages of the cell cycle.
Only a few studies have utilised flow cytometry to measure plg binding (Miles et al 1991; Felez et al 1993; Reinartz et al 1993). Two shortcomings were apparent in these studies: firstly, flow cytometry formed a minor part of each study - the development of these flow cytometric assays was not adequately described or considered; secondly, single-colour fluorescence only was utilised, i.e. samples were not stained with PI to differentiate between viable and non-viable cells which is a standard component of flow cytometric analysis.

Irrespective of any other work, observations described in the previous chapter indicated that data generated using $^{125}$I-plg binding assays were highly variable (Fig 2.3.3B). HCT116 cells were found to specifically bind relatively high levels of plg (judged by the ability of TA to inhibit $^{125}$I-plg binding), however very low and variable levels of plg-Rs were isolated from plasma membrane preparations (Fig 2.3.4-6). This variability in the level and types of plg-Rs isolated could be a product of the variability in the initial binding assay - i.e plg-R expression varies due to cellular attributes which cannot be determined using $^{125}$I-plg binding assays. Collectively these data warrant the development of other methods for measuring cellular plg binding.

There are numerous advantages in using flow cytometry to measure plg-R expression (i.e plg binding) compared to other techniques such as $^{125}$I-plg binding assays:
(1) There are no health or safety issues associated with handling and disposing of radioactive material.

(2) Unbound plg does not need to be removed (by washing) as only cell-associated plg is detected. This is achieved by ‘gating’ specific subpopulations of cells based on some parameter such as viability (i.e. PI uptake) and then measuring plg binding only on that population. This is a significant advantage due to the low affinity of plg - removing unbound plg by washing (a feature of all radioligand binding assays) reduces the concentration of free plg which could cause cell-bound plg to dissociate in order to re-establish equilibrium binding.

(3) Plg binding can be measured on specific subpopulations of cells, for example viable versus non-viable cells or cells in different stages of the cell cycle. This is important particularly if plg-R expression varies between subpopulations of cells within the sample.

(4) Plg binding can be measured simultaneously with other cellular attributes (e.g. cell size, expression of differentiation antigens etc.).

(5) Plg binding is measured on intact cells. Any damage or cellular changes occurring during experimental manipulation can be determined.

The aim of this chapter was to develop a robust and reliable flow cytometric plg binding assay that can be used to measure plg-Rs (i.e. specific plg binding) on a variety of cells (i.e. adherent and non-adherent cells).
3.2 MATERIALS AND METHODS

3.2.1 Materials

All cell culture reagents were obtained from Trace BioSciences (Sydney, NSW, Australia) except for FCS which was obtained from CSL, (Melbourne, Victoria, Australia). Human glu-plg was either purified according to the method of Grant (1990) or was generously provided by American Diagnostica (ADI) (Greenwich CT, USA). Human lys-plg was obtained commercially (ADI, Greenwich CT, USA). NAP-10 columns and Lysine-Sepharose 4B were obtained from Amrad Pharmacia Biotech (Melbourne, Victoria, Australia). TA, BSA, PI, dimethyl sulphoxide (DMSO), H₂O₂, hydroxylamine, Hepes, and FITC were obtained from Sigma (Sydney, NSW, Australia). Carboxypeptidase B was obtained from Calbiochem-Novachem (Sydney, NSW, Australia). PMA was obtained from Boehringer Mannheim (Sydney, Australia). TNFα was generously provided by Darren Saunders (Department of Biological Sciences, University of Wollongong). FACS tubes were obtained from Becton Dickinson.

3.2.2 Cell lines and cell culture

The human monocytic leukemic cell line U937 was obtained from the American Type Culture Collection. The human colon cancer cell lines HCT116, LIM1215 and KM12SM were provided by Dr M Baker (Dept. Biological Sciences, University of Wollongong, Australia). All cell lines were cultured in RPMI1640/10% FCS at 37°C in a humidified atmosphere of 5% CO₂ Adherent
cells were passaged using 0.05% (w/v) trypsin/5mM EDTA/PBS (pH 7.4) to lift them into suspension.

3.2.3 Labelling plg with fluorescein isothyocyanate (FITC)

Glu-plg (2-5 mg/ml) was dialysed against 0.1 M carbonate buffer (pH 9) and a 50-fold molar excess of FITC added after being dissolved in DMSO. Plg was mixed for 16h at 4°C in the dark and subsequently treated with 0.01% hydroxylamine as for 30min at 4°C to remove all labile FITC:plg bonds. Unincorporated FITC was separated from FITC-plg by gel filtration through a NAP-10 column using HBSS (phenol red free) containing 20mM Hepes, 1mM CaCl₂, 1mM MgCl₂ (pH 7.4). In some instances, FITC-plg was adsorbed to lysine-Sepharose 4B, eluted with 0.2M TA, then dialysed against HBSS. FITC-plg was aliquoted and stored at -80°C until required. The number of molecules of FITC conjugated to each plg molecule was determined using the following formula:

\[
\text{FITC/plg molecule} = \frac{A_{495\text{nm}} \times \text{dilution factor}}{68,000 \text{ mg/ml plg}} \times 92,000
\]

where \( A_{495\text{nm}} \) is the absorbance maxima of FITC; 60,000 is the molar extinction coefficient (\( \text{cm}^{-1}\text{M}^{-1} \)) of conjugated fluorescein and 92,000 is the molecular weight of plg.

3.2.4 Flow cytometric plg binding assay

Cells were cultured for 24h in RPMI1640/10% plg-depleted FCS (prepared as described in section 2.2.5). Adherent cells were lifted into suspension by firstly
washing them for 5min at RT with HBSS and then treating them with 5mM EDTA in HBSS (Ca and Mg free) for 10min at 37°C. Cells (2x10^5) were washed twice with HBSS and resuspended in HBSS/0.1% BSA containing 0.5μM FITC-plg ± 1mM TA in a total volume of 200μl (the concentration of FITC-plg and TA was altered in certain experiments) and incubated for 60min at 4°C in the dark. PI (5 μg/ml) was added for 5min in order to differentiate viable from non-viable subpopulations of cells. Cell-associated fluorescence was detected using a FACSort (Becton-Dickinson, Australia) using the following instrument settings: FSC (forward scatter – cell size) (E00 Voltage, 1.00 AmpGain, Lin Mode); SSC (side scatter – granularity) (360 Voltage; 1AmpGain, Lin Mode); FL-1 (630 Voltage, 1 AmpGain, Log Mode); FL-2 (475 Voltage. 1AmpGain, Log Mode); Compensation (FL-1: 0.8% FL-2: 0%). Twenty thousand events were acquired for each sample. Viable and non-viable subpopulations of cells were 'gated' based on PI uptake (i.e. from FL-2 histograms). Cell-associated fluorescence due to FITC-plg binding was determined from FL-1 histograms. Lysine-dependent (specific) plg binding was calculated as the difference in mean fluorescence between cells incubated solely with FITC-plg (total binding) and cells incubated with FITC-plg and 1mM TA (non-specific binding). 1mM TA was experimentally found to be the optimal concentration for maximally inhibiting FITC-plg binding (Fig 3.3.12). All experimental measurements were performed in triplicate and each experiment repeated at least twice.
3.2.5 Carboxypeptidase B treatment of cells

The effect of carboxypeptidase B treatment on plg binding to cells was assessed as previously described (Miles et al 1991). Briefly U937 cells were cultured in RPMI/10% plg depleted FCS for 24h at 37°C, washed thrice with HBSS, then treated with 10, 25 and 50 U/ml carboxypeptidase B in HBSS for 30min at 37°C. Cells were washed thrice with HBSS and plg binding measured as described in 3.2.4.

3.2.6 PMA treatment of cells

A stock solution of PMA was made by dissolving PMA in DMSO, aliquoting and storing at -20°C. Cells were treated with PMA as previously described (Felez et al 1990) by culturing cells for 48h in 0, 0.1, 10, 20, 40 and 80mM PMA. Cells were washed thrice with HBSS then plg binding assessed as described in 3.2.4.

3.2.7 Peroxide treatment of cells

Cells were washed thrice with HBSS (Ca/Mg-free) then incubated in PBS containing 0, 0.1, 1, 5, 10, and 20mM H₂O₂ for 30min/37°C in the dark. Cells were washed thrice with HBSS then plg binding assessed as described in 3.2.4.

3.2.8 TNFα treatment of cells

Cells were cultured in the presence of 0 and 10ng/ml TNFα for 24h. Cells were washed thrice with HBSS then plg binding assessed as described in 3.2.4.
3.2.9 Data analysis

All flow cytometric data was analysed using CellQuest (v3.1, Becton-Dickinson, Sydney Australia). Each experiment was performed in duplicate or triplicate and each experiment repeated at least twice. Unless indicated all results are expressed as means ± 1 SEM and each experiment repeated at least twice. To test for statistical differences data were analysed using JMP statistical analysis software (v6). Statistically significant differences between two treatments were assessed using a student's t-test. Differences between treatments were considered significant when p<0.05.

3.3 RESULTS

3.3.1 FITC-labelling of plg

Plg was labelled with FITC via its reactive amino groups (lysine) according to a well established procedure (Goding 1976) and purified by size exclusion chromatography or lysine-Sepharose 4B chromatography. The mean number of FITC molecules conjugated to each plg molecule was 3.72 ± 1.220 (N=9) which is consistent with previous studies that have described 2-4 FITC molecules per plg (Felez et al 1990).

3.3.2 Gating strategy for analysing plg binding by flow cytometry

One of the key aspects of developing any flow cytometric assay is determining how the cells should be analysed - i.e. what cellular attributes are important
and how different subpopulations of cells should be distinguished from other subpopulations of cells. This is achieved by placing a border or marker around the subpopulation of cells - called a 'gate'. As some adherent cells tend to clump together it is important to differentiate single cells from clumps of cells. The other two important cellular characteristics are cell viability and obviously plg binding.

The gating strategy for measuring plg binding on non-adherent cells is illustrated in Fig 3.3.1 using U937 cells as the example. Fig 3.3.1A is a density plot of FSC versus SSC and shows the general physical characteristics of U937 cells - a single dense population of cells is apparent. For U937 cells it was unnecessary to place a gate around this population as there were no other subpopulations of cells. When U937 cells were stained with PI (to differentiate viable from non-viable cells) two distinct subpopulations of cells were evident as shown Fig 3.3.1B: these were viable (no PI uptake) and non-viable cells (maximum PI uptake). Viable (Fig 3.3.1C) and non-viable cells (Fig 3.3.1D) were gated and analysed separately for plg binding. Cellular autofluorescence is represented by the shadowed profile - autofluorescence of non-viable cells was noticeably higher than that of viable cells. Fluorescence peaks due to total FITC-plg binding (dark line) and non-specific FITC-plg binding (light line) are also shown in both histograms. Markers were placed over both total and non-specific binding peaks and the mean fluorescence intensity calculated using CellQuest software. The difference between total FITC-plg and non-specific
Fig 3.3.1: Gating strategy for measuring plg binding on U937 cells. U937 cells were cultured in plg-free media for 24h then incubated with 0.5μM FITC-plg + 1mM TA for 60min at 4°C. PI (5μg/ml) was added prior to flow cytometric analysis to differentiate viable from non-viable subpopulations of cells. A. Density plot of forward scatter (size) versus side scatter (granularity). B. PI histogram showing viable and non-viable cells. C. FITC-plg binding to viable U937 cells (shaded histogram = autofluorescence; dark line = total plg binding; light line = non-specific plg binding. D. FITC-plg binding to non-viable cells.
FITC-plg binding was a measure of specific FITC-plg binding. The gating strategy for adherent cells was similar to that for non-adherent cells except that cells were gated firstly on size in order to discriminate between single cells and clumps of cells (Fig 3.3.2).

3.3.3 Establishing a relationship between cell viability and plg binding

The next step in the development of the flow cytometric plg binding assay was to select a suitable buffer that would maintain cell integrity for up to several hours. The viability (level of PI uptake) of U937 and HCT116 and LIM1215 cells was assessed after two hours incubation in PBS, HBSS or RPMI1640. In all cell lines, cell viability (i.e the proportion of viable cells) was highest in RPMI1640, followed by HBSS and then PBS (Fig 3.3.3). Based on this information PBS was excluded as an incubation buffer. RPMI1640 was also excluded because it contains small concentrations of lysine and arginine which could potentially interfere with plg binding. By a process of elimination HBSS, containing 1mM Mg, 1mM Ca and 0.1% BSA, was chosen as the binding buffer. U937 cells appeared to be the most robust of the cell lines studied followed by LIM1215 and then HCT116.

When U937 cells were stained with PI and analysed by flow cytometry two distinct subpopulations of cells were evident (Fig 3.3.4A): these were viable (negligible PI uptake) and non-viable (full PI uptake). Typically, 90-95% of cells were viable and 5-10% were non-viable. The addition of 0.5 μM FITC-plg (Fig
Fig 3.32: Gating strategy for measuring plg binding on HCT116 cells. HCT116 cells were cultured in plg-free media for 24h then incubated with 0.5μM FITC-plg ± 1mM TA for 60min at 4°C. PI (5μg/ml) was added prior to flow cytometric analysis to differentiate viable from non-viable subpopulations of cells. A. Density plot of forward scatter (size) versus side scatter (granularity) showing single cells and clumps of cells. B. PI histogram showing viable and non-viable cells.

C. FITC-plg binding to viable U937 cells (shaded histogram = autofluorescence; dark line = total plg binding; light line = non-specific plg binding. D. FITC-plg binding to non-viable cells.
Fig 3.33: Effect of various buffers on the viability (PI uptake) of U937 (A), LIM1215 (B) and HCT116 cells (C). Cells were cultured in pig-free media, washed and incubated in PBS (pH 7.4), HBSS (pH 7.4) or RPMI1640 for 2h at 4°C. PI (5µg/ml) was added for 5min at 4°C prior to flow cytometric analysis where the proportion of viable and non-viable cells were visualised on FL-2 histograms.
Fig 3.3.4: Relationship between cell viability (PI uptake) and plg binding on U937 cells. U937 cells were cultured in plg-free media for 24h then incubated with 0.5μM FITC-plg ± 1mM TA for 60min at 4°C. PI (5μg/ml) was added prior to flow cytometric analysis to differentiate viable and non-viable subpopulations of cells. A. Density plot of control cells stained solely with PI and showing distinct subpopulations of viable and non-viable cells. B. Density plot of cells incubated with FITC-plg and PI (total plg binding). C. Density plot of cells stained with FITC-plg + 1mM TA and PI (non-specific plg binding).
3.3.4B) caused a marked increase in fluorescence associated with viable and non-viable cells compared to their respective controls (Fig 3.3.4A), indicating that plg binds to both subpopulations. Non-viable cells exhibited dramatically higher (~100-fold) fluorescence than did viable cells, indicating an enormous increase in their ability to bind plg. The presence of 1mM TA resulted in the marked reduction of the fluorescence associated with non-viable cells (~95%) but this was not evident with viable cells (Fig 3.3.4C).

An almost identical result was observed on HCT116 cells (Fig 3.3.5). When HCT116 cells were stained with PI and analysed by flow cytometry viable and non-viable cells were also evident (Fig 3.3.5A). HCT116 cells were relatively fragile and thus the typical proportion of viable cells was 75-80% and non-viable cells was 20-25%. Additionally a diffuse intermediate PI staining population was evident. The addition of 0.5µM FITC-plg (Fig 3.3.5B) also caused a marked increase in fluorescence on viable and non-viable cells compared to their respective controls (Fig 3.3.5A), indicating that plg binds to both subpopulations of HCT116 cells. Like U937 cells, non-viable HCT116 cells exhibited dramatically higher (~100-fold) fluorescence than did viable cells, indicating an enormous increase in their ability to bind plg. The presence of 1mM TA resulted in the marked reduction of the fluorescence associated with non-viable HCT116 cells (~75%) but this was not evident with viable cells (Fig 3.3.5C).
Fig 3.3.5: Relationship between cell viability (PI uptake) and plg binding on HCT116 cells. HCT116 cells were cultured in plg-free media for 24h then incubated with 0.5μM FITC-plg ± 1mM TA for 60min at 4°C. PI (5μg/ml) was added prior to flow cytometric analysis to differentiate viable and non-viable subpopulations of cells. A. Density plot of control cells stained solely with PI and showing distinct subpopulations of viable and non-viable cells. B. Density plot of cells incubated with FITC-plg and PI (total plg binding). C. Density plot of cells stained with FITC-plg + 1mM TA and PI (non-specific plg binding).
These observations on both U937 and HCT116 cells indicate that non-viable cells bind magnitudes more FITC-plg than viable cells and that a large proportion of this binding is lysine-dependent. In contrast, plg binding to viable cells appeared to be essentially lysine-independent. Collectively these data suggest that there is a direct relationship between cell viability (PI uptake) and the magnitude of lysine-dependent plg binding.

3.3.4 Pretreatment of adherent cells

One of key features of developing a ligand binding assay is to ensure that both the culture and manipulation of cells does not modify the level of receptors. In the current study, all cells were cultured in plg-free conditions to minimise the occupation of plg-Rs with bovine plg. Adherent cells are usually passaged using trypsin/EDTA to lift them into suspension. This treatment is likely to digest plg-Rs as they have previously been shown to be susceptible to trypsin treatment (Camacho et al 1989). To confirm this observation, HCT116 cells were detached from tissue culture flasks with either 0.05% (w/v) trypsin or 5mM EDTA in HBSS (Ca and Mg free). Passaging HCT116 cells with trypsin reduced the level of plg-Rs (i.e. specific plg binding) on non-viable cells by 14.0 + 3.8% (N=3) (Fig 3.3.6B) compared to cells passaged using 5mM EDTA. Although no specific plg binding was detected on viable HCT116 cells, total plg binding was decreased by 11.1 + 0.42% (N=3) and non-specific binding increased by 6.9 + 1.7% (N=3) when cells were passaged using trypsin (Fig 3.3.6B). Passaging HCT116 cells with trypsin also increased the number of non-viable
Fig 3.3.6: Effect of passaging cells with trypsin or 5mM EDTA on plg binding to viable (A) and non-viable (B) HCT116 cells. HCT116 cells were cultured in plg-free media for 24h then passaged using either 0.05% (w/v) trypsin or 5mM EDTA. Cells were washed and plg binding measured by incubating cells with 0.5μM FITC-plg ± 1mM TA at 4°C for 60min. PI (5μg/ml) was added prior to flow cytometric analysis to differentiate viable from non-viable cells. Each bar represents the mean of triplicate determinations ± 1 SEM. The absence of error bars indicates that the error was smaller than the graphical symbol.
Fig 3.3.7: Effect of passaging cells with trypsin versus EDTA (versene) on the proportion of viable (A) and non-viable cells (B). HCT116 cells were cultured in pig-free media for 24h then passaged using either 0.05% (w/v) trypsin or 5mM EDTA. Cells were washed and incubated with PI (5µg/ml) prior to flow cytometric analysis in order to determine the proportion of viable and non-viable cells. Each bar represents the mean of triplicate determinations ± 1 SEM. The absence of error bars indicates that the error was smaller than the graphical symbol.
cells by 28.4 ± 4.6% (Fig 3.3.7B).

When HCT116 cells were treated with higher concentrations of trypsin (i.e 0.1-0.5% w/v) a different affect occurred with trypsin reducing total and non-specific plg binding on both viable and non-viable cells (Fig 3.3.8). On non-viable HCT116 cells, specific plg binding was increased by 22.9 ± 6.58% (N=3) following mild trypsin treatment (i.e 0.1% w/v) (Fig 3.3.8B) which contrasts with the above affect of 0.05% (w/v) trypsin (Fig 3.3.6B), however it is consistent with previous studies (Camacho et al 1989; Ouimet et al 1994; Gase et al 1996). Treatment of HCT116 cells with 0.25% (w/v) trypsin was found to also increase specific plg binding to non-viable cells by 56 ± 7.02% (Fig 3.3.6B). Surprisingly, 0.5% (w/v) trypsin was found to cause a slight reduction in specific plg binding to non-viable HCT116 cells (Fig 3.3.8B). Collectively these observations indicate that: (1) adherent cells should be detached using 5mM EDTA prior to flow cytometric plg binding assays, and (2) the affect of trypsin on plg-R expression is concentration dependent.

3.3.5 Establishing the time of equilibrium plg binding

One of the key features in any ligand binding study is the time required to reach equilibrium binding as this indicates the incubation time necessary for completion of the assay and also provides some information on the relative affinity of the ligand for its cellular receptor/s. U937 and HCT116 cells were incubated with 0.5μM plg for various time periods until equilibrium binding
Fig 3.3.8: Effect of increasing concentrations of trypsin on plg binding to viable (A) and non-viable (B) HCT116 cells. HCT116 cells were cultured in plg-free media for 24h, removed using 5mM EDTA treated with 0-0.5% (w/v) trypsin for 30min/RT. Cells were washed and plg binding measured by incubating cells with 0.5µM FITC-plg ± 1mM at 4°C for 60min. PI (5µg/ml) was added prior to flow cytometric analysis to differentiate viable from non-viable cells. Each bar represents the mean of triplicate determinations ± 1 SEM. The absence of error bars indicates that the error was smaller than the graphical symbol.
Fig 3.3.9: Timecourse of plg binding to viable (A) and non-viable (B) U937 cells. U937 cells were cultured in plg-free media for 24h, washed and incubated with 0.5μM FITC-plg ± 1mM TA for 0-120min at 4°C. At various timepoints PI (5μg/ml) was added prior to flow cytometric analysis to differentiate viable from non-viable cells. Each point represents the mean of triplicate determinations ± 1 SEM. The absence of error bars indicates that the error was smaller than the graphical symbol.
Fig 3.3.10: Timecourse of plg binding to viable (A) and non-viable (B) HCT116 cells. HCT116 cells were cultured in plg-free media for 24h, washed and incubated with 0.5μM FITC-plg ± 1mM TA for 0-120min at 4°C. At various timepoints PI (5μg/ml) was added prior to flow cytometric analysis to differentiate viable from non-viable cells. Each point represents the mean of triplicate determinations ± 1 SEM. The absence of error bars indicates that the error was smaller than the graphical symbol.
was attained. Specific plg binding reached equilibrium on non-viable U937 and HCT116 cells at approximately 60min (Fig 3.3.9B & Fig 3.3.10B respectively). Although no specific plg binding was detected on viable cells, total and non-specific plg binding to viable U937 and HCT116 cells reached equilibrium at approximately 30min (Fig 3.3.9A & Fig 3.3.10B). Based on these results, 60min was used as the standard incubation period for flow cytometric plg binding assays on all cell lines.

3.3.6 Effect of removing unbound plg on the measurement of plg binding

One of the key advantages of using flow cytometry to study low affinity receptor:ligand interactions (such as plg binding) is that unbound ligand does not need to be removed in order to measure cell-associated ligand. To determine if removing unbound plg by washing cells had any affect on plg binding, HCT116 cells were incubated with plg, washed with HBSS up to four times and the level of plg binding measured. Plg binding to both viable and non-viable cells was markedly reduced when cells were washed once (Fig 3.3.11). Both total and non-specific plg binding to viable cells was reduced by approximately 70% when washed once (Fig 3.3.11A). Increasing the number of afterwashes did not further reduce the level of total and non-specific binding on viable cells (Fig 3.3.11A). Total plg binding was reduced by 32 ± 3.4% (N=3) on non-viable cells with a single afterwash. Non-specific binding to non-viable cells remained constant regardless of the number of afterwashes. Importantly, specific plg binding to non-viable cells was reduced by 59.5 ± 3.9 % with a single
Fig 3.3.11: Effect of removing unbound plg on the detection of plg binding to viable (A) and non-viable (B) U937 cells. U937 cells were cultured in plg-free media for 24h, washed and incubated with 0.5μM FITC-plg ± 1mM TA for 60min at 4°C. Cells were then washed up to 4 times with HBSS prior to flow cytometric analysis. PI (5μg/ml) was added to all samples in order to differentiate viable from non-viable cells. Each point represents the mean of triplicate determinations ± 1 SEM. The absence of error bars indicates that the error was smaller than the graphical symbol.
afterwash with a small but steady decrease evident with increased afterwashes (Fig 3.3.11B). These results indicate that afterwashing removes more than half of the plg specifically bound to non-viable cells and consequently it should not be employed in plg binding assays. These results also suggest that two types of specific plg binding occur on non-viable cells - a low affinity interaction which is easily removed by afterwashing, and a higher affinity interaction which is reasonably resistant to afterwashing. Based on these results unbound FITC-plg was not removed by washing prior to flow cytometric analysis.

3.3.7 Specificity of cellular plg binding

It was clear that specific plg binding was restricted to non-viable cells. The next series of experiments were aimed at determining the specificity of plg binding to viable and non-viable subpopulations of cells. U937 cells were incubated with FITC-plg in the presence of either increasing concentrations of TA or unlabelled glu or lys-plg. FITC-plg binding to viable U937 cells could not be inhibited with up to 100mM TA (Fig 3.3.12A) or 100-fold molar excess of unlabelled glu or lys-plg (Fig 3.3.13A & 3.3.14A) confirming that plg binding to viable cells is a lysine-independent (i.e non-specific) interaction. In contrast, 1mM TA maximally inhibited FITC-plg binding to non-viable cells, while increasing the concentration of TA beyond 1mM reduced the level of inhibition (Fig 3.3.12B). This observation indicated that the level of inhibition of FITC-plg binding to non-viable cells was dependent on the concentration of TA. TA concentrations below 1mM did not display a higher capacity to inhibit
Fig 3.3.12: Inhibition of FITC-plg binding to viable (A) and non-viable (B) U937 cells with increasing concentrations of TA. U937 cells were cultured in plg-free media for 24h, washed and incubated with 0.5μM FITC-plg ± 0-100mM TA for 60min at 4°C. PI (5μg/ml) was added prior to flow cytometric analysis to differentiate viable from non-viable cells. Each point represents the mean of triplicate determinations ± 1 SEM. The absence of error bars indicates that the error was smaller than the graphical symbol.
Fig 3.3.13: Inhibition of FITC-plg binding to viable (A) and non-viable (B) U937 cells with increasing concentrations of unlabelled glu-plg. U937 cells were cultured in plg-free media for 24h, washed and incubated with 0.5μM FITC-plg + 0-100-fold molar excess of unlabelled plg for 60min at 4°C. PI (5μg/ml) was added prior to flow cytometric analysis to differentiate viable from non-viable cells. Each point represents the mean of triplicate determinations ± 1 SEM. The absence of error bars indicates that the error was smaller than the graphical symbol.
Fig 3.3.14: Inhibition of FITC-plg binding to viable (A) and non-viable (B) U937 cells with increasing concentrations of unlabelled glu-plg. U937 cells were cultured in plg-free media for 24h, washed and incubated with 0.5μM FITC-plg + 0-100-fold molar excess of lys-plg for 60min at 4°C. PI (5μg/ml) was added prior to flow cytometric analysis to differentiate viable from non-viable cells. Each point represents the mean of triplicate determinations ± 1 SEM. The absence of error bars indicates that the error was smaller than the graphical symbol.
FITC-plg binding (data not shown). Additionally, FITC-plg binding to non-viable U937 cells was inhibited over a range of concentrations of unlabelled glu and lys-plg (Fig 3.3.13B and 3.3.14B). For example, a 100-fold molar excess of glu and lys-plg inhibited FITC-plg binding to non-viable U937 cells by 30 ± 1.3% and 80 ± 7.5% respectively. These results confirmed that specific plg binding was restricted to non-viable U937 cells.

3.3.8 Proteinacious C-terminal lysine residues function as plg-Rs on non-viable cells

Proteinacious C-terminal lysine residues have previously been shown to function as plg-Rs and are consequently susceptible to carboxypeptidase B treatment. The aim of this experiment was to examine the effect of carboxypeptidase B treatment on plg binding to viable and non-viable subpopulations of U937 cells. When U937 cells were pretreated with increasing concentrations of carboxypeptidase B, specific FITC-plg binding to non-viable cells was reduced (Fig 3.3.15B) indicating that proteins with C-terminal lysine residues function as plg-Rs on non-viable cells. For example, pretreatment of U937 cells with 50U/ml carboxypeptidase B reduced plg binding to non-viable cells by 37 ± 2.4%. A student’s t-test indicated that 25 (p<0.05) and 50 (p<0.05) but not 10U/ml (p>0.05) carboxypeptidase B caused a statistically significant reduction in FITC-plg binding on non-viable cells. As no specific (i.e. lysine-dependent) plg binding occurred on viable U937 cells carboxypeptidase B had no effect (3.3.15A).
Carboxypeptidase B Concentration U/ml

Fig 3.3.15: Effect of carboxypeptidase B on plg binding to viable (A) and non-viable (B) U937 cells. U937 cells were cultured in plg-free media for 24h, washed and treated with 0-50U/ml carboxypeptidase B for 30min at 37°C. Cells were then washed and incubated with 0.5μM FITC-plg ± 1mM TA for 60min at 4°C. PI (5μg/ml) was added prior to flow cytometric analysis to differentiate viable from non-viable cells. Each bar represents the mean of triplicate determinations ± 1 SEM. The absence of error bars indicates that the error was smaller than the graphical symbol.
3.3.9 Saturability of plg binding to viable and non-viable cells

U937 cells were incubated with increasing concentrations of plg in order to determine the saturating concentration of plg and subsequently its affinity (i.e. $K_d$). Scatchard analysis is currently not possible using flow cytometry as suitable standards (i.e. beads with defined numbers of lysine binding sites) are unavailable. Although plg binding to viable U937 cells is a non lysine-dependent interaction, both total and non-specific plg binding were saturated at approximately $4\mu$M (Fig 3.3.16A) which translates to a $K_d$ of $2\mu$M. This is both the concentration of plg in serum and the average reported $K_d$ in the scientific literature. Specific plg binding to non-viable U937 cells reached an apparent maximum at $1-2\mu$M ($K_d = 0.5-1\mu$M), however binding did not appear to reach a stable equilibrium as increasing concentrations of plg resulted in a progressive reduction in the level of binding (Fig 3.3.16B). Collectively these results confirm that specific plg binding is restricted to the surface of non-viable U937 cells and has at least a 4-fold higher affinity for non-viable cells than the non-lysine-dependent binding detected on viable cells.

3.3.10 Can any specific plg binding be generated on viable U937 cells?

All evidence indicated that plg-Rs were absent on viable U937 and HCT116 cells, and that a loss of cell viability (detected as an increase in PI uptake) increased plg-R expression. The next series of experiments were undertaken in an attempt to generate plg-Rs on viable cells without causing a loss in cell viability.
Fig 3.3.16: Plg concentration curves for viable (A) and non-viable U937 cells. U937 cells were cultured in plg-free media for 24h then incubated with 0-
600µg/ml FITC-plg ± 1mM TA for 60min at 4°C. PI (5µg/ml) was added prior to
flow cytometric analysis to differentiate viable from non-viable cells. Each
point represents the mean of triplicate determinations ± 1 SEM. The absence of
error bars indicates that the error was smaller than the graphical symbol.
U937 cells were cultured for 48h in media containing 0-80mM PMA as PMA has previously been shown to increase plg-R expression on U937 cells (Felez et al 1990). No specific plg binding could be detected on viable U937 cells that had been treated with increasing concentrations of PMA (Fig 3.3.17A). Although the level of specific plg binding to non-viable U937 cells fluctuated following PMA treatment no clear pattern was evident (3.3.17B). These observations indicate that PMA does not increase plg-R expression on viable U937 cells. Additionally, PMA had no affect on cell viability (data not shown).

TNFα is an inflammatory mediator which has previously been shown to increase uPAR expression in U937 cells (Lu et al 1988), peripheral blood monocytes (Kirchheimer et al 1988) and colon cancer cells (Wang et al 1988). To investigate the affect of TNFα on plg-R expression, U937 cells were cultured in 10μg/ml TNFα for 48h and the level of plg binding measured. Plg-Rs (specific plg binding) could not be detected on viable U937 cells that had been treated with TNFα for 48h (Fig 3.3.18A). A 52.6 ± 4.33% (N=3) reduction in specific plg binding was observed on non-viable U937 cells that had been treated with TNFα (Fig 3.3.18B) despite the fact that TNFα caused an approximate 5-fold increase in the proportion of non-viable cells (Fig 3.3.18C). These results indicate that TNFα does not increase plg-R expression on viable U937 cells.
Fig 3.3.17: Effect of PMA on plg binding to viable (A) and non-viable U937 cells. U937 cells were cultured for 48h in plg-free media that contained 0-80mM PMA. Cells were washed and incubated with 0.5μM FITC-plg ± 1mM TA for 60min at 4°C. PI (5μg/ml) was added prior to flow cytometric analysis to differentiate viable from non-viable cells. Each bar represents the mean of triplicate determinations ± 1 SEM. The absence of error bars indicates that the error was smaller than the graphical symbol.
Fig 3.3.18: Effect of TNFα on plg binding to viable (A) and non-viable (B) U937 cells. U937 cells were cultured for 24h in plg-free media that contained 10μg/ml TNFα. Cells were washed and incubated with 0.5μM FITC-plg + 1mM TA for 60min at 4°C. PI (5μg/ml) was added prior to flow cytometric analysis to differentiate viable from non-viable cells. A. Effect of TNFα on plg binding to viable cells. B. Effect of TNFα on plg binding to viable cells. C. Effect of TNFα on cell viability (PI uptake). Each bar represents the mean of triplicate determinations ± 1 SEM. The absence of error bars indicates that the error was smaller than the graphical symbol.
In a further attempt to generate plg-Rs on viable cells, U937 cells were treated with 0-20mM peroxide for 30min and then plg binding measured. The rationale for this approach was to attempt to modify cell-surface proteins using an oxidative agent with the aim of generating plg binding sites. Peroxide treatment did not generate any plg-Rs (i.e specific plg binding) on viable U937 cells (Fig 3.3.19A) and also appeared to have no affect on specific plg binding to non-viable cells (3.3.19B). Additionally, peroxide treatment had no affect on cell viability (data not shown).

A further attempt was made to generate plg-Rs on viable cells by quiescing them for 24h in the absence of serum (i.e.). The rationale for this approach is that FCS could contain factors (e.g. cytokines or growth factors) that prevent plg-R expression on viable cells. No specific plg binding was detected on viable U937 cells that had been cultured in the absence of FCS for 24h (Fig 3.3.20A). Additionally, serum starvation did not modify specific plg binding (i.e plg-R expression) on non-viable U937 cells (Fig 3.3.20B) or affect cell viability (data not shown).

3.3.10 Comparison of plg binding on several cell lines

It was clear that plg binding was dramatically elevated on non-viable cells with no specific plg binding detected on viable U937 and HCT116 cells. To confirm these observations, plg binding was measured on two other colon cancer cell lines, LIM1215 and KM12SM. Additionally plg binding to three breast
Fig 3.3.19: Effect of peroxide on plg binding to viable (A) and non-viable (B) U937 cells. U937 cells were cultured for 48h in plg-free then treated with 0-20mM peroxide for 30min. Cells were washed and incubated with 0.5μM FITC-plg ± 1mM TA for 60min at 4°C. PI (5μg/ml) was added prior to flow cytometric analysis to differentiate viable from non-viable cells. Each bar represents the mean of triplicate determinations ± 1 SEM. The absence of error bars indicates that the error was smaller than the graphical symbol.
Fig 3.3.20: Effect of serum starvation on plg binding to viable (A) and non-viable (B) U937 cells. U937 cells were cultured for 24h in serum-free media, washed and incubated with 0.5μM FITC-plg ± 1mM TA for 60min at 4°C. PI (5μg/ml) was added prior to flow cytometric analysis to differentiate viable from non-viable cells. Each bar represents the mean of triplicate determinations ± 1 SEM. The absence of error bars indicates that the error was smaller than the graphical symbol.
carcinoma cell lines, MDA-MB-231, MCF-7 and T47D was independently measured by Ranson et al (1998) using the flow cytometric plg binding assay developed in this chapter. No specific plg binding was detected on the surface of viable U937, HCT116, LIM1215 and KM12SM cells while specific plg binding to non-viable cells has highly elevated on all cells lines (Fig 3.3.21). The level of plg-R expression on non-viable cells varied with U937 and HCT116 cells exhibiting the highest level of specific plg binding (Fig 3.3.21B). LIM1215 cells expressed approximately half the level of plg-Rs as U937 and HCT116 cells while KM12SM exhibited the lowest level of specific plg binding. Low levels of specific plg binding were detected on viable MCF-7, T47D and MDA-MB-231 cells with MDA-MB-231 cells expressing the highest level of plg-Rs (Ranson et al 1998). Specific plg binding was also dramatically elevated on non-viable breast carcinoma cells (Ranson et al 1998). These comparative data confirm that the dramatic increase in plg-R expression on non-viable cells is a widespread phenomenon with the level of plg-Rs dependent on the cell type. Although no specific plg binding was detected on any viable cell types within the current study some viable breast cancer cells have subsequently been shown to possess plg-Rs (Ranson et al 1998).

3.4 DISCUSSION

A novel flow cytometric plg binding assay was developed to enable the measurement of plg binding on a range of cell types (i.e adherent and non-adherent) and on various subpopulations of cells (e.g viable and non-viable).
Fig 3.3.21: Comparison of plg binding to various viable (A) and non-viable (B) cell lines. U937, HCT116, LIM1215 and KM12SM cells were cultured for 24h in plg-free media. HCT116, LIM1215 and KM12SM cells were detached using 5mM EDTA and all cells washed prior to the addition of 0.5μM FITC-plg ± 1mM TA for 60min at 4°C. Each bar represents the mean of triplicate determinations ± 1 SEM. The absence of error bars indicates that the error was smaller than the graphical symbol.
Dual-colour staining of cells with both FITC-plg and PI revealed that a direct relationship exists between cell viability and plg binding with non-viable cells binding up to 100-fold more plg than viable cells (Fig 3.3.4 & 3.3.5). Comparative studies confirmed this observation on several cell lines including U937, HCT116, LIM1215, KM12SM, MCF-7, T47D and MDA-MB-231 cells [Fig 3.3.21 and Ranson et al (1998)]. These results indicated that increased plg-R expression on non-viable cells is a widespread phenomenon, however some non-viable cell types have a greater capacity to specifically bind plg than others do.

Although a few recent studies have utilised flow cytometry to detect cellular plg binding (Miles et al 1991; Felez et al 1993; Reinartz et al 1993), the majority of studies have exclusively employed $^{125}$I-plg radioligand binding assays. The observations described in this chapter clearly indicate that radioligand binding assays are no longer a satisfactory method of examining plg binding due to the direct relationship between cell viability (PI uptake) and lysine-dependent plg binding. By using $^{125}$I-plg binding assays specific plg binding to viable cells could easily be overestimated due to the increase in plg-Rs on non-viable cells. Care should also be taken when examining the effect of any factor (e.g protease, growth) on plg binding as the treatment itself may cause a loss of cell viability and consequently artifically elevate plg binding. For example, in the current study TNFα (Fig 3.3.18) and mild trypsin treatment (Fig 3.3.7) were shown to
increase the proportion of non-viable U937 cells by approximately 5-fold and non-viable HCT116 cells by 1.3-fold respectively.

The disparity between the observation that viable U937 cells express no plg-Rs and previous studies employing the same cell line but using $^{125}$I-plg binding assays (Plow et al 1986; Felez et al 1990; Edelberg et al 1990; Gonzalez Gronow et al 1990), can be explained simply by the failure of radioligand binding assays to discriminate plg binding on viable and non-viable cells. It should be noted that plg-Rs are present on some viable cell types and can be detected using the current flow cytometric assay as validated by Ranson et al (1998) who found that specific plg binding occurs on viable breast carcinoma cells.

In the current study plg bound to viable U937 cells in a non-lysine dependent manner as TA could not inhibit binding (Fig 3.3.12A), despite the observation that binding was saturable (Fig 3.3.16A). Plg binding to viable U937 cells was not inhibited by either unlabelled glu or lys-plg (Fig 3.3.13A & 3.3.14A) further confirming that plg binding to viable U937 cells appears to be a non-specific interaction. The absence of C-terminal lysine residues on viable U937 cells was confirmed when carboxypeptidase B treatment failed to reduce plg binding (Fig 3.3.15A). Although plg binding to viable U937 cells was non-specific it was saturated at a plg concentration of $4\mu M$ plg ($K_d = 2\mu M$) (Fig 3.3.16A) which is comparable with previous affinity measurements generated using scatchard analysis and $^{125}$I-plg binding assays (refer Table 1.5).
Plg binding to non-viable U937 cells was specific and predominantly lysine-dependent with binding inhibited with both TA (Fig 3.3.12B) and unlabelled glu and lys-plg (Fig 3.3.13B & 3.3.14B). Additionally, lys-plg was more affective than glu-plg at competing with FITC-plg for non-viable binding sites suggesting that lys-plg has a higher affinity for cellular plg-Rs, as has previously been reported (Ellis et al 1986; Silverstein et al 1988; Kuusela & Saksela 1990). An unexpected observation was that 100-fold molar excess of unlabelled glu-plg poorly inhibited FITC-plg binding to non-viable cells (Fig 3.3.13B). A possible explanation for this result is that FITC-plg has a higher affinity for plg-Rs on non-viable cells than unlabelled glu-plg.

Low concentrations of TA strongly inhibited cellular plg binding while higher concentrations were less inhibitory (Fig 3.3.12B). Plg has been shown to undergo a large conformational change upon binding to lysine analogues such as TA (Markus et al 1978; Mangel et al 1990; Christensen 1991). This conformational change is likely to expose hidden LBS in the plg molecule such as the one located in K4 (Vali & Patthy 1981). Thus low concentrations of TA are affective at blocking plg binding because they interact with a single strong LBS in K5. Higher concentrations of TA are less affective because they interact with more than one LBS due to the TA-induced conformational change. A TA concentration should be reached which saturates all of the five LBS in the plg molecule, however as shown in the present study, plg binding to non-viable U937 cells could not be completely abolished by either TA or unlabelled plg.
This result suggests that a proportion of cellular plg binding (~10-30%) involves non lysine-dependent mechanisms which is consistent with previous observations showing that a relatively small proportion of cellular plg binding is non lysine-dependent (Hajjar et al 1986; Campbell et al 1994).

Equilibrium plg binding was reached on both U937 and HCT116 cells at approximately 60min. An interesting observation was that specific plg binding to non-viable HCT116 cells (Fig 3.3.10B) appeared to be less stable than that occurring on non-viable U937 cells (Fig 3.3.9B) with the level of specific plg binding fluctuating over time. This result suggests that the mechanism of plg binding to non-viable HCT116 cells is different to that on U937 cells possibly due to the presence of different plg-Rs.

Carboxypeptidase B pretreatment of U937 cells reduced plg binding to non-viable cells (Fig 3.3.15B) clearly implicating proteins with C-terminal lysine residues as plg-Rs. Plg binding to non-viable cells was never completely abolished by treatment with carboxypeptidase B again suggesting that not all plg-Rs are susceptible to carboxypeptidase B (Miles et al 1991; Felez et al 1993). Plg had a relatively high affinity for non-viable cells compared to viable cells with apparent saturation occurring at a plg concentration of 1µM plg ($K_a = 0.5\mu M$) (Fig 3.3.16B).
The increase in plg-R expression on non-viable cells suggests that plg-Rs might play a role during cell death. In support of this observation are independent histochemical studies showing that plg binding is increased on dead or damaged epithelial cells (Green 1978; Burgos et al 1982; Jenkins et al 1983) and on necrotic breast carcinoma tissue (Burtin et al 1985). The dramatic increase in plg-R expression on non-viable cells could be the result of the direct upregulation of plg-Rs during death. Intracellular stores of uPAR exist in some cells that do not express cell-surface uPAR (Jardi et al 1996) and it is also possible that intracellular stores of plg-Rs exist which could become externalised during death. In support of this hypothesis is the fact that several plg-R candidates such as annexin II (Hajjar et al 1994), α-enolase (Miles et al 1991), actin (Dudani et al 1996) and G3PDH (Broder et al 1991) are intracellular proteins.

Another explanation for the increase in plg-Rs on non-viable cells is the exposure of cryptic C-terminal lysine residues during death. In the current study, trypsin treatment was shown to have varying effects on the level of specific plg binding to non-viable HCT116 cells. A relatively low concentration of trypsin (that would normally be used to passage cells) caused a reduction in specific plg binding to non-viable cells (Fig 3.3.8B) presumably by degrading plg-Rs (i.e lysine residues). In contrast, higher concentrations of trypsin caused an increase in specific plg binding (Fig 3.3.8B) which is consistent with several previous studies (Camacho et al 1989; Ouimet et al 1994; Gase et al 1996). This
observation suggests that trypsin can either degrade or generate plg-Rs on non-viable HCT116 cells depending on its concentration. These results illustrate that the level of plg-Rs (i.e. specific plg binding) on non-viable cells can be altered by proteolytic modification of the cell surface.

Plg-R expression on viable U937 cells could not be induced by PMA (Fig 3.3.17A), TNFα (Fig 3.3.18A), peroxide (3.3.19A) or serum starvation (Fig 3.3.20). PMA has previously been shown to increase plg-R expression on U937 cells (Felez et al 1990) and uPAR on a variety of cells (Stopelli et al 1985; Estreicher et al 1989; Picone et al 1989; Lund et al 1991; Wang et al 1994; Jardi et al 1996) however no evidence of any modulation of plg-R expression was observed on PMA-treated U937 cells. The observation made by Felez et al (1990) that PMA increases plg-R expression could have been due to PMA causing cell death thereby elevating plg binding. TNFα has been shown to increase uPAR expression on U937 cells (Lu et al 1988) as well as several other cell lines (Kirchheimer et al 1988). An interesting observation made during the current study was that TNFα appeared to decrease plg-R expression on non-viable U937 cells (Fig 3.3.18B). This effect of TNFα could have been due to the inhibition of pathways that increase plg-R expression on non-viable cells (such as the translocation of plg-Rs to the cell surface) or to the actual assembly/synthesis of the receptor/s. It is possible that other factors (e.g. cytokines, hormones etc.)
could induce plg-R expression on viable cells and would be worthy of further investigation.

In summary, the development of a novel flow cytometric plg binding assay has enabled the measurement of plg binding on specific subpopulations of cells (i.e viable and non-viable). This assay has proven to be a superior technique to $^{125}$I-plg binding assays due to the direct relationship between cell viability (PI uptake) and lysine-dependent (i.e specific) plg binding. It is envisaged that this assay could be easily adapted for the analysis of plg-R expression in relation to other cellular processes such as cell adhesion, differentiation, division, growth and migration. Furthermore these studies have shown that plg-R expression is dramatically elevated on non-viable cells and suggest that plg-Rs could play a role during cell death (either necrosis or apoptosis).
Chapter 4

Measurement of Plasminogen Receptors on Apoptotic Cells
4.1 INTRODUCTION

Observations described in the previous chapter showed that plg-Rs were highly elevated on non-viable cells which suggested that they might play a role during cell death. Two types of cell death are known to occur in the body. As described in Section 1.6, necrosis is a non-specific form of cell death which is induced by a sudden loss in cellular homeostasis and is characterised by a loss in membrane integrity and cellular lysis. The physiological outcome of necrosis is inflammation which often leads to tissue damage. In contrast, apoptosis is a specific, highly regulated form of cell death characterised by an ordered progression of morphological and biochemical changes. Apoptosis does not cause inflammation or tissue damage.

A subpopulation of non-viable cells could consist of apoptotic and/or necrotic cells. In order to assess the involvement of plg-Rs in either apoptosis or necrosis it is necessary to differentiate between the two subpopulations of non-viable cells. There are a variety of morphological and biochemical differences between apoptotic and necrotic cells that can be measured flow cytometrically (Darzynkiewicz et al 1992).

Several components of the PA system have been found to change during apoptosis. uPA mRNA is increased in apoptotic granulosa cells (Johnson et al 1997) with uPA antigen increased on apoptotic hepatocytes (Santonirugiu et al 1996). In contrast, other studies have shown that tPA, PAI-1, PAI-2 and uPAR
levels decrease during endothelial cell apoptosis (Zoellner et al 1998). Evidence is also accumulating that PAI-2 protects cells from certain apoptotic stimuli (Dickinson et al 1995; Gan et al 1995; Antalis et al 1998). Based on these studies and data generated in the previous chapter, it is logical to hypothesise that plg-Rs and other components of the PA system may be modulated during apoptosis.

The aim of this chapter was to: (1) measure plg binding on apoptotic U937 cells; (2) visualise plg binding to apoptotic cells by fluorescence microscopy; and (3) measure uPA and uPAR expression on apoptotic U937 cells.

4.2 MATERIALS AND METHODS

4.2.1 Materials

All cell culture reagents were obtained from Trace BioSciences (Sydney, NSW, Australia) except for FCS which was obtained from CSL, (Melbourne, Victoria, Australia). Human glu-plg was either purified according to the method of Grant (1990)] or was generously provided by American Diagnostica (ADI) (Greenwich CT, USA). Murine antibodies to human uPAR (#3936) and uPA (#394) were purchased from ADI (Greenwich CT, USA). FITC conjugated anti-mouse secondary antibody was obtained from Silenus (Melbourne, Victoria, Australia). NAP-10 columns and Lysine-Sepharose 4B were obtained from Amrad Pharmacia Biotech (Melbourne, Victoria, Australia). TA, BSA, PI, 7-
amino-actinomycin D (7-AAD), dimethyl sulphoxide (DMSO), hydroxylamine, cycloheximide (CHX), Hepes, and FITC were obtained from Sigma (Sydney, NSW, Australia). Annexin-V-fluos (FITC-labelled) annexin V was purchased from Boehringer Mannheim (Sydney, Australia). TNFα was generously provided by Darren Saunders (Department of Biological Sciences, University of Wollongong, Australia), FACS tubes were obtained from Becton Dickinson. 400ASA film was purchased from Kodak (Sydney, Australia).

4.2.2 Cell lines and cell culture

The human monocytic leukemic cell line U937 was obtained from the American Type Culture Collection and cultured in RPMI1640/10% FCS at 37°C in a humidified atmosphere of 5% CO₂.

4.2.3 Induction of apoptosis in U937 cells

U937 cells were routinely induced to undergo apoptosis using the protein synthesis inhibitor CHX. Cells were cultured in RPMI1640/10% pig depleted FCS overnight and fresh medium containing 10µg/ml CHX added for 24h at 37°C. In some experiments cells were induced to undergo apoptosis by culturing them for 24h in media containing 10ng/ml TNFα.
4.2.4 Differentiation of viable, apoptotic and late apoptotic/necrotic cells by nuclear staining and flow cytometry

Apoptotic cells were differentiated from viable and necrotic/late apoptotic cells by staining with 7-AAD (Schmid et al. 1994) or by supravital exposure to PI (Zamai et al. 1996). Cells were stained with either 5μg/ml 7-AAD or 10μg/ml PI (both in HBSS) for 15min/4°C and then analysed immediately by flow cytometry. 7-AAD and PI staining were measured using FL-3 and FL-2 detectors respectively. Apoptotic cells were gated as an intermediate staining population on FL-2 and FL-3 histograms. PI was routinely used in most experiments as it more clearly differentiated apoptotic cells from viable and necrotic cells than 7-AAD.

4.2.5 Measurement of internucleosomal DNA fragmentation (DNA laddering)

U937 cells (1x10⁶/ml) were cultured for 24h in 10μg/ml CHX and 4.5x10⁶ cells washed thrice in HBSS then quickly resuspended in 400μl pre-heated (50°C) 0.5M Tris (pH 9.0), 2mM EDTA, 10mM NaCl, 2% w/v SDS and 0.3mg/ml proteinase K. Cells were incubated overnight at 50°C then three successive phenol/chloroform extractions performed followed by one chloroform extraction. One tenth the volume of 3M sodium acetate and 2.5 volumes of ice cold 100% ethanol were added, mixed by inversion and incubated at -20°C overnight. DNA was pelleted by centrifugation at maximum speed in a microfuge and washed once with 80% v/v ethanol. DNA was pelleted once
again then air-dried for 1h/RT. DNA was resuspended in 50-100µl TE buffer [10mM Tris (pH 7.4), 1mM EDTA, 0.1% v/v Tween-20].

The concentration of DNA was determined using a microplate assay using a SpectroMax microplate reader and utilising Softmax™Pro (Molecular Devices, Sydney Australia). Water (100µL) was added to each well of a SPECTRAplate-disposable and background absorbance corrected. Each well was then ‘spiked’ with a 2µl DNA sample (2µl buffer for the control) and the amount of DNA determined using Softmax™Pro which utilises the formula: DNA/2µl sample = OD260nm x (100/0.006) ng/2µl sample. Each 10µg of DNA was treated with 1µl RNase for 30min/RT. DNA sample buffer was added and incubated at 60°C/5min. Samples were then placed immediately on ice until required. Each sample was loaded into a 1.6% agarose gel and electrophoresed at 60V for 4-6h. Gels were stained with ethidium bromide for 30min/RT then destained for 20min in water. Gels were visualised and photographed using a gel documentation system (NovaLine, Sydney, Australia).

4.2.6 Measurement of phosphatidylserine (PS) exposure

PS exposure was measured using FITC-annexin-V (Annexin-V-Fluos) according to the manufacturer’s instructions (Boehringer Mannheim, Sydney Australia). Briefly, cells were washed twice with HBSS and once with incubation buffer [10mM Hepes/NaOH (pH 7.4), 140mM NaCl, 5mM CaCl₂] then incubated in 50µl Annexin-V-Fluos labelling solution (20µl Annexin-V-
Fluos diluted in 1000µl incubation buffer) for 15min/4°C. Cells were diluted to 400µl with incubation buffer (containing 10µg/ml PI) and analysed by flow cytometry. The percentage of PS positive cells was calculated from FL-1 histograms.

4.2.7 Fluorescence microscopy

Cells were incubated with HBSS/0.1% BSA containing 50µg/ml FITC-plg ± 1mM TA for 45min at 4°C in the dark. PI (10 µg/ml) was added and the cells incubated for a further 15min at 4°C. Cells were washed thrice with HBSS and seeded into 96-well tissue culture plates and allowed to settle for 10min at room temperature. Cell associated fluorescence due to FITC-plg binding and PI uptake were viewed using a Leitz DMIL fluorescence microscope. Photographs were taken using a MPS45 photoautomat (Wild Heerbrugg, Switzerland) and 400ASA film (Kodak) with an exposure time of 1min.

4.2.8 Flow cytometric plg binding assay

Plg binding to apoptotic cells was measured as described in 3.2.4. with the exception that apoptotic cells were differentiated from viable and apoptotic cells as described in 4.2.4.

4.2.9 Measurement of uPAR on apoptotic cells

U937 cells were cultured for 24h in media containing 10µg/ml CHX. To remove any cell-surface uPA, cells were treated with 50mM glycine-HCl (pH 3)
containing 0.1 M NaCl for 3min/RT. This was neutralised with one-fifth the volume of 0.5M Hepes (pH 7.5) containing 0.1M NaCl. Cells were washed twice with HBSS (pH 7.4) and incubated with 20μg/ml murine anti-uPAR antibody (#3936) for 30 min/4°C. Control cells were incubated with 20μg/ml of an isotype control. Cells were washed thrice with HBSS and then incubated with a FITC conjugated anti-mouse immunoglobulin for 30min/4°C in the dark. Cells were washed thrice with HBSS and resuspended in 200μl of HBSS containing 10μg/ml PI for 15min in the dark at 4°C prior to flow cytometric analysis. Specific uPAR expression was calculated as the difference in the fluorescence of cells incubated with the isotype control and the fluorescence of cells incubated with anti-uPAR antibody.

4.2.10 Measurement of uPA on apoptotic cells

U937 cells were cultured for 24h in media containing 10μg/ml CHX, washed thrice with HBSS, then incubated with HBSS/0.1% BSA containing 20μg/ml murine anti-uPA antibody (#394) for 30min/4°C. Cells were washed thrice with HBSS and incubated with HBSS/0.1% BSA containing 20μg/ml FITC conjugated anti-mouse immunoglobulin for 30min/4°C in the dark. Control cells were incubated with 20μg/ml of an isotype control. Cells were washed thrice with HBSS and resuspended in 200μl of HBSS, containing 10μg/ml PI, and incubated for 15min in the dark/4°C. Specific uPA expression was calculated as the difference in the fluorescence of cells incubated with the isotype control and the fluorescence of cells incubated with anti-uPA antibody.
4.2.11 Data Analysis

All experimental measurements were performed in triplicate and each experiment repeated at least twice. Unless indicated all results are expressed as means ± 1 SEM. To test for statistical differences data were analysed using JMP statistical analysis software (v6). Statistically significant differences between two treatments were assessed using a student's t-test. Significant differences among greater than two treatments were assessed by one-way analysis of variance (ANOVA) with a Tukey-Kramer multiple comparison test. Differences between treatments were considered significant when p<0.05.

4.3 RESULTS

4.3.1 Apoptotic cells can be differentiated from viable and late apoptotic/necrotic cells using 7-AAD or PI staining

In order to measure plg-Rs on apoptotic cells it was necessary to differentiate them from viable and late apoptotic cells/necrotic cells. This was achieved by measuring 7-AAD or PI uptake by apoptotic cells using flow cytometry. When U937 cells were cultured in the presence of an apoptotic inducer such as CHX or TNFα and stained with 7-AAD, apoptotic cells were differentiated as an intermediate staining subpopulation of cells (Fig 4.3.1). Density plots of forward scatter (FSC) (size) versus 7-AAD uptake on control cells revealed two distinct subpopulations of cells: viable cells which were large (i.e. had high FSC) but did not stain with 7-AAD; and dead cells which were smaller (i.e. had low
FSC) and stained positive with 7-AAD (Fig 4.3.1A). The corresponding plot for CHX-treated U937 cells (Fig 4.3.1B) shows the presence of an intermediate staining population of apoptotic cells which were also smaller than both viable and necrotic cells. Apoptotic cells were also distinguishable on FL-3 histograms of 7-AAD uptake as an intermediate-staining population (Fig 4.3.1 D). This histogram also illustrates the overlap in staining of viable, apoptotic and necrotic cells which is one of the limitations to using 7-AAD to differentiate apoptotic cells.

A similar staining pattern was achieved using PI, however PI was observed to more clearly differentiate apoptotic cells from viable and necrotic cells (Fig 4.3.2). As illustrated in both the density plot of FSC versus PI uptake (Fig 4.3.2B) and the FL-2 histogram of PI uptake (Fig 4.3.2D), minimal overlap in PI staining of viable, apoptotic and necrotic cells was evident. In all subsequent experiments apoptotic cells were differentiated from viable and necrotic cells based solely on PI uptake (i.e. from FL-2 histograms) (Fig 2.3.2D).

4.3.2 CHX induces apoptosis in U937 cells

In order to establish an optimised model system for analysing plg binding during apoptosis, the induction of apoptosis with various concentrations of CHX or TNFα over time was investigated. The effect of each concentration of CHX or TNFα on the percentage of viable, apoptotic and dead cells was
Fig 4.3.1: Differentiation of apoptotic cells from viable and necrotic/late apoptotic cells by 7-AAD staining. A. Density plot of forward scatter (size) versus 7-AAD uptake of control cells showing viable and dead subpopulations of cells. B. Density plot of forward scatter (size) versus 7-AAD uptake of CHX-treated cells showing viable, apoptotic and dead subpopulations of cells. C. and D. 7-AAD histograms for control and CHX-treated cells showing viable, apoptotic and dead subpopulations of cells.
Fig 4.32: Differentiation of apoptotic cells from viable and necrotic/late apoptotic cells by PI staining. A. Density plot of forward scatter (size) versus PI uptake of control cells showing viable and dead subpopulations of cells. B. Density plot of forward scatter (size) versus PI uptake CHX-treated cells showing viable apoptotic and dead cells. C. and D. Histogram plots of PI uptake for control and CHX-treated cells showing viable, apoptotic and dead subpopulations of cells.
Fig 4.3.3: Induction of apoptosis in U937 cells over time with CHX or TNFα. U937 cells were cultured for 48h in the presence of 10, 50 and 100µg/ml CHX or 10, 50 and 100ng/ml TNFα. At various times the proportion of viable (A.), apoptotic (B.) and necrotic/late apoptotic (C.) cells was determined by PI staining. Each point represents the mean of triplicate determinations ± 1 SEM. The absence of error bars from certain points is due to the error being smaller than the graphical symbol.
measured, based on differential PI uptake, and indicated that CHX was a much better inducer of apoptosis than TNFα (Fig 4.3.3). The lowest concentration of CHX tested (10µg/ml) was found to induce the fastest rate of apoptosis over 48h as shown by the rate of disappearance of viable cells (Fig 4.3.3A) and the concomitant increase in the proportion of apoptotic (Fig 4.3.3B) and late apoptotic/necrotic cells (Fig 4.3.3C). Higher concentrations of CHX (i.e. 50 and 100µg/ml) were actually found to be less affective at inducing apoptosis over 48h (Fig 4.3.3). Low concentrations of TNFα (10ng/ml) appeared to induce low levels of necrosis as shown by the absence of any intermediate PI staining cells (Fig 4.3.3B) and a the presence of a small percentage of necrotic/dead cells (Fig 4.3.3C). Higher concentrations of TNFα (50 and 100ng/ml) induced low level apoptosis in U937 cells over 48h (Fig 4.3.3). Based on these data a 24h exposure to 10µg/ml CHX was chosen to routinely induce apoptosis in U937 cells as this resulted in the best spread of cells over each subpopulation (~ 55% viable, 25% apoptotic and 20% dead).

To confirm that U937 cells treated for 24h with 10µg/ml CHX were apoptotic, internucleosomal DNA fragmentation and PS exposure were measured as they are two of the distinguishing characteristics of apoptosis. Genomic DNA was extracted from control and CHX-treated U937 cells and analysed by agarose gel electrophoresis. A clear pattern of DNA laddering was evident in the CHX-treated cells compared to control cells where no laddering was observed (Fig 4.3.4). The presence of DNA laddering confirmed that CHX could induce
Fig 4.3.4: Agarose gel showing evidence of DNA laddering in CHX-treated U937 cells. U937 cells were cultured for 24h in the presence or absence of 10μg/ml CHX. Genomic DNA was extracted as described in section 4.2.5, and DNA laddering visualised by agarose gel electrophoresis. Lane 1 = DNA from U937 cells cultured in the absence of CHX; Lane 2 = DNA from U937 cells cultured in the presence of 10μg/ml CHX; Lane 3 = 100bp DNA ladder.
apoptosis in U937 cells. PS exposure was measured by measuring the binding of Annexin V to cells by flow cytometry. Dual-colour staining of control cells with Annexin-V-Fluos and PI revealed two distinct subpopulations of cells: these were viable (negative for both PI uptake and Annexin V binding) and dead (positive for both PI uptake and Annexin V binding) (Fig 4.3.5 A). CHX-treated U937 cells exhibited both viable and non-viable subpopulations of cells in addition to an early apoptotic subpopulation that was negative for PI uptake but positive for PS exposure (Fig 4.3.5 B). PS exposure could also be represented by FL-1 histograms of Annexin-V-Fluos binding (Fig 4.3.5). Control cells displayed a viable cell peak and a minor PS positive peak (apoptotic and dead cells). CHX-treated cells exhibited a significantly larger PS positive peak. These results confirm that some (~30%) CHX-treated U937 cells are apoptotic because they exhibit both DNA laddering and PS exposure whilst failing to take up PI.

4.3.3 Plg-Rs are elevated during apoptosis

Having confirmed that CHX can induce apoptosis in some U937 cells and that apoptotic cells can be differentiated from viable and dead cells by PI uptake, attention was focussed on measuring plg-Rs (i.e. plg binding) on apoptotic cells. Specific plg binding was found to be dramatically elevated on both apoptotic and necrotic cells while no binding was detected on viable cells (Fig 4.3.6). A student’s t-test indicated that there was no significant difference in plg-R expression between apoptotic and necrotic cells (t=2.01, df=4, P>0.05).
Fig 4.3.5: Phosphatidylserine (PS) exposure in CHX-treated U937 cells. U937 cells were cultured for 24h in the presence of 10µg/ml CHX and the level of PS exposure measured using Annexin-V-Fluos staining. A. Density plot of control cells stained with Annexin-V-Fluos and PI showing viable and dead subpopulations of cells. B. CHX-treated cells stained with Annexin-V-Fluos and PI showing the presence of an early apoptotic subpopulation of cells. C. FL-1 histogram showing the difference in PS exposure on control and CHX-treated U937 cells.
Fig 4.3.6: Plg-R expression on viable, apoptotic and late apoptotic/necrotic U937 cells. CHX-treated U937 cells were incubated with 0.5μM FITC-plg ± 1mM TA for 60min and the level of plg binding measured by flow cytometry. PI (10μg/ml) was added prior to flow cytometric analysis to differentiate apoptotic from viable and necrotic subpopulations of cells. Each bar represents the mean of triplicate determinations ± 1 SEM.
Fig 4.3.7: Visualisation of FITC-plg binding to CHX-treated U937 cells by fluorescence microscopy. CHX-treated U937 cells were incubated with 0.5μM FITC-plg ± 1mM TA for 60min and the level of plg binding visualised by fluorescence microscopy. Some samples were dually-stained with FITC-plg and PI (10μg/ml). A. control cells (bright field); B. CHX-treated cells (bright field); C. control cells stained with PI; D. CHX-treated cells stained with PI; E. Control cells stained with FITC-plg; F. CHX-treated cells stained with FITC-plg; G. Control cells stained with FITC-plg and TA; H. CHX-treated cells stained with FITC-plg and TA; I. Control stained with FITC-plg and PI; J. CHX-treated cells stained with FITC-plg and PI; K. Control cells stained with FITC-plg and TA and PI; L. CHX-treated cells stained with FITC-plg and TA and PI. (NB: Fields of view were selected and photographed which most clearly illustrated a particular result).
To visualise plg binding on apoptotic cells, CHX-treated cells were stained for both plg binding and viability (PI uptake) and examined by fluorescence microscopy. CHX-treated U937 cells exhibited the classic morphological changes characteristic of apoptosis when viewed under bright field (Fig 4.3.7B) compared to control cells (Fig 4.3.7A) including cell shrinkage, membrane blebbing, invagination of the plasma membrane and condensation of nuclear material. PI staining of CHX-treated cells clearly showed nuclear fragmentation and blebbing which are also characteristic of apoptosis (Fig 4.3.7D). Control cells failed to take up any PI except for the occasional (<5%) dead cell (Fig 4.3.7C).

Apoptotic cells bound high levels of FITC-plg (Fig 4.3.7F) whereas control cells exhibited negligible fluorescence (Fig 4.3.7E). Plg appeared to bind evenly over the surface of apoptotic cells and could be abolished with TA (Fig 4.3.7H) while control cell fluorescence remained negligible (Fig 4.3.7G). There appeared to be variation in the level of plg-Rs expressed on CHX-treated cells with some cells binding more FITC-plg than others (Fig 4.3.7F). Dual colour staining of apoptotic cells with FITC-plg and PI confirmed that high levels of plg binding were restricted to apoptotic cells (Fig 4.3.7J): the even green fluorescence associated with FITC-plg binding and the fragmented, globular PI-stained nuclei are clearly visible. Some CHX-treated cells bound high levels of FITC-plg but were negative for PI staining suggesting that elevated plg binding might precede PI uptake during cell death. Control cells were consistently negative for both FITC-plg binding and PI staining (Fig 4.3.7J). The presence of TA dramatically inhibited (~90%) plg binding to apoptotic cells that were doubly
stained with FITC-plg and PI (Fig 4.3.7L) while control cells exhibited negligible fluorescence (Fig 4.3.7K). These results confirmed that plg-Rs are elevated on apoptotic U937 cells and that CHX can induce apoptosis in U937 cells.

4.3.4 uPA and uPAR expression are altered on apoptotic U937 cells

Several independent studies have shown that both uPA and uPAR levels are altered during apoptosis and consequently experiments were undertaken to determine if uPA and uPAR levels, like plg-Rs, change during CHX-induced apoptosis in U937 cells.

CHX-treated U937 cells were probed for cell surface uPA with a murine anti-uPA antibody. All subpopulations of cells (i.e. viable, apoptotic and necrotic) expressed cell-surface uPA as assessed by flow cytometry (Fig 4.3.8). An approximately 30-fold increase in uPA levels occurred on apoptotic cells while necrotic cells exhibited a 24.3 ± 0.35% reduction in uPA expression relative to viable cells. A one-way ANOVA indicated that uPA levels were significantly higher on apoptotic cells compared to viable and necrotic cells (F=1195.26, df=2, p<0.05; Tukey-Kramer). Additionally, uPA expression on necrotic cells was significantly lower than that on viable cells (p<0.05). These observations provide evidence that cell-surface uPA is significantly increased during CHX-induced apoptosis in U937 cells.

CHX-treated U937 cells were then probed for uPAR by firstly pretreating cells
Fig 4.3.8: uPA expression on viable, apoptotic and late apoptotic/necrotic U937 cells. CHX-treated U937 cells were probed for uPA expression using MAb #394. PI (10μg/ml) was added prior to flow cytometric analysis to differentiate apoptotic from viable and necrotic subpopulations of cells. uPA expression was determined as the difference between the fluorescence of cells incubated with #394 and cells incubated with an isotype control. Each bar represents the mean of triplicate determinations ± 1 SEM.
Fig 4.3.9: Effect of acid pretreatment on the proportion of viable, apoptotic and late apoptotic/necrotic U937 cells. Control and CHX-treated U937 cells were incubated with glycine-HCl (pH 3) buffer for 3 min then neutralised with 0.5M Hepes/0.1M NaCl. Cells were stained with PI then analysed by flow cytometry to determine the proportion of viable, apoptotic and late apoptotic/necrotic cells. A. Control cells. B. CHX-treated cells. Each bar represents the mean of triplicate determinations ± 1 SEM.
with low pH (pH 3) buffer to dissociate any receptor-bound uPA that could interfere with uPAR detection. This treatment had a marked affect on the proportion of viable, apoptotic and dead cells (Fig 4.3.9). Acid elution of uPA from on control cells reduced the proportion of viable cells by 15.8 ± 0.80%, with a concomitant equivalent increase in the proportion of dead cells (Fig 4.3.9A). A student’s t-test confirmed that acid elution significantly reduced the proportion of viable cells (t=19.83, df=4, p<0.05) and increased the proportion of dead cells (t=25.00, df=4, p<0.05) compared to their respective controls. Acid elution of uPA from CHX-treated cells almost doubled the proportion of apoptotic cells (Fig 4.3.9B) which was also determined to be statistically significant when compared to the control (t=12.86, df=4, P<0.05). Collectively these data show that utilising a low pH buffer to remove receptor bound uPA has a significant impact on cell viability.

When CHX-treated cells were probed with a murine anti-uPAR antibody, uPAR was found to be expressed on all subpopulations of cells (viable, apoptotic and dead) with apoptotic and necrotic cells displaying a 5-fold and 2.7-fold decrease in uPAR expression compared to viable cells (Fig 4.3.10). A one-way ANOVA indicated that uPAR expression was significantly different between all subpopulations of cells (i.e viable, apoptotic and necrotic cells) (F=2844.41, df=2, p<0.05; Tukey-Kramer). These observations provide evidence that uPAR expression is altered during CHX-induced apoptosis in U937 cells.
Fig 4.3.10: uPAR expression on viable, apoptotic and late apoptotic/necrotic U937 cells. CHX-treated U937 cells were probed for uPAR expression using MAb #3936. PI (10\(\mu\)g/ml) was added prior to flow cytometric analysis to differentiate apoptotic from viable and necrotic subpopulations of cells. uPAR expression was determined as the difference between the fluorescence of cells incubated with #3936 and cells incubated with an isotype control. Each bar represents the mean of triplicate determinations \(\pm 1\) SEM. The absence of error bars indicates that the error was smaller than the graphical symbol.
4.4 DISCUSSION

Although previous studies have indicated that plg binding is increased on necrotic cells (Green 1978; Burtin et al 1985) no studies have directly measured plg binding during apoptosis. In the current investigation both plg-Rs (i.e. specific plg binding) and cell-surface uPA were increased on apoptotic U937 cells which suggests that PA may play a role in apoptosis.

Numerous studies have utilised U937 cells to examine both plg (Plow et al 1986; Felez et al 1990; Edelberg et al 1990; Gonzalez-Gronow et al 1991; Felez et al 1993) and uPA binding (Cubellis et al 1986; Plow et al 1986; Felez et al 1990). This coupled with the fact that U937 cells have been used extensively to study apoptosis justified the use of this cell line to investigate the expression of components of the PA system during apoptosis.

U937 cells were induced to undergo apoptosis using the protein synthesis inhibitor CHX which has previously been shown to cause apoptosis in U937 cells (Kochi & Collier 1993; Bicknell et al 1994; Dini et al 1996). An advantage of using CHX is that any cellular changes that occur during apoptosis are not due to new protein synthesis. Microscopic examination of cells (Fig 4.3.7) in addition to the measurement of PI/7-AAD uptake (Fig 4.3.1, 2 & 3), DNA fragmentation (Fig 4.3.4) and PS exposure (Fig 4.3.5) confirmed that CHX could induce apoptosis in U937 cells. TNFα also appeared to induce apoptosis in U937 cells but was much less potent than CHX (Fig 4.3.3).
Flow cytometric analysis revealed that plg-Rs (i.e. specific plg binding) were dramatically elevated on apoptotic U937 cells (Fig 4.3.6). The level of plg-Rs on apoptotic cells was similar that on late apoptotic/dead cells which suggests that increased plg-R expression is maintained throughout the apoptotic process. These results are the first reported evidence that plg-Rs (i.e specific plg binding) are increased during apoptosis. Dual-colour fluorescence microscopy of apoptotic cells confirmed that elevated plg binding was predominantly restricted to apoptotic U937 cells (Fig 4.3.7), however there was some evidence that plg-Rs were increased on some PI negative cells (Fig 4.3.7J). This later result suggests that plg-R expression may precede changes in cells relative permeability to PI during apoptosis.

Other components of the PA cascade, namely uPA and uPAR were also found to be modulated during CHX-induced apoptosis. A large increase in cell-surface uPA was measured on apoptotic U937 cells with uPA levels dropping sharply on late apoptotic/necrotic cells (Fig 4.3.8). This transient increase in cell-surface uPA contrasts with the stable increase in plg-Rs during apoptosis. The increase in uPA during apoptosis is supported by the recent finding that uPA mRNA is increased in granulosa cells undergoing apoptosis (Johnson et al 1997) and on apoptotic dysplastic hepatocytes (Santonirugiu et al 1996). TNFα, which can induce either apoptosis or necrosis (Laster et al 1989), has also been shown to stimulate uPA production by endothelial cells (Niedbala & Stein 1991) and uPA mRNA in ovarian cancer cells (Simonitsch & Krupitza 1998).
uPAR expression was decreased during apoptosis with low levels present on both apoptotic and late apoptotic/necrotic cells (Fig 4.3.10). It is difficult to reconcile the fact that cell-surface uPA increased during CHX-induced apoptosis without a similar increase in uPAR. However, a recent study (Zoellner et al 1998) reported a decrease in uPAR on apoptotic endothelial cells while uPA levels remained unchanged. It is possible that uPA may be bound to sites other than uPAR on apoptotic cells such as gangliosides which have been shown to bind both uPA and plg (Miles et al 1989). The uPAR data needs to be interpreted with caution as the acid wash that was used to remove any uPA-occupied uPAR markedly affected the proportion of viable, apoptotic and dead cells (Fig 4.3.9).

Collectively these results show, for the first time, that both plg-Rs (i.e. specific plg binding) and cell-surface uPA are dramatically elevated on U937 cells undergoing CHX-induced apoptosis. This begs the question as to whether PA is similarly elevated on apoptotic cells considering that the two components necessary for activation (i.e plg and uPA) are present. In order to test this hypothesis it would be necessary to measure plg activation (i.e. plm) on apoptotic cells. As this has not been done before it would require the development of a flow cytometric PA assay.
Chapter 5

Development of a Flow Cytometric Plasminogen Activation Assay
5.1 INTRODUCTION

Results from the previous chapter indicated that plg-Rs and cell surface uPA were increased on apoptotic U937 cells. Although this suggested that components of the PA system might play a role in CHX-mediated apoptosis in U937 cells, in order to give these results any physiological credence it is necessary to measure PA on apoptotic cells. If plm were found to be generated on apoptotic cells then this would support the hypothesis that PA plays a role in apoptosis.

The conventional method for measuring cellular PA is to analyse plm formation (i.e. plm activity) using a chromogenic or fluorogenic plm substrate. Examples of commonly used chromogenic plm substrates are H-D-valyl-L-leucyl-L-lysine-p-nitroanilide dihydrochloride (S-2251) and H-D-norleucyl-hexahyrotyrosyl-lysine-p-nitroanilide diacetate salt (Spectrozyme PL). These substrates are cleaved by plm to release p-nitroanilide which is then detected spectrophotometrically. The major limitation to chromo/fluorogenic plm assays, like $^{125}$I-plg binding assays, is they do not allow the measurement of plm on specific subpopulations of cells. There is clearly a need then to develop alternative cellular plm assays which enable the measurement of PA on different subpopulations of cells.

The trypsin-like serine protease inhibitor aprotinin has previously been labelled with FITC and used in non-cytometric plm assays with data generated
shown to be equivalent to that generated using S-2251 (Ellis et al 1987). Additionally, the relatively small size of aprotinin (6kDa) make it a suitable probe for cell-surface plm (i.e. PA).

The aim of this chapter was to develop a flow cytometric plg activation assay, using FITC-aprotinin, to measure plm formation on the surface of viable, apoptotic and late apoptotic/necrotic U937 cells.

5.2 MATERIALS AND METHODS

5.2.1 Materials

All cell culture reagents were obtained from Trace BioSciences (Sydney, NSW, Australia) except for FCS which was obtained from CSL, (Melbourne, Victoria, Australia). Human glu-plg was either purified according to the method of Grant (1990)] or was generously provided by American Diagnostica (ADI) (Greenwich CT, USA). NAP-10 columns and Lysine-Sepharose 4B were obtained from Amrad Pharmacia Biotech (Melbourne, Victoria, Australia). TA, BSA, PI, dimethyl sulphoxide (DMSO), hydroxylamine, cycloheximide (CHX), Heps, and FITC were obtained from Sigma (Sydney, NSW, Australia). The specific uPA inhibitor glu-gly-arg-chloromethylketone (EGR-CMK) and the specific plm inhibitor valine-phenylalanine-lysine-chloromethylketone (VFK-CMK) were obtained from Calbiochem-Novachem (Sydney, NSW, Australia).
Human PAI-2 and Spectrozyme™ PL were obtained commercially (ADI, Greenwich CT, USA).

5.2.2 Cell lines and cell culture

The human monocytic leukemic cell line U937 was obtained from the American Type Culture Collection and cultured in RPMI1640/10% FCS at 37°C in a humidified atmosphere of 5% CO₂.

5.2.3 Induction of apoptosis with CHX

U937 were induced to undergo apoptosis with CHX as described in 4.2.3.

5.2.4 Labelling of aprotinin with FITC

Aprotinin (5 mg/ml) was dissolved in 0.1 M carbonate buffer (pH 9) and a 50-fold molar excess of FITC added after being dissolved in DMSO. Aprotinin was mixed for 16h at 4°C in the dark and subsequently treated with 0.01% hydroxylamine for 30min/4°C to remove all labile FITC:aprotinin bonds. Unincorporated FITC was separated from FITC-aprotinin by gel filtration through a NAP-10 column using Hank's balanced salt solution (HBSS) containing 20mM Hepes, 1mM CaCl₂, 1mM MgCl₂ (pH 7.4). Typically, 2-4 FITC molecules were bound to each aprotinin molecule. FITC-aprotinin was aliquoted and stored at -80°C until required. The number of molecules of FITC conjugated to each aprotinin molecule was determined using the following formula:
FITC/aprotinin molecule = $A_{495\text{nm}} \times \text{dilution factor} \times 6,000$

68,000 mg/ml aprotinin

where $A_{495\text{nm}}$ is the absorbance maxima of FITC; 60,000 is the molar extinction coefficient (cm$^{-1}$M$^{-1}$) of conjugated fluorescein and 6,000 is the molecular weight of aprotinin.

5.2.5 Spectrozyme™ PL microplate assay

Spectrozyme™ PL was reconstituted to a 5mM stock solution in MilliQ water according to the Manufacturer’s instructions (ADI, Greenwich CT, USA), aliquoted and stored at -80°C until required. 0.5μM plm ± equivalent molar concentrations of aprotinin or FITC-aprotinin in PBS (pH 7.4) (200μl total volume) was incubated for 30min/RT in triplicate wells of a microplate. Spectrozyme™ PL was added to a final concentration of 0.5mM and the plate incubated for a further 45min/37°C. The plate was read at 405nm using a SpectroMax microplate reader (Molecular Devices, Sydney, Australia). Control absorbances (wells containing only Spectrozyme™ PL in PBS) were subtracted from all samples.

5.2.6 Flow cytometric PA assay

U937 cells were cultured in RPMI1640/10% plg-depleted FCS for 24h at 37°C, washed thrice with HBSS and resuspended in HBSS/0.1% BSA ± 2μM plg. Cells were incubated for 60min at 37°C, washed thrice with ice cold HBSS then resuspended in HBSS/0.1% BSA containing 2μM FITC-aprotinin ± 100-fold
molar excess of unlabelled aprotinin, and incubated for 45 min at 4°C in the dark. Cells were washed thrice in HBSS and resuspended in HBSS containing 10μg/ml PI. Specific FITC-aprotinin binding (indicating the presence of cell-surface plm) was calculated as the difference between the fluorescence of cells incubated solely with FITC-aprotinin (total binding) and cells incubated with FITC-aprotinin and 100-fold molar excess of unlabelled aprotinin (non-specific binding). In all experiments, preincubation of cells with plg was required for specific FITC-aprotinin binding to occur.

5.2.7 Data analysis

All experimental measurements were performed in triplicate and repeated at least twice. Unless indicated all results are expressed as means ± 1 SEM. To test for statistical differences data were analysed as described in section 4.2.11.

5.3 RESULTS

5.3.1 Cytotoxicity of Spectrozyme™ PL

Cellular PA is typically measured using artificial plm substrates with little consideration for the potential cytotoxicity of these compounds. To investigate the toxicity of Spectrozyme™ PL, U937 cells were incubated with 0-0.5mM Spectrozyme™ PL for up to 2h and the proportion of viable and non-viable cells assessed flow cytometrically (i.e. by measuring PI uptake). The effect of Spectrozyme™ PL on cell viability was striking with 0.1mM Spectrozyme™ PL
increasing the proportion of non-viable cells by approximately 3-fold, 0.25mM increasing the proportion of non-viable cells 16-fold and 0.5mM increasing the proportion of non-viable cells 20-fold (Fig 5.3.1B). In all samples the increase in non-viable cells was mirrored by an equivalent decrease in viable cells (Fig 5.3.1A). These results indicate that Spectrozyme™ PL is highly cytotoxic and is thus unsuitable for any cellular-based PA assays where cell death is being examined.

5.3.2 FITC-aprotinin can be used to detect cell-surface plm

Aprotinin was labelled with FITC according to an established procedure (Goding 1976) with 2.5 ± 1.7 (N=4) molecules of FITC conjugated to each molecule of aprotinin. This level of conjugation is a magnitude higher than that previously reported (Ellis et al 1987). The capacity of FITC-aprotinin to inactivate plm was investigated utilising the Spectrozyme™ PL assay. FITC-aprotinin was equally affective as unlabelled aprotinin at inhibiting plm (Fig 5.3.2). This was confirmed using a student’s t-test which indicated that there was no significant difference in the inhibitory ability of aprotinin or FITC-aprotinin (t=0.96, df=4, p>0.05). These results indicated that conjugating FITC to aprotinin does not significantly alter its plm-inhibitory activity.

Feasibility studies were undertaken to assess the use of FITC-aprotinin in detecting cell-surface plm (i.e PA) by flow cytometry. Apoptosis was induced in U937 cells with CHX and the amount of total, non-specific and specific FITC-
Fig 5.3.1: Effect of Spectrozyme™ PL on the viability of U937 cells. U937 cells were incubated with 0, 0.1, 0.25 and 0.5mM Spectrozyme™ PL and at various times up to 2h the proportion of viable and non-viable cells determined flow cytometrically by measuring PI uptake. A. Effect of various concentrations of Spectrozyme™ PL on the % of viable U937 cells over time. B. Effect of various concentrations of Spectrozyme™ PL on the % of non-viable U937 cells over time. Each point represents the mean of triplicate measurements ± 1 SEM. The absence of error bars indicates the error was smaller then the graphical symbol.
Fig 5.3.2: The effect of FITC conjugation on the ability of aprotinin to inhibit plm. 0.5μM plm ± 0.5μM aprotinin or FITC-aprotinin was incubated for 30min at RT in triplicate wells of a microplate. Spectrozyme™ PL was added to a final concentration of 0.5mM and the plate incubated for a further 45min at 37°C. The plate was read at 405nm using a SpectroMax microplate reader (Molecular Devices, Sydney, Australia). Control absorbances (wells containing only Spectrozyme™ PL in PBS) were subtracted from all samples. Each bar represents the mean of triplicate measurements ± 1 SEM. The absence of error bars indicates the error was smaller than the graphical symbol.
aprotinin binding (indicating the presence of active trypsin-like serine proteases) measured by flow cytometry. To determine whether any endogenous cell-surface plm (or other trypsin-like proteases) was present, cells were either preincubated with or without plg. Additionally the experiment was performed at 4 and 37°C to assess the temperature dependence of PA.

Viable U937 cells did not bind any FITC-aprotinin either specifically or non-specifically at 4 or 37°C regardless of whether cells had been pretreated with plg (Fig 5.3.3). These results indicated that PA does not occur on viable U937 cells and that no endogenous trypsin-like proteolytic activity is present on these cells. Apoptotic U937 cells (i.e. cells taking up an intermediate level of PI) that had not been pretreated with 2μM plg at both 4 and 37°C failed to specifically bind FITC-aprotinin indicating the absence of plm and hence PA on the cell surface (Fig 5.3.4). In contrast, apoptotic U937 cells that had been pretreated with 2μM plg for 60min could specifically bind FITC-aprotinin (as shown by the inhibition of FITC-aprotinin binding with a 25-fold molar excess of unlabelled aprotinin) at both 4 and 37°C (Fig 5.3.4). These results indicate that although no endogenous trypsin-like activity is found on apoptotic cells exposure to physiological concentrations of plg can lead to PA at 4 and 37°C. These results also indicate that pretreatment of cells with plg is an absolute requirement for PA to occur. Virtually no specific FITC-aprotinin binding was detected on necrotic cells when U937 cells were preincubated with plg. Additionally necrotic cells possessed no endogenous trypsin-like activity (Fig 5.3.5).
Fig 5.3.3: Histogram plots showing the absence of FITC-aprotinin binding to viable U937 cells. U937 cells cultured in plg-free conditions for 24h were treated with or without 2μM plg for 1h at 4 or 37°C then incubated with 2μM FITC-aprotinin + a 25-fold molar excess of unlabelled aprotinin for 45min at 4°C. PI (10μg/ml) was added prior to flow cytometric analysis to differentiate viable from apoptotic and necrotic/late apoptotic cells. A. Viable cells incubated in the absence of plg at 4°C. B. Viable cells incubated in the absence of plg at 37°C. C. Viable cells incubated with 2μM plg at 4°C. D. Viable cells incubated with 2μM plg at 37°C. (NB: specific and non-specific FITC-aprotinin binding overlap).
cells + FITC-aprotinin (total binding)

- cells + FITC-aprotinin + 25-fold excess unlabelled aprotinin (non-specific binding)

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**Fig 5.3.4: Histogram plots showing FITC-aprotinin binding to apoptotic U937 cells.**

U937 cells were cultured in plg-free conditions in the presence of 10μg/ml CHX for 24h then treated with or without 2μM plg for 1h at 4 or 37°C then incubated with 2μM FITC-aprotinin + a 25-fold molar excess of unlabelled aprotinin for 45min at 4°C. PI (10μg/ml) was added prior to flow cytometric analysis to differentiate apoptotic from viable and necrotic/late apoptotic cells. A. Apoptotic cells incubated in the absence of plg at 4°C. B. Apoptotic cells incubated in the absence of plg at 37°C. C. Apoptotic cells incubated with 2μM plg at 4°C. D. Apoptotic cells incubated with 2μM plg at 37°C. The difference between total FITC-aprotinin binding (dark line) and non-specific FITC-aprotinin binding (light line) indicates specific FITC-aprotinin binding (i.e pm).
Fig 5.3.5: Histogram plots showing FITC-aprotinin binding to necrotic U937 cells. U937 cells were cultured in plg-free conditions in the presence of 10μg/ml CHX for 24h then treated with or without 2μM plg for 1h at 4 or 37°C then incubated with 2μM FITC-aprotinin ± a 25-fold molar excess of unlabelled aprotinin for 45min at 4°C. PI (10μg/ml) was added prior to flow cytometric analysis to differentiate apoptotic from viable and necrotic/late apoptotic cells. A. Necrotic cells incubated in the absence of plg at 4°C. B. Necrotic cells incubated in the absence of plg at 37°C. C. Necrotic cells incubated with 2μM plg at 4°C. D. Necrotic cells incubated with 2μM plg at 37°C. The difference between total FITC-aprotinin binding (dark line) and non-specific FITC-aprotinin binding (light line) indicates specific FITC-aprotinin binding (i.e plm).
The comparative activation of plg on viable, apoptotic and dead cells is presented in Fig 5.3.6. The top density plot of PI uptake (cell viability) versus FITC-aprotinin binding (PA) shows viable, apoptotic and dead cells - apoptotic cells taking up an intermediate level of PI. Apoptotic cells bound the highest level of FITC-aprotinin, followed by dead cells and viable cells bound none. The presence of 100-fold molar excess of unlabelled aprotinin significantly reduced FITC-aprotinin binding to both apoptotic and dead cells. These results confirm that PA is predominantly restricted to apoptotic U937 cells.

5.3.3 Optimisation of the flow cytometric PA assay

With preliminary experiments indicating that PA (i.e plm generation) could be measured by flow cytometry and that PA seemed to be predominantly restricted to apoptotic U937 cells, studies were undertaken to optimise the flow cytometric PA assay. For example, the time necessary for FITC-aprotinin binding to reach equilibrium was determined by incubating CHX-treated U937 cells with FITC-aprotinin for various times up to 90min. The binding of FITC-aprotinin to apoptotic U937 cells (i.e. cells taking up an intermediate level of PI) was relatively fast with the majority of binding occurring within the first 5min (Fig 5.3.7). There was a gradual increase in FITC-aprotinin binding from 5min until a plateau was reached at approximately 45min. Based on these results cells were routinely incubated with FITC-aprotinin for 45min.

The optimal concentration of FITC-aprotinin was determined by incubating
Fig 5.3.6: Density plots showing simultaneous measurement of PA on viable, apoptotic and necrotic subpopulations of U937 cells. CHX-treated U937 cells were treated with 2µM plg for 1h then probed for plm with FITC-aprotinin + 100-fold molar excess of unlabelled aprotinin. PI (10µg/ml) was added prior to flow cytometric analysis to differentiate viable, apoptotic and necrotic/late apoptotic subpopulations of cells. A. Density plot of PI uptake versus FITC-aprotinin binding for cells incubated solely with FITC-aprotinin (total binding). B. Density plot of PI uptake versus FITC-aprotinin binding for cells incubated with FITC-aprotinin and 100-fold molar excess of unlabelled aprotinin (non-specific binding). The difference between total FITC-aprotinin binding (A) and non-specific FITC-aprotinin binding (B) is a measure of specific FITC-aprotinin binding (i.e. plm formation).
Fig 5.3.7: Timecourse of FITC-aprotinin binding to apoptotic U937 cells. CHX-treated U937 cells were pretreated with 2μM plg for 1h then incubated with 2μM FITC-aprotinin for up to 90min. At various times the level of FITC-aprotinin binding to apoptotic cells was measured by flow cytometry. Each point represents the mean of triplicate measurements ± 1 SEM. The absence of error bars indicates the error was smaller than the graphical symbol.
CHX-treated U937 cells, that had been pretreated with 2μM plg, with increasing concentrations of FITC-aprotinin. FITC-aprotinin bound to apoptotic cells in a linear fashion up to a concentration of 2μM whereupon a plateau was reached (Fig 5.3.8). Assuming that all of the plg added to cells was converted to plm, these results suggest that the stoichiometry of FITC-aprotinin binding to cell-surface plm was a 1:1 interaction. The specificity of FITC-aprotinin binding to apoptotic cells was determined by inhibiting FITC-aprotinin binding to apoptotic cells with increasing concentrations of unlabelled aprotinin.

FITC-aprotinin binding to apoptotic cells was highly specific as the presence of 0-100 fold molar excess of unlabelled aprotinin caused a progressive decrease in FITC-aprotinin binding (Fig 5.3.9). For example, a 1-fold molar excess of unlabelled aprotinin reduced FITC-aprotinin binding to apoptotic cells by $73 \pm 0.58\%$ (N=3). In all subsequent experiments 100-fold molar excess of unlabelled aprotinin was used to assess non-specific FITC-aprotinin binding as it maximally inhibited FITC-aprotinin binding [$90 \pm 0.21\%$ (N=3)].

To determine the kinetics of cell-surface PA on apoptotic U937 cells CHX-treated cells were incubated with 2μM plg at 37°C and cell-surface plm measured at various times over 1h. Plm formation appeared to be rapid with near maximal levels of specific FITC-aprotinin binding detected within 10min (Fig 5.3.10). The level of cell-surface plm appeared to remain relatively constant over 60min with some slight fluctuations observed between 10 and
Fig 5.3.8: Concentration curve of FITC-aprotinin binding to apoptotic U937 cells. CHX-treated U937 cells were pretreated with 2μM plg for 1h then incubated with various concentrations of FITC-aprotinin up to 5μM for 45min. PI (10μg/ml) was added prior to flow cytometric analysis to differentiate apoptotic from viable and necrotic/late apoptotic cells. Each point represents the mean of triplicate measurements ± 1 SEM. The absence of error bars indicates the error was smaller than the graphical symbol.
Fig 5.3.9: Inhibition of FITC-aprotinin binding to apoptotic U937 cells by increasing molar excesses of unlabelled aprotinin. CHX-treated U937 cells were incubated with 2μM plg at 37°C for 60min then probed for cell-surface plm with 2μM FITC-aprotinin ± 0-100-fold molar excess of unlabelled aprotinin. PI (10μg/ml) was added prior to flow cytometric analysis to differentiate apoptotic from viable and necrotic/late apoptotic cells. Each point represents the mean of triplicate measurements + 1 SEM. The absence of error bars on certain points indicates the error was smaller than the graphical symbol.
Fig 5.3.10: Kinetics of cell-surface plm formation on U937 cells. CHX-treated U937 cells were incubated with 2μM plg and at various times over 1h the level of plm formation measured. PI (10μg/ml) was added prior to flow cytometric analysis to differentiate apoptotic from viable and necrotic/late apoptotic cells. Specific FITC-aprotinin binding (indicating the presence of active plm) was calculated as the difference in the fluorescence of cells incubated solely with FITC-aprotinin (total binding) and residual fluorescence of cells incubated with FITC-aprotinin and 100-fold molar excess of unlabelled aprotinin (non-specific binding). Each point represents the mean of triplicate measurements ± 1 SEM. The absence of error bars indicates the error was smaller than the graphical symbol.
60min. These results show that exogenously added plg is rapidly converted to plm on the surface of apoptotic U937 cells.

The time over which plm remains active or bound to the cell surface could indicate the time over which this serine protease would be able to participate in extracellular proteolytic events. To determine the stability of cell-surface plm once generated, CHX-treated U937 cells were incubated with plg and at various times probed for plm. Plm could be detected on the surface of apoptotic cells with FITC-aprotinin both immediately and for up to 2h after activation had occurred. From 1h post activation the level of plm appeared to begin to steadily decrease (Fig 5.3.11). These results suggest that plm generated on the surface of apoptotic U937 cells is relatively stable and remains active for several hours.

5.3.4 PA can be blocked by inhibiting plg binding

The presence of plg-Rs and thus specific plg binding is considered to be essential for PA to proceed. Logically then PA could be stopped by inhibiting plg binding. CHX-treated U937 cells were preincubated with plg and 1 or 10mM TA or 10% (v/v) FCS and the level of specific FITC-aprotinin binding (i.e. PA) measured. PA was blocked with 1 and 10mM TA by $63 \pm 11.5\%$ and $96 \pm 1.2\%$ (N=3) respectively (Fig 5.3.12). 10% (v/v) FCS (expected to contain bovine plg) was also found to inhibit PA by $48 \pm 17\%$ (N=3) (Fig 5.3.12). These results show that inhibiting plg binding can block PA and supports the essential role for plg-Rs during PA.
Fig 5.3.11: Stability of plm activity generated on the surface of apoptotic U937 cells. CHX-treated U937 cells were incubated with 2μM plg for 1h, washed and then incubated at 37°C in HBSS for 2h. At various times the level of active cell-surface plm was measured flow cytometrically using FITC-aprotinin. PI (10μg/ml) was added prior to flow cytometric analysis to differentiate apoptotic from viable and necrotic/late apoptotic cells. Specific FITC-aprotinin binding (indicating the presence of active plm) was calculated as the difference in the fluorescence of cells incubated solely with FITC-aprotinin (total binding) and residual fluorescence of cells incubated with FITC-aprotinin and 100-fold molar excess of unlabelled aprotinin (non-specific binding). Each point represents the mean of triplicate measurements ± 1 SEM. The absence of error bars indicates the error was smaller than the graphical symbol.
Fig 5.3.12: Inhibition of cell-surface PA on apoptotic U937 cells with TA or FCS. CHX-treated U937 cells were incubated with 2μM plg in the presence of 1 or 10mM TA or with 10% (v/v) FCS. The level of active cell-surface plm was measured flow cytometrically using FITC-aprotinin. PI (10μg/ml) was added prior to flow cytometric analysis to differentiate apoptotic from viable and necrotic/late apoptotic cells. Specific FITC-aprotinin binding (indicating the presence of active plm) was calculated as the difference in the fluorescence of cells incubated solely with FITC-aprotinin (total binding) and residual fluorescence of cells incubated with FITC-aprotinin and 100-fold molar excess of unlabelled aprotinin (non-specific binding). Each point represents the mean of triplicate measurements ± 1 SEM. The absence of error bars indicates the error was smaller than the graphical symbol.
5.3.5 Inhibiting uPA can block PA

As uPA was previously shown to be highly elevated on apoptotic U937 cells (Fig 4.3.8) experiments were undertaken to determine if PA on apoptotic U937 cells could be blocked with uPA inhibitors. When CHX-treated U937 cells were incubated with plg in the presence of PAI-2 or the specific uPA inhibitor EGR-CMK, PA (i.e. specific FITC-aprotinin binding) was reduced by $21 \pm 1.4\%$ and $65 \pm 2.0\%$ respectively (N=3) (Fig 5.3.13). A student’s t-test indicated that EGR-CMK was significantly better at inhibiting PA than PAI-2 ($t=5.09$, df=4, p<0.05), however neither were observed to totally inhibit PA. These results indicate that in this system, uPA plays an important role in the activation of plg on the surface of apoptotic U937 cells.

5.3.6 FITC-aprotinin binding to apoptotic U937 cells can be inhibited with a specific plm inhibitor.

Observations thus far indicated that the presence of plg was essential for plm generation (specific FITC-aprotinin binding) on the surface of apoptotic U937 cells (Fig 5.3.4) and also that uPA appeared to be crucial for this process to occur (Fig 5.3.13). In order to eliminate the possibility that other trypsin-like serine proteases were involved in PA on apoptotic U937 cells, CHX-treated U937 cells were incubated with plg and then probed with FITC-aprotinin with or without the specific plm inhibitor VFK-CMK (1mM). FITC-aprotinin binding to apoptotic U937 cells was completely blocked with 1mM VFK-CMK (Fig 5.3.14). This experiment indicates that FITC-aprotinin binding to apoptotic U937 cells is
Fig 5.3.13: Inhibition of cell-surface PA with EGR-CMK or PAI-2. CHX-treated U937 cells were incubated with 2μM plg in the presence of 1mM EGR-CMK or 1nM PAI-2 for 1h at 37°C. The level of active cell-surface plm was measured flow cytometrically using FITC-aprotinin. PI (10μg/ml) was added prior to flow cytometric analysis to differentiate apoptotic from viable and necrotic/late apoptotic cells. Specific FITC-aprotinin binding (indicating the presence of active plm) was calculated as the difference in the fluorescence of cells incubated solely with FITC-aprotinin (total binding) and residual fluorescence of cells incubated with FITC-aprotinin and 100-fold molar excess of unlabelled aprotinin (non-specific binding). Each bar represents the mean of triplicate measurements ± 1 SEM. The absence of error bars indicates the error was smaller than the graphical symbol.
Fig 5.3.14: Inhibition of FITC-aprotinin binding to apoptotic and necrotic/dead U937 cells with the specific plm inhibitor VFK-CMK. CHX-treated U937 cells were incubated with 2μM plg then probed for cell-surface plm with FITC-aprotinin ± 1mM VFK-CMK. PI (10μg/ml) was added prior to flow cytometric analysis to differentiate apoptotic from viable and necrotic/late apoptotic cells. Each bar represents the mean of triplicate measurements ± 1 SEM. The absence of error bars indicates the error was smaller than the graphical symbol.
solely due to the presence of cell-surface plm and not due or derived from some other trypsin-like serine protease.

5.3.7 PA on viable, apoptotic and dead cells

With the flow cytometric PA assay developed on U937 cells attention was then directed toward actually measuring PA on viable, apoptotic and dead subpopulations of U937 cells. As shown in Fig 5.3.15, plm formation was dramatically elevated on apoptotic U937 cells, low levels of plm were detected on dead cells and none on viable cells. A one-way ANOVA confirmed that plm generation was significantly different among all subpopulations of cells (i.e viable, apoptotic and dead) (F=3689.42, df=2, p<0.05). These results lend support to the hypothesis that PA plays a role during CHX-induced apoptosis in U937 cells.

5.4 DISCUSSION

Numerous studies have utilised chromogenic plm substrates to measure PA on a variety of both pro and eukaryotic cells. A major disadvantage of using these chromogenic-based assays is that PA cannot be measured on specific subpopulations of cells. Additionally, a commonly used plm substrate, Spectrozyme™ PL, has been shown in this study to be highly cytotoxic at concentrations routinely used to measure cell-generated plm (Fig 5.3.1). In the current study, a novel flow cytometric PA assay was developed which utilised FITC-aprotinin to measure plm on specific subpopulations of cells (i.e viable,
Fig 5.3.15: Measurement of PA on the surface of viable, apoptotic and necrotic U937 cells. CHX-treated U937 cells were incubated with 2μM plg then probed for cell-surface plm with FITC-aprotinin ± 100-fold molar excess of unlabelled aprotinin. PI (10μg/ml) was added prior to flow cytometric analysis to differentiate apoptotic from viable and necrotic/late apoptotic cells. Each bar represents the mean of triplicate measurements ± 1 SEM. The absence of error bars indicates the error was smaller than the graphical symbol.
apoptotic and necrotic). The development of this assay was crucial in order to test the hypothesis that PA is involved in CHX-induced apoptosis in U937 cells. FITC-aprotinin has proved to be a useful probe for cell-surface plm due to a number of characteristics: its relatively small size (6kDa); other specific proteinaceous plm inhibitors, such as α2-MG and α2-AP, cannot inhibit cell-surface plm (Plow et al 1986; Hall et al 1991); it has been used previously in non-cytometric plm assays (Ellis et al 1986); it remains active after FITC conjugation (Fig 5.3.2); it is specific for trypsin-like serine proteases. Although FITC-aprotinin could potentially detect any trypsin-like protease (e.g. trypsin, chymotrypsin, kallikrein, plm) the specific binding of FITC-aprotinin to apoptotic U937 cells in the current study was entirely due to plm because: (1) cells absolutely required pretreatment with plg in order for specific FITC-aprotinin binding (plm formation) to occur (Fig 5.3.3-5); (2) specific FITC-aprotinin binding (plm formation) was almost completely blocked by inhibiting plg binding with TA (Fig 5.3.12); (3) specific FITC-aprotinin binding (plm formation) was markedly reduced when cells were treated with uPA inhibitors PAI-2 and EGR-CMK (Fig 5.3.13); and (4) specific FITC-aprotinin binding was completely blocked with the specific plm inhibitor VFK-CMK (Fig 5.3.14).

The binding of FITC-aprotinin to plg-treated apoptotic U937 cells was rapid (Fig 5.3.7), saturable (Fig 5.3.8) and specific (Fig 5.3.9). PA was also observed to be rapid with the majority of plm generated within 10min after the addition of exogenous plg (Fig 5.3.10). This is in contrast with previous cellular studies,
using chromogenic plm substrates, which have shown a steady increase in plm generation over several hours (Felez et al 1990; Osada et al 1991; Lopez-Alemany et al 1995).

When PA was measured on viable, apoptotic and dead cells, using the newly developed PA assay, striking differences were seen in the level of specific FITC-aprotinin binding (Fig 5.3.6 & 5.3.15). No binding was detected on the surface of viable U937 cells (Fig 5.3.6 & 5.3.15), in contrast to previously reported data (Ellis et al 1989; Felez et al 1990) which was generated using chromogenic-based plm assays. Like ¹²⁵I-plg binding assays, chromogenic plm assays do not allow discrimination of PA on specific subpopulations of cells (i.e viable apoptotic and dead) which is the most likely explanation for the disparity between these results and the current study. A dramatic increase in PA (specific FITC-aprotinin binding) was observed on apoptotic U937 cells while only very low levels of activation were detected on dead cells (Fig 5.3.6 & 5.3.15). If one considers the data in Fig 5.3.6 it appears that PA is switched on as cells undergo apoptosis and is then switched off as cells progress into late apoptosis/necrosis. Cell-surface plm was detected by the addition of FITC-aprotinin for at least 2h after activation (Fig 5.3.11). These data suggest that plm generated on the surface of apoptotic U937 is relatively stable.

The observation that TA could almost completely abolish PA (Fig 5.3.12), when incubated simultaneously with plg, on apoptotic U937 cells indicates that
specific plg binding (i.e. plg-Rs) is paramount for PA to proceed. This result is consistent with previous studies which have shown that lysine analogues can inhibit PA on U937 cells (Ellis et al. 1989), corneal fibroblasts (Lopez-Alemany et al. 1995) and keratinocytes (Reinartz et al. 1993). In support of this premise is the fact that PA did not occur on viable U937 cells (Fig. 5.3.3, 6 & 15) because no plg-Rs were present, irrespective of the presence of uPA (refer Fig. 4.3.8). Based on these observations it would appear that plg-Rs are a key determinant of whether PA proceeds on U937 cells. Hence it would be important to examine when plg-Rs are increased during apoptosis. uPA was also shown to be crucial for PA during apoptosis as activation was inhibited with the specific uPA inhibitor EGR-CMK and also PAI-2. EGR-CMK and PAI-2 were never observed to completely block PA which suggests that other proteases might participate in PA on apoptotic U937 cells. These data also indicate that PAI-2 can inhibit cell-bound uPA as previously described (Baker et al. 1990; Stephens et al. 1990).

Although the flow cytometric PA assay developed in this chapter was utilised to measure plm generation on viable, apoptotic and necrotic U937 cells it could easily be adapted to measure PA on other cell lines (e.g. adherent or transfected cell lines) and other subpopulations of cells (e.g. cells in various stages of the cell cycle). For example, Ranson et al. (1998) used the current assay to measure plm formation on viable breast carcinoma cell lines.
In summary, a dramatic transient increase in PA was detected on apoptotic U937 cells using a novel flow cytometric PA assay. This observation is consistent with previous data showing that both plg-Rs (i.e. specific plg binding) and uPA are elevated during CHX-induced apoptosis (Fig 4.3.6 & 4.3.8 respectively) and provides evidence that PA plays a physiological role during apoptosis. Plm could feed into a broader proteolytic network prevalent during apoptosis and degrade a range of cellular targets or activate other protease classes. As PA appears to be confined to the cell surface, its role may be restricted to extracellular proteolytic events, for example, the separation of apoptotic cells from neighbouring cells (i.e. anoikos) or the extracellular matrix within a specific tissue location.
Chapter 6

The Role of Plasminogen receptors in Apoptosis
6.1 Introduction

PA is involved in numerous cell-surface proteolytic events including tumour cell invasion, angiogenesis, tissue remodelling and inflammation. An adjunct to these roles is increasing evidence that PA, or components of the PA system, play a role in cell death. Plg and tPA-deficient mice exhibit resistance to kainic acid-induced neurodegeneration implicating the PA system in neuronal death (Tsirka et al 1997a & b). Overexpression of plg can be cytotoxic to mammalian cells due to the generation of intracellular plm (Busby et al 1991). This ‘toxicity’ can be prevented via the co-expression of physiological levels of α₂-AF (Busby et al 1991). PAI-2 has also been shown to protect cells against TNFα-induced apoptosis (Dickinson et al 1995), the cytopathic effects of alphavirus infection (Antalis et al 1988) and infection of human macrophages with Mycobacterium avium (Gan et al 1995). Evidence suggesting a possible protective role for components of the PA system is provided by the observation that plg can protect neuronal cells against nitric oxide-induced apoptosis (Toku et al 1998).

Observations described in previous chapters indicated that relatively high levels of plm are generated on the surface of apoptotic U937 cells as a consequence of a concomitant increase in plg-Rs (which facilitated an increase in specific plg binding) and cell-surface uPA. These results suggested that PA plays a role in CHX-induced apoptosis in U937 cells where plm could degrade structural proteins and/or activate other protease classes. It was also apparent
from these studies that the increase in plg-Rs (detected as an increase in specific plg binding) was critical for PA to occur (Fig 5.3.12).

Although apoptosis can be initiated by a diversity of molecules (refer Wertz & Hanley 1996) the execution and degradative phases are characterised by an ordered progression of morphological and biochemical changes that are largely regulated by proteases (Martin & Green 1995). One of the earliest morphological changes occurring during apoptosis is the externalisation of PS from the inner to the outer leaflet of the plasma membrane (Martin et al 1995; Zwaal & Schroit 1997). In some circumstances, the generation of cell-surface serine proteolytic activity has been shown to require PS. For example, thrombin activity can be generated on apoptotic vascular smooth muscle cells following PS exposure (Flynn et al 1997). The most likely explanation for this phenomena is that prothrombin binds to PS as 5μM Annexin V could significantly reduce thrombin generation. Based on this observation it seems plausible to hypothesise that PS exposure could be important for plm generation on apoptotic U937 cells. This hypothesis is supported by the observation that plg can bind to immobilised PS via a lysine-dependent mechanism (Nicholas Andronicus, Dept Biological Sciences, University of Wollongong, Pers. Comm.).

To elucidate the role of plg-Rs during CHX-induced apoptosis in U937 cells it is important to establish the temporal relationship of plg-R expression to other
apoptotic changes such as PS exposure, internucleosomal DNA fragmentation and differential PI uptake. The aim of this chapter then was to investigate the temporal relationship of plg-R expression to other apoptotic changes during CHX-induced apoptosis in U937 cells and to ascertain whether plg plays a cytoprotective or cytodestructive role in this model. In addition, the possible cellular relevance of the in vitro binding of plg to PS was investigated.

6.2 MATERIALS AND METHODS
All materials, cell lines, methods and data analysis used were as described in sections 3.2.1 4.2.1, 4.2.5, 4.2.6, 5.2.3 and 4.2.11.

6.3 RESULTS

6.3.1 Plg binding to PS
The binding of plg to cell-surface PS was investigated by examining the ability of physiological concentrations of plg (2μM) to inhibit Annexin-V-Fluos binding to apoptotic (CHX-treated) U937 cells. When CHX-treated cells were simultaneously incubated with Annexin-V-Fluos and 2μM plg a 52.6 ± 2.3% (N=3) increase in Annexin-V-Fluos binding was observed (Fig 6.3.1). A student's t-test indicated that the level of Annexin-V-Fluos binding to apoptotic U937 cells was significantly higher in the presence of 2μM plg (T=6.01, df=10, p<0.05). This suggests that plg does not compete with Annexin-V-Fluos for binding to PS on the surface of apoptotic U937 cells. Alternatively plg could
Fig 6.3.1: Effect of 2μM plg on the binding of Annexin-V-Fluos to apoptotic U937 cells. CHX-treated U937 cells were incubated with Annexin-V-Fluos in the absence (control) or presence of 2μM. Bars represent the mean of triplicate determinations ± 1 SEM.
cause an increase in PS exposure in some way or could facilitate the binding of Annexin-V-Fluos to cell-surface PS.

6.3.2 Increased plg-R expression is a late apoptotic event

U937 cells were induced to undergo apoptosis with CHX and at various times the level of PS exposure, PI uptake, DNA fragmentation and plg binding measured. Internucleosomal DNA fragmentation was apparent in CHX-treated U937 cells over time with an increase appearing at 36h (Fig 6.3.2A). As reported by Martin et al (1995) and Zwaal and Schroit (1997) PS exposure was found to be a relatively early apoptotic event with approximately 40% of cells expressing PS at 4h (Fig 6.3.2B). After 4h there was a steady increase in PS exposure albeit at a lower rate until approximately 70% of the population displayed PS. The level of PI uptake mirrored the level of plg binding over 36h with both displaying a steady increase over time but at approximately half the rate as PS exposure (Fig 6.3.2B). Although previous microscopic analysis had suggested that some PI negative cells could bind high levels of plg (Fig 4.3.7), the current observations clearly indicated that in the majority of cells, the increase in plg-R expression during CHX-induced apoptosis is coincident with PI uptake.
Figure 6.3.2: Temporal relationship of plg-R expression (plg binding) to internucleosomal DNA fragmentation, PS exposure and PI uptake during CHX-induced apoptosis in U937 cells. U937 cells were cultured in the presence of 10µg/ml CHX for 36h and at various times the level of DNA laddering, PS exposure, PI uptake and plg binding measured. A. Agarose gel showing the increase in DNA laddering over 36h. B. Graph showing the % of U937 cells displaying PS exposure, PI uptake and plg-R expression (plg binding). Each point represents the mean of triplicate determinations + 1 SEM. The absence of error bars is due to the error being smaller than the graphical symbol.
6.3.3 Effect of plg on the rate of CHX-induced apoptosis in U937 cells

Under certain conditions plg has been shown to act as either a cytotoxin (Busby et al 1991) or a cytoprotectant (Toku et al 1998). To determine whether physiological levels of plg can accelerate or reduce CHX-induced cell death, U937 cells were cultured in plg-free media containing either CHX, plg or both CHX and plg for up to 36h. At various times the percentage of cells with PS exposure and the percentage of cells positive for PI uptake were determined. The percentage of cells expressing PS (i.e both early and late apoptotic) was determined from FL-1 histograms of FITC-Annexin-V binding while the percentage of PI positive cells (i.e both apoptotic and late apoptotic/necrotic) was determined from FL-2 histograms.

The relative percentage of U937 cells that were measured as PS or PI positive remained constant in control samples over 36h in the absence of the apoptotic stimuli CHX (Fig 6.3.3A & B open circles respectively). In contrast, the addition of CHX resulted in a progressive increase in the percentage of U937 cells measured as PS or PI positive (Fig 6.3.3A & B open triangles respectively). Equally, the presence of 2μM human plg (-CHX) also increased (by ~10%) the relative proportion of PS positive U937 cells, with the most rapid rise seen between 12h and 36h (Fig 6.3.3A open squares). In contrast the presence of plg did not result in as obvious increases in relative percentages of cells that took up PI (Fig 6.3.3B open squares). By far the most dramatic effect on PS and PI positive cell numbers was seen when cells were cultured in the presence of
Fig 6.3.3: Effect of physiological levels of plg on the rate of CHX-induced apoptosis in U937 cells. U937 cells were cultured in plg-free media containing either 2μM plg, 10μg/ml CHX or both 2μM plg and 10μg/ml CHX for 36h. Control cells were incubated solely in plg-free media. At various times the percentage of cells with PS exposure or positive for PI uptake were determined flow cytometrically. A. percentage of U937 cells with PS exposure (i.e both early and late apoptotic cells) over time. B. Percentage of PI positive U937 cells (i.e both apoptotic and late apoptotic/necrotic) over time. Each point represents the mean of triplicate determinations ± 1 SEM. The absence of error bars is due to the error being smaller than the graphical symbol.
both plg and CHX. The rate and level of PS exposure and PI uptake when cells were cultured in the presence of both plg and CHX was almost double that of CHX alone (Fig 6.3.3A & B open diamonds respectively).

Statistical analyses were performed on the above data to ascertain the significance of the effect of plg on the level of CHX-induced apoptosis (i.e PS exposure and PI uptake). One-way ANOVAs indicated that PS exposure on cells in all treatments was significantly different at 12 (F=141.91, df=3, p<0.05; Tukey-Kramer HSD), 24 (F=849, df=3, p<0.05; Tukey-Kramer HSD) and 36h (F=564.9681, df=3, p<0.05; Tukey-Kramer HSD). These results confirm that the presence of 2µM plg significantly increases the level CHX-induced apoptosis in U937 cells and that plg can increase PS exposure in the absence of CHX. One-way ANOVA's also indicated that PI uptake was significantly different in all treatments at 12 (F=74.57, df=3, p<0.05; Tukey-Kramer HSD) and 24h(F=397.27, df=3, p<0.05; Tukey-Kramer HSD), while at 36h significant differences in PI uptake were detected between all treatments (F=269.79, df=3, p<0.05; Tukey-Kramer HSD) except between cells cultured in plg-free media (i.e control) and cells cultured with 2µM plg (t=2.053, df=4, p>0.05). These analyses confirm that the presence of physiological levels of plg enhances the rate at which U937 cells enter CHX-induced apoptosis (i.e PI uptake and PS exposure) and warrant further investigation of how the presence of plg increases PS exposure in the presence of apoptotic stimuli.
To investigate whether plg can accelerate apoptosis once initiation has begun, U937 cells were pre-exposed to CHX for 12 or 24h in plg-free media then exposed to 2µM plg for an hour. PS exposure (Annexin-V-Fluos binding) and PI uptake increased by 36 ± 1.2 % (N=3) and 31 ± 2.7% (N=3) respectively on controls cells (i.e no-CHX treatment) when exposed to 2µM plg for 1h (Fig 6.3.4). Statistical analysis of these data using student t-test's indicated that 2µM plg significantly increased both PS exposure (t=75.58, df=4, p<0.05) and PI uptake (t=24.43, df=4, p<0.05) on U937 cells in the absence of CHX. The presence of 2µM plg increased PS exposure and PI uptake by 3.4 ± 0.13 % (N=3) and 20 ± 1.4% (N=3) respectively on 12h CHX-treated U937 cells (Fig 6.3.4). This effect of plg on PS exposure was not significant (t=1.60, df=4, p>0.05) while the effect of plg on PI uptake was highly significant (t=17.86, df=4, p<0.05). The presence of 2µM plg had no significant effect on PS exposure (t=1.73, df=4, p>0.05) yet had a slightly significant effect on PI uptake (t=4.56, df=4, p<0.01) in U937 cells that had been exposed to CHX for 24h (Fig 6.3.4). These observations suggest that physiological levels of plg can increase PS exposure (i.e apoptosis) and PI uptake (i.e cell death) in U937 cells in the absence of apoptotic stimuli which is consistent with earlier observations (Fig 6.3.3). Additionally this effect of plg on the level of PS exposure and PI uptake is diminished the longer the cells are exposed to an apoptotic stimuli. These results indicate that cells that have been sensitised with an apoptotic stimuli (CHX) in the absence of plg are not 'nudged' further along the apoptotic pathway if they are then exposed to plg. These results also suggest that the persistent presence of plg during CHX-
Fig 6.3.4: Effect of short-term exposure to physiological concentrations of plg on PS exposure (A) and PI uptake (B) on U937 cells cultured in CHX for 12 or 24h. U937 cells were cultured in the presence of 10μg/ml CHX for 12 or 24h then incubated with 2μM plg for 1h. Control cells were cultured in plg-free media only. A. Percentage of U937 cells with PS exposure (i.e both early and late apoptotic cells) over time. B. Percentage of PI positive U937 cells (i.e both apoptotic and late apoptotic/necrotic) over time. Each bar represents the mean of triplicate determinations ± 1 SEM. The absence of error bars is due to the error being smaller than the graphical symbol.
induced apoptosis is necessary for the observed effects.

6.4 DISCUSSION

Apoptosis is a complex process involving a myriad of structural and biochemical changes ultimately leading to the resorption of cells by the body in a manner that avoids inflammation. Although apoptosis can be initiated by a diversity of molecules the consensus of scientific opinion is that these 'death signals' converge into a single pathway leading to the characteristic cellular changes associated with apoptosis. Extensive evidence implicates the involvement of various protease systems in the execution and degradative phases of apoptosis including PA. Various experiments have indicated that intracellular PAI-2 can protect against TNFα, viral or bacterial apoptosis (Dickinson et al 1995; Gan et al 1995; Antalis et al 1998). Studies have shown a role for tPA-catalysed PA in various types of neuronal death (Tsirka et al 1997a & b). Other studies have revealed that uPA is increased during apoptosis in heptocytes (Santonirugiu et al 1996), granulosa cells (Johnson et al 1997) and ovarian cells (Simonitsch & Krupitza 1998). In contrast, endothelial cell apoptosis is characterised by a decrease in tPA, PAI-1, PAI-2 and uPAR while uPA is unchanged (Zoellner et al 1998). These disparate roles for PA in cell death on different cell types can be explained by the hypothesis that the role of PA is cell-specific and is influenced by the manner in which death is initiated (i.e the death signal).
Thrombin activity has previously been shown to be generated on apoptotic vascular smooth muscle cells following PS exposure (Flynn et al 1997) most likely due to the binding of pro/thrombin to PS. In the current study physiological levels of plg could not compete with Annexin V for binding to cell-surface PS during CHX-induced apoptosis (Fig 6.3.1). In fact, exposure to plg appeared to increase the level of Annexin-V-Fluos binding to CHX-treated U937 cells. One explanation for this observation is that plg could indirectly mediate the binding of annexin V to the cell surface. It is possible that while most Annexin V binds to exposed PS some could bind to receptor-bound plg following the addition of plg. Another possibility is that plg increases the level of PS exposure on apoptotic cells which facilitates increased annexin V binding. This later proposal is corroborated by consistent observations showing that physiological concentrations of plg increase PS exposure (Annexin-V-Fluos binding) by 5-10% on both viable and apoptotic cells (Fig 6.3.3 & 4).

The temporal relationship of plg-R expression (i.e specific plg binding) to established apoptotic markers (PS exposure, DNA laddering and differential PI uptake) during CHX-induced apoptosis was examined in order to determine the likely apoptotic stage (i.e initiation, execution or degradation) that plg-Rs play a role. PS exposure on CHX-treated U937 cells was found to be the earliest apoptotic event which is supported by previous evidence showing that PS exposure is an early and widespread event during apoptosis of a variety of human and murine cells (Martin et al 1995). Low levels of internucleosomal
DNA fragmentation (i.e. DNA laddering) could be detected between 4-12h but by far the majoratory was detected at 24 and 36h. Of the three markers, only PI uptake was shown to be coincident with plg-R expression (Fig 6.3.2B) and is supported by previous data in this thesis showing that there is a direct relationship between PI uptake and the magnitude of lysine-dependent (i.e. specific) plg binding (Fig 3.3.4 & 3.3.5).

Observations reported in this chapter suggest that changes to the plasma membrane of apoptotic U937 cells that make them moderately permeable to PI co-occur with the increase in plg-R expression. The data does not indicate if these two cellular events are linked. The exact mechanism of PI uptake by apoptotic cells is unknown although evidence indicates that it is not due to membrane damage (Vitale et al 1993). Therefore it is likely that membrane damage is not responsible for the increase in plg-Rs on apoptotic cells. PI uptake is generally considered to be a late apoptotic event associated with the degradative phase of apoptosis and generally occurs at or around the same time as DNA fragmentation (Vitale et al 1993; Mower et al 1994). Collectively these observations suggest that plg-Rs are increased during late-stage cell death where they are likely to contribute to the degradative phase of apoptosis. Future studies could be focussed at examining the temporal relationship of other components of the PA cascade, such as uPA, uPAR, and PAI-2 during apoptosis.
The presence of physiological levels of plg was found to dramatically increase the rate and level of CHX-induced apoptosis in U937 cells (as measured by PS exposure and PI uptake) (Fig 6.3.3). Studies examining the rates of DNA fragmentation when physiological levels of plg are present remain to be undertaken. These observations suggest that plg contributes to cell death in a manner additive with apoptotic inducers (e.g CHX). The simultaneous exposure of cells to both plg and CHX was necessary for the observed additive effect as U937 cells pre-exposed to CHX were not noticeably 'pushed' along the apoptotic pathway when exposed to physiological levels of plg (Fig 6.3.4).

An interesting observation was that plg itself could cause some death (PS exposure and PI uptake) in cells cultured in the absence of CHX (Fig 6.3.3 6.3.4) which is consistent with a previous study showing that high levels of plg are cytotoxic to mammalian cells (Busby et al 1991). The mechanism of this cytotoxicity was via the generation of plm and it is thus possible that the effect observed in the current study was also due to plm. A major weakness to this explanation is that cell-surface PA was shown to be absent on viable U937 cells (Fig 5.3.3, 5.3.6 & 5.3.15) so either plm is generated by some other mechanism (e.g in solution) or plg cytotoxicity is due to a non-plm mechanism. Alternatively, contaminating traces of plm in plg preparations could have made a direct contribution to cell death.
Numerous intracellular proteases (e.g. caspases, endonucleases, serine proteases) are involved in apoptosis so the role of cell-surface plm remains a puzzle. It was previously shown that plm generated on the surface of apoptotic U937 cells remains there for at least two hours so it is unlikely that plm is internalised (Fig 5.3.11). Several independent studies have suggested that PA might be involved in anoikis (cell detachment) during apoptosis (Matsuoka et al 1998; Simonitsch & Krupitza 1998). Although this suggestion is attractive it does not provide a direct explanation for the role of PA during apoptosis of a non-adherent cell line (i.e. U937). As it is well established that PA can act in concert with other proteases during processes such as ECM degradation, it is tempting to speculate that plm could feed into the broader proteolytic network that is prevalent during apoptosis. Whatever the role of plg during apoptosis the current study indicates that it is not essential as CHX-induced apoptosis proceeded in its absence albeit at a lower rate (6.3.3).

In conclusion, it is hypothesised that substrates such as proteases or structural proteins can be activated or degraded respectively by the elevated levels of plm generated on apoptotic cells in a manner which accelerates death. Without plg these processes still occur but at a slower rate. Additionally, evidence is provided that plg-Rs are increased on the cell surface during late-stage CHX-induced apoptosis in U937 cells suggesting that PA is likely to play a role in the degradative phase of apoptosis.
Chapter 7

General Discussion
The focus of this thesis has been to extend our understanding of the manner in which plg interacts with cancer cells. Initially, the binding of plg to the monocytic leukemic cell line U937, the colon cancer cell line HCT116 and the breast carcinoma cell lines MDA-MB-231 and MCF-7 was studied with the aim of establishing a relationship between plg binding and metastatic potential. Using the 'established' approach of radioligand binding assays it was difficult to repeatedly measure plg binding to these cell lines (Fig 3.3.3). Although $^{125}$I-plg binding studies indicated the presence of plg-Rs on the highly metastatic colon cancer cell line HCT116 (Fig 3.3.3), the isolation and identification of proteinacious receptors was unsuccessful (Fig 3.3.4-6). The variability of data generated using $^{125}$I-plg binding assays and the apparent discrepancy between binding data and receptor isolation studies warranted the development of a more reliable and robust technique for measuring cellular plg binding.

Flow cytometry was utilised to analyse cellular plg binding as it enabled the measurement of plg binding on discreet subpopulations of cells, for example viable and non-viable cells. A flow cytometric plg binding assay was developed (Chapter 3) in order to enable the measurement of plg binding on specific subpopulations of cells (i.e viable and non-viable). Repeated experimentation clearly indicated that no specific (i.e lysine-dependent) plg binding occurred on a range of viable cell types including U937 cells and the colon carcinoma cell lines LIM1215, HCT116 and KM12SM (Fig 3.3.21). Using U937 cells as a model system it was shown that specific plg binding was restricted entirely to non-
viable cells (Fig 3.3.4). In fact, plg binding to non-viable cells exhibited all of the characteristics that have previously been attributed to viable cells (gathered using $^{125}$I-plg binding assays) including saturability (Fig 3.3.9, 3.3.10), reversibility (Fig 3.3.11), inhibition by lysine analogues such as TA (Fig 3.3.12) and cold plg (Fig 3.3.13, 3.3.14), and susceptibility to carboxypeptidase B treatment (3.3.15). Plg-Rs could not be generated on viable U937 cells even following treatment with PMA (Fig 3.3.17), TNFα (Fig 3.3.18), trypsin (Fig 3.3.8) or peroxide (Fig 3.3.20). A direct relationship was also found to exist between cell viability (PI uptake) and the magnitude of lysine-dependent plg binding (Fig 3.3.4, 3.3.5).

The consequence of not utilising an assay (such as the flow cytometric assay developed in this thesis) that enables the concurrent measurement of plg binding and viability, would be to overestimate plg-R expression on any 'viable' cell type if even small numbers of non-viable cells are present. Increased plg binding to non-viable cells appears to be a widespread phenomena and has subsequently been confirmed by Ranson et al (1998) on several breast carcinoma cell lines including MCF-7, MDA-MB-231 and T47D cells. Ranson utilised the assay that was developed in this thesis to show the occurrence of specific plg binding to viable MDA-MB-231 cell. This independently validates the flow cytometric plg binding assay and shows that specific plg binding (i.e plg-R expression) does occur on some viable cell types.
The current flow cytometric studies cast a shadow over previous plg binding data generated using $^{125}$I-plg binding assays considering that plg binding to non-viable cells can not be accounted for using this technique. The question raised is whether previous observations are valid considering the direct relationship between cell viability and plg binding (Fig 3.3.4 & 5). If a cell line were particularly fragile then one would expect that using $^{125}$I-plg binding assays would yield a less accurate result than a cell line that was more robust due to the increased proportion of non-viable cells generated during experimental manipulation. It is clear from the current studies that $^{125}$I-plg binding assays are no longer a satisfactory means of studying plg binding and it is recommended that all future plg binding studies employ flow cytometry. The differential expression of plg-Rs on other subpopulations of cells could also be investigated using the assay described in this thesis - for example, examining plg-R expression during different stages of the cell cycle. The specific quantitation of plg-Rs by scatchard analysis is currently not possible using flow cytometry because suitable flow cytometric standards (i.e beads with specific numbers of lysine binding sites) are unavailable. One improvement to the current flow cytometric assay would be to synthesise suitable lysine coated beads, with a defined number of lysine binding sites, which would allow the direct quantitation of cellular plg-Rs.

No studies have directly examined changes in plg-R expression during apoptosis. Previous unrelated studies, which utilised morphological
examination or PI staining/fluorescence microscopy to detect dead cells, indicated that plg binding was increased on necrotic cells (Green 1978; Burgos et al 1982; Jenkins et al 1983; Burtin et al 1985). A model system for studying plg binding during CHX-induced apoptosis in U937 cells was developed (Fig 4.3.1-5). Plg-Rs (i.e. specific plg binding) were found to be dramatically elevated on apoptotic cells (Fig 4.3.6 & 7) along with a large transient increase in cell-surface uPA (Fig 4.3.8) which indicated that PA could play a role during CHX-induced apoptosis in U937 cells. The mechanism for the increase in plg-Rs and uPA is unclear, however it is likely that it does not involve any new protein synthesis (as cells were treated with the protein synthesis inhibitor CHX) suggesting that intracellular stores of uPA and plg-Rs are externalised during death. The increase in plg-Rs during apoptosis was not linked to PS exposure (Fig 6.3.2).

The logical outcome for the increase in specific plg binding and uPA expression on apoptotic U937 cells is the generation of cell-surface plm. Consequently a novel flow cytometric PA assay was developed using FITC-aprotinin to detect plm generation on the surface of specific subpopulations of cells (viable, apoptotic and late apoptotic/necrotic) (Chapter 5). PA was found to be highly elevated on apoptotic cells (Fig 5.3.4, 6 & 15) which supports the hypothesis that plg binding and activation play a role in CHX-induced apoptosis in U937 cells. The importance of both plg-Rs (i.e specific plg binding) and uPA was shown by the inhibition of PA with TA (Fig 5.3.12) and uPA inhibitors PAI-2 and EGR-CMK (Fig 5.3.13). Although the majority of PA appeared to be due to uPA it is
possible that other proteases, such as tPA could activate plg on apoptotic cells. Future studies could investigate whether other components of the PA system (e.g. tPA, PAI-2 etc) are modulated during CHX-induced apoptosis or whether apoptotic proteases (such as the caspases) can generate plm. The flow cytometric PA assay described in this thesis could be utilised to measure plm generation on the surface of any cell type or subpopulation of cells. Ranson et al (1998) have utilised this assay to measure plm generation on the surface of the breast carcinoma cell line MDA-MB-231.

Analysis of the temporal relationship of plg-R expression to several established apoptotic markers (i.e. PS exposure, PI uptake and DNA laddering) indicated that the increase in plg-Rs was a late apoptotic event that was co-incident with PI uptake (Fig 6.3.2). This suggested that plg-Rs are likely to play a role in the degradative phase of CHX-induced apoptosis in U937 cells. Further experimentation indicated that physiological levels of plg were not essential for apoptosis to proceed, but if present, could greatly accelerate both the rate and level of death (Fig 6.3.3 & 4). Future experiments could be directed toward examining the temporal variation of other components of the PA system during CHX-induced apoptosis and also to establish the fate of cell-surface plm. It is possible that cell-surface plm could contribute to the anoikis of dying cells. Alternatively plm could activate other proteases thereby feeding into the broader proteolytic network that is prevalent during apoptosis. Another possibility is that plm could contribute to the dismantling of the cellular
architecture by directly degrading structural proteins. Clearly the observations reported in this thesis are only the beginning of an exciting new area of study involving the PA system.

In summary, this thesis describes the development of novel flow cytometric plg binding and activation assays which were utilised to analyse plg binding and activation during cell death. These assays are a valuable technical advancement and will facilitate the analysis of PA on a diversity of other cell types and during other cellular processes. Plg-Rs were dramatically elevated on a variety of non-viable cells including a subpopulation of apoptotic U937 cells that were generated following CHX treatment. The presence of both plg-Rs (i.e specific plg binding) and uPA was critical for the high levels of plm formation on the surface of these cells. Although the function of this plm remains to be determined it is hypothesised that substrates can be activated or degraded by the elevated levels of plm on apoptotic cells and that this is responsible for the accelerated rate at which they die. Without plg these processes were shown to still occur but at a much reduced rate. Observations also indicated that the role of plg-Rs is most likely confined to the degradative phase of apoptosis. Collectively these data provide strong evidence that the PA system plays a role in CHX-induced apoptosis in U937 cells and lends support for the important role of flow cytometry in plg binding and activation studies.
REFERENCES


type 2 protects against viral cytopathic effects by constitutive interfern alpha/beta priming. J. Exp. Med. 187(11): 1799-1811


Kariko, K., Malkowicz, S. B., Li, W. J., Kuo, A. And Barnathan, E. S. (1993). Invasive uroepithelial cells express high levels of urokinase receptor and plasminogen receptor. *Int. J. Onco* 3(6): 1089-1095


Binding of plasminogen to corneal fibroblasts and their extracellular matrix. Evidence for a
receptor in cell membranes. Fibrinolysis. 9: 223-229


Lu, H., Mirshahi, M., Krief, P., Soria, C., Soria, J., Mishal, Z., Bertrand, O., Perrot, J. Y., Li, H.,
receptors for plasminogen and urokinase by interferon gamma on U937 cells. Biochem-Biophys.

Enhanced expression of urokinase activity on U937 cell line by 1,25-dihydroxyvitamin D3


Lund, L. R., Riccio, A., Andreasen, P., Nielsen, L. S., Kristensen, P., Laiho, M., Saksela, O., Blasi,
F. and Dano, K. (1987). Transforming growth factor-beta is a strong and fast acting positive
regulator of the level of type-1 plasminogen activator inhibitor mRNA in WI-38 human lung


activator inhibitor (PAI-1) and tissue-type plasminogen activator (tPA). *Thromb. Haemost.* 58:65


serum-inducible genes. Different patterns of gene regulation during G0 to S and G1 to S progression. 
*J. Cell. Sci.* 107: 227

Wilson, E. L., Jacobs, P. and Dowdle, E. B. (1983). The secretion of plasminogen activators by 


Wiman, B. and Collen, D. (1979). On the mechanism of the interaction between human $\alpha_2$-


