THE ROLE OF PLASMINOGEN ACTIVATOR RECEPTOR IN CANCER INVASION AND DORMANCY

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Abstract
Urokinase plasminogen activator receptor (uPAR) has been identified some 15 years ago and the anticipation was that its presence on the cell surface will provide a focus for anchoring uPA and possibly protect the enzyme from native inhibitors. The studies of the last decade have shown that uPA localized to the surface of cells by uPAR is indeed an important factor in the process of cancer cell invasion and metastasis. We developed a chick embryo model in which we showed that uPAR is crucial in invasion of stroma and in intravasation (breaching of the blood vessels walls). More recently and unexpectedly, uPAR - a protein anchored in the outer leaflet of the plasma membrane, has been shown to initiate signal transduction events and affect cell migration. We have shown that uPAR co-associates with fibronectin binding integrin, α5β1, activates them and that this interaction leads to a greatly increased level of active ERK. When the association between uPAR and integrin or integrin and fibronectin are interrupted either by reduction of surface uPAR expression, or by other means, human carcinoma cells enter a state of protracted dormancy. We show that very high levels of active ERK are required to keep cancer cells proliferating in vivo.

Key words: urokinase plasminogen activator (uPA), uPA receptor (uPAR), cancer dormancy, invasion, intravasation, ERK, α5β1 integrin

Biochemical characterization of uPAR and its interaction with uPA

Urokinase-type plasminogen activator (uPA) interacts with a specific plasma membrane receptor, discovered in 1985 (Vassalli et al., 1985; Stoppelli et al., 1985). The receptor was cloned from several species including human (Roldan et al., 1990; Kristensen et al., 1991). During post-translational processing a 22 amino acid C-terminal domain is removed and a glycosyl-phosphatidyl-inositol moiety (Ploug et al., 1991) membrane anchor is added. The mature, heavily glycosylated protein is composed of 3 homologous, independently folded extracellular domains, that bind uPA with high affinity (Kd of 0.1 to 1nM). uPA binds to domain 1 of uPAR, but interdomain interactions contribute significantly to the binding affinity (Ploug et al., 1994; Behrendt et al., 1996).

uPA is secreted in the form of a pro-enzyme and is converted to an active enzyme which is composed of two-chain held by a disulfide bond. Although, under normal conditions plasmin activates pro-uPA, other means of activation must exist, as the level of active uPA is similar in wild type and plasminogen null mice (Bugge et al., 1995). Activation of pro-uPA by plasmin and activation of plasminogen by uPA proceed much faster on the surface of cells (in association with receptors) than in fluid phase (Ellis et al., 1989; Ellis et al., 1991). In addition, surface-bound plasmin generated by this interaction is believed to be at least partially shielded from inhibition by native, high molecular weight plasma inhibitors (Stephens et al., 1989).

The role of uPAR-associated uPA in invasion and metastasis

The presence of uPAR bound uPA is important for efficient generation of surface-bound plasmin which, through its direct degradation of matrix proteins as well as activation of pro-MMPs (Mazzieri et al., 1997), has been shown to be important in cancer cell invasion (Dani et al., 1985; Mignatti et al., 1986; Ossowski, 1988a; Cajot et al., 1989; Schlechte et al., 1989; Testa and Quigley, 1990; Ossowski et al., 1991; Mignatti and Rifkin, 1993; Blasi, 1993; Stahl and Mueller, 1994) as well as in lung colonization and spontaneous metastasis (Ossowski and Reich, 1983; Hearing et al., 1988; Axelrod et al., 1989; Crowley et al., 1993; Kobayashi et al., 1994). It appears that when the uPA concentration is limiting, the enzyme is most efficiently utilized when receptor-bound (Ossowski, 1988b; Ossowski et al., 1991; Quax et al., 1991).
Following is a description of the experimental model we developed and used to establish the role of uPA/uPAR in different stages of the metastatic spread:

1. **Tumorigenicity.** Cells are inoculated into the artificial air sac on the wounded chorioallantoic membranes (CAMs) of 10 day old chick embryos and incubated for 7 days. The size of nodules produced on the CAMs is measured and recorded and the presence of tumor cells in the nodules is assessed microscopically.

2. **Metastasis.** Cells inoculated onto a wounded chorioallantoic membrane (CAM) of a chick embryo rapidly grow and disseminate through the vasculature into embryo organs. Lungs, the most predictable target for metastasis, are removed, minced and reinoculated on fresh CAMs for an additional week of growth. Metastatic cells present in the original lung «mince» are sufficiently enriched by the second cycle of growth and can be easily detected either microscopically or by measurement of human specific uPA levels (Ossowski and Reich, 1980; Ossowski and Reich, 1983; Ossowski 1988a,b).

3. **Local invasion of connective tissue.** Wounded CAMs, that were allowed to reseal in vivo, are inoculated with cells labelled with $^{125}$I-labeled for 24 hrs in culture. After 24 hrs of incubation, the cells which did not invade are removed by extensive wash and trypsinization and the excised CAMs, containing cells which penetrated into the tissue, and are protected from trypsin action, are counted in a gamma counter, (Ossowski, 1988b).

4. **Extravasation.** Extravasation is tested by injecting $^{125}$I-labeled cells intravenously and measuring their arrest in organs. (Ossowski, 1988a).

5. **Intravasation.** HEP3 tumors were dissociated with collagenase, plated at high density (3 x 10$^6$ cells per 100 mm dish) and passaged once 24 to 48 hrs prior to the experiment, detached from the culture dish with 2mM EDTA in PBS, resuspended in 50ml of PBS with Ca++ and Mg++ and inoculated (usually at 10$^6$ cells) onto a CAM of a 9 day old chick embryo in which an artificial air sac was created. After 50 hrs of incubation the lower half of the CAM was removed, and snap-frozen in liquid nitrogen. The genomic DNA was extracted and amplified by PCR using primers designed to recognize human alu sequences. The reaction mixture contained $^{32}$P-dCTP and PCR products were separated on PAGE, the gels dried and autoradiographed. (Kim et al., 1998).

**uPAR in malignant tumors**

Malignant tumors over-express urokinase type plasminogen activator (uPA) and its receptor (uPAR). Depending on the type of cancer, tumor cells, stroma or infiltrating cells were shown to be the source of these proteins. Both uPA and uPAR have been shown to be predictive of early recurrence of malignancy.

Immunocytochemical and in situ hybridization analyses of human cancer tissue sections indicate that most types of malignant tumors acquire uPA either through auto- or paracrine interactions (DeBruin et al., 1987; Janicke et al., 1990; Duffy et al., 1990; Grönndahl-Hansen et al., 1991). Similarly, the in vivo expression of uPAR has been shown in many tumors (Pyke et al., 1991; Bianchi et al., 1994; Carriero et al., 1994). These include colon and breast carcinomas (Pyke et al., 1993; Suzuki et al., 1998; Bianchi et al., 1994), several types of brain tumors (Mohanam et al., 1994; Gladson et al., 1995), melanoma and squamous cell carcinoma (De Vries et al., 1994). The prevalence of surface-bound uPA in cancer cells, and the experimental results implicating uPA in mechanisms underlying this disease suggest that surface proteolysis may represent a potentially attractive target for directed therapy.

**Approaches to therapy**

The accumulated evidence from experimental models, and the tight correlation that exists between the level of uPA/uPAR and the aggressiveness of cancer show that the uPA/uPAR complex plays an important role in cancer invasion. Most experimental approaches taken to date are either aimed at interfering with the uPA association with uPAR or at directly reducing the levels of uPAR. The latter may be a more effective approach as uPAR itself may have pro-malignant influence. We first tested whether anti-human uPA antibodies that inhibited the catalytic activity of uPA will have an effect on malignancy. This treatment blocked metastasis in a chick embryo model (Ossowski and Reich, 1983). Prostate cancer metastasis (PC3) in nude mice was shown to be inhibited by overexpression in these cells of a non-cleavable form of uPA (Crowley et al., 1993). To block uPA binding to its receptor a random peptide bacteriophage display library (Goodson et al., 1994) was examined and one peptide with an IC$_{50}$ of 10 nM was identified, but peptide with higher affinity would probably be needed for therapeutic applications. Several additional approaches have also been tried, including the use of amino terminal fragment of uPA, or DFP-inactivated uPA to displace active uPA from its receptor. Most have been found effective in experimental models.

To directly down-regulate uPAR expression antisense oligonucleotides, degradation of the mRNA by a specific ribozyme, or proteolytic cleavage of the receptor uPA-binding domain have been tried. "Switching off" the uPAR gene expression by an anti-messenger oligo-nucleotide have been shown to reduce invasion through Matrigel in human fibroblasts transformed with SV-40 virus (Quattrone et al., 1994). A successful delivery of anti-human uPAR-RNA ribozyme to cultured human
osteosarcoma cells has been described (Kariko et al., 1994) but no follow-up to this approach has been published.

Our own choice of intervention was to stably disable the uPAR gene expression by transfecting a highly malignant human epidermoid carcinoma cells, HEP3, (Toolan, 1954), with a construct producing uPAR antisense RNA. We prepared a construct which contained a 300 bp PCR-amplified 5’ fragment of uPAR-cDNA, which included the ATG codon, in antisense orientation. Several clones with reduced uPAR level were tested and found not to differ in their rate of growth in culture, and the amount and type of protease content; all clones produced high levels (~ 0.5 Ploug Units/10^6 cells) of uPA and 2 gelatinases, the 72 and 92 kDa. Therefore, except for blocking of uPAR, the transfection of the clonal selection did not alter other specific phenotypic properties usually associated with malignancy. The reduction in pro-uPA binding paralleled reduced levels of uPAR-protein and uPAR mRNA (Yu et al., 1997).

We found that the diminished expression of surface-uPAR leads to a severe reduction in invasiveness of tumor cells.

### uPAR in signal transduction

In addition to its matrix-degrading function, the binding of uPA or pro-uPA to its receptor has been shown to stimulate chemotaxis and/or chemokinesis in a variety of cell types such as neutrophils, tumor cells and endothelium (Gudewicz and Gilboa, 1987; Fibbi et al., 1988; Odekon et al, 1992; Del Rosso et al., 1993; Busso et al., 1994; Stahl and Mueller, 1994, Resnati et al., 1996).

Not unlike the case for other GPI-linked proteins, which are known to transduce signals through as yet unidentified pathways, the possibility of several signal transduction pathways through this receptor has also been considered. These involve both tyrosine phosphorylation, PKC, de novo DAG generation etc. (Del Rosso et al., 1993; Dumler et al., 1993; Anchini et al., 1994; Dumler et al., 1994, 1998; Busso et al., 1994; Koshelnick et al., 1996).

### uPAR in tumor dormancy

Clinical experience in cancer patients indicates that some primary cancers and most metastatic lesions un-

![Diagram](image-url)
Undergo a period of dormancy prior to entering a stage of progressive growth. Although this may be the most crucial step in cancer progression, the mechanisms underlying the conversion from a dormant to an actively growing state have not been elucidated. A prevalent hypothesis envisions that in order to grow, cancer cells must acquire the ability to induce angiogenesis (Folkman, 1995) and that the pro-angiogenic phenotype must be dominant. Another model of tumor dormancy involves experimental B-cell lymphoma in which antibodies directed to surface immunoglobulins act as agonists of signal transduction pathways leading to a block in cancer cell growth (Marches et al., 1998).

We discovered that uPAR down-regulation renders a human epidermoid carcinoma, HEp3, dormant (Kook, et al., 1994; Yu et al., 1997). These cancer cells, expressing less than 50% of the normal level of uPAR, grew indistinguishably from parental cells in culture but, when inoculated onto the chorioallantoic membrane (CAM) of chick embryos and maintained by serial in vivo passages, entered a period of protracted (~4 months) dormancy. We determined that dormancy was the result of a reduced proliferation rate rather than an increased apoptotic rate (Yu et al., 1997). These and other published results allow us to propose that, in addition to the well established role of uPA/uPAR as an activator of proteolytic cascade, this complex also participates in signal transduction. In this capacity it affects cell motility, (Nguyen et al., 1998) and, as we recently showed, (Aguirre Ghiso et al., 1999) it affects tumor dormancy. We found that a reduction in surface uPAR expression in HEp3 carcinoma cells, that induces the state of protracted dormancy (Yu et al., 1997), causes G1/G0 arrest of the cell cycle in vivo. The uPAR-rich, tumorigenic cells had a much higher basal level of active ERK1/2 that could be further activated by incubating the cells with single chain uPA (scuPA). The response to scuPA of cells with reduced uPAR (dormant) was weak and substantially delayed. Several reports have shown that binding of uPA to uPAR in tumor or endothelial cells activates the mitogen activated protein kinase (MAPK), ERK1 and 2 (Konakova et al., 1998; Nguyen et al., 1998; Tang et al., 1998). However, these reports neither addressed how uPAR activates signaling nor considered its in vivo consequence. In trying to identify the possible signal responsible for ERK activation we found that in cells expressing the full complement of uPAR, α5β1 integrins were in an active conformation and that these cells adhered efficiently to fibronectin (FN) inducing a robust and persistent activation of ERK, and a growth stimulation not present in uPAR-deficient cells. We hypothesized that interactions between uPA/uPAR and α5β1 integrin mediate the FN-dependent ERK-activation, and that a threshold level of uPA/uPAR/α5β1 complex had to be surpassed for FN-dependent ERK-activation to stimulate in vivo growth. In support of this hypothesis we found that anti-β1 antibodies co-precipitated uPAR, that soluble uPAR induced ERK activation in uPAR-deficient cells, and that anti-uPAR antibodies and a peptide that hinders uPAR/β1 interactions, diminished FN-induced ERK activation in uPAR-rich cells. Moreover, we found that in uPAR-rich malignant cells high ERK activation persisted in vivo, and that blocking the signal to ERK by anti-uPAR or anti-β1 antibodies, transiently inhibited tumor growth. We conclude that by rendering α5β1 integrins inactive, uPAR downregulation reduces FN-dependent ERK activation, forcing cancer cells into dormancy.

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References


