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Biological significance of the expression of urokinase-type plasminogen activator receptors (uPARs) in brain tumors

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1. ABSTRACT

The urokinase-type plasminogen activator receptor (uPAR) plays a critical role in the regulation of cell-surface plasminogen activation in several physiological and pathological conditions. Recent evidence suggests that the uPAR is also involved in processes that are not related to plasminogen activation, including cell adhesion and transmission of extracellular signals across the plasma membrane. The uPAR influences cell migration and spreading both *in vivo* and *in vitro* through the cell-surface activation of plasminogen. The uPAR can bind to vitronectin, an adhesive extracellular matrix protein that contains the Arg-gly-Asp (RGD) cell adhesion domain and that serves as a ligand for several integrin receptors. uPAR also forms complexes with β1, β2, and β3 integrins, thereby allowing mutual interactions and regulation between cell adhesion and proteolysis. Recently, uPAR has been shown to have strong prognostic value for predicting disease recurrence and overall survival in certain types of cancer. We discuss here the biological significance of uPAR in the glioblastoma invasion process. Strong correlations found between elevated uPAR levels in glioblastoma cells and tumor invasiveness have led to uPAR being selected as a target for therapy in experimental animal models. Using antisense vectors to down regulate uPAR expression at the level of the mRNA and protein in glioblastoma cells, has been shown to inhibit tumor formation in nude mice. These results provide a potential basis from which to develop novel therapeutic strategies to direct the expression of antisense uPAR and to evaluate the efficiency of this technique for cancer gene therapy in patients with brain tumor.

2. INTRODUCTION

Extracellular proteolytic enzymes have been implicated in the spread of cancer; release of proteolytic enzymes from tumors is thought to facilitate cancer cell invasion into the surrounding normal tissue through the breakdown of basement membranes and extracellular matrix (ECM). This degradation is accomplished through the integrated action of several enzyme systems, including the generation of plasmin, a serine protease, through the urokinase pathway of plasminogen activation. Plasminogen activation is considered central to the regulation of pericellular proteolysis that occurs under both normal and pathological conditions. One example of the latter is cancer invasion, which requires tissue destruction and cell migration. The urokinase-type plasminogen activator (uPA) and the tissue-type plasminogen activator (tPA) are both efficient activators of plasminogen, are products of distinct genes (1-3), and differ mostly in their molecular structure and in the function of their non-catalytic regions. uPA is fibrin-independent and largely receptor-bound whereas tPA is fibrin dependent and primarily intravascular (4). Plasmin generated by either tPA or uPA degrades several protein constituents of the ECM directly, as well as activating certain other protease zymogens and latent growth factors. Of the two profibrinolytic enzymes, uPA has been studied more thoroughly in relation to tumor invasion (5).

3. THE uPA RECEPTOR

The receptor for urokinase-type plasminogen activator (uPAR), also called CD87 (6), is related to members of the Ly-6 superfamily, which include CD59, murine Ly-6, squid glycoprotein Sgp-2, and a variety of snake-venom toxins (7). Identical to the macrophage differentiation antigen Mo3 (8), uPAR is a cell membrane-anchored binding protein for uPA and accumulates plasminogen activator activity at the cell surface (9). The human uPAR gene located on chromosome 19q13, consists of 7 exons and 6 introns extending over 23 kb of genomic DNA (10). The promoter region of the human uPAR contains sequences related to consensus *cis*-acting elements for the activator protein-1, nuclear factor-κB and Sp-1 transcription factors. Most of the promoter activity is contained within the first 180 bp upstream of the

transcriptional start site (11, 12). Human uPAR is encoded as a 1.4 kb mRNA transcript (13). cyclic AMP (cAMP) induces uPAR expression exclusively through activation of gene transcription in which a preexisting transcriptional factor may be involved, whereas phorbol myristate acetate (PMA) both transcriptionally and posttranscriptionally regulates uPAR gene expression in PL-21 human myeloid leukemia cells (14).

Growth factors and cytokines can also induce the uPAR expression (15, 16). The number of uPAR molecules per cell varies markedly, and the K_d values for ligand binding for both the two chain uPA and the pro-uPA are reportedly in the range of 0.1-1 nM (17, 18). The increase in receptor number induced by PMA always seems to be accompanied by a reduction in receptor affinity for uPA (19), whereas the ligand-binding affinity of receptors that are induced by cAMP remains unchanged (20).

The uPAR recognizes pro-uPA and uPA at its EGF-like amino-terminal domain (21, 22), a heavily glycosylated, 55- to 65- kDa, 313 amino acid protein containing 5 potential N-glycosylation sites (13). The hydrophobic carboxy-terminal domain of uPAR is processed during biosynthesis and substituted by a glycosyl-phosphatidylinositol (GPI) anchor that allows it to be attached to the membrane (23, 24). The receptor-binding domain of uPA has been localized to a region in the growth-factor domain between residues 20-and 32 (25). Recently Asn22, Asn27, His29, and Trp30 in human uPA have been identified as being key determinants in the species-specific binding of uPA to uPAR (26).

The mature uPAR receptor protein consists of three homologous domains of approximately 90 residues each (27); early sequencing results led to the assumption that these repeats are autonomous structural entities. The binding region for uPA resides within the first amino-terminal repeat of the uPAR. Chemical modification of tyrosine residues in the uPAR identified Tyr57 as being intimately engaged in the interaction with uPA (28), suggesting that the region around amino acids 50-60 of Domain 1 in human uPAR constitutes an important interaction site for uPA. However, the affinity of isolated Domain1 for the ligand is ~1500-fold lower than that of the intact molecule (29), indicating that the uPAR domain 1 does not contain all of the determinants necessary for high-affinity uPA binding. Another ligand-contact site identified recently within Domains 2 and 3 seems to have even lesser affinity for uPA than does the isolated Domain 1 (30). Further studies with transfected mutants missing either Domain 2 or Domain 3 suggested that some combination of Domain 1 with Domain 2 or Domain 3 is required for optimal ligand-binding affinity (31). The interaction of uPA with its receptor exhibits some species specificity; human uPA does not bind to murine uPAR, and murine uPA does not recognize human uPAR (32, 33). However, human uPA does interact with bovine (34) and hamster (35) cells with high affinity.

uPAR is shed from the surface of uPAR-expressing cells either constitutively or in response to certain soluble stimuli (6). Soluble uPAR (suPAR) has been found in plasma and other body fluids from patients with inflammatory conditions; moreover, this suPAR could bind exogenous uPA (36). suPAR also has been detected in sera from normal individuals (37), and in higher concentrations in sera from patients with lung cancer (38) and paroxysmal nocturnal hemoglobinuria (37). Pederson et al. found high concentrations of suPAR in the ascitic fluid of patients with ovarian carcinoma (39). Tumor cells release suPAR as indicated by the full-length suPAR found in culture supernatant from human HT-1080 fibrosarcoma cells (40). In another study, recombinant suPAR bound in a specific fashion to leukemic cells, blood polymorphonuclear neutrophils and monocytes; its binding was variably influenced by the presence uPA ligand and did not affect the cell-associated plasmin activation. The nature of the cellular binding sites for suPAR remains unknown (41). The potential physiological significance of suPAR is shown by the ability of recombinant suPAR to inhibit the proliferation and invasion of tumor cells in vitro, a finding that implies a scavenger function for this soluble receptor (42).

uPA can cleave uPAR between Domains 1 and 2 *in vitro* (43). An N-terminally truncated form of uPAR comprising Domains 2 and 3 has been found in cystic fluid from ovarian cancer (44) and from normal and tumorous thyroid cells (45) indicating that this cleavage also can take place *in vivo*. Indeed, spliced uPAR cDNAs that lack the part encoding the GPI attachment site also have been detected in mouse (46), rat (47), and human tissues (48). uPAR is not phosphorylated after binding its ligand and the formation of receptor-ligand complexes does not result in internalization of the complex or receptor down-regulation unless receptor bound uPA is complexed to its specific inhibitors.

uPA is produced from cells as a single-chain proenzyme that is converted by limited proteolysis into an active two chain molecule. The uPAR binds to either inactive proenzyme form (pro-uPA) or active two-chain form without affecting its proteolytic activity (49). Binding is saturated by autocrine and paracrine mechanisms in cancer cells. Binding to uPAR regulates uPA activity by either accelerating its conversion from pro-uPA to the active form or by inhibiting the active enzyme through the involvement of specific inhibitors. When active uPA is bound to the receptor, it is not internalized and remains on the cell surface. On the other hand, receptor-bound uPA is complexed to its specific inhibitors, plasminogen activator inhibitor-1 (PAI-1), PAI-2, or protease nexin-1 (PN-1) that complex is then internalized and degraded (50-53). Thus the uPAR plays a dual role in controlling uPA-catalyzed proteolysis, either by functioning to focus uPA activity at the leading front of migrating cells or by participating as a "clearance" receptor for uPA: PAI complexes.

Acting with the α2-macroglobulin receptor/low density lipoprotein-related protein (α2MR/LRP), uPAR can internalize the uPA/PAI-1 complex and then recycle itself to the cell surface (54). However, this recycling does not provide a means of down regulating the uPAR. However, uPAR does bind to the 275-kD cation-independent,

mannose 6-phosphate/insulin-like growth factor II [IGF-II] receptor (CIMPR), which does participate in uPAR down regulation via lysosomal degradation (55). The binding epitope on the CIMPR is different from those that bind Man-6-P and IGF-II. Binding between uPAR and CIMPR seems to involve Domains 2 and 3 of the uPAR protein moiety but not the GPI anchor (55). The uptake of uPAR by CIMPR seems to be independent of both receptor-bound uPA and uPA-PAI-1 complexes.

Targeted deletion of the uPAR gene in mice significantly reduced uPA-mediated activation of plasminogen in peritoneal macrophages (56, 57), confirming the central role of uPAR in cell-surface plasminogen activation. Moreover, uPAR deficiency did not compromise fertility, development, or homeostasis in homozygous uPAR-/- mice (56). Bugge et al. found fibrin deposits in the livers of mice deficient in both uPAR and tPA but not in animals lacking only uPAR or tPA (58) indicating that plasminogen activation focused at the cell surface by uPAR is important for fibrin surveillance in the liver. Experiments with cultured smooth muscle cells from uPAR-deficient mice have shown that uPAR-dependent plasminogen activation is also involved in the activation of latent transforming growth factor beta (TGF-β) (59).

4. TUMOR CELL INVASION

uPAR-dependent cell-surface plasminogen activation contributes to the invasiveness and metastastic behavior of tumor cells. Inhibition of uPAR expression by using an antisense approach has been shown to inhibit tumor cell invasiveness (60), prevent metastasis (61), reverse invasive behavior (62), and increase the tumor latency period (63). Conversely, overexpression of uPAR in human neuroglioma (64), osteosarcoma (65), epidermoid carcinoma (66) and rat breast cancer cells (67) resulted in increased invasiveness and tumorigenicity *in vivo* and *in vitro*. Bacteriophage peptide display has allowed peptides that inhibit the uPA/uPAR interaction to be generated (68); these peptides have been shown to inhibit angiogenesis and primary tumor growth in syngeneic mice (69). Soluble recombinant uPAR also inhibited the invasiveness of ovarian cancer cells by "scavenging" uPA (42).

For some tumors, high levels of uPAR seem to be a marker of poor prognosis. In colorectal malignancies, high levels of uPAR constitute an independent prognostic marker for poor survival of the patients (70). Breast cancer biopsy samples containing high levels of uPAR were associated with a worse prognosis than samples with low levels of the receptor (71-74); uPAR was undetectable in normal breast tissue (71). Furthermore, high uPAR levels were significantly associated with a shortened overall survival in patients with squamous cell lung carcinoma (75). As for the soluble form of the receptor, the presence of suPAR in plasma from both healthy humans and cancer patients suggests that phospholipases or proteases could cleave uPAR's glycolipid anchor protein, near the carboxy terminus. Patients with lung (38), ovary (39), breast and colorectal cancer (76) have higher plasma levels of suPAR than do healthy individuals. Whether suPAR is a prognostic marker in plasma of patients with cancer remains inconclusive.

5. CELL ADHESION

The uPAR has also been found to be a high-affinity receptor for vitronectin suggesting that uPAR plays a role in cell adhesion (77, 78). The uPAR binds to both the cleaved form of vitronectin present in plasma and the full-length form (79). The vitronectin-binding site on uPAR is distinct from the uPA binding site; vitronectin binding to uPAR and uPAR-mediated adhesion to vitronectin is not blocked by the RGD peptide and does not depend on divalent cations. The latter characteristics distinguish this interaction from known integrin-vitronectin interactions. Competition studies with synthetic peptides suggest that the region between residues 364 and 380 close to the carboxy-terminus of vitronectin may be involved in uPAR binding (80). The vitronectin-uPAR binding is enhanced by the simultaneous binding of urokinase, but is inhibited by active- but not by latent or cleaved- PAI-1 (81, 82). The simultaneous recognition of vitronectin by uPAR and the αvβ3 integrin co-localizes these two receptors to adhesion structures and directs the proteolytic activity of plasminogen systems to the matrix (83, 84). On the other hand, when PAI-1 binds to vitronectin, it interferes with vitronectin recognition by integrin αvβ3, thereby stimulating release of cells from the matrix and supporting cell migration (85, 86). Competition studies with purified uPAR domains suggest that more than one domain is involved in the vitronectin binding reaction and perhaps that the intact uPAR is required for efficient binding (79). Vitronectin has been shown to concentrate the uPA/suPAR-complex to cell surfaces and ECM sites (87), which leads to the accumulation of plasminogen activator activity required for cell migration and tissue remodeling.

The β2 subfamily of integrins plays a critical role in cell adhesion and migration. Association of uPAR with the β2-integrins, CD11b/CD18 (also known as Mac-1and complement receptor-3 [CR3]) has been found in studies of co-capping and co-immunoprecipitation with neutrophils and monocytes (88, 89). Incubating monocytes with antibodies to CR3 impaired uPAR-dependent adhesion to vitronectin, and engagement of uPAR by uPA or vitronectin inhibited CR3 dependent fibrinogen degradation (90). Down-regulation of uPAR with antisense oligonucleotides in monocytes reduced the adhesiveness of CR3 (91). uPA did not induce Ca2+ signalling or superoxide production in neutrophils from patients with leukocyte adhesion deficiency, in which the β2-chain integrins are lacking; moreover, NIH3T3 transfectants mobilized Ca2+ upon uPA incubation only when both uPAR and CR3 were expressed (92). These results support the concept that integrins may function as signal transducers for plasminogen activation. The interaction of β2 integrins with the T-cell antigen-receptor complex has been shown to increase uPAR mRNA and protein and to promote migration of T cells through an ECM *in vitro* (93). Leukocytes treated with phosphatidylinositol-specific phospholipase C (PI-PLC) lost their ability to adhere to

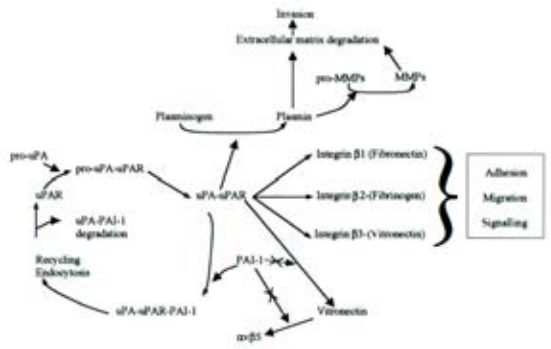

Figure 1. Schematic representation of uPAR mediated cellular events on the cell surface. Binding of pro-uPA to uPAR provides the cell surface with a potential plasmin dependent proteolytic activity determining matrix degradation. uPA bound uPAR interaction with PAIs influences endocytosis whereas uPAR interaction with vitronectin and subclasses of integrins results in modulation of cell adhesion and migration.

endothelium via β2-integrins, whereas maximal cell-to-cell contact was reconstituted by the addition of suPAR (94). uPAR that was bound with a monoclonal antibody increased the adhesion of monocytic cells and neutrophils to vascular endothelium 6- to 8-fold, whereas inactive uPA that was bound to uPAR significantly reduced cell-to-cell adhesion (94). In an *in vivo* study, the β2 integrin-dependent recruitment of leukocytes to the inflamed peritoneum of uPAR deficient mice was significantly reduced relative to that in wild-type animals (94). These latter findings indicate that β2 integrin-mediated leukocyte-endothelial cell interactions and recruitment to inflamed areas both require the presence of uPAR. The association between uPAR and the β2 integrins clearly initiate a variety of direct and indirect regulatory mechanisms for both adhesive and proteolytic processes (95).

In nonhematopoietic cells that do not express β2 integrins, the uPAR seems to interact with β1 and β3 integrins. For example, in HT 1080 fibrosarcoma cells, uPAR and β1 integrins associated together on tumor cells that adhere to fibronectin, laminin and vitronectin, and uPAR and β3 integrin associated together on tumor cells that adhered to vitronectin (96, 97). These and other results suggest that uPAR specifically associates with certain members of the β1 and β3 integrin families on adherent tumor cells, and that ECM components induce specific integrin-uPAR associations to enable directional proteolysis for tumor cell migration and invasion. In contrast to the uPAR interaction with the β2 integrins, those with β1 and β3 integrins can be induced through the binding of integrins by ECM components. In a recent study, adhesion to vitronectin of human 293 embryonic kidney cells that overexpressed cell-surface uPAR was enhanced and adhesion to fibronectin was reduced (83). Both events were reversed when uPAR was shed from cell surfaces by treating the cells with PI-PLC. In the same study, expression of uPAR did not affect cell surface expression of β1 integrin but altered its function as follows. A "17 mer" peptide that could bind and disrupt the uPAR/integrin complex was found to abrogate the uPAR-induced alteration of integrin function in these cells; however, suPAR was unable to do so (83).

6. THE uPAR AND ITS ROLE IN GLIOMAS

Malignant gliomas, the most common primary brain tumors are highly aggressive and have a high rate of recurrence even when treated aggressively with surgery, radiation, and chemotherapy. Local invasive infiltration, growth, and vascularity are key features of malignant brain tumors, and it is well documented that they not only invade normal brain tissue but also cause destruction and replacement of normal anatomical structures within the brain. The molecular sequence underlying brain tumor invasiveness is complex and involves a series of sequential steps. Proteolytic degradation of the ECM is thought to be the first step in the process of tumor cell invasion and vascularization. The role of the plasminogen activation system in this process has attracted much interest. Localization of uPA activity on the cell surface has been shown to be a prerequisite for the invasive capability of tumor cells; this process is facilitated by the expression of uPAR on the cell surface. uPAR mediated pericellular proteolysis or its interactions with adhesion molecules results in variable adhesion or detachment events and is pivotal for the control of cell migration and invasion (figure 1). Increases in the expression of components of the plasminogen activation system have been found in malignant astrocytomas (96, 98). Increased uPA activity has been found in the more malignant phenotypes of astrocytoma cell lines *in vitro* (99-101). uPA activity also was increased drastically in human malignant brain tumors *in vivo* (102-105) and was correlated with poor prognosis (106, 107). We also found that human glioblastoma cell lines expressed uPARs that could contribute to their invasive capability (96, 108, 109).

Because of its importance in accelerating the activation of uPA, several studies have examined the expression of the uPAR in primary brain tumors (96, 110). uPAR expression was significantly higher in anaplastic astrocytoma and glioblastoma than in normal brain tissues or in low-grade gliomas (96, 110). In the study of human gliomas, *in situ* hybridization revealed that uPAR mRNA was found within astrocytomas especially at the leading edges of the tumors (96, 110). In U251MG glioblastoma cells, uPAR is localized at cell/ECM focal contacts, together with the αvβ3 integrin (96). α2MR/LRP efficiently mediates the internalization of uPA in complex with uPAR and PAI-1. α2MR/LRP was recently found to be overexpressed in malignant astrocytomas, especially glioblastomas (111). Moreover, the increased expression of α2MR/LRP seems to correlate with both the expression of uPAR and the malignancy of the astrocytoma suggesting that α2MR/LRP plays a role in facilitating the invasiveness of glioblastomas by regulating cell-surface proteolytic activity.

The selective inhibition of the uPA/uPAR interaction is considered a therapeutically feasible approach

to the treatment of malignant brain tumors. *In vitro* studies in glioblastoma cells using antisense expression vectors to down-regulate uPAR expression significantly decreased their migration and invasiveness (60). Co-culture of tumor spheroids with fetal rat-brain aggregates showed that the cells transfected with antisense constructs to reduce uPAR expression did not invade the fetal rat-brain aggregates (60). Furthermore, glioblastoma cells expressing antisense uPAR cDNA did not invade or form tumors in the brains of nude mice when injected intracerebrally (112); and expression of the integrin $\alpha 3\beta 1$ but not integrin $\alpha 5\beta 1$ was increased in cells grown in culture (113). In our study, reduced expression of uPAR in human glioma cells led to morphological changes associated with decreased spreading and cytoskeletal disorganization suggesting that coordinated expression of uPAR and integrins may be involved in the spreading of glioma cells transfected with antisense uPAR (113).

7. PERSPECTIVE

The uPAR is a multifunctional protein that seems to be involved not only in regulation of local proteolytic activity that governs matrix degradation and the extracellular proteolysis that leads to invasion of cells, but also in modulation of cell adhesion by interacting with integrins and matrix components. The interactions between uPAR and integrins may constitute a major signalling pathway for uPAR to regulate adhesion which in turn influences the interface between cells and their extracellular matrices. Investigations in cancer patients indicate that uPAR can be valuable as a prognostic marker. Up-regulation of uPAR in established glioblastoma cell lines and brain tumor tissues suggest that uPAR may play a role in tumor invasiveness and malignancy, indicating uPAR is a candidate for targeted gene therapy. Using antisense constructs to down-regulate uPAR in glioblastoma cells has been shown to decrease both migration and invasion *in vitro* as well as tumor formation in nude mice. In this context, interfering with uPAR expression may offer an effective therapeutic strategy against malignant glioma.

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Abbreviations: cAMP, cyclic AMP; CIMPR, cation-independent, mannose 6-phosphate/insulin-like growth factor receptor; CR3, complement receptor3; ECM, extracellular matrix; GPI, glycosylphosphatidylinositol; α2MR/LRP, α2-macroglobulin receptor/ low density lipoprotein-related protein; PMA, phorbol myristate acetate; PAI-1, plasminogen activator inhibitor-1; PN-1, protease nexin-1; suPAR, soluble uPAR; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; uPAR, uPA receptor.

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