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Structure, function and antagonists of urokinase-type plasminogen activator

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1. ABSTRACT

Urokinase (uPA) is a serine protease which converts plasminogen to plasmin, a broad-spectrum protease active on extracellular matrix (ECM) components. Like many components of the blood coagulation, fibrinolytic and complement cascades, uPA has a modular structure, including three conserved domains: a growth factor-like domain (GFD, residues 1 – 49), a kringle domain (residues 50 – 131), linked by an interdomain linker or "connecting peptide" (CP, residues 132 – 158) to the serine protease domain (residues 159 – 411). Although direct molecular interactions with urokinase receptor and integrins have been extensively described, the function of single uPA domains is not completely understood. Because of the causal involvment of uPA in cancer invasion and metastasis, the blockade of uPA interactions and activity with specific inhibitors is of interest for novel strategies in cancer therapy. New inhibitors derived from the interdomain linker or "connecting peptide" are coming into focus. This review summarizes the recent findings on the uPA structure-function relationship and provides further information on existing inhibitors of uPA multiple functions.

2. INTRODUCTION

The serine protease urokinase (uPA) is secreted as a 411 aminoacids single-chain zymogen form (pro-uPA) which becomes activated by plasmin cleavage in the extracellular milieu (1). In turn, active uPA converts inactive plasminogen into the active serine protease plasmin, able to degrade most of the ECM components and activate latent collagenases and growth factors. Pro-uPA consists of a growth factor-like domain (GFD, residues 1 – 49), a kringle domain (residues 50 – 131), an interdomain linker or "connecting peptide" (CP, residues 132 – 158), and a serine protease domain (residues 159 – 411) (Figures 1 and 2). Activation of the pro-enzyme occurs by proteolytic cleavage at Lys ^{158}-Ile^{159}, thus generating a two-chain molecule linked by a disulfide bridge between Cys^{148} and Cys^{279} (HMW uPA). A further proteolytic cleavage releases an amino-terminal fragment (ATF, residues 1 – 135) and a small region linked to a large C-terminal proteolytic domain (LMW uPA) (2). *In vivo*, pro-uPA may be activated by plasmin and also by other proteases, including glandular kallikrein mGK-6, as shown by the analysis of urine from plasminogen-deficient mice (3). Hepsin is an example of type II transmembrane protease

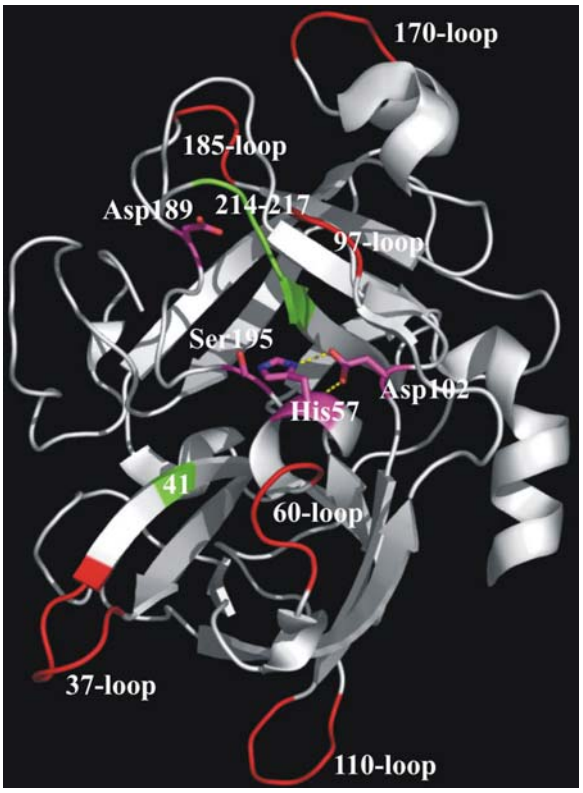

Figure 1. The 3-dimensional structure of human uPA. The figure shows the catalytic triad Ser195, His57, and Asp102, and Asp189 in the S1 pocket (pink); the main chain binding area around residues 214-217 and 41 (green); and uPA's specific surface loops (red) (Reproduced with permission of P.A. Andreasen).

which is able to convert pro-uPA into an active enzyme (4). Pro-uPA may undergo several post-translational modifications, such as glycosylation of Asn^{302}, phosphorylation on $Ser^{138/303}$ and fucosylation of Thr^{18} (5-6). In particular, the latter two modifications modulate the catalytically-independent chemotactic and mitogenic activities of uPA, respectively (5, 7). Pro-uPA is encoded by a 6.4 kb gene which is located on the long arm of chromosome 10 (8-9). The TATA-box containing minimal promoter ensures a low level of uPA basal expression and includes a GC-rich region and a CAAT sequence binding to the ubiquitous factors Sp1 and CTF, respectively. An upstream enhancer is located at –2 kb and requires the cooperation between an Ets(PEAIII)/AP1 and an AP1 site to up-regulate uPA mRNA transcription (10). This cooperation is mediated by a 74-bp protein-binding domain, the cooperation mediator element (11). It is noteworthy that transcription factors binding to the Ets/AP-1 sites are activated by the Ras/MAPK-dependent pathway, which is triggered by a variety of stimuli like growth factors, cytokines, uv light and is hyper-activated in most tumors (12). Transcriptional regulation may be mediated also by the NF-kB complex acting through binding of Rel/p65 and p65/p50 to a specific sequence located at –1583 bp (13). Interestingly, the overall analysis of the exon-intron organization of the pro-uPA gene strongly suggests that exons encoding single functional domains were exchanged between different genes by intronic recombination events (8, 14). This observation well agrees with the finding that single uPA domains are structurally and functionally autonomous (15).

3. THE MULTI-DOMAIN PROTEASE UROKINASE

3.1. The growth factor-like domain

Like bood coagulation factors VII, IX and X and the anticoagulant protein C, uPA features a conserved growth factor-like module which is homologous to the EGF-like protein family (16). The EGF-like domain includes six cysteine residues which are engaged in disulfide bonds. The structure of several EGF-like domains has been solved. In all cases, a two-stranded beta-sheet is followed by a loop and a C-terminal short two-stranded sheet (17). Consolidated evidence shows that GFD mediates the binding of uPA to a glycosylphosphatidylinositol (GPI)-anchored cell surface molecule, also known as uPAR, which was discovered over twenty years ago (18-19). The receptor binding ability of uPA is retained by a peptide corresponding to residues 12-32 of the human sequence (16). A mutagenic analysis of the residues involved in the full molecule shows that Lys23, Tyr24, Phe25, IIe28, and Trp30 are essential for uPAR binding (20). Recent evidence reports the occurrence of intra-molecular interactions between kringle and GFD which stabilize uPA/uPAR complexes. This is supported by the fact that a pro-uPA variant lacking the kringle domain binds to soluble uPAR with a similar "on-rate" but with a faster "off-rate" than wild-type pro-uPA (21). Pro-uPA, uPA and ATF exhibit a similar K_D for binding to uPAR, in the range of 100 - 500 pM (22). Receptor-bound uPA is proteolytically active and can be inhibited by plasminogen activator inhibitor type-1 (PAI-1). As shown by kinetic studies, cell surface plasminogen activation exhibits an overall increase of kcat/Km of 5.7-fold with respect to the same reaction with soluble components. Therefore, receptor-bound uPA endows cells with an efficient matrix-degrading ability (23). Although it is not the subject of this review, most of the described biological outcomes following uPA/uPAR association are proteolytically independent and relate to cell migration, adhesion and proliferation (24). Remarkably, the mitogenic activity of a peptide corresponding to residues 4 – 43 and full-length uPA on human SaOS-2 osteosarcoma cells is dependent on fucosylation of Thr^{18}. On the contrary, motogen and anti-apoptotic activities of uPA are retained by GFDp, a peptide corresponding to the amino-acids 12-32, showing that post-translational modifications in this region are not relevant to these functions (25-26).

3.2. The kringle domain

Kringles are autonomously folding domains retaining similar architecture during evolution. A likely possibility is that kringles of prothrombin, plasminogen, tissue plasminogen activator, coagulation factor XIIa and uPA may have evolved from a common ancestral module (27). The uPA kringle and kringle 2 of tissue-type plasminogen activator (tPA) domains exhibit similar global folds (28). Unlike the tPA kringle 2 which exhibits a lysine

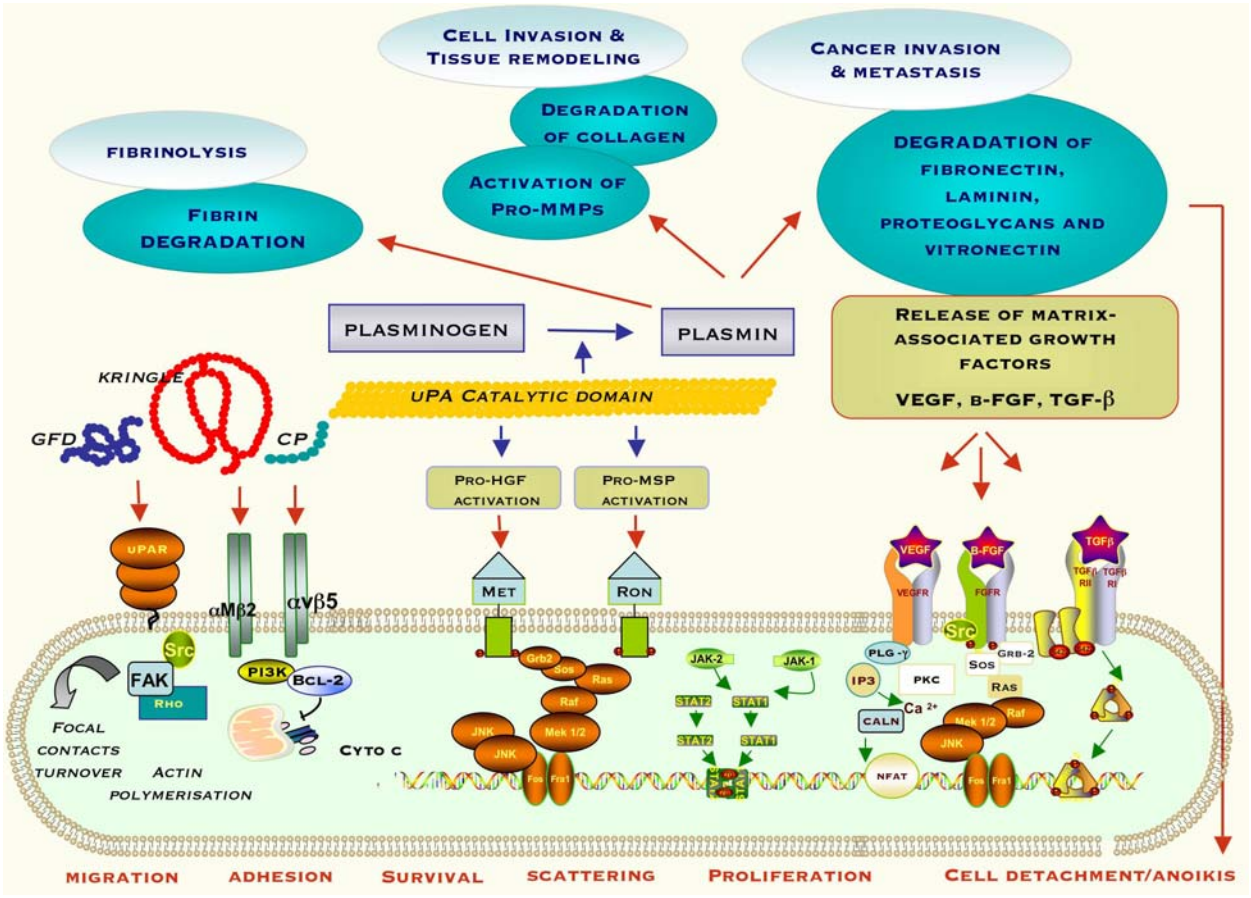

Figure 2. Interactions and functional effects of the urokinase single domains. Urokinase may affect cell physiology by a variety of molecular mechanisms: first of all through its non-catalytic amino-terminal region (consisting of a growth factor-like domain or GFD, a kringle domain and a "connecting peptide" or CP) which interacts with uPAR and integrins. Second, through a proteolytically-dependent mechanism by cleaving, at least, three substrates like plasminogen, pro-MSP (precursor to macrophage stimulating protein) and pro-HGF (pro-hepatocyte growth factor). The catalytic activity of uPA results in fibrin and matrix degradation as well as in the direct, or plasmin-mediated, activation of several growth factors, thus affecting cell migration, adhesion, scattering, survival/apoptosis and proliferation.

binding site, isolated kringle fragments of uPA bind polyanions like heparin trough a basic cluster of Arg108, Arg109, and Arg110 (29). By phage display technology, a putative uPA kringle-binding consensus sequence BXXSSXXB (where B represents a basic amino-acid and X represents any amino-acid), was identified and subsequently found in the gp130 signal transducer protein (30). Interestingly, the uPA kringle binds to (alpha)v(beta)3 integrin and promote migration, which can be blocked by plasminogen kringles 1-3 or 1-4 (angiostatin), a known integrin antagonist (31). Pluskota *et al.* report that pro-uPA binding to (alpha)M(beta)2 integrin is critical for the (alpha)M(beta)2-mediated enhancement of plasmin generation. Interestingly, this binding was lost by a kringle-less pro-uPA mutant (lacking amino-acids 47 – 135), showing that kringle is required for this interaction (32). Another study reports that an isolated uPA fragment corresponding to residues 43 – 156 promotes smooth muscle cell migration at nanomolar concentrations in a uPAR-independent manner (33). Acute pro-inflammatory effects of the mouse uPA region spanning residues 48 – 144 have been observed in the lungs of mice exposed to LPS which appear to be mediated by Akt and NF-kB activity (34). The search for cryptic activities of uPA which could be applied to anti-cancer therapy shows that FGF-stimulated proliferation and VEGF-induced migration of endothelial cells may be prevented by human recombinant kringle domain (UK1, residues 45 – 135), possibly via an unknown uPAR-independent mechanism (35).

3.3. The connecting peptide region

Evidence for a role of the connecting peptide was originally suggested by the finding that uPA may be phosphorylated on Ser138 and/or Ser303 with clearcut functional consequences (5). This post-translational modification occurs in A431 human carcinoma cells prior to secretion and is inhibited by protein kinase C inhibitors (36). Phosphorylated uPA (P-uPA) retains full catalytic ability and uPAR binding whereas it lacks the ability to stimulate cell adhesion and migration. Substitution of the critical Ser with Glu residues results in uPA variants with weak or no chemotactic activity (5). Interestingly, Ser138 is

conserved among different mammalian species, like mouse, rat, yellow baboon, bovine and orangutan, suggesting a relevant functional role (Votta and Stoppelli, unpublished). Further evidence indicating the relevance of the connecting peptide region is based on the effects of Å6, a peptide corresponding to uPA residues 135-143 which inhibits tumor progression and angiogenesis (37). Recently, an isolated peptide corresponding to uPA residues 135-158 was reported to bind to (alpha)v(beta)5 integrin with high affinity, to induce chemotaxis at picomolar concentrations, and to stimulate the association of uPAR and (alpha)v(beta)5 integrin (26). Interestingly, this binding was not dependent on the GFD-uPAR interaction, but potentiates the functional effects of GFD on chemotaxis. The most ready interpretation of these observations is that the linker of uPA induces a conformational change in (alpha)v(beta)5 integrin, which impacts on cell migration and invasion. These findings suggest that uPA may affect cell function by bridging uPAR and integrins, thus fully stimulating migration.

3.4. The catalytic domain

The crystal structure of the uPA catalytic domain has been determined and shows that the enzyme has the expected topology of a trypsin-like serine protease. The enzyme has an S1 specificity pocket, an S2 hydrophobic pocket and an S3 pocket; insertions of extra-residues in loop regions create unique surface areas. A specific triad, His 204, Asp 255, and Ser 356 is responsible for the catalytic mechanism (17). The protease-specific surface loops surrounding the active site are also involved in exosite interactions of uPA with its substrates. It has long been known that active uPA catalyzes the conversion of the inactive zymogen plasminogen to the broad-spectrum protease plasmin by cleaving an R560-V561 peptide bond (38). However, uPA can also activate the precursor to the scatter factor/hepatocyte growth factor pro-HGF, which exhibits extensive homology with plasminogen, and the macrophage stimulating protein, thereby indirectly controlling cell proliferation, ECM invasion and prevention from apoptosis (39). There are two uPA inhibitors, namely PAI-1 and PAI-2 (plasminogen activator inhibitor type-2), belonging to the serpin (serine protease inhibitors) family. PAI-1 reacts more quickly that PAI-2 with uPA and causes a non-reversible inhibition of the enzyme (38). Covalent uPA-PAI-1 complexes are cleared by uPAR-dependent internalization, if the protease is receptor-bound (40-41).

Receptor-mediated membrane localization of uPA is of relevance to the proteolytic efficiency of the whole system. In particular, kinetic studies have shown that receptor-bound uPA exhibits a 40-fold lower Km than soluble uPA and a concurrent 6-fold reduction in kcat, thus resulting in an overall increase of catalytic efficiency (23). Since plasminogen can become membrane-bound, the occurrence of receptors for plasminogen and uPA on the same cell results in the enhanced formation of surface-associated plasmin. This machinery generates broad-spectrum proteolytic activity, which is restricted to cell surface and protected by circulating inhibitors, such as (alpha)2-antiplasmin (42). The proteolytic activity of receptor-bound uPA may contribute to signaling by removing D1 domain and exposing uPAR SRSRY chemotactic epitope, which interacts with fMLP receptors (43). Most of the proteolytically driven effects of uPA are mediated by plasmin which may down-regulate the local concentration of fibrin, fibronectin, laminin, vitronectin and proteoglycans. In addition, plasmin activation of latent metalloproteases leads to collagen degradation (38). In vascular pathologies, such as the rupture of aortic aneurysms, plasmin is an important activator of pro-metalloproteases (44). A previously unrecognized cellular effect of the degradation of ECM molecules is cell detachment, which may be followed by anoikis (corresponding to detachment-induced apoptosis). In this respect, the generation of plasmin activity may be pro-apoptotic, whereas the expression of inhibitors, such as protease nexin-1 may favour cell survival (45). A novel role for plasmin in the differentiation of pancreatic precursor cells has been proposed, raising the possibility that islet formation by pancreatic precursor cells is triggered by plasminogen activation and regulated by endogenous PAI-1 (46). Plasmin activity may also subvert growth factor signalling. In fact, following ECM degradation there is an increased availability of active bFGF and VEGF as well as the direct activation of latent TGF-beta (47). *In vivo*, the multiple effects of plasmin activity emerge from the analysis of plasminogen-deficient mice, which survive embryonic development but are impaired in growth, fertility and survival. In the same mice, skin wound healing is severely impaired, suggesting that plasmin activity is also required for tissue regeneration (48). In humans the absence of plasminogen is associated to a pathological condition, known as ligneous conjunctivitis. In non-severe cases, the application of plasminogen-containing eye drops may reduce symptoms (49).

4. FUNCTIONAL PROPERTIES OF UROKINASE

4.1. Regulatory mechanisms of urokinase synthesis, localisation and activity

The expression level, activity and localisation of uPA is strictly controlled in a variety of physiological and pathological conditions. Expression of uPA may be regulated at transcriptional level through the activity of its promoter region and at the level of mRNA stability through mRNA-protein interactions. As described in the Introduction, the uPA promoter region is highly responsive to phorbol esters, growth factors, steroid hormones, cytokines and uv light through the Ras/MAPK-dependent pathway impinging on the Ets/AP-1 binding sites (12). In breast carcinomas, the Ets-1 and Ets-2 transcription factors bind to uPA and MMP-9 promoters, in response to epidermal growth factor, thus enhancing invasion and metastasis (50). The important role of histone modifications in regulating uPA gene expression is established by recent findings highlighting the induction of uPA expression by histone deacetylase inhibitors trichostatin A and sodium butyrate in human cancer cells (51). uPA expression may be controlled also at the level of mRNA stability by many factors, such as protein synthesis inhibitors, PKC activity, glucocorticoids. This regulatory mechanism involves one (AU)-rich sequence within the 3'-UTR of uPA mRNA. The (AU)-rich sequence-dependent degradation mechanism is

impaired in breast carcinoma cell lines and the half-life of uPA mRNA is markedly longer than in normal cells (52). Constitutive expression of uPA leading to a highly metastatic phenotype is achieved by an increase in mRNA stability of MDA-MB-231 breast carcinoma cells (53). At molecular level, phosphorylation of mitogen-activated protein kinase-activated protein kinase 2 (MK2) results in an enhanced binding of HuR proteins to the AU-rich elements in the 3' untranslated regions of uPA and uPAR, thus leading to mRNA stabilization (54). Among the factors controlling uPA synthesis is LPA (lysophosphatidic acid), which is an important intercellular signaling molecule participating to pathogenesis of many human diseases. LPA upregulates uPA secretion via p38(MAPK) signaling pathway in ovarian cancer cells (55). uPA increased expression may be also a consequence of a decreased cell-cell adhesion. Disruption of E-cadherin-dependent cell-cell adhesion by a soluble E-cadherin fragment or by the function-blocking antibody Decma results in Src-dependent Erk activation and in uPA induction (56).

The regulation of uPA localization is achieved by binding to uPAR which focalizes protease activity on cell surface and mediates uPA-dependent signaling (23). Early results showed that in human fibroblasts receptor-bound uPA is mainly found at focal contact sites, suggesting that uPA may restrict uPAR lateral mobility (57). UPAR clustering is induced in differentiating myelomonocytic U937 cells by pre-incubation with uPA or ATF (58). The enzymatic activity of soluble and receptor-bound uPA may be controlled by the specific soluble inhibitors PAI-1 (plasminogen activator inhibitor type 1) and PAI-2. (plasminogen activator inhibitor type-2). Although both molecules are strong inhibitors of uPA proteolytic activity, they seem to play additional roles in cell biology. First of all, PAI-1 binds with high affinity to matrix vitronectin, thus affecting cell adhesion and is reported to be anti-apoptotic. Remarkably, PAI-1 is one of the most informative biochemical markers of a poor prognosis in several human cancer types. Unexpectedly, recent studies on cells expressing PAI-1 in human tumors and data generated in PAI-1 deficient mice support the possibility that PAI-1 promotes tumor growth and spread (59). Regarding PAI-2, the fact that only a small percentage of PAI-2 is secreted argues for alternative roles of this serpin. Consistently with this observation, PAI-2 alters gene expression, influences the rate of cell proliferation and differentiation, and inhibits apoptosis in a uPA-independent manner (60).

4.2. Urokinase and human pathology

Consolidated evidence shows that uPA has a relevant role in many physiological conditions such as intravascular fibrinolysis, angiogenesis, tissue regeneration and immune response. Different pathological conditions turned out to causally involve a dysregulated production of uPA, such as cancer growth and metastasis. The deep molecular knowledge of the uPA system is helping to define the molecular players acting under these pathological conditions.

The activity of uPA may be required for an efficient tissue regeneration in diseases involving inflammation and tissue repair. For example, uPA-/- mice had a severe regeneration defect, with decreased recruitment of blood-derived monocytes to the site of injury and with persistent myotube degeneration. In addition, uPA-deficient mice accumulated fibrin in the degenerating muscle fibers (61). Acute viral myocarditis is an important cause of cardiac failure in humans. In mouse models of acute viral myocarditis, it has been observed an increased expression of uPA and MMP-9. Interestingly, loss of uPA or MMP activity reduces the cardiac inflammatory response, thereby protecting against cardiac injury, dilatation, and failure during virally-induced myocarditis (62).

Available data support the hypothesis that plasminogen could play a role in certain skin diseases. Transgenic mice overexpressing uPA and uPAR in basal epidermis develop extensive alopecia due to involution of hair follicles, epidermal thickening and sub-epidermal blisters, a phenotype due to receptor-bound uPA catalytic activity (63).

The plasminogen activation system represents a potent mechanism of extracellular proteolysis and is an essential component of normal wound healing, as shown in mice models. In particular, a functional cooperation between the plasminogen/plasmin and the metalloprotease cascade is suggested by the finding that complete inhibition of the healing process requires both plasminogen deficiency and metalloprotease inhibition (64). Urokinase is regarded as an important trigger of the fibrinolytic process, via plasminogen activation. Although uPA-deficient mice do not have major thrombotic disorders, double uPA–tPA-knockout mice show extensive extracellular fibrin deposition very similar to that observed in plasminogen-knockout mice (65). One evidence is that uPA expression in atherosclerotic arteries contributes to intimal growth and constrictive remodeling leading to lumen loss, thus accelerating atherogenesis. Antagonists of uPA activity might, therefore, be useful in limiting intimal growth and preventing constrictive remodeling (66). Interestingly, in a mouse model of glomerulonephritis, a reduced uPA-mediated proteolysis correlates with excessive fibrin deposition (67). In the case of infection, uPA might first favor the recruitment and activation of immune cells, activate latent pro-inflammatory cytokines, and modulate T-lymphocyte responses (68). Mice deficient in uPA fail to generate type 1 immune responses during infection with Cryptococcus neoformans (69). Data show that the uPA-deficient mice are more susceptible than controls to botryomycosis, a staphylococcal infection, suggesting that uPA contributes significantly to the immune response (70).

Urokinase has been shown to play crucial roles in tumor progression as a soluble or membrane-associate protease. Experiments with cell cultures and animal models have strongly indicated a pivotal biological role of uPA-catalysed plasminogen activation in tumor progression (1, 2, 38). Most recently, the results from studies involving uPA-deficient mice ($uPA^{-/-}$ mice) were all in agreement with the idea that uPA-catalysed plasmin generation is rate-

limiting for tumor growth, local invasion and metastasis (71-72). In principle, a high level of uPA in extracts of primary breast carcinomas was reported to predict an early relapse (73-74). Consistent with this possibility, the expression level of uPA and uPAR in breast carcinomas are higher than in the normal counterparts (75-76). This relationship was later confirmed with other cancer types (77) The concept of uPAR-dependent pericellular proteolysis in breast cancer is supported by the finding that fibroblast-derived stromal uPA enhances epithelial tumor cell invasion by up-regulating uPA and (alpha)v(beta)5 vitronectin receptors (78). The role of uPA should be considered in view of the cross-talk of primary tumors with the surrounding tumor stroma, which in different tumors has been shown to secrete uPA and MMPs (79). The hypothesis of the importance of stromal uPA is consistent with the findings that a genetically mammary gland tumor, in which uPA is expressed mainly by stromal cells, disseminated slower in $uPA^{-/-}$ mice than in wild type mice (72). *In vivo*, the proteolytic activity of plasmin seems to be required for Polyoma T-induced vascular tumor formation, as the combined loss of uPA and tPA leads to a significantly reduced tumor growth rate (80). Indirect evidence that uPA proteolytic activity is relevant to tumor angiogenesis is provided by studies in mice deficient in the PAI-1 showing that PAI-1 is pro-angiogenic at physiological concentrations through its anti-proteolytic activity (81). In the emerging picture, the uPA has the ability to support the malignant phenotype through several mechanisms. First of all, by virtue of its matrix degrading ability, which favors tumor dissemination; second, by stimulating cell motility through the association with uPAR and integrins; third, by stimulating cell proliferation and by protecting cells from apoptosis, thus enhancing tumor cell survival in a uPAR-dependent manner. The uPAR itself, other than concentrating uPA proteolytic activity on cell surface and being a mediator of most ligand-dependent effects on growth, motility and apoptosis, is itself an anti-apoptotic factor (25). This information support the possibility that dysregulation of uPA level and/or localisation might cause or favor different diseases, and suggest that interference with uPA-activity could block disease. More remains to be elucidated to understand how to manipulate the uPA–uPAR system in pathology, without hampering their physiological function in fibrinolysis and tissue remodeling.

5. ANTAGONISTS TO UROKINASE

In principle, there are several ways to interfere with uPA function: one approach is the identification of inhibitors blocking the protease catalytic activity with high affinity and selectivity. Another possibility is to antagonise the interaction of GFD with the uPAR. A third possibility is to interfere with uPA-integrin interaction, which has been recently described as a property of the kringle and connecting peptide regions. Finally, uPA mRNA expression could be targeted by the use of RNA interference technology.

5.1. Antagonists of growth factor-like domain

Given the crucial role of the uPA/uPAR interaction in tumor invasion and metastasis, specific antagonists can be regarded as cancer therapeutics. Early evidence showed that a peptide corresponding to residues 18 – 32 of the human uPA is the minimal sequence allowing binding to uPAR (16). More recently, Magdolen *et al.* have identified a synthetic cyclic peptide covering the residues 19–31 (cyclo19,31[D-Cys19] peptide) as a potent inhibitor of uPA/uPAR association (82). However, it has to be taken into consideration that peptides spanning the uPAR binding domain, like GFD, may stimulate cell migration and signalling at nanomolar concentrations (26). An alternative approach is directed to the inhibition of the uPA-uPAR interaction using small synthetic peptide antagonists isolated by combinatorial chemistry. Å 9-mer peptide was a potent inhibitor of uPAR binding and reduced the intra-vasation of HEp-3 cancer cells by approx. 60% in a chicken chorioallantoic membrane assay (83).

5.2. Antagonists of kringle domain and connecting peptide region

One of the first monoclonal antibodies directed to uPA recognizes an epitope located between GFD and kringle domains and has been widely employed for affinity purification of uPA, ATF and pro-uPA (84). Although 5B4 monoclonal antibody reduces uPAR binding, it is unknown whether it affects uPA-integrin interaction. Anti-kringle monoclonal antibodies which block the binding of uPA to polyanions have been subsequently developed (29). Given the recent reports showing the association of the kringle and connecting peptide regions with several integrins, it would be interesting to assess whether any of these monoclonals may block these interactions and the relative functional effects. The chemotactic and integrin-binding abilities of the uPA interdomain linker region emerged quite recently, both in the full length molecule and in the isolated form, as CPp (residues 135-158) (26). Previous evidence was based on the analysis of the uPA serine phosphorylation occurring on Ser138, located in the CP region. Interestingly, Glu-substituted variants are chemotactically inactive and may be considered antagonists of the linker region (5). More recent evidence shows that uPA^{S138E} and $uPA^{S138E/303E}$ prevent migration induced by wild type uPA and by other chemoattractants (Franco, Vocca and Stoppelli, unpublished).

The activity of peptides corresponding to sequences of the connecting peptide region is exemplified by Å6, a 8-mer capped peptide (residues 135 – 143). This peptide inhibits tumor cell invasion as well as angiogenesis *in vitro* and *in vivo* (37). Å6 has been reported to inhibit dissemination of Mat B-III rat breast cancer cells and formation of new blood vessels, the effects being greater in the presence of Å6 and tamoxifen (85). Similarly, Å6 has been shown to inhibit growth and neo-vascularisation of human U87MG glioblastoma cells growing on nude mice, particularly when combined with cisplatin (86). Furthermore, Å6 has been reported to suppress retinal neo-vascularisation in an animal model (87). The mechanism of action of Å6 has not been fully elucidated. Surface plasmon resonance analyses showed that Å6 acts at $1x10^{-6}$M concentrations and may interfere with the uPA-uPAR

interaction (37). However, recent data show that that CPp (corresponding to residues 135-158 of human uPA) retains the uPA ability to associate with (alpha)v(beta)5 integrin at nanomolar concentrations, suggesting that Å6 may act through integrins (26). However, further experiments are needed to clarify this issue. Recently, a phase I clinical trial of Å6 in gynaecologic malignancies has been completed and indicated neither toxicity nor any immunogenic response (88).

5.3. Inhibitors of enzymatic activity

Proteolytic activity generated by uPA is relevant to metastasis and angiogenesis. Thus, the inhibition of uPA is a promising strategy in cancer. In the '80s, polyclonal anti-uPA antibodies were obtained to prevent uPA activity *in vitro* and in a chicken tumor model (89-90). Subsequently, several monoclonal antibodies recognizing different epitopes in the uPA molecule were generated (91-92). In the last ten years, many potent and selective molecules inhibiting the catalytic activity of uPA have been obtained. Available structural information of enzyme-inhibitor complexes has greatly accelerated the optimisation process of in silico designed inhibitors (93). These data were used to identify a class of uPA inhibitors based on the 2-naphthamidine template that exhibit a remarkable selectivity for uPA over trypsin-like enzymes (94). Based on the peptide sequence of an optimal uPA substrate, phenethylsulfonyl-D-Ser-Ala-D-aminomethyl benzamidine was characterised as a selective uPA inhibitor with a K_i value of 3.1 nM (95-96). A similar inhibitor prevented lung metastases and prolonged survival of nude mice injected with HT1080 fibrosarcoma cells (97). WX-UK1, a novel inhibitor of uPA derived from 3-amidinophenylalanine, inhibits matrigel invasion of breast and cervical carcinoma cells as well as spreading of orthotopically transplanted BN472 breast tumors in rats, thus suggesting a novel adjuvant antimetastatic therapy (98-99). Among the 1-isoquinolinylguanidines which are potent and selective inhibitors of uPA (Ki 10 nM), UK-371,804 is the most characterised. Following local administration, this compound effectively inhibited uPA in a porcine acute excisional wound model. In particular, UK-371,804 is recommended in the treatment of chronic dermal ulcers, which are characterised by excessive uncontrolled proteolytic degradation resulting in ulcer extension, loss of functional matrix molecules (e.g., fibronectin), and retardation of epithelialization (100). The uPA inhibitor WXC-340 prevents metastatic growth following surgery in a murine colorectal carcinoma model, suggesting that perioperative uPA inhibition with WXC-340 may represent a novel anti-tumor strategy (101). A 4-oxazolidinone analogue (UK122) which inhibits uPA activity with a K_i of $0.2x10^{-6}$M was developed. UK122 significantly inhibits pancreatic cancer cell migration and invasion capability (102). By screening phage-displayed random peptide libraries with uPA as the target, the disulfide bridged sequence CSWRGLENHRMC (upain-1) was isolated (103). A similar strategy yielded a specific inhibitor for mouse uPA (104). Although both molecules specifically inhibit plasminogen activation, their effect in mouse cancer models remains to be investigated.

Taken together, these findings suggest that uPA enzymatic activity may be controlled *in vivo* by many potent and selective inhibitors which may be employed in the control of tumor dissemination, wound healing and other pathologies caused by excess uPA activity. Finally, an interesting possibility is to take advantage of uPA activity to target cancer cells. Recently, an engineered anthrax toxin that is selectively activated by cell-surface uPA was generated. The novel recombinant anthrax toxin, PrAgU2/FP59, composed of the uPA-activated protective antigen and a fusion protein of Pseudomonas exotoxin and lethal factor showed anti-lung cancer efficacy in an *in vivo* human tumor model (105).

5.4. Inhibitors of urokinase expression

High level expression of uPA has been well documented in a variety of tumors and correlated to a poor prognosis. Therefore, inhibition of uPA expression may be regarded as an anti-tumor strategy. In recent years, RNA interference (RNAi) technology has been recognised as a post-transcriptional effective methodology to silence specific genes (106). In principle, the inhibition of uPA synthesis can be achieved by a stable transfection with an antisense uPA vector or by transfection with small interfering RNA molecules (siRNA). According to Arens N. *et al*, the use of specific siRNAs provides a more efficient down-regulation of uPA mRNA than the introduction of an antisense uPA vector (107). Several groups have explored the use of siRNAs to target uPA and/or uPAR for therapeutic applications in cancer treatment. An early report shows that SKHep1C3 epatocarcinoma cells transfected with a plasmid coding for an RNA composed of two identical 19-nucleotide sequence motifs forming an hairpin dsRNA showed a consistently decreased level of uPA protein as well as a reduction of migration, invasion, and proliferation (108). The simultaneous targeting of uPA and uPAR is a powerful approach, as it results in a decrease in the phosphorylation of the Ras-activated pathway molecules such as FAK, p38MAPK, JNK and ERK1/2, as well as the MEK-activated phosphatidylinositol 3-kinase (PI3k) pathway in glioma cells (109). SKHep1C3 cells stably transfected with a plasmid coding for an siRNA directed to human uPA exhibited a consistent reduction of migration, invasion, and proliferation (108). Another approach to silence uPA expression is based on the fact that DNA demethylation is a common mechanism underlying the abnormal expression of uPA. Histone deacetylase (HDAC) inhibitor trichostatin A stimulates uPA expression and cancer cell invasion, raising the possibility that HDAC inhibitors employed as cancer therapeutics may favour metastasis by up-regulating uPA (51). Inhibition of demethylation of uPA promoter with S-Adenosyl-l-methionine results in uPA silencing and inhibition of MDA-231 cells tumor cell invasion *in vitro* and tumor growth and metastasis *in vivo* (110-111).

Furthermore, nucleic acid molecules with high affinity for a target transcription factor can be introduced into cells as decoy cis-elements to bind these factors and alter gene expression. For instance, a peptide nucleic acid chimera containing an Sp1 binding sequence, has been investigated to down modulating the expression of Sp1

target genes, and thereby, preventing uPAR-dependent breast tumor invasion (112). Although encouraging, these studies are still preliminary and will have to be extended to animal models which mimic tumor progression using clinically acceptable modes of nucleic acid delivery.

6. PERSPECTIVES

Because uPA is reported to be differentially expressed in cancer tissues compared to normal tissues, this protease has the potential to be developed as prognostic and/or therapeutic target. These studies are likely to contribute to the development of molecularly-driven targeted therapies in the near future.

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Abbreviations: uPA, Urokinase Plasminogen Activator; ECM, Extra Cellular Matrix; pro-uPA, single-chain zymogen form of uPA; uPAR, Urokinase Receptor; CP, uPA connecting peptide region (residues 132-158); GFD, uPA growth factor-like domain (residues 1-49); ATF, Amino-Terminal Fragment; GPI, glycosylphosphatidylinositol; P-uPA Phosphorylated uPA; PAI-1, Plasminogen Activator Inhibitor type-1; PAI-2, Plasminogen Activator Inhibitor type-2; tPA, tissue-type Plasminogen Activator; serpin, serine protease inhibitor.

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