Anti-Cancer Drugs 2001, **12,** pp. 387–400

Review paper

The urokinase plasminogen activator receptor (uPAR) as a target for the diagnosis and therapy of cancer

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The identification and characterization of validated molecular targets for cancer drug and diagnostic development is rapidly changing the way that promising new anti-cancer compounds are developed and evaluated. A significant body of *in vitro* and *in vivo* data has established the urokinase plasminogen activator (uPA) system as a promising target for cancer drug development. The uPA system has been demonstrated to have pleiotropic activities in the development of tumors, and in tumor progression and angiogenesis. There are multiple ways to target this system, the most straightforward being the development of small molecule active site inhibitors of the serine protease, uPA. However, compounds of this type have not entered into clinical trials, and issues related to selectivity and specificity of this class of inhibitors have yet to be satisfactorily resolved. Recent evidence suggests that in addition to uPA, its specific cell surface receptor (uPAR) may also be a suitable target for the design and development of cancer therapeutic and diagnostic agents. uPAR is central to several pathways implicated in tumor progression and angiogenesis. The binding of the uPA zymogen (scuPA) to uPAR appears to be a pre-requisite for efficient cell-surface activation of scuPA to the active two-chain form (tcuPA) by plasmin, and simple ligand occupancy of uPAR by scuPA initiates various signaling pathways leading to alterations in cell motility and adhesion. One therapeutic rationale that is currently being investigated is the simple displacement of scuPA or tcuPA from suPAR, which may effectively inhibit both the proteolytic and signal-transducing cascades. In addition, other approaches to the modulation of the activity of this system that may also be useful include blocking the interaction of uPAR with integrins and extracellular matrix proteins as well as strategies to down-regulate the expression of uPA and uPAR in target cells. This review will summarize these approaches, and also describe the targeting of uPAR for diagnosis and imaging. [© 2001 Lippincott Williams & Wilkins.]

Key words: Angiogenesis, cancer diagnostics, cancer therapeutics, metastasis, urokinase, urokinase plasminogen activator receptor.

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Introduction

A paradigm shift is currently underway in the discovery of anti-cancer agents. The recent availability of data implicating specific molecules and pathways in tumor progression has led investigators to begin developing therapeutic and diagnostic agents specifically against validated targets. This approach contrasts markedly with the traditional approach to cancer drug development, which has focused on the identification of molecules with cytotoxic activity against tumor cells. It has become evident that cytotoxic molecules, identified simply on the basis of their ability to poison as many cancer cells (and often, normal cells) as possible, are insufficient and, in some cases, undesirable to combat the progression of many tumors. Novel approaches to cancer therapy have begun to focus on the modulation of tumor characteristics other than tumor cell proliferation directly as a means of suppressing tumor growth and metastasis. These approaches include attempting to inhibit tumor neovascularization (angiogenesis), extracellular matrix (ECM) remodeling (e.g. during local invasion) and responsiveness of the tumor to growth factors as well as attempting to increase the rate of tumor cell apoptosis.

The urokinase plasminogen activator (uPA) system is central to many of the activities mediated by tumor-associated cells and has been implicated in angiogenesis, growth factor activation and mobilization, ECM remodeling, invasion and metastasis.[1–3] The uPA system is comprised of several protein members: uPA, a three-domain serine protease; uPAR, the three-domain cell surface receptor for uPA; plasminogen, the substrate protease zymogen that is activated through the catalytic activity of uPA and PAI-1 (plasminogen activator inhibitor-1), an endogenous inhibitor of uPA activity that may also contribute to tumor progression.[4] The activities of

the uPA system are initiated through a cascade of events originating with uPAR (Figure 1), and involve both protease-dependent, extracellular and protease-independent, intracellular events. Initiation of these cascades occurs when the zymogen form of uPA (scuPA) binds to uPAR. The scuPA–suPAR complex itself is able to activate plasminogen[5] in the absence of activation of scuPA to uPA (the active, two-chain form). In addition, several proteases[6,7] have demonstrated the ability to activate scuPA as well, although the physiological relevance of these proteases is still questionable. Plasmin remains as the most likely physiological activator of scuPA.[8] Once scuPA is activated to uPA, it is able to convert plasminogen to its active form, plasmin.[9] Each molecule of plasmin can amplify this cascade by activating many more molecules of scuPA, a process that is made more efficient by the fact that plasminogen and scuPA (by binding to uPAR) are localized to the cell surface.[10] In addition, plasmin can activate downstream proteases such as proMMP-3, leading to the activation of proMMP9[11] and the remodeling of ECM by both MMP-3 and MMP-9 as well as by plasmin directly.[12] Plasmin is also able to process or release ECM bound growth factors such as transforming growth factor (TGF)-β, vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF)-2, which can contribute to tumor progression and angiogenesis.[13–15] uPA catalytic activity is quenched by PAI-1. However, the role of PAI-1 in tumor progression will not be discussed in detail in this review.

A second cascade mediated through uPAR leads to cellular differentiation and is initiated by the binding of uPA to uPAR.[16–18] This pathway requires only the interaction of the N-terminal domain of uPA with uPAR and is not dependent on the catalytic activity of uPA. However, the extracellular proteolytic cascade and the intracellular signaling cascade leading to differentiation may act in concert to contribute to cell motility. uPAR appears to be a central molecule in regulating events related to angiogenesis, cell motility, differentiation and tumor progression, suggesting that this receptor is a valid target for anti-cancer therapy. In this review, I will discuss evidence that validates uPAR as a novel target for anti-cancer therapy as well as the various approaches that currently hold potential for therapeutically targeting uPAR.

uPAR expression in cancer and angiogenesis

uPAR in human cancer tissue

The expression of uPAR has been evaluated extensively in sections obtained from human cancer tissue biopsies using immunohistochemical techniques as well as *in situ* hybridization. uPAR is expressed by several cell types in tumors including the tumor cells themselves,[19] endothelial cells[20] and macrophages.[21] However, the prevalent cell types expressing uPAR may vary in different cancer types. In addition, the use of different antibodies raised against uPAR by various investigators has given rise to different patterns of

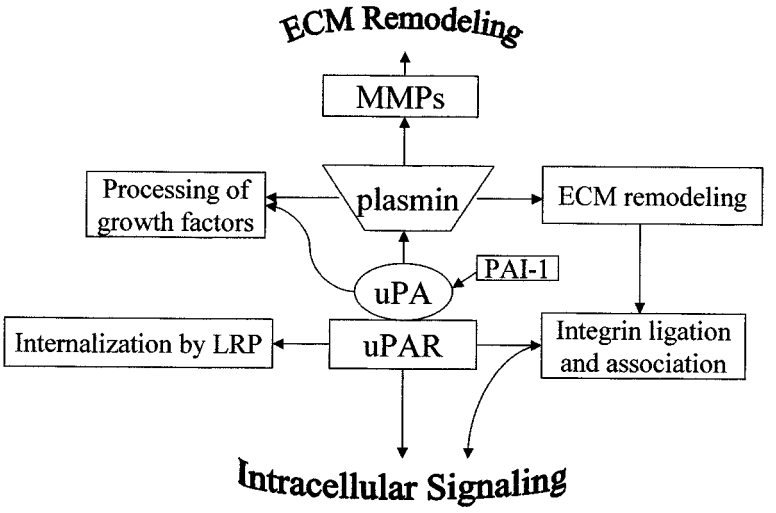

Figure 1. uPAR is central to many of the activities implicated in tumor progression. uPA, urokinase plasminogen activator; uPAR, urokinase plasminogen activator receptor; MMPs, matrix metalloproteases; PAI-1, plasminogen activator inhibitor-1, LRP, α_2-macroglobulin receptor.

immunostaining in tissues obtained from the same tumor type. This has resulted in substantial controversy as to the exact pattern of uPAR distribution, especially in colon[19,22] and breast cancer,[20,23] and given rise to significant disagreement between different laboratories evaluating these tissues. Nevertheless, several general paradigms have begun to emerge. For example, an increase in uPAR expression in the tumor cells themselves is correlated with advanced disease and the presence of distal metastases. This has recently been demonstrated in tumor samples obtained from patients with colon[19] and breast cancer.[24] In addition, the presence of uPAR-positive tumor cells in the circulation of patients that have been resected for gastric carcinoma predicts for a dramatically poorer prognosis.[25] Further, increased uPAR expression in the primary tumor cells also correlates with a highly invasive phenotype and poor prognosis in several tumor types.[26] This suggests that uPAR is expressed in tumor cells with the most invasive phenotype. High levels of uPAR expression have also been observed in tumor-associated macrophages in numerous tumors including breast,[23] primary liver cancer[21] and colon.[22] Many human tumors have an inflammatory component that may actually stimulate angiogenesis and macrophages have been demonstrated to be associated with regions of high microvessel density in human cancer.[27] Macrophages express numerous angiogenic factors including VEGF and IL-8,[28,29] and, thus, uPAR may regulate angiogenesis indirectly by mediating macrophages invasion and adhesion in tumors. There is strong *in vitro* evidence for uPAR being involved in monocyte and macrophages adhesion and chemotaxis.[30,31] Experiments with uPAR-deficient mice ($uPAR^{-/-}$) have demonstrated the impaired ability of these mice to recruit neutrophils,[32] but the effect on macrophages *in vivo* and specifically macrophages in tumors has not yet been addressed.

VEGF expressed by tumor-associated macrophages or tumor cells can up-regulate uPAR expression in endothelial cells.[33] uPAR expressed on endothelial cells may have a direct role in angiogenesis by mediating endothelial cell migration and differentiation. A role for uPAR in angiogenesis has been established using *in vitro* tube formation assays. In a Matrigel system, the binding of uPA and fragments of uPA augments tube formation by HUVEC and uPAR expression on these cells is regulated by factors contained in the Matrigel.[34] In fibrin matrices, tumor necrosis factor (TNF)-α stimulates tube formation by human microvascular endothelial cells and this tube formation is dependent on uPAR. uPAR blocking monoclonal antibodies completely inhibit formation of the tubes in this assay.[35] In this system, only proteolytically active uPA contributes to tube formation and the blocking antibody leads to a disruption of the uPA–uPAR cell surface complex. Endothelial cells over-expressing uPA release matrix-bound angiogenic growth factors, another activity that may be mediated by uPA–uPAR cell surface complexes. uPAR expression is also up-regulated in endothelial cells in response to hypoxia *in vitro*,[36] which has been suggested to initiate angiogenesis in tumors *in vivo*.[37] The possible role of the uPA system in angiogenesis has been confirmed further *in vivo* where the release of FGF-2 from binding sites in the ECM has recently been shown to be mediated by uPA.[38] In breast cancer patients with lymph node metastasis, uPAR expression has been localized to the leading edge of migrating endothelial cells,[39] emphasizing the correlate of uPAR expression with advanced disease. In clinical colon carcinoma samples, both VEGF and uPAR were more highly expressed in tumors with increased blood vessel invasion (neovascularization) than in tumors lacking vascularity.[40] Immunohistochemical examination of these tissues demonstrated that uPAR was most highly expressed on tumor cells invading the microvessels (intravasation). Finally, using a novel polymerase chain reaction assay to quantitate intravasated cells in a CAM model, Kim *et al.* have also confirmed a requirement for uPAR in tumor cell intravasation.[41]

Hypoxia may also stimulate the motility of the tumor cells themselves. MDA-MB-231 breast cancer cells cultured under hypoxic conditions were more highly invasive than the same cells cultured under normoxic conditions. Hypoxia stimulated higher cell-surface expression of uPAR and a concomitant increase in cell-surface uPA-dependent proteolytic activity due to additional recruitment of uPA by the uPAR over-expressing cells.[42] The increased invasiveness of the MDA-MB-231 cells cultured under hypoxic conditions was completely abrogated using an anti-uPAR antibody that could displace uPA. Hypoxia has been shown to increase both the transcription as well as the stability (post-transcriptional regulation) of the uPAR message in MCF-7 cells, indicating multiple levels of control for uPAR expression.[43]

Control of uPAR expression

Many of the signaling pathways and molecules that have historically been implicated in tumor progression and angiogenesis lead to the up-regulation of uPAR expression. These same pathways may also play a role in the activation of integrins, and the association of uPAR with mutiple integrin heterodimers may be a central event required for cell motility, invasion and uPAR-dependent signaling, as will be described later in

this review. For example, uPAR expression in endothelial cells is up-regulated by angiogenic growth factors such as FGF-2[44] and VEGF.[33] VEGF has recently been implicated in the physiological activation of integrins (earlier studies on integrin activation involved artificial activation with divalent cations).[45] VEGF alters the avidity of various integrins for their ligands while at the same time increasing the expression of uPAR, which may be required for modulating the activity of integrins during cellular migration and adhesion. Several proteases that have been implicated in the remodeling of the ECM also regulate the expression of uPAR. uPA itself regulates uPAR expression in several different cell lines and this effect depends only on the binding of uPA to uPAR.[46] In another study, an inhibitor of uPA catalytic activity suppressed uPAR expression in a breast tumor model *in vivo*.[47] Neither of these results is particularly surprising since many of the pathways that control uPAR expression are also activated through uPAR. The catalytic activity of uPA may initiate an autocrine loop whereby the formation of plasmin by uPA leads to the mobilization of growth factors sequestered in the ECM[13] or the processing of these growth factors to active forms.[14,15] These factors then feedback in an autocrine fashion and up-regulate uPAR expression. Type I collagenase has also been reported to affect the expression of uPAR and the suppression of Type I collagenase expression in melanoma cells using an antisense strategy decreased uPAR expression in these same cells.[48] uPAR expression is also up-regulated through constitutively active Src[49] and K-Ras-dependent[50] pathways in tumor cells, suggesting a possible mechanism for how an invasive phenotype may be induced in transformed cells. Induction of uPAR through Src results in a highly invasive phenotype and this invasiveness could be inhibited using a small cyclic peptide antagonist of the binding of uPA to uPAR.[49] Similarly, targeted disruption of K-Ras in a colon carcinoma cell line led to a decrease in uPAR expression with a concomitant decrease in invasiveness.[50] Since uPAR expression is regulated by extracellular cytokines and growth factors, which often signal through Src and Ras, it is not surprising that p38 Map kinase has also been demonstrated to regulate uPAR expression. BT549 breast cancer cells, which contained a constitutively active p38α, had increased uPAR expression and were highly invasive.[51] A specific inhibitor of p38 kinase activity, SB203580, suppressed the expression of uPAR and inhibited the ability of these cells to invade Matrigel. Decreased expression of uPAR resulted from a de-stabilization of uPAR mRNA. Shetty and Idell[52] have recently demonstrated that uPAR (as well as uPA and PAI-1) expression is regulated at least in part at the post-transcriptional level and identified a specific uPAR mRNA binding protein that is involved in the de-stabilization of the uPAR message.

The same pathways that lead to the up-regulation of uPAR expression are also involved in uPAR-dependent signaling. Signaling through uPAR has been demonstrated to activate several pathways depending on the cell type. In MCF-7 breast cancer cells, uPAR signaling requires focal adhesion kinase (FAK), *src* and *shc*.[53] FAK associates with integrin β chains and the participation of *src* in this pathway can activate Ras and downstream mitogen-activated (MAP) protein kinases.[54,55] Src family kinases are also activated in other cell types such as U937 monocyte-like cells, where different members of this kinase family regulate both adhesion and migration.[56] Tyrosine phosphorylation of FAK and MAPK is also regulated through uPAR in endothelial cells.[57]

uPAR structure and function

uPAR is a 45–65 kDa glycoprotein that is localized to the outer layer of the plasma membrane of cells.[58] Protein contributes about 31.5 kDa to the mass of uPAR, the rest being contributed by carbohydrate. There are five *N*-linked glycosylation sites in uPAR, and the glycosylation pattern and the affinity of cell-surface uPAR for uPA can be altered by differentiating agents such as phorbol myristyl acetate (PMA).[59] uPAR is localized to the outer layer of the membrane via a glycosyl phosphatidylinositol linkage (GPI anchor) and does not possess a transmembrane domain.[60] Despite this fact, signal transduction through uPAR does occur and the existence of adaptor molecules capable of coupling the binding of uPA to uPAR to transmembrane signaling has been proposed.[61] Various candidate integrins may fulfill this adaptor function, and co-localization studies have demonstrated an association of uPAR with β_1 and β_2 integrins from tumor cells and neutrophils.[62,63] Other integrins, including $\alpha_5\beta_1$[64], $\alpha_v\beta_3$[62] and $\alpha_v\beta_5$,[65] have been demonstrated to be required for uPAR-dependent cell motility on various matrices and also for tumor cell survival. uPAR also associates with α_M[66] and Tarui *et al*. have demonstrated that uPAR is a ligand for multiple integrin types and can associate with integrins on neighboring cells *in trans*, suggesting that uPAR may also be involved in cell–cell adhesion.[67] uPAR has also been localized to caveolae. The membrane protein, caveolin, which binds to several signaling molecules that are linked to integrin function such as *src*, is required for the formation of integrin/uPAR complexes and uPAR-

dependent adhesion.[68] Thus, uPAR appears to play a central role in integrin-mediated signal transduction and adhesion.

uPAR is comprised of three domains with structural homology based on the spacing of the disulfide bonds.[58] There is very little other homology in the primary sequence. Domain 1 (Figure 2) contains most of the determinants required for the binding of uPAR to uPA.[69] Alanine scanning mutagenesis of residues within domain 1 completely abolishes the ability of uPAR to bind to uPA.[70] However, isolated domain 1 binds to uPA with 100-fold less affinity than the intact uPAR.[69] Thus, additional determinants in either domain 2 or domain 3 are required to realize the full affinity of the binding. Photoaffinity labeling experiments using a peptide-based affinity ligand of uPAR label residues in both domain 1 and domain 3, suggesting that domains 1 and 3 form a composite binding site for uPA.[71] Further, alanine scanning mutagenesis of domain 2 has identified a 9 amino acid segment of uPAR that appears to be critical for the binding of uPAR to uPA, thus also implicating domain 2 in the composite binding site for uPA.[72] Domains 2 and 3 (D23) have also been implicated in the interaction of uPAR with kininogen,[73] vitronectin[74] and integrins.[67] Finally, the connecting region between domain 1 and domain 2 is chemotactic for monocytic cells, and can be exposed through proteolysis or through a putative conformational change that occurs when uPA binds to uPAR.[75] This processing may be physiologically relevant as both D1 and D23 fragments have been detected in the urine of cancer patients.[76] Thus, uPAR may present several potential targets for interfering with its activity.

Validation of uPAR as a therapeutic and diagnostic target

Numerous approaches have been used to validate uPAR as a target for cancer therapy and diagnostics, and many of these approaches provide a basis for translation into clinical use. The majority of validation studies have focused on assessing the effect of blocking the uPA–uPAR interaction. However, it is clear that the interactions of uPAR that occur downstream from uPA are also important to the biological function of uPAR and thus present novel targets for therapy. Although the effects of blocking these downstream interactions of uPAR (e.g. with integrins) have not been investigated extensively *in vivo*, several studies *in vitro* suggest that this approach could have utility for inhibiting adhesion and migration. For example, Simon *et al.* identified a region of the integrin subunit, α_M, that was homologous to a uPAR binding peptide identified by phage display.[66] A peptide based on this region, M25, did not block the binding of uPA to uPAR but nevertheless inhibited

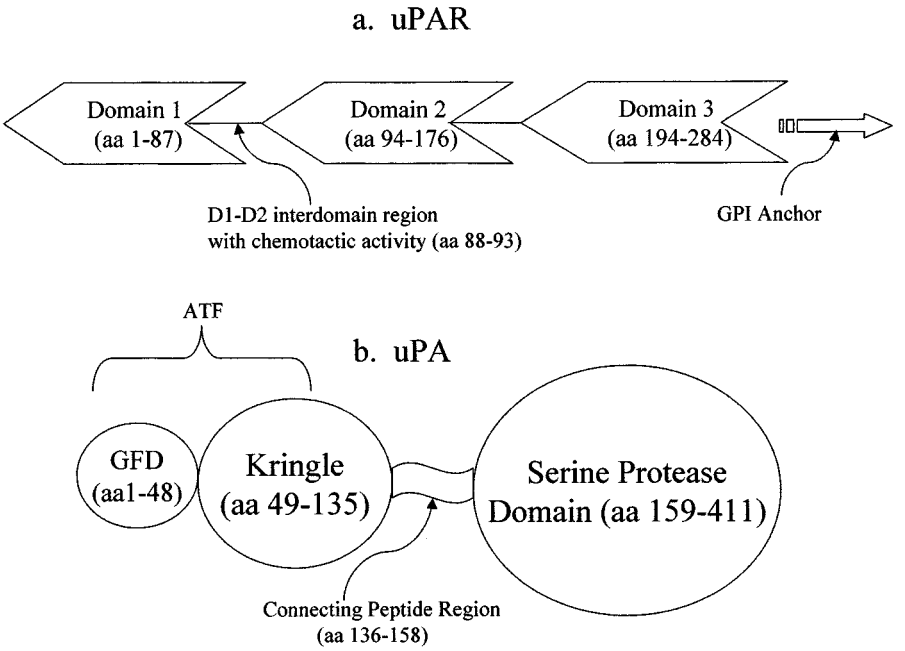

Figure 2. Schematic representation of (a) uPAR and (b) uPA. GPI, glycosyl phosphatidylinositol; GFD, growth factor domain.

leukocyte adhesion to fibrinogen, vitronectin and endothelial cells. Studies using antisense approaches, which led to decreased uPAR protein levels and affect both ligand (uPA)-dependent and ligand-independent (downstream) activities of uPAR have demonstrated substantial anti-tumor effects in several systems. Aguirre Ghiso *et al.*[64] demonstrated that transfection of HEp3 human epidermoid carcinoma cells with uPAR antisense reduced uPAR expression by 70% and led to a protracted state of dormancy in a CAM model. Reduction in uPAR levels in these cells decreased the association of uPAR with the integrin $\alpha_5\beta_1$, resulting in a decreased avidity for this integrin and the decreased adhesion of the transfected cells to fibronectin. Adhesion of HEp3 cells to fibronectin was also inhibited by the M25 peptide, emphasizing the potential importance of non-ligand mediated uPAR interactions. Mohan *et al.* used adenovirus-mediated delivery of antisense targeting uPAR to generate glioblastoma cell lines that failed to form tumors when inoculated intracranially into nude mice.[77] These same investigators also demonstrated that injection of these adenoviral constructs into pre-established glioblastoma tumors induced the regression of these tumors *in vivo*.

Several studies have also validated targeting the uPA–uPAR interaction directly. Crowley *et al.* transfected prostatic carcinoma tumor cells with a cDNA construct that expressed catalytically inactive uPA, then inoculated these cells s.c. into nude mice to assess the effects of this construct on metastasis.[78] Animals that were transfected with the catalytically inactive uPA demonstrated almost complete inhibition of metastasis. In effect, these investigators generated a uPAR antagonist (e.g. catalytically inactive uPA) *in situ*, which could only bind to uPAR on the tumor cells (which were of human origin). There is a human–rodent species barrier that prevents the high-affinity binding of uPA from one species to uPAR from the other species and this has been one of the challenges in validating uPAR as a target since most strategies at inhibition focus on human uPAR, but most models are rodent based. In the Crowley study, the investigators could only evaluate the effects of blocking tumor cell uPAR, which would tend to under-represent the potential activity (as well as potential toxicity) of a uPAR antagonist. Li and colleagues recently addressed this issue in an elegant study using an adenoviral vector to deliver and express the murine uPAR binding domain (the ATF) from murine uPA.[79] Intratumoral injection into either a pre-established human tumor (MDA-MB-231 breast cancer) or into a pre-established mouse tumor (Lewis Lung) arrested tumor growth in both models. Neovascularization of the tumor and the region surrounding the tumor was also suppressed. Finally, distal metastasis was inhibited as well. Several other studies have also validated the anti-tumor effects of inhibiting the uPA–uPAR interaction using ATF hybrid molecules. Ignar and co-workers demonstrated that a daily injection of an ATF–mouse IgG fusion protein extended the latencies of unstaged experimental melanoma and colon carcinoma tumors in nude mice.[80] The growth of staged tumors (200 mm^3) was unaffected, although experimental metastasis could be inhibited by as much as 95%. Taken together, there is a significant body of evidence that supports the targeting of uPA for cancer therapy.

Possible modes of therapy targeting uPAR

Peptide and small molecule antagonists of the uPA–uPAR interaction

Several approaches using peptides to inhibit the uPA–uPAR interaction have been reported. Most of these approaches have focused on using peptides based on the sequence of the growth factor domain of uPA (GFD), which mediates the binding of uPA to uPAR. Kobayashi *et al.*[81] used linear peptides spanning amino acids 17–34 of mouse uPA to inhibit lung metastasis in a Lewis lung model. More recently, several investigators have attempted to cyclize the uPAR binding region of uPA using either disulfide bridge formation to cyclize a peptide spanning amino acids 19–31 of uPA[82] or an amino acid bridge to cyclize amino acids 20–30 of uPA.[83] Both approaches produced cyclic peptides that were able to compete with the binding of uPA to uPAR on cells with an $IC_{50} \sim 10$–20 nM. A cyclic peptide derived using the second approach (Å5) was able to completely inhibit laminin degradation by a colon carcinoma cell line transfected to constitutively express Src, which induced up-regulation of uPAR in these cells.[49] Unfortunately, despite the promise of this approach, it has not been validated thus far *in vivo*. Linear peptides based on the sequence of uPA lack potency, and are predicted to have poor pharmacological properties and lack of stability due to susceptibility to exoprotease degradation in the plasma. Similar problems may exist with cyclic peptides although they would be expected to have increased plasma stability as compared to their linear counterparts.

Goodson *et al.* reported the discovery of high-affinity peptide ligands of uPAR using phage display.[84] The most potent of these, peptide 20, was able to inhibit the binding of uPA to uPAR with an $IC_{50} \sim 10$ nM, which was at least 1 order of magnitude

better than previously reported for any other linear peptide. Peptide 20 has some homology to the GFD of uPA and Ploug *et al.* used this peptide as the base sequence for peptide optimization approaches.[71] These investigators describe substitution at certain positions using uncommon amino acids and peptoids, leading to optimized peptides which inhibited the binding of uPA to uPAR with an $IC_{50} \sim 2$ nM (only 2-fold less than the IC_{50} for uPA, the native ligand, in their competition assay). However, these more potent peptides have not yet been tested in animal studies. To some extent, a more potent peptide may circumvent some of the potential pharmacological problems typically associated with peptide therapeutics and would be expected to localize to a tumor. Nevertheless, a significant plasma half-life will still be necessary for any antagonist of the uPA–uPAR interaction as a significant pool of uPAR-bound uPA will need to be displaced from the tumor surface for an inhibitor of this type to be effective.

Several moderate and weak affinity non-peptidic, small molecule antagonists of uPA binding have also been described. Suramin (as well as other polysulfonated, dinaphthyl compounds such as Trypan blue) inhibited the binding of uPA to U937 cells in a non-competitive manner with an $IC_{50} \sim 30$ μM.[85] However, suramin has been demonstrated to interact with many molecules implicated in cancer progression and angiogenesis such as FGF, IGF, TGF-β and others,[86,87] and this promiscuous activity may be responsible for the significant toxicity observed with suramin in cancer patients.[88] Oligo-thiophene inhibitors of uPA binding to uPAR have also been reported,[89] the most potent of these having an $IC_{50} \sim 2$ μM. No other biological data has been obtained using this class of inhibitors.

Peptide and small molecule antagonists of uPAR interactions that are down-stream from uPA binding

uPAR interactions with integrins mediate cellular signaling and differentiation leading to changes in adhesion and motility of tumor cells, angiogenic endothelial cells and inflammatory cells. Thus, the inhibition of uPAR–integrin interactions may lead to decreased motility of these cells making this inhibition a viable therapeutic hypothesis. There has been no direct validation for the utility of this approach *in vivo* although the down-regulation of uPAR expression had dramatic effects on tumor growth of experimental glioblastoma in mice[77] and HEp3 tumors in a CAM model.[64] These dramatic effects could be modulated in part through a decrease in uPAR interactions with integrins although decreased binding of uPA or decreased interactions of uPAR with other unidentified molecules could also explain these effects. Wei *et al.*[90] demonstrated that a peptide identified through phase display (M25) was able to bind to uPAR and inhibited cellular adhesion to fibronectin. This peptide did not inhibit the binding of uPA to uPAR. Recently, Simon *et al.* identified M25 as having homology to the integrin α_M[66] and demonstrated that this peptide was able to inhibit adhesion to fibrinogen, vitronectin and cytokine-stimulated endothelial cells.

The identification of small molecule lead compounds that inhibit either uPA binding to uPAR or uPAR interactions with integrins presents several challenges. Protein–protein interactions such as these are difficult to target in the absence of structural data since they require inhibition that must occur over a fairly large protein surface. Although ligand–receptor interactions are often driven by a hot spot of 4 or 5 amino acid side chains,[91] the same may not be true for an interaction such as uPAR with an integrin. Screening for inhibitors of uPA binding to uPAR or uPAR binding to an integrin are also unlikely to yield viable lead compounds. Most compound libraries and combinatorial libraries are comprised of molecules with a molecular mass in the 200–300 Da range and these may be too small to demonstrate activity in a screening assay designed to evaluate the interaction of uPAR with a particular integrin. This hypothesis is supported to some extent by the non-peptide antagonists of the uPA–uPAR interaction, which are in the range of 500–650 Da.[89] Further, although a peptide approach is useful for providing leads (as has been described above), it may be difficult to obtain peptides that are potent inhibitors and also possess drug-like properties. Cyclization may be required to increase the potency of a peptide inhibitor and this may make large-scale synthesis impractical or extremely costly. Structural information on uPA–uPAR or uPAR–integrin interactions could provide data that would be useful for drug design. However, generating a *de novo* lead from the structure of a protein complex remains the holy grail of structure-based drug design and has never been achieved. Nevertheless, there are recent reports of peptidomimetics being designed based on the complex of a SH2 domain bound to a phosphopeptide, thus the application of structure-based drug design to targets involving protein–protein interactions may be realistic in the near future. In addition, the advent of high-throughput structural approaches such as structure–activity relationships by crystallography[92] may also lead to the identification of novel lead inhibitors of the uPA–uPAR and uPAR–integrin interactions.

Regulation of uPAR expression

Geldanamycins. The geldanamycins are a class of benzoquinoid ansamycin antibiotics that have potent tumor cell cytotoxic activity.[93] Unfortunately, despite potent tumoricidal activity, the parent geldanamycins also exhibit an undesirable toxicity profile, which has limited their clinical utility.[94] Recently, analogs of geldanamycin with improved toxicity profiles have been identified and one of these, 17-AAG, is currently in phase I clinical trials as a novel cytotoxic agent.[95] The cytotoxic activity of the geldanamycins appears to be mediated through the inhibition of HSP90, which forms complexes with several oncogene products and signaling molecules such as $pp60^{v\text{-}src}$, p185erbB2, mutant p53 and Raf-1.[96] Inhibition of HSP90 by geldanamycin may lead to dissociation of HSP90–signaling molecule complexes.[97]

There may be other, more subtle activities for the geldanamycins that were not initially observed in tumor cell cytotoxicity assays. Webb *et al.* recently reported[98] that geldanamycin treatment of cells *in vitro* led to the down-regulation of c-Met [the cellular receptor for hepatocyte growth factor (HGF)] expression, which in turn induced the down-regulation of uPAR and uPA expression. The effect on uPAR was especially pronounced and geldanamycin reduced HGF-stimulated uPAR expression to levels similar to those observed in non-stimulated cells. These effects occurred at concentrations (nanomolar) of geldanamycins that were several orders of magnitude lower than those required for cytotoxic effects. Further, the geldanamycins inhibited HGF-mediated plasminogen activation at even lower (fentomolar) concentrations. HGF and c-Met are major mediators of epithelial cell motility,[99] and HGF has also been demonstrated to be a pro-angiogenic factor.[100] These activities probably occur at least partially through the regulation of the uPA system. In the study by Webb *et al.*, geldanamycin inhibited HGF-mediated cell motility and invasion in the same concentration range required for inhibition of uPAR expression. This suggests that geldanamycins might be useful as anti-angiogenic and anti-invasive/anti-metastatic agents at doses well below their MTD and that these effects in part could be mediated through the down-regulation of uPAR expression.

Anti-sense and gene therapy. Down-regulation of uPAR expression has also been achieved in pre-clinical models using anti-sense and gene therapy approaches leading to increased tumor dormancy,[64] decreased tumorigenesis,[77] the inhibition of angiogenesis[79] and increased survival.[101] Although these approaches have been used successfully in pre-clinical studies, limitations of delivery have limited the utility of this approach in the clinic. Nevertheless, there are early signs that anti-sense targeting of other gene products [PKC-α by ISIS 3521, which is showing early signs of activity in non-small cell lung cancer (NSCLC)] may have therapeutic utility, and once the delivery and stability problems of these therapeutic vectors is overcome, uPAR would become a viable target for this approach.

Recent studies have also described the regulation of uPAR expression at the post-transcriptional level through the interaction of uPAR mRNA with a specific uPAR mRNA binding protein.[52] The binding of this 50 kDa protein to a 51 nucleotide stretch in the coding region of the uPAR mRNA destabilizes the message by increasing its degradation rate. Strategies aimed at promoting the interaction of the uPAR mRNA binding protein with uPAR mRNA (e.g. by increasing the expression of the binding protein) or by mimicking the binding of the uPAR mRNA binding protein with a peptide or synthetic compound that could be delivered inside the cell could lead to down-regulation of uPAR expression. Unfortunately, these strategies would have to overcome many barriers such as delivery and design/discovery of suitable compounds and thus may not be practical to pursue in the short term.

Monoclonal antibodies

The use of monoclonal antibodies to interfere with the various activities of uPAR has not yet been exploited at the therapeutic level. Anti-uPAR antibodies could potentially inhibit the binding of uPAR to uPA, integrins, matrix components or other uPAR accessory molecules. In addition, it may be possible to generate subtypes of anti-uPAR IgG that are capable of recruiting an inflammatory/immune response directed only at a tumor or the tumor-associated vasculature. This opsonization could result in tumoricidal effects, potentially leading to tumor regression. Another strategy would be to develop antibodies directed against the ATF portion of uPA (Figure 2). This could lead to direct antagonists of uPA binding to uPAR. Opsonization using ATF as the target would probably not be a viable strategy since uPA, independent of uPAR, is required for normal function in several compartments (e.g. the alveoli of the lung or in the renal tubules) and one would not want to generate an immune response against the uPA in these compartments. In addition, the generation of immunologic memory against uPA is not desirable as this could interfere with the physiological functions of this

enzyme. Thus, tremendous care in selection of the IgG subtype would be required if targeting the ATF half of the uPA–uPAR interaction.

Conjugates and targeted therapy

Since uPAR expression is up-regulated in tumor and tumor-associated endothelial cells, targeting uPAR may represent a specific way in which to selectively deliver therapeutic and diagnostic agents to a tumor. uPAR targeting may be accomplished using uPAR-binding peptides, antibodies, small molecules or even uPA or fragments of uPA. Rajagopal and Kreitman recently described several ATF–toxin (a truncated form of the *Pseudomonas* exotoxin) fusion proteins that were cytotoxic to some tumor cells at concentrations as low as 0.3 pM.[102] Similar approaches could also be used to target radio-therapeutic and radio-diagnostic moieties to cells expressing uPAR. These approaches would also be expected to have anti-angiogenic activity and might be useful if delivered using metronomic dose scheduling as recently described by Folkman[103] and Kerbel.[104]

Potential for toxicity in targeting uPAR

There is substantial evidence that uPAR expression is up-regulated only during tissue remodeling (e.g. during wound healing), in cell motility (e.g. invasion of macrophages) or during pathology (e.g. in cancer, pathological angiogenesis, etc.). A recent study by Solberg *et al.*[105] demonstrated that murine uPAR is expressed primarily in tissues undergoing active remodeling. If this result could be extrapolated to the human situation, selectivity would be implied for targeting uPAR as a cancer therapeutic or diagnostic since most human tissues are quiescent and would thus not be expected to express uPAR. uPAR null mice do not have any phenotype and develop and reproduce normally and this suggests that inhibition of uPAR would not lead to significant mechanism-based side effects. Of course, this also raises the question of what the physiological role of uPAR really is and what importance it has in tumor biology. The major drawback of the null mice is that they were bred and developed with a uPAR$^{-/-}$ background. Thus, compensatory mechanisms consistent with survival may inaccurately reflect the requirement (or lack thereof) for uPAR under certain conditions in an adult organism. Neutrophil recruitment is impaired in uPAR$^{-/-}$ mice but not to an extent sufficient to lead to mortality. Very little else has been done to challenge these mice or to evaluate the role of uPAR in tumorigenesis or tumor progression in the null models. There are several transgenic models (TRAMP prostate carcinoma model, Rip-Tag pancreatic carcinoma model) that lead to spontaneous tumor formation and it would be interesting to cross these mice with the uPAR$^{-/-}$ mice. Unfortunately, these experiments may still yield a negative result since uPAR expression is correlated with metastasis and later stage tumor progression, which the transgenic models never really achieve. Thus, uPAR$^{-/-}$ mice suggest that the inhibition of uPAR may not lead to significant mechanism-based toxicity but this remains to be proven with uPAR-specific compounds.

Drug toxicity often results from the interaction of a drug with proteins related to the drug target. For example, inhibitors of matrix metalloproteases have significant toxicity that arises from the undesired inhibition of related family members in addition to the family members implicated in cancer.[106] uPAR does not have any known protein isoforms and thus toxicity due to cross-talk with another isoform of the receptor would not be expected. Although uPAR populations on cells can appear to be fairly heterogeneous due to the existence of glycosylation isoforms, there appears to be only one uPAR protein expressed on the surface of human cells (although an alternatively spliced soluble variant has been described[107]). There is no evidence to suggest that particular uPAR glycosylation isoforms participate in specific functions and the approach thus far has been to inhibit the bulk population of uPAR. Clearly, toxicity issues need to be addressed for each specific set of inhibitors but *a priori* the approach of targeting uPAR seems to hold promise for relatively little mechanism-based toxicity.

Diagnostics based on uPAR

A significant body of data in the literature has established the potential for uPAR as a prognostic marker in multiple tumor types.[108,109] Recently, the presence of soluble forms of uPAR (suPAR) in patients with acute myeloid leukemia[110] and renal cell carcinoma[111] has been demonstrated to correlate with poor prognosis, and may be indicative of increased tumor burden. The shedding of suPAR into the plasma may also correlate with increased intra-tumoral proteolysis and thus may be indicative of an aggressive tumor phenotype. The majority of studies to evaluate the expression of uPAR in cancer have focused on establishing the levels of suPAR in biopsied samples using ELISA,[112] Western blot or immunohistochemistry,[113] or in plasma using ELISA.[108,109] However, uPAR is not expressed uniformly throughout a tumor and may be restricted to tumor cells at the invasive edge of a tumor, adjacent to neovessels or in the adjacent

neovessels themselves.[26] The presence of uPAR in gastric cancer[28] and in colorectal cancer[26] has been demonstrated to be a marker of metastasis. Further, in breast cancer patients with nodal metastasis, uPAR expression has been demonstrated to be strongly up-regulated in the neovessels of the primary tumor and to correlate with concomitant up-regulation of VEGF, again suggesting that uPAR expression correlates with metastasis and progressive disease.[27] Thus, one can hypothesize that the detection of uPAR may be useful in identifying those patients that will progress to metastatic disease or those that might already have metastatic foci present. The selective up-regulation of uPAR on the surface of tumor and tumor-associated endothelial cells provides a means of targeting imaging and diagnostic agents to tumors *in vivo* and this could provide a highly sensitive method for detecting the presence of cells expressing uPAR. Although monoclonal antibodies have some practical issues associated with their use as targeting agents, they can be modified to make them more suitable for this application (in fact, the same anti-uPAR antibody could be used to target either a therapeutic or a diagnostic moiety to a tumor). Similarly, peptides or small molecules could be conjugated to perform the same function.

Diagnostic and therapeutic agents targeting uPAR would ideally be used in conjunction with each other. A diagnostic agent could be used to select patients for anti-uPAR therapy (e.g. those that have high levels of uPAR in their tumor or those that display multiple uPAR-positive metastases) followed by the use of an anti-uPAR therapeutic agent. Cyclic peptides capable of carrying either diagnostic or therapeutic moieties and target uPAR with high affinity have recently been described in the patent literature.[114] However, no such agent is currently being developed for clinical trials.

Future directions and conclusion

Although there is a significant amount of data validating uPAR as a diagnostic and therapeutic target, there is a paucity of agents that target or inhibit uPAR activity in pre-clinical development or even approaching development. Some of the issues regarding development of such agents have focused on the difficulty of antagonizing the uPA–uPAR interaction, methodological difficulties in delivering anti-sense or gene therapy vectors (as has been true for many other targets as well) and the historical reluctance to use immunotherapy. Several commercial ventures are focusing on this system as a therapeutic and diagnostic target but are still several years away from putting a reasonable candidate into clinical trials. Unfortunately, the importance of uPAR as a target will not be completely established until agents suitable for use in cancer patients are tested.

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(Received 27 February 2001; accepted 12 March 2001)