[Frontiers in Bioscience 8, s294-304, May 1, 2003]

PLASMINOGEN BINDING AND CANCER: PROMISES AND PITFALLS

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1. ABSTRACT

The urokinase plasminogen activation system is a key modulator of the tissue remodeling processes required for tumor cell invasion and metastasis. Malignant progression is characterised by inappropriately high cell surface levels of receptor- bound active urokinase. This enhances the rate of plasminogen activation resulting in markedly increased levels of cell surface plasmin. The repercussions of this are significant and include the activation of growth factors and signaling pathways, and the degradation of extracellular matrices, either directly or indirectly, via the activation of matrix metalloproteinases. Native, circulating plasminogen binds in a lysine- and/or carbohydrate-dependent manner to tumor and endothelial cells with low affinity but high capacity and a heterogeneous group of plasminogen receptors have been identified. This heterogeneity underscores the complexity of the mechanisms responsible for the regulation of cell-surface plasminogen binding. This review summarizes the literature on known plasminogen receptor candidates and shows that they can be subdivided into three classes based on their mode of interaction with plasminogen. We also aim to emphasize the notion that in the tumor environment the known intrinsic functional relationship between plasminogen conformation and activation is essentially connected to cellular binding. This allows plasminogen to be co-localised in an activation-susceptible form with the enhanced uPA levels seen in malignancy and together furnishes tumor cells with elevated tissue remodeling capacity. In addition, some of the pitfalls and strategies encountered when conducting plasminogen receptor experiments are also addressed.

2. INTRODUCTION

The acquisition of the malignant phenotype requires a multitude of complex processes. This includes a) loss of control of cell proliferation, b) loss of replicative senescence and, c) the ability of otherwise anchored and immobile cells to metastasize (a process involving local invasion, entry into lymphatic or blood vessel and implantation at sites distant to tissue of origin) (1-3). The latter process appears to be accounted for by deregulation of expression of so-called "metastasis genes" (3). These encode transmembrane receptors and their ligands, proteinases and their inhibitors which, in conjunction with associated signal transduction molecules, usually mediate physiological stress responses related to cell adhesion and migration (e.g., inflammation and wound healing) (2-6).

Overexpression of the genes encoding the urokinase plasminogen activator (uPA) system is a key event in malignant tumor progression (4,7,8). The aberrant expression of these (and other) metastasis genes are mediated *in vitro* by growth factors, hormones, phorbol esters and cytokines, and by cell adhesion molecule interactions via the mitogen activated protein kinase (MAPK) and/or JNK/SAPK group of signaling pathways (9,10). In the uPA system, uPA is synthesized and secreted as a zymogen (pro-uPA) (11), whose activation is markedly accelerated upon binding with high affinity (~ 0.1 – 1 nM) to specific cell surface uPA receptors (uPAR) (12-14). Receptor density varies depending on cell type (~10^3 - 10^6 sites/cell) (15). Active, receptor bound uPA then efficiently converts the cell surface-bound zymogen plasminogen into the highly active, serine protease plasmin (16). Receptor-bound plasmin is protected from the circulating inhibitors and thus promotes tissue degradation and remodeling of the local extracellular environment. This is achieved by initiating a cascade of pericellular proteolysis which can directly and indirectly (via activation of pro-metalloproteases) degrade integral extracellular matrix (ECM) molecules (2,4,7). Plasmin is also responsible for the release of latent growth/angiogenic factors from ECM (17). While plasminogen is also activated by tissue-type plasminogen activator (tPA), this function is primarily the maintenance of vascular integrity rather than pericellular

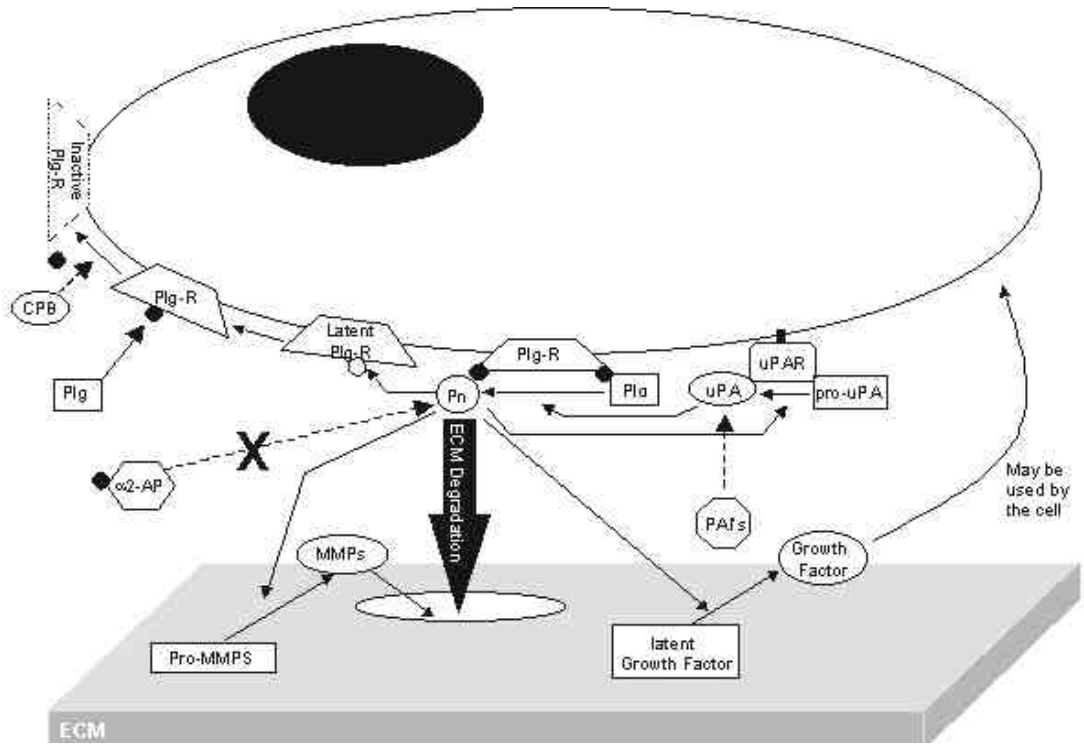

Figure 1. A potential scheme for tumor cell-surface associated plasminogen binding and activation. Schematic representation of the cell surface assembly of the urokinase plasminogen (Plg) activation cascade focusing on the role plasminogen receptors (Plg-R) play in the acquisition of cell-surface plasmin (Pn). Once present receptor-bound plasmin can directly degrade the extracellular matrix (ECM), activate metalloproteinases (MMPs) and activate growth factors. Receptor-bound plasmin is also shielded from inactivation by α2-antiplasmin (α2-AP) as the lysine binding sites of plasmin are occupied. Digestion of cell surface proteins by plasmin may result in an increase in the total number of C-terminal lysine residues present at the cell surface and thereby increasing the number of plasminogen binding sites of the cell. O represents a pre-existing C-terminal lysine residue on a class 1 plasminogen receptor (see section 4.1 for explanation of receptor classes). ● represents a plasmin cleavable lysine residue from a latent or class 2 plasminogen receptor. Class 3 receptors are not shown for simplicity. Solid arrows represent activation reactions or binding events whereas broken arrows represent inhibition events. CPB = carboxypeptidase B; PAIs = plasminogen activation inhibitors 1 or 2.

proteolysis (4,7). The proteolytic activity of uPA is regulated by plasminogen activator inhibitors type 1 and 2 (PAI-1 and PAI-2) (18). PAI-1 and uPA can also modulate uPAR interaction with vitronectin and various integrins thus affecting cell adhesion and, via the integrin mediated signaling pathways, regulate cell migration (6,19,20). This functional duality of the uPA system underscores its critical role in metastatic processes as cell adhesion, migration and proteolysis can be co-ordinately controlled by one system.

The inappropriate and unregulated generation of extensive proteolytic activity via plasmin is a key feature of tumor cell metastasis. Three critical events are required for efficient cell-surface plasminogen activation: 1) plasminogen binding to cell surface receptors, 2) binding-dependent conformational change of plasminogen to an activation-susceptible form, and 3) co-localization with uPA (Figure 1). Satisfaction of all these criteria at the surface of tumor cells facilitates efficient plasmin-mediated pericellular proteolysis, which in turn contributes to the invasive and metastatic capacity of these cells. The purpose of this review is to firstly overview plasminogen structure as it relates to its binding and activation function. We will then summarize the interaction with mammalian cells and the characteristics of the various isolated plasminogen receptors. Several bacterial plasminogen receptors have also been identified but will not be discussed here (reviewed in (21)). Secondly, we will build on the importance of plasminogen receptors in cancer by highlighting the evidence that binding not only stabilizes an activation-susceptible conformation, but that cellular plasminogen binding and activation capacity is inextricably linked to elevated uPA/uPAR. Differentiated, non-invasive/migratory cells would not be expected to bind and activate cell surface plasminogen and indeed, as discussed in section 6, these cells do not overexpress uPA/uPAR. Thus, by analogy to the fibrin clot which acts as a template for binding and co-localization of both plasminogen and tPA and greatly stimulates plasminogen activation, receptor binding co-localizes plasminogen with uPA and therefore is necessary for the enhanced, unregulated pericellular plasmin activity evident in malignant tissues.

3. PLASMINOGEN CONFORMATION AND ACTIVATION: STRUCTURE FUNCTION RELATIONSHIPS

The activation of plasminogen to plasmin is greatly modified by factors that affect the conformation of the zymogen, which circulates in a mostly closed, activation-resistant form (reviewed in (22)). This section briefly summarizes plasminogen structure in order to highlight the intrinsic functional relationship between plasminogen conformation and activation. In the cellular environment this relationship is essentially connected to plasminogen receptor binding. The latter process will be discussed in detail in following sections.

Plasminogen is secreted as a single chain 791 amino acid glycoprotein that has an apparent molecular weight of 92 kDa and an amino-terminal glutamic acid (reviewed in (23,24)). Glu-plasminogen is present in the plasma at a concentration of approximately 2 μM (25), the source being primarily hepatic (25) though many extra-hepatic sites express the plasminogen gene (26). Two glycoforms exist. Both glycoform I and II are glycosylated at Thr_{346}, while glycoform I is also glycosylated at Asn_{289} (23). This difference in carbohydrate content affects the functional properties of the zymogen ((27) see section 4). In addition, plasminogen is phosphorylated at Ser_{578} (28). When plasminogen is activated by uPA or tPA, the Arg_{561}-Val_{562} peptide bond is cleaved to give rise to twin chain plasmin (29) comprising an amino-terminal heavy (approximately 65 kDa) and carboxy-terminal light chain (25 kDa) covalently joined by two disulfide bridges (23,24). The light chain contains the protease domain and contains the typical His/Asp/Ser catalytic triad found in many serine proteases (23,24). The heavy chain region of plasminogen contains an amino-terminal peptide domain (NTP; amino acids 1-77) as well as five triple-disulfide-bonded kringle structures which mediate binding to other molecules (23,24). All of the kringle domains have been characterised with respect to their affinities for small lysine analogues that structurally resemble either the amino- or carboxy-terminal, or internal lysine residues of proteins, with kringles 1, 4 and 5 having highest affinity interactions (23). The crystal structures of the individual kringles 1(30), 4 (31,32), and 5 (33) domains have been solved and demonstrate that they contain preformed, surface exposed lysine binding site motifs. These motifs mediate the lysine-dependent binding of plasminogen to fibrin and to cell-surface receptors (see sections 4 and 5). In addition, plasmin activity is inhibited by alpha2-antiplasmin in a two-step process that involves lysine-dependent binding with plasminogen via kringles 1, 4 and 5, followed by classic serpin inhibition of the protease (34).

The crystal structure of glu-plasminogen has not been solved but a combination of physical and molecular modeling techniques have provided insights into the tertiary structure of the molecule. Circulating glu-plasminogen exhibits a closed, right-handed, spiral conformation (35-37). This closed conformation of glu-plasminogen is maintained by the binding of multiple kringle lysine binding site motifs with internal lysine residues (38,39), such as Lys_{50} and Lys_{62} located in the NTP of glu-plasminogen (39). Indeed, removal of the NTP from glu-plasminogen by plasmin results in the formation of lys-plasminogen (86 kDa) (40,41) which has a more open, U-shaped conformation than glu-plasminogen (42,43). The intramolecular lysine binding network can also be disrupted by the coordinated binding of exogenous lysine analogues (42,44,45) resulting in a rapid conformational change ($t_{1/2}$ = 0.01 s) (44) that is stabilized in the open form. This suggests that a complex, multivalent interaction is required for the complete conformational change of glu-plasminogen, as we found using a recombinant receptor protein for plasminogen (alpha-enolase) (46). Furthermore, the binding interaction also stabilizes the activation-susceptible conformation ((46); discussed further in section 5.1).

The functional significance of such a transition in glu-plasminogen conformation in the presence of lysine analogues or binding moieties containing accessible lysine residues (e.g. fibrin, ECM, cells) is that the rate of conversion to plasmin is significantly accelerated by its activators (22). In fact, both the conformations (42,43,46) and activation rates (22) of lys-plasminogen and liganded glu-plasminogen are comparable. The functional significance derived from all these data is that a binding step is essential for rapidly generating plasmin as it induces a conformation that presents glu-plasminogen as a better substrate for uPA or tPA. Thus, plasminogen binding, particularly when it co-localizes the zymogen with either uPA or tPA, is physiologically relevant in facilitating plasminogen activation during processes requiring pericellular proteolysis.

4. PLASMINOGEN BINDING TO THE CELL SURFACE

Plasminogen has been shown to bind to resting and thrombin-stimulated platelets (47) and a wide range of nucleated adherent and non-adherent eukaryotic cells including; circulating blood cells (48), keratinocytes (49), endothelial cells, fibroblasts, hepatocytes, neuronal cells, and a variety of tumor cell lines (50-53). In addition, plasminogen has been immunologically localised to the basal layer of normal human epidermis (54), to cell surfaces in human mammary carcinoma tissue (55,56), and to the invasive front of cutaneous melanoma lesions (57). The cellular binding parameters of glu-plasminogen appear to be dependent upon cell type and/or assay technique. However, some key features of glu-plasminogen binding to cells are consistent including: (1) saturable, low affinity (K_d ~ 0.1 - 2 μM), lysine-dependent interactions with a high number of binding sites/cell (10^4-10^7 sites/cell) (51,52); (2) the facilitation of glu-plasminogen activation (22,58); (3) protection of cell-surface plasmin from inactivation by alpha2-antiplasmin and alpha2-macroglobulin (59,60); and (4) increased catalytic efficiency of cell-surface plasmin compared to solution-phase plasmin (61). Facilitated activation results from co-localization of activators via either uPAR-bound uPA or plasminogen/tPA co-receptors. The latter appears to be a feature mostly of endothelial cells and melanoma cells (see below). Using a number of techniques with breast (52) and

other carcinoma cell lines (Ranson, unpublished data), we have shown that elevated cell-surface plasminogen binding and activation is strongly associated with metastatic capacity and other parameters (uPA/uPAR status as well as EGFR/*erb*B-2 and ER status) commonly associated with malignancy. We have also shown that binding stabilizes an activation-susceptible conformation of glu-plasminogen on the cell surface (62).

Efficient cell binding is mediated largely through C-terminal lysine residues at the cell surface as demonstrated by the reduced plasminogen binding capacity of cells following the addition of carboxypeptidase B, an enzyme which cleaves C-terminal lysine residues (63-65). Indeed, plasma basic carboxypeptidases may play a role in regulating plasminogen binding to leukocytes (64). Furthermore, limited proteolysis of human mammary carcinoma cells has been shown to increase their plasmin/ogen binding capacity (61,63). Treatment of the cells with cycloheximide does not diminish this increased plasmin binding capacity indicating that such enhancement is not due to de-novo synthesis of binding proteins (63). These studies suggest that active cell-surface plasmin may cleave existing latent cell-surface proteins, exposing new C-terminal lysine residues, thereby generating new cell-surface plasminogen binding proteins. Indeed several plasminogen receptors that lack pre-existing C-terminal lysines have been identified (discussed in section 5). These mechanisms by which glu-plasminogen may interact with a tumor cell surface are summarized in Figure 1.

As has been shown with small lysine analogues and fibrin (22), variant forms of plasminogen have different binding parameters in the presence of cells. Lys-plasminogen (66) and plasmin (67-70) bind with higher affinity to cells (K_d ~ 0.001 - 0.1 μM) than glu-plasminogen. This differential affinity of plasminogen variants for the cell surface is probably due to the conformational differences between glu- and lys-plasminogen in which the latter, minus the NTP, exists in the open conformation that favors binding (see section 2). Incubation of glu-plasminogen with U937 but not human umbilical vein endothelial cells induces the formation of lys-plasminogen via proteolysis by endogenous plasminogen activators (71,72), and this may provide a mechanism for enhanced plasmin generation at the cell surface. Alternatively, receptor-bound glu-plasmin may undergo auto-catalysis following activation yielding receptor-bound lys-plasmin and unbound NTP.

Binding studies using elastase fragmented plasminogen identified kringles 1-3 as a primary recognition site, and kringle 4 and mini-plasminogen (kringle 5-protease domain) as secondary recognition sites for plasminogen binding on stimulated and unstimulated platelets (66). Using fragments of plasminogen, epsilon-aminocaproic acid and benzamidine, Burge *et. al.*, (54) demonstrated that glu-plasminogen binding to basal cell layers of the epidermis is primarily mediated by the kringle 5 lysine binding site motif as well as by a second epsilon-aminocaproic acid -dependent site. Clearly, the binding interaction may be mediated by different and/or multiple kringle domains depending on the cell type and receptor. However, the use of plasminogen fragments negates the intramolecular lysine-binding network that maintains the closed, physiologically relevant conformation of glu-plasminogen and this may distort the relative importance of the various kringle lysine binding site motifs responsible for receptor binding.

Another parameter that influences cell-surface plasminogen binding is the glycosylation status of the zymogen. The more heavily glycosylated glu-plasminogen (type 1) binds to U937 cells with a lower affinity than its less glycosylated variant type 2 (73). Though less distinct, plasminogen carbohydrate side chains also affects binding on glioma cells and hepatocytes (74). While both plasminogen glycoforms when studied in isolation bind to 1-LN human prostate cancer cells with similarly high affinity (K_d ~ 4 - 25 nM), it appears that the carbohydrate side chains regulate the binding to separate receptors (see section 5.1 (53,75)). Furthermore, the various glycoforms of glu-plasminogen type 2, which differ in their sialic acid content, appear to bind to these cells via two distinct mechanisms - the less sialylated forms binding via their lysine binding sites while the more heavily sialylated forms bind via their carbohydrate chains (53).

5. MAMMALIAN CELL SURFACE PLASMINOGEN RECEPTORS

5.1. Multiple, distinct proteins may act as plasminogen receptors

The high number of plasminogen binding sites/cell together with the lysine- and carbohydrate-dependent binding mechanisms indicate that more than one receptor is responsible for localizing glu-plasminogen to the eukaryotic cell surface. Indeed multiple proteins with glu-plasminogen binding activity are detectable by ligand blotting in membrane preparations of breast cancer cell lines (52), and a heterogeneous group of 11 proteinaceous putative cellular plasminogen receptors has been identified (Table 1). In addition, the plasma membrane glycosphingolipids, gangliosides, may also contribute to cell surface plasminogen binding (76). Furthermore, thrombin stimulated platelets express cell surface fibrinogen and thrombospondin, both of which can bind plasminogen (77). Clearly, a range of potential plasminogen receptors may singly or in combination define the total plasminogen binding capacity of a cell. Moreover, various ECM proteins can generally bind plasminogen (K_d ~ 10 nM) with higher affinity than cell-surface receptors (78,79). Melanoma cells secrete large amounts of tPA into the pericellular environment which can co-bind with plasminogen to certain ECM molecules, thus resulting in enhanced pericullar proteolysis (reviewed in (9)). It is conceivable that a juxtaposition of cellular uPAR-bound uPA and ECM bound plasminogen may also be a mechanism by which tumor cells generate pericellular proteolytic activity.

Surface receptors that bind glu-plasminogen in a lysine-dependent manner may be classified into two classes. Class 1 binding proteins possess a pre-existing C-

Table 1. Cellular plasminogen receptors, previous known functions and evidence of cell surface expression

Class[1]	Protein identified as a plasminogen receptor[2]	Previously known function[3]	Cell types with cell surface expression	Known secretion/surface targeting mechanism[3]	K_d(μM)[4]
1	Alpha-enolase(81)	Intracellular glycolytic enzyme	U937(81) Rat neuronal cells (99)	No	1.9*(95) 1.4*(98) 1.0**(46)
	Cytokeratin 8(83)	Intermediate filament	Hepatocytes and MCF-7 cells(83)	No	0.16*
	TIP4a(84)	Nuclear ATPase and DNA helicase activity	ND[5]	No	0.57*
2	Actin(85)	Microfilament	Endothelial cells (97) MCF-7 and MDA-MB-231 cells (62)	No	ND
	Amphoterin(86)	Multiple biological functions associated with nervous system	Neuronal cells (86)	yes	ND
	Annexin II(87)	Ca^{++}/phospholipid binding protein	Endothelial cells (87)	Yes	0.16*(88)
	Glycoprotein IIIb/IIa(78)	integrin	Platelets (78) Synovial fibroblasts complexed with alpha2-macroglobulin (89)	Yes	ND
	GP330(90)	Autoantigen associated with Heymann nephritis	glomerular epithelial cells (90)	Yes	~0.25*
	Tetranectin(92,93)	c-type lectin	Colocalised with plasminogen in ECM of colonic tumor(103), melanoma tissue (57)	Yes	0.5*
	Extracellular domain of tissue factor apoprotein(94)	Integral membrane glycoprotein initiates extrinsic pathway of coagulation	Colocalised with plasminogen in luminal epithelial and smooth muscle cells within a human atherosclerotic plaque (94)	Yes	0.1 and 2**
3	Tissue factor(76)	" "	1-LN human prostate cancer cells (76)	Yes	0.017*
	Dipeptidyl peptidase IV(53)	Multifunctional cell surface glycoprotein	1-LN human prostate tumor cell line (53)	Yes	ND

[1] See text for explanation of classification. [2] Only references where protein first reported as a plasminogen receptor are cited. [3] See references listed under Protein Identification column for details. [4] Values are for glu-plasminogen mixtures or isolated glu-plasminogen glycoforms binding to purified native or recombinant proteins by either endpoint (*) or real-time assays (**). References are only cited if different from reference where protein was first identified as a plasminogen receptor. [5] Not determined

terminal lysine residue and include alpha-enolase (80), cytokeratin 8 (81,82), and TIP49a (83). Class 2 proteins do not have a pre-existing C-terminal lysine residue and include actin (84), amphoterin (85), annexin II (86,87), glycoprotein IIIb/IIa (77,88), GP330 (89,90), tetranectin (91,92), and extracellular domain of tissue factor apoprotein (93). For actin and annexin II partial plasmin digestion reveals a new carboxy-terminal lysine residue resulting in maximal plasminogen binding and activation (86,94). Thus, by analogy to the mechanism that enhances plasminogen binding to fibrin/fibrinogen, plasmin may act in a positive feedback loop to activate the plasminogen binding capacity of pre-existing, latent cell-surface binding proteins. The mechanism/s of binding to the other class 2 receptors remain unresolved but it is known that plasminogen can bind to the internal lysine-containing a1/a2 repeat in the streptoccocal group A plasminogen receptor PAM in the absence of protease digestion (95).

A third class of plasminogen binding proteins involves a lysine-independent interaction. This class includes dipeptidyl peptidase IV which binds the more highly sialylated plasminogen 2 glycoforms via their O-linked carbohydrate side chains on 1-LN prostate cancer cells (53). Binding of highly sialylated plasminogen 2 glycoforms by this mechanism elicits a calcium signaling response leading to enhanced expression of metalloproteinase-9. The receptor for the plasminogen 1 glycoform on 1-LN prostate cancer cells was recently reported to be tissue factor (75). However, unlike

dipeptidyl peptidase IV, binding is mediated by both the N-linked oligosaccharide side chain and the lysine binding site motifs of plasminogen glycoform 1 (75). While binding via this mechanism also initiates a calcium signaling response, the downstream effect of this response has yet to be characterized.

The physico-chemical plasminogen binding and activation properties of many of these receptors as either isolated and purified, or recombinant proteins have been analysed using solid-phase endpoint assays (Table 1). In general, binding is characterised as a single, low affinity interaction which results in the acceleration of glu-plasminogen activation by either uPA or tPA, and protection of receptor bound plasmin from inactivation by alpha2-antiplasmin (51,96). However, using surface plasmon resonance technology which allows the analysis of protein:protein interactions in real-time, we found that the binding of glu-plasminogen to immobilized recombinant alpha-enolase is a complex, multivalent interaction (46). The dissociation of glu-plasminogen from alpha-enolase was mediated by at least two components with apparent dissociation rate constants of $k_{d1}=4.7\times10^{-2}s^{-1}$ and $k_{d2}=1.6\times10^{-3}s^{-1}$. The second slower dissociation event reflects an increase in the stability of the complex. Global analysis of the interaction suggested a two-state conformational change reaction, with an overall K_d of 1 μM. Using similar technology, Fan et al. (1998) found that the binding process between glu-plasminogen and immobilized purified extracellular domain of tissue factor apoprotein reflects either multiple binding sites with different affinities, co-operativity or more complex models. Therefore, it is possible that the binding mechanisms may have been oversimplified in previous studies using end-point assays.

5.2. A bona-fide receptor must be shown to be at the cell-surface

A bona-fide receptor must be shown to be at the cell-surface orientated towards the extracellular environment where it can bind plasminogen so as to be efficiently activated. Other than immunocytochemical localization, a definitive study showed that CHO cells engineered to express cell surface tissue factor bound 70% more plasminogen than control CHO cell lines (93). Furthermore, care must be taken to ensure that receptor isolation procedures, especially when using whole cell lysates, do not simply result in the isolation of intracellular proteins that fortuitously bind plasminogen. For example, it was shown that both plasminogen glycoforms can bind to a lysine-rich sequence of the lectin concanavalin A as acetylation of the lysine groups greatly diminished the lysine-dependent interaction (97). This suggests that plasminogen has the capacity to bind to purified proteins containing a suitable lysine-rich region, including those without any apparent physiological relevance.

This has important repercussions when it is considered that many of the putative plasminogen receptors (eg., alpha-enolase, actin, cytokeratin, TIP49a) have well characterised intracellular biological roles (Table 1) and may be present in abundance in the cell cortex and/or within the cytoplasm in general. Also, none of these molecules contain classical secretion signals nor is it known how they are anchored to the cell surface. Since several of these putative plasminogen binding proteins are significantly more abundant in the intracellular compartment compared to the cell surface, even a small proportion of damaged cells in an assay will greatly alter the average plasminogen binding capacity of the total cell population. Using a dual-color flow cytometric technique which distinguishes between viable and non-viable cells together with ligand histochemistry, we confirmed that the specific plasminogen binding capacity of non-viable or deliberately permeabilized human breast cancer cell lines was 100 times greater compared to that of intact cells (52). Therefore, it is paramount that the plasminogen binding assay (and receptor isolation) procedure be able to discriminate between intact or non-viable (i.e. damaged plasma membrane) cells so as to not overestimate the true cell-surface binding capacity of the cell. Many of the cellular plasminogen parameters described in the literature (reviewed in (51)) were obtained using plasminogen binding assays (usually with I^{125}-plasminogen) which have no provision for discounting intracellular binding due to the presence of non-viable cells. Thus, it must be considered that the number of binding sites per cell using such techniques may be overestimations of the true number. This would also have ramifications for estimations of affinity constants.

We have used various immunocytochemistry techniques to determine whether putative plasminogen receptors alpha-enolase, actin, annexin II, cytokeratin 8 and tetranectin are cell-surface localised in breast cancer cell lines (62). While both actin and annexin II were the only proteins detectable on the surface of these cells only actin appears to play a role in plasminogen binding (62). This is not the case for endothelial cells where both actin and annexin II acts as non-competitive co-receptors for plasminogen and tPA (50,98). By acting as a co-receptor for tPA and plasminogen, actin and/or annexin II co-localizes enzyme and substrate for maximal activation. Since the secretion of tPA appears to be restricted to endothelial cells in normal tissues and to melanoma and neuroblastoma (11), it is unlikely that actin or annexin II act as a co-receptor for tPA and plasminogen on carcinoma cell lines.

In contrast to annexin II, whose role is likely restricted to maintaining vascular integrity by utilising tPA and plasminogen (50), we found that actin co-localizes with a fraction of the total cell-surface localised plasminogen on the high binding capacity MDA-MB-231 cell line (62). By analogy to the actin/tPA/plasminogen and annexinII/tPA/plasminogen ternary complexes on endothelial cells, a ternary complex between actin, plasminogen and uPAR-bound uPA may be a possible mechanism for facilitating the generation of excess plasmin by metastatic carcinoma cells. This in turn would enhance plasminogen binding possibly by revealing new binding sites after plasmin modification of the cell surface.

Using an affinity-purified polyclonal antibody to recombinant alpha-enolase (96) we could only detect the

protein on U937 cells (Andronicos and Ranson, unpublished data) and not on carcinoma cells (62). According to Redlitz *et al*. (99), alpha-enolase represents only 10% of plasminogen binding capacity of U937 cells. Thus, it appears that alpha-enolase cell surface expression is restricted to monocytoid (80,99) and rat neuronal (100) cells. Cytokeratin 8 has been identified on the surfaces of hepatocytes (81) and MCF-7 breast cancer cells (81,82). Using a commercially available monoclonal antibody to cytokeratin 8, we were unable to detect any cell surface expression either by flow cytometry or immunohistochemistry on either MCF-7 or MDA-MB-231 cells (62). The antibody was suitable as it was able to detect intracellular cytokeratin 8 in these and other carcinoma cell lines by either western blotting of whole cell lysates or by immunohistochemistry of permeabilized cells (Andronicos and Ranson, unpublished data). While tetranectin localizes within a wide variety of tissues and cells (101-103), there is evidence to suggest that tetranectin is a secreted, ECM-associated plasminogen binding protein rather than a cell-surface receptor (102,103). Indeed, we found no evidence of cell-surface associated tetranectin on breast cancer cell lines (62).

6. REGULATION OF PLASMINOGEN BINDING CAPACITY IS LINKED TO UPAR-BOUND UPA LEVELS ON TUMOR CELLS

The inappropriate and unregulated generation of extensive proteolytic activity via plasmin is a key feature of tumor cell metastasis and is linked to overexpression of uPA/uPAR. This is supported by a large body of experimental and clinical data showing that directly or indirectly abrogating plasmin activity significantly reduces tumor cell proliferation, invasion *in vitro* and/or metastasis or angiogenesis in *in vivo* models. This has been shown using specific inhibitors or antibodies against uPA, plasmin, or uPAR, antisense inhibition of uPA/uPAR, and recombinant or synthetic analogues of uPA or uPAR (8,104-107) Immunohistochemical and *in situ* hybridization studies have shown that the strongest and most consistent expression of the uPA system universally occurs in primary carcinoma tissue with high tumor grade, and in some cases has been further localised to cells at the invasive margins (108-114). In contrast, benign lesions (e.g. fibro adenoma) and normal tissue were either negative or with occasional weak/diffuse staining for all markers in the majority of studies that included such tissue for comparison. Clinical data from several groups measuring activity and/or antigen levels in breast tumor extracts show that uPA and PAI-1 are strong and statistically significant, independent prognostic markers for identifying node-negative patients at high risk of relapse (115-118) and are proving to be useful selection criteria for adjuvant chemotherapy in such patients (117,118). The emerging hypothesis for the role of elevated PAI-1 levels in tumors may be related to integrin mediated cell adhesion and migration, rather than inhibition of uPA activity (19,20). Not surprisingly, there has been a concomitant rapid expansion in the number of studies utilizing uPA-targeted drug delivery strategies for cancer therapy (7), including our own (104-106).

The co-localization of an activation susceptible conformation of glu-plasminogen (via binding) and uPAR-bound active uPA is an important requirement in generating cell-surface plasmin. Since plasma uPA (~ 20 pM) is mostly complexed with PAI-1 (4), this suggests that efficient cellular uPAR occupancy results only from autocrine and/or paracrine synthesis and secretion of uPA. Synthesis of uPA, uPAR and inhibitors are subject to regulation by local or systemic signals, expressed appropriately as in the case of inflammation, angiogenesis, or wound healing, or inappropriately as in the case of tumor invasion (4). In breast carcinoma cell lines uPA/uPAR over-expression is also associated with a metastatic phenotype which is clearly linked to an enhanced capacity to generate plasmin (52,119,120). One study suggests that uPAR-bound uPA may act not only as a plasminogen activator, but may also facilitate binding via a non-active site interaction (121).

Plasmin cleavage of pro-uPA at the Lys_{158}-Ile_{159} activation bond results in Lys_{158} becoming the C-terminal lysine residue of the A-chain (containing the receptor binding domain) of uPA which is connected to the B-chain (serine protease domain) by a single disulfide bond (11). Using surface plasmon resonance technology, Ellis *et al.* (121) showed that glu-plasminogen can bind to immobilized active-site-blocked uPA in a lysine-dependent manner and with an apparent K_d of 50 nM. These authors identified the sequence uPA149-158 (GQKTLRPRFK) as being involved in a non-active site-dependent interaction between uPA and plasminogen that results in the efficient activation of cell-surface plasminogen. Furthermore, uPA isolated from the urine has phenylalanine as the A-chain C-terminal residue (phe-uPA). There is a 1.6 fold decrease in the K_m for the *in vitro* activation of glu-plasminogen by lys-uPA compared to phe-uPA (122). This suggests that the binding of glu-plasminogen to the C-terminal lysine residue of the uPA A-chain induces a lysine-dependent conformational change in glu-plasminogen which transforms it into a better substrate for activation. The anti-uPA antibody used in the co-localization studies is directed against the B-chain of uPA and does not inhibit glu-plasminogen binding to the MDA-MB-231 cells (62). This supports the notion that glu-plasminogen may bind directly to cell surface bound uPA via an active site-independent interaction. The binding of cell-surface associated plasminogen to uPAR-bound uPA via a non-active site interaction may sterically facilitate the activation of plasminogen by uPA by juxtaposing the active site of uPA and the activation cleavage site of plasminogen (121). This hypothesis is supported by evidence that plasminogen co-localizes with receptor-bound uPA on the surface of MDA-MB-231 cells and that it is this pool only of plasminogen that is readily activated to plasmin (62). It is unlikely that uPAR interacts directly with plasminogen, as it has been demonstrated that uPAR-uPA function can be substituted by cell-surface glycolipid anchored uPA or an anti-pro-uPA monoclonal antibody bound to pro-uPA (123,124).

Thus, a potential sequence of events leading to high plasmin levels on metastatic cells can be hypothesized as follows. As tumor cells acquire the malignant phenotype

the expression patterns of various metastasis genes are deregulated. This includes the constitutive overexpression of uPAR and pro-uPA, leading to the binding of pro-uPA to its receptor and the association of the complex at focal contacts (4,125). In conjunction, or as a result of this association, the cell surface expression patterns of a series of other proteins may be affected. Plasminogen may then be able to bind to exposed internal or existing C-terminal lysine residues on proteins that are proximal to uPAR-bound pro-uPA. Since pro-uPA has some intrinsic activator activity (126), trace amounts of plasmin are formed which kick-starts a positive feedback amplification loop by generating the formation of a plasminogen binding/activator complex. That is, plasmin acts on pro-uPA, activating it to twin-chain (active) uPA and potentially other latent receptors to expose new C-terminal lysines. Plasminogen can efficiently bind to these sites, and is then very efficiently activated by the juxtaposed active uPA to generate more plasmin, hence more C-terminal lysines. This would eventually result in the high plasminogen binding and activation capacity observed on malignant tumor cells. Concomitantly, plasminogen may also bind via its carbohydrate side chain to transmembrane proteins such as dipeptidyl peptidase IV, which may lead to enhanced metalloproteinase expression (53). Our confocal data indicate that other unidentified plasminogen binding moieties exist remote from uPA/actin sites (62). The significance of this is that plasminogen may be activated by uPA derived from adjacent stromal cells (127). This may also account for enhanced plasmin generation in malignant tissues. Hence, the invasive cell is armed with high levels of plasmin activity.

7. PERSPECTIVES

Clearly, the uPA system plays a key role in tumor progression and metastasis as it is involved in cellular adhesion, chemotaxis/migration and proteolytic degradation of tissue barriers. However, the mechanisms that result in elevated plasminogen binding, a crucial step that allow malignant cells to acquire such proteolytic activity, are still very unclear. Biological and chemical agents that cause a loss/gain of cell bound uPA are correlated with decreased/increased plasmin activity and/or invasion. Yet, there are still only a few studies that show a direct effect between cell surface uPA/uPAR and plasminogen binding. One earlier study showed that glucocorticoids indirectly caused a significant loss of plasmin binding that was correlated with a clear decrease in cell bound uPA activity (69). We have shown a strong association between high uPA/uPAR levels and plasminogen binding capacity in malignant breast cell lines (52). It may be that the uPAR status, and more specifically, the levels of uPAR-bound uPA on human breast carcinoma cells are primarily responsible for enhanced plasminogen binding and activation capacity. This would provide a direct link between the expression of the metastasis genes uPA/uPAR, enhanced cellular proteolytic activity and invasive capacity of metastatic cells.

8. ACKNOWLEDGEMENTS

Support for this work was provided by the National Health and Medical Research Council of Australia Project Grant #970808. The authors gratefully acknowledge Dr. Darren Saunders for his critical reading of the manuscript and for his helpful discussions.

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Key Words: Plasminogen, Receptor, UPA, Tumor, Metastasis, Review

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