Chapter 12
The Urokinase Plasminogen Activator Receptor as a Target for Cancer Therapy

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Abstract Proteolytic processes are necessary for normal physiological functions in the body, including normal blood vessel maintenance, clot formation and dissolution, bone remodeling, and ovulation. The same enzyme system for the above roles is also used by the cancer cells for their growth and spread. These enzymes are produced by the tumor cells or cells surrounding them and can degrade the basement membrane and extracellular matrix (ECM) which consist of several components including collagens, glycoproteins, proteoglycans, and glycosaminoglycans. A major protease system responsible for ECM degradation is the plasminogen activation system, which generates the potent serine protease plasmin. The subject of this chapter, the urokinase plasminogen activator (uPA) receptor, plays an impressive range of distinct but overlapping functions in the process of cancer invasion and metastasis. Indeed, overexpression of this molecule is strongly correlated with poor prognosis in a variety of malignant tumors. Impairment of uPAR function, or inhibition of its expression, impedes the metastatic potential of many tumors. Several approaches have been employed to target uPAR with the aim of disrupting its ligand-independent action or interaction with uPA, Vn, or integrins, including the more recent antisense technology. This chapter also discusses the *in vivo* and *in vitro* use of antisense approaches and other similar techniques for downregulating uPAR as a potential therapy for cancer.

Introduction

The major cause of death in patients with malignant solid tumors such as carcinomas is the ability of cancer cells to invade surrounding tissues and form distant metastases. The spread of cancer cells from the primary site to a distant location is

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known to follow a sequence that requires their detachment from the primary site, migration through the local stroma, invasion into and then extravasation from the vascular tree, before finally migrating toward, adhering to and proliferating at a distant site to form a metastatic tumor.

A major determinant for the invasive and metastatic potential of tumor cells is their ability to proteolytically degrade extracellular matrix (ECM) and the basement membrane surrounding the primary tumor, which facilitates local invasion and intravasation, leading to distant dissemination of the disease (Muehlenweg et al. 2001, Romer et al. 2004). Although several extracellular protease systems have been implicated in the tissue degradation and remodeling that often accompanies cancer invasion, several studies show that the urokinase plasminogen activator (uPA) system is central to these processes, as reviewed previously (Blasi and Carmeliet 2002, Mazar 2001, Mazzieri and Blasi 2005, Romer et al. 2004, Wang et al. 2001). Components of the uPA system thus represent promising candidates for targeted cancer therapies.

The uPA/uPAR System

The uPA/uPAR system is involved in a variety of cell functions, including extracellular proteolysis, adhesion, proliferation, chemotaxis, neutrophil priming for oxidant production and cytokine release, all of which variously contribute to the development, implantation, angiogenesis, inflammation, and metastasis of tumors (Ge and Elghetany 2003).

The uPA/uPAR system consists mainly of the serine protease uPA, its cell membrane-associated receptor (uPAR), a substrate (plasminogen), and plasminogen activator inhibitors (PAI-1 and PAI-2) (Mazar et al. 1999, Wang 2001). uPA is produced and secreted as single-chain polypeptide—a zymogen known as pro-uPA—that lacks plasminogen-activating activity. Upon binding of pro-uPA to uPAR, it is cleaved by various proteases into an active two-chain uPA molecule. This active uPA enzyme then converts the zymogen plasminogen to the active serine protease plasmin, which is involved in the degradation of the ECM and basement membranes by either direct proteolytic digestion or by activation of other zymogen proteases, such as pro-metalloproteases and pro-collagenases, thereby promoting tumor migration (Mazzieri and Blasi 2005, Wang 2001). Binding of uPA to uPAR provides an inducible, transient, and localized cell-surface proteolytic activity (Mazzieri and Blasi 2005, Wang 2001).

Studies have suggested that among the uPA/uPAR system components, uPAR might have a more crucial role in the metastasis process (Wang 2001). Many of the activities of uPA, including its activation by plasmin, are dependent on its binding to uPAR (Mazar et al. 1999). However, uPAR knock-out mice do not show any evidence of deficient fibrinolysis, even when in combination with the $tPA^{-/-}$ mutation, which suggests that pro-uPA activation takes place naturally also in the absence of uPAR, at least under nonstimulated conditions.

uPAR

The human uPAR cDNA encodes a 335 amino acid polypeptide, which during the cell surface sorting is posttranslationally modified in several ways to generate the mature receptor. An amino-terminal signal peptide and a carboxyterminal GPI-anchor peptide are removed during cell surface sorting and processing for GPI anchoring. Finally, the protein is extensively glycosylated. The mature uPAR protein consists of 3 homologous cysteine-rich repeats of about 90 amino acids each. Three-dimensional structure of uPAR and the details of the uPA binding site are discussed in Chapter 34 by Jacobson et al.

uPAR Cleavage and Shedding

Cell surface uPAR has been shown to undergo two major types of covalent modifications which alter the function of the receptor. The first type of uPAR modification is either a proteolytic cleavage close to the GPI anchor or a hydrolysis of the GPI-anchor by a phospholipase (Pedersen et al. 1993, Sier et al. 1998). This cleavage releases the entire receptor from the cell surface (suPAR), with concomitant functional changes, but does apparently not alter the ligand-binding properties of the receptor notably. We refer to this process as uPAR shedding. The second type is a proteolytic cleavage in the linker region connecting DI and DII and results in the release of the D1 fragment from the rest of the receptor. As described below, this cleavage changes the biochemical properties of uPAR completely. We will refer to this phenomenon as uPAR cleavage.

Recent advances in the study of uPAR shedding and cleavage supports the possibility that these processes are important in the malignant process of tumor invasion and metastasis. The GPI-anchoring of uPAR renders the protein prone to release from the cell surface and soluble forms of uPAR can indeed be found both *in vitro* and *in vivo*. The mechanism of uPAR shedding may be catalyzed by cellular phosphatidylinositol-specific phospholipase D (PIPL-D), which is able to release GPI-anchored proteins, including uPAR, from the cell surface (Metz et al. 1994, Wilhelm et al. 1999). Also plasmin appears to shed uPAR, although the mechanism is still not clear (Beaufort et al. 2004). Shedding of uPAR releases the receptor from the cell surface but does not apparently alter the affinity for its two major ligands, uPA and Vn (Ronne et al. 1994, Wei et al. 1994). Shedding occurs both *in vitro* and *in vivo*: suPAR is present in the conditioned medium from a variety of cultured cells (Chavakis et al. 1998, Holst-Hansen et al. 1999, Lau and Kim 1994, Ploug et al. 1991, Sidenius et al. 2000), as well as in biological fluids such as plasma, urine, cerebrospinal fluid, ascites, and ovarian cyst fluid (Garcia-Monco et al. 2002, Mustjoki et al. 2000a, Pedersen et al. 1993, Sier et al., 1999, Stephens et al. 1997, Wahlberg et al. 1998). The uPAR linker region connecting DI and DII is prone to hydrolysis by a variety of different proteases. This region may be cleaved by trypsin, chymotrypsin and, physiologically more important, uPA, plasmin,

neutrophil elastase, as well as by a number of different matrix metalloproteinases (MMPs) (Andolfo et al. 2002, Behrendt et al. 1991, Hoyer-Hansen et al. 1997a, Koolwijk et al. 2001, Ploug et al. 1994). While the uPA-catalyzed cleavage of suPAR *in vitro* is independent of the uPA/uPAR interaction (Hoyer-Hansen et al., 1992), cleavage of cell-surface uPAR is accelerated through a mechanism which requires the binding of uPA to uPAR (Hoyer-Hansen et al. 1997b). The reason for this acceleration of uPAR cleavage is not clear, but evidence has been presented that GPI-anchored and soluble uPAR may have different conformations (Andolfo et al. 2002, Hoyer-Hansen et al. 2001) with the GPI-anchored form being more susceptible to cleavage (Andolfo et al. 2002, Hoyer-Hansen et al. 2001). Besides uPA, also MMPs are capable of cleaving uPAR in cell culture (Koolwijk et al. 2001) and *in vitro* (Andolfo et al. 2002). The protease(s) responsible for uPAR cleavage *in vivo* has not been determined. Cleaved uPAR [DIIDIII] has been found on the surface of many cells including endothelial cells, lymphocytes, and in several different cancer cell lines (Holst-Hansen et al. 1999, Hoyer-Hansen et al. 1992, Ragno et al. 1998, Sidenius et al. 2000, Solberg et al. 1994). Cleaved uPAR has also been identified in detergent extracts of human tumors xenografted in nude mice and in the Lewis Lung tumor in mouse (Holst-Hansen et al. 1999, Solberg et al. 1994). Cleavage of uPAR releases D1 to the surroundings and this fragment can indeed be found in the conditioned medium from cells with cleaved uPAR on the surface (Koolwijk et al. 2001, Mustjoki, Sidenius and Vaheri 2000b, Sidenius et al. 2000). Soluble forms of the DIIDIII fragment are also found in culture medium of cancer cells (Mustjoki et al. 2000b, Sidenius et al. 2000), in the fluid from human malignant ovarian cysts (Wahlberg et al. 1998), in urine from healthy individuals and cancer patients (Mustjoki et al. 2000a, Sidenius et al. 2000, Sier et al. 1999), and in blood from patients with acute myeloid leukemia (Mustjoki et al. 2000a).

uPA/uPAR Interactions and Signal Transduction

The important role of uPAR in tumor cell adhesion, migration, invasion, and proliferation makes this receptor an attractive drug target in cancer treatment; however, this is complicated by the extent of the published uPAR "interactome." For this reason, the most important question becomes which of the many molecular interactions are really essential to mediate uPAR function. Recently, the crystal structures of uPAR in complex with a peptide antagonist (Llinas et al. 2005) and with the N-terminal fragment of uPA (Barinka et al. 2006) were presented, providing the first rational basis toward understanding how uPAR may organize its multiple molecular interactions.

The second well-characterized ligand for uPAR is Vn, a glycoprotein produced in the liver and present at high concentrations in plasma (Preissner 1989). The uPAR binding site in Vn has been mapped to the amino-terminal somatomedin B domain of Vn (Deng et al. 1996, Okumura et al. 2002), a region which also contains the binding sites for PAI-1 and for the integrin Vn receptor ($\alpha V\beta 3$) (Hoyer-Hansen

et al. 1997a, Sidenius and Blasi 2000). Several antibodies against D1 inhibit the interaction between uPAR and Vn (Hoyer-Hansen et al. 1997a, Kanse et al. 1996, Sidenius and Blasi 2000). The binding to Vn is connected to the occupancy of uPAR by uPA (Hoyer-Hansen et al. 1997a, Sidenius and Blasi 2000, Waltz and Chapman 1994, Wei et al. 1994) and is, at least *in vitro*, controlled by uPAR dimerization (Sidenius et al. 2002). The interaction between uPA, Vn, and uPAR is profoundly altered by receptor cleavage. The released D1 fragment has a >1,000-fold reduced affinity for uPA as compared to the intact receptor and the DIIDIII fragment has no measurable affinity at all (Ploug et al. 1994). Also the uPAR interaction with Vn is lost as none of the generated fragments binds with measurable affinity (Hoyer-Hansen et al. 1997a, Sidenius and Blasi 2000). Sidenius and colleagues (2007) have also demonstrated that a direct Vn interaction is both necessary and sufficient to initiate uPAR-induced changes in cell morphology, migration, and signaling independently of other direct lateral protein–protein interactions. Their data suggest that the single interaction between uPAR and Vn may be responsible for many of the proteolysis-independent biological effects initiated by uPAR (Madsen et al. 2007).

Most of the cellular responses modulated by the uPA/uPAR system, including migration, cellular adhesion, differentiation, and proliferation (Blasi and Carmeliet 2002) require transmembrane signaling, which cannot be mediated directly by a GPI-anchored protein such as uPAR. For this reason, besides the well-established interactions with uPA and Vn, uPAR has been reported to entertain direct contacts with a variety of extracellular proteins and membrane receptors, such as integrins (Chapman and Wei 2001, Ossowski and Aguirre-Ghiso 2000), epidermal growth factor (EGF) receptor (Liu et al. 2002), high molecular weight kininogen (Colman et al. 1997), caveolin, and the G-protein-coupled receptor FPRL1 (Resnati et al. 2002). As a result, uPAR activates intracellular signaling molecules such as tyrosine- and serine-protein kinases, Src, focal adhesion kinase (FAK), and extracellular-signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK).

The interaction of uPAR with integrins is supported by co-immunoprecipitation experiments and by the effect of uPAR-binding peptides isolated from phages libraries (Aguirre-Ghiso et al. 1999, Bohuslav et al. 1995, Carriero et al. 1999, Tarui et al. 2001, Wei et al. 1996). Although uPAR can interact with many integrins, it appear to have the highest affinity for the fibronectin receptors $\alpha 3\beta 1$ and $\alpha 5\beta 1$ (*see* Chapter 23 by Degryse). Ligand-induced signaling necessary for normal $\beta 1$ integrin function requires caveolin and is indeed regulated by uPAR. Caveolin and uPAR may operate within adhesion sites to organize kinase-rich lipid domains in proximity to integrins, promoting efficient signal transduction (Wei et al. 1999). Furthermore, disruption of uPAR-integrins association by uPAR-binding peptides broadly impairs integrin function, suggesting a novel strategy for regulation of integrins in the settings of inflammation and tumor progression (Simon et al. 2000). The best characterized uPAR-dependent signaling pathway is the one described by Aguirre-Ghiso and colleagues. They propose that even cancer cells with multiple mutations may use the uPAR surface receptor and ECM components to regulate signaling pathways that control cell cycle progression

and/or arrest (Aguirre-Ghiso et al. 2003). They describe a uPAR-dependent mechanism by which the majority of tumor cells modulate the activity ratio between the proliferation inducer ERK (Hoshino et al. 1999) and the negative growth regulator p38 (Chen et al. 2000). On the basis of the study of ten different cell lines, their results show how uPAR and α5β1 activate the EGFR in a EGF-independent but FAK-dependent manner (Liu et al. 2002) and generate high ERK and low p38 activity necessary for the *in vivo* growth of cancer cells. A positive loop is activated in which high ERK activity increases uPAR and uPA expression (Aguirre-Ghiso et al. 2001, Lengyel et al. 1995, 1997, 1996) and the high uPAR level maintains high ERK activity by activating α5β1 (Aguirre-Ghiso et al. 1999, Liu et al. 2002). The cancer cell proliferation loop can be interrupted by a reduction of uPAR level by cleavage of its domain 1, important for the uPAR/α5β1 interaction and activation (Aguirre-Ghiso et al. 1999, Liu et al. 2002, Montuori et al. 2002) or by loss of uPA and/or FN.

uPAR in Cell Motility

uPAR plays a role in the migration of a variety of cell types, and evidence is accumulating that uPAR-dependent migration is mediated through integrins (*see* Chapter 23, Degryse). In addition to β1- and β2-integrin involvement in the uPAR-dependent adhesion and migration of leukocytes (Aguirre-Ghiso et al. 1999, Gyetko et al. 1995, 1994; Liu et al. 2002, Montuori et al. 2002, Sitrin et al. 1996, Wong et al. 1996), the interaction of uPAR with integrins has also been demonstrated on tumor cells. Xue et al. have demonstrated the interaction of uPAR on HT1080 cells with various α and β integrins including β1 and β3 and αv, α3, α5, and α6 (Xue et al. 1997). Migration, but not adhesion on Vn, of FG cells, which express av β5, was uPA–uPAR dependent. However, migration of several melanoma cell lines, which express only αvβ3 occurred independently of uPAR (Yebra et al. 1996). The adhesive and pro-migratory effects of uPAR, as well as the identity of the integrin adapter, depend on the cell type and the ECM component in question. The interactions of uPAR in cell migration and invasion may change as matrix barriers are remodeled or as the cell migrates through areas of different matrix composition. Several signaling pathways have been implicated in uPAR-mediated cell migration *in vitro*. uPAR-dependent signaling via the JAK/STAT pathway may be involved in the migration of vascular smooth muscle cells (Dumler et al. 1998). A second, uPAR-dependent signaling pathway involving Src-like protein tyrosine kinases has also been described in these cells, although the functional relevance of this second pathway is not yet understood. The JAK/STAT pathway was also activated by clustering the uPA–uPAR complex using a monoclonal antibody in the human kidney epithelial cell line TCL-598, resulting in the migration of this cell line (Koshelnick et al. 1997, Nguyen et al. 1998). In MCF-7 breast cancer cells, uPAR occupancy resulted in cell migration, which occurred through the activation of ERK1/ERK2. An inhibitor targeting MAPK kinase, a member of the JAK family of kinases, suppressed ligand-induced uPAR-dependent

activation of ERK1/ERK2 in these cells (Nguyen et al. 1998). The MAPK pathway is activated in cytokine-mediated signaling and has been implicated as a major signal-transducing pathway in angiogenesis (stimulated by vascular endotheliel growth factor (VEGF) and basic fibroblast growth factor (bFGF) (D'Angelo et al. 1995, Jones et al. 1998).

Chymotrypsin-cleaved suPAR is a potent chemoattractant for several different cell lines (Fazioli et al. 1997, Resnati et al. 1996). The chemotactic response and kinetics of p56/59hck phosphorylation induced by proteolytically inactive uPA derivatives and that of cleaved suPAR are similar, suggesting that the same signaling pathway may be activated by these molecules (Resnati et al. 1996). Inhibitors of tyrosine kinases and heterotrimeric G proteins block the chemotactic response and the induction of phosphorylation of p56/59hck. The fact that pertussis toxin inhibits chemotaxis and the phosphorylation of p56/59hck suggests that heterotrimeric G proteins are involved and that they act upstream of the tyrosine kinase in the signaling pathway. The fact that cleaved soluble uPAR and peptides containing the uPAR chemotactic epitope are strong chemokine-like molecules strongly suggests the existence of one or more membrane "adapter" molecule(s) capable of transmitting the chemotactic signal over the membrane (Fazioli et al. 1997, Resnati et al. 1996). Indeed, it was shown that the DIIDIII fragment generated by chymotrypsin cleavage of suPAR interacts with, and signals through, the FPRL1/LXA4R chemokine receptor (Resnati et al. 2002). Both GPI-anchored and soluble forms of cleaved uPAR have been observed on different cell types and in diverse biological fluids. The strong chemotactic properties of suPAR fragments, together with the fact that similar fragments are found at high concentrations in cancer, suggests that the chemotactic activities of these fragments may play a role in the process of tumor invasion, maybe as an autocrine or paracrine signal for tumor cell motility.

uPAR and Cell Proliferation

In addition to regulating cell migration, uPAR also regulates cell proliferation. Work by Ossowski and co-workers has described a mechanism by which the uPAR/integrin interaction may not only affect tumor growth and invasion through its regulation of extracellular proteolysis and integrin-dependent cell migration but also by directly promoting tumor cell proliferation (Liu et al. 2002). The model cell system used by these researchers is the HEp3 cell line, which is highly malignant, grows rapidly *in vivo* on the chicken chorioallantoic membrane, expresses uPAR, and has a high level of active ERK (Aguirre-Ghiso et al. 1999). Downregulation of uPAR expression in these cells, by antisense technology or prolonged culture *in vitro*, results in strong reduction in the level of ERK activation and causes concomitant tumor dormancy. ERK activation by uPAR in HEp3 cells is dependent upon the interaction between uPAR and $\alpha 5\beta 1$-integrin and is maximal when both of these receptors are engaged by their respective ligands (uPA and Fn). Because ERK is a downstream effector molecule of both integrins and the EGF receptor (EGFR),

which is expressed in HEp3 cells, Ossowski and colleagues proceeded to analyze the possible role of EGFR in uPAR-mediated ERK activation and tumor growth. Indeed, they succeeded in demonstrating that in HEp3 cells the EGFR, independently of EGF, mediates the uPA/uPAR/α5β1/Fn-induced tumor growth pathway. Interestingly, the uPAR-mediated growth promoting signaling pathway did not require high levels of EGFR expression, distinguishing it from the fibronectin-dependent integrin-mediated EGFR-activation previously described (Moro et al. 1998). uPAR-dependent EGFR-activation requires high levels of intact uPAR and is paralleled by a physical association between the EGFR and α5β1 in a FAK-dependent manner. These data present, to our knowledge, the first example of how carcinoma cells can utilize a normal expression level of a EGFR and a high expression level of uPAR to activate a growth factor-independent mitogenic pathway.

uPAR and Cancer

uPAR is expressed across a variety of tumor cell lines and tissues, including colon, breast, ovary, lung, kidney, liver, stomach, bladder, endometrium, and bone (Ge and Elghetany 2003, Mazzieri and Blasi 2005, Wang 2001). uPAR expression is not confined to the tumor cells themselves: several tumor-associated cell types, including macrophages, mast cells, endothelial cells, NK cells, and fibroblasts, are all capable of uPAR expression in various tumor types (Mazar et al. 1999, Mazar 2001, Sidenius and Blasi 2003). Indeed, the involvement of stromal cells in the generation of extracellular proteolysis argues that cancer invasion is the result of an interaction between cancer cells and stromal cells. It is not only the cancer cells that invade but also a mixed cell population. Tha cancer cells are the initiators and probably the organizers, but each cell type contributes in a distinct way to the overall process.

Several experimental evidences support the importance of the uPA/uPAR system in cancer with respect to its ability to modulate cell migration and cell adhesion and therefore determine the invasive and metastatic properties of tumor cells both *in vitro* and *in vivo* (for review, *see* Sidenius and Blasi 2003). uPAR levels have been strongly correlated with metastatic potential and advanced disease, which has been demonstrated in tumor samples obtained from patients with colon and breast cancer (Ge and Elghetany 2003, Dano et al. 2005). For example, uPAR is overexpressed in malignant breast cancer tissues but not in normal and benign breast tumors. uPAR has been found to be particularly abundant at the leading edge of tumors, that is, in those areas where tumor cells invade normal tissue (Lindberg et al. 2006, Skriver et al. 1984, Yamamoto et al. 1994). Tumor angiogenesis, a necessary event in tumor progression to sustain tumor growth and metastasis dissemination, is also modulated by the uPA/uPAR system (Carmeliet and Jain 2000, Jain and Carmeliet 2001). It requires a finely regulated cell proliferation, differentiation, and migration. After activation, endothelial cells express increased amount of uPA and uPAR at their leading migratory front to modulate ECM degradation, redeposition and cell adhesion (Blasi and Carmeliet 2002). The increased levels of uPAR expression typically associated with tumor tissue, its relative absence from normal, quiescent tissue, and

its central role in regulating angiogenesis and tumor progression suggest that uPAR represents an attractive target for cancer therapy. Most experimental strategies have been focused on reducing pericellular uPA-mediated plasminogen activation, a goal which (in theory) may be obtained by a variety of approaches. First, by reducing the expression of uPA and/or uPAR expression. Second, by interfering with the uPA/uPAR interaction. Third, by a direct inhibition of uPA activity. Alternative approaches to interfere with the uPA-system includes interference with the uPAR/Vn and uPAR/integrin interactions. Direct approaches aimed at directly killing the tumor cells using toxins targeting one or more of the components of the uPA-system have also been developed and hold great promise.

There are several reasons why pharmacological targeting of uPA/uPAR may be attempted without major side effects. First, animal models suggest that the uPA-system is not essential for fertility or survival under physiological conditions. Second, the thrombosis risk of nocturnal hemoglobinuria patients does not appear to depend on the lack of uPAR (Bessler et al. 2002).

Antisense Therapeutic Strategies for Downregulation of uPAR In Vivo

Kook and co-workers were the first to evaluate the effect of antisense inhibition of uPAR on invasion and metastasis in human squamous cell carcinoma. Using a vector that is capable of expressing an antisense uPAR transcript, Kook et al. (1994) demonstrated that in highly malignant human squamous carcinoma cells downregulation of uPAR reduced the invasive potential. Furthermore, the tumors that developed from antisense clones were less invasive and nontumorigenic in chick embryos 7 days after injection and less invasive when injected in nude mice. This was the seminal *in vivo* demonstration that tumor growth, invasion, and metastasis could be inhibited by an antisense approach through Watson–Crick base-specific complementarity.

Downregulation of uPAR can also be obtained by the classic antisense oligodeoxynucleotide (asODN) technology, which consists of the injection of antisense DNA strands complementary to uPAR mRNA, or the antisense RNA technology, based on transfection with a vector capable of expressing the antisense transcript complementary to uPAR mRNA. Margheri et al. (2005) and D'Alessio et al. (2004) both investigated the antimetastatic and/or antitumor potential of the same 18mer phosphorothioate asODN in two different experimental models. Using a rodent model of bone metastasis, Margheri et al. (2005) injected malignant human prostate carcinoma cells into the heart (left ventricle) of CD1 nude mice. The animals were then subjected to daily intraperitoneal injections of asODNs and analyzed at 28 days after the heart injection or at the first signs of serious distress. Treatment with the asODN resulted in complete inhibition of bone metastases in 80% of the mice as well as complete inhibition of lymphonode and lung metastases.

D'Alessio et al. (2004) injected human melanoma cells with high metastatic potential into the hind leg muscles of CD1 male nude mice. Four days after cell

implant, when a mean tumor mass of 350 mg was evident, the mice were treated intravenously with the asODN for five consecutive days. A second and third cycle of treatment was administered at 2-day intervals, and the mice were sacrificed and analyzed 25 days after tumor implantation. The asODN treatment resulted in 45% reduction of primary tumor mass and 78% reduction of lung metastases. Thus, these two studies show the ability of uPAR downregulation to reduce tumor growth in two types of cancers, which was effective both when administration was intraperitoneal and by the more clinically relevant intravenous way.

Similarly, Nozaki et al. (2005) showed that injection of highly malignant human oral squamous carcinoma cells pretreated with an 18mer phosphorothiate uPAR asODN into the chorioallantoic membrane vein of 10-day-old chick embryos yielded 86% inhibition of liver metastasis. They also showed that orthotopic implantation of cells pretreated with the asODN into the submucosa of the oral floor of 6-week-old female BALB/c immunocompromised mice inhibited the invasive capacity of these cells.

Research groups investigating antisense RNA technology for downregulation of uPAR *in vivo* have employed both plasmids (Dass et al., 2005, Go et al. 1997, Kook et al. 1994, Wang et al. 2001) and adenovirus (Gondi et al. 2004a, Lakka et al. 2001, Mohan et al. 1999) constructs for this purpose. Go et al. (1997) produced their plasmid construct by cloning the same 300 bp fragment as (Kook et al. 1994), corresponding to the 5′ end of uPAR in an antisense orientation. Human glioma cells stably transfected with the antisense construct were injected intracerebrally into 7-week-old female athymic nude mice. Animals were sacrificed and analyzed 1, 2, 3, and 4 weeks postinjection. Stable transfectants failed to form tumors and were negative for uPAR expression.

Wang et al. (2001) constructed plasmid expression vectors containing either a 585 bp 3′ uPAR cDNA fragment or a 498 bp 5′ uPAR cDNA fragment in the antisense orientation. Human colon cancer cell lines were transfected with each of the antisense clones and injected into the dilated lateral tail veins of 3–4-week-old athymic mice. At 9–12 weeks postinjection, the mice were sacrificed and examined. Metastasis of cells to the lungs was observed in 63% to 78% of mice injected with the parental colon cancer or control cells. By contrast, only 19% and 9% pulmonary metastases were observed in mice injected with the 3′ and 5′ antisense clones, respectively.

Downregulation of uPAR levels by an antisense strategy using adenovirus constructs resulted also in inhibition of growth and invasion of human glioblastoma (Gondi et al. 2004b, Mohan et al. 1999) and human lung cancer cells (Lakka et al. 2001). These three studies used the same adenovirus construct for the two types of cancers, highlighting its cross-cancer potency.

Mohan et al. (1999) also performed tumor regression experiments by injecting human glioblastoma cells subcutaneously into nude mice and then injecting the mice every other day with the antisense-containing adenovirus construct when the tumor size had reached 4–5 mm (after 8–10 days). They showed that injection of the antisense construct into pre-established tumors in nude mice caused regression of those tumors. Mohan et al. showed complete inhibition of tumor formation

after intracranial injections of glioma cells transfected with the antisense construct, Gondi et al. (2004a) reported a reduction in intracranial tumor growth of 60% or greater.

The discovery of RNA interference (RNAi) has provided new opportunities for cancer therapy. Small interfering RNAs (siRNAs) are more potent inhibitors of gene expression compared with ribozymes and deoxyribozymes (Beale et al. 2003). However, this evaluation was only performed *in vitro* using human squamous carcinoma cells A431 and EGFR as targets, and the findings might not be directly relevant to the *in vivo* efficiency of these constructs. Surprisingly, not much has been accomplished to compare different gene downregulation systems, and more studies should be devoted to this examination.

Nevertheless, investigators have already used an shRNA-based RNAi plasmid system for the downregulation of uPAR in prostate cancer (Pulukuri et al. 2005) and glioblastoma cells (Gondi et al. 2004a, Lakka et al. 2005). These researchers have all used a plasmid construct expressing the same small hairpin RNA (shRNAs), which they also refer to as siRNAs, targeted to uPAR. Importantly, a clear majority of *in vivo* studies looking at the downregulation of uPAR have used sequences antisense to the 5′ region of the uPAR mRNA.

For the above studies, human glioblastoma cells were intracranially injected into athymic male and female nude mice. Eight to ten days after tumor growth, sustained-release infusion of 150 μg of the shRNA-expressing plasmid construct was performed into the brain of each animal. The mice were sacrificed and analyzed at the end of the 5-week follow-up period or when the control mice started showing symptoms. Gondi et al. (2004b) reported a 65% regression of pre-established intracranial tumor growth. These findings were supported by Lakka et al. (2005) who reported 70% inhibition of pre-established intracranial tumor growth.

uPAR Downregulation may Affect Cellular Function and Signal Transduction

Researchers that have used either antisense or siRNA technologies for the successful *in vivo* downregulation of uPAR in various cancers have concurrently tested these same technologies in *in vitro* biological assays. Evaluation of the results of these *in vitro* assays reveals that downregulation of uPAR has led, in most cases, to inhibition of invasion (D'Alessio et al. 2004, Dass et al. 2005, Lakka et al. 2005, 2001; Margheri et al. 2005, Mohan et al. 1999), migration (Dass et al. 2005, Lakka et al. 2001), adhesion (Dass et al. 2005), and proliferation (D'Alessio et al. 2004, Lakka et al. 2005, Margheri et al. 2005). In addition, reduced uPAR levels lead to inhibition of tumor-induced angiogenesis (Lakka et al. 2005) and ECM degradation (Nozaki et al. 2005, Wang et al. 2001).

As stated earlier, some of the biological functions of uPAR, such as proliferation, are facilitated by the regulation of several different signaling molecules. In an attempt to understand and/or elucidate the involvement of uPAR in downstream

signaling pathways, studies have investigated the effect of uPAR downregulation on components of the relevant signaling pathways. D'Alessio et al. (2004) reported that melanoma cells exhibited a strong decrease in ERK1/2 activation when an 18mer asODN was used to downregulate uPAR. Using this same asODN for the downregulation of uPAR in prostate cancer cells, Margheri et al. (2005) reported a strong decrease of FAK/JNK/Jun phosphorylation (thereby causing a decrease in the activation of the FAK/JNK/Jun pathway). At the same time, the synthesis of cyclins A, B, D1, and D3 was inhibited, and these prostate cancer cells accumulated in the G2 phase of the cell cycle. The downregulation of uPAR by a plasmid construct expressing shRNA for uPAR resulted in significantly reduced levels of the phosphorylated forms of MAPK, ERK, and AKT signaling pathway molecules (Lakka et al. 2005).

However, the majority of studies applying uPAR downregulation for cancer *in vivo* failed to look at which signaling pathways are perturbed as a result. In any case, different laboratories choose to elucidate effects on different pathways and, although there is an abundance of literature looking at individual pathways *in vitro*, it is difficult to compare results from separate studies because various parameters, including cell line, passage number, minor technical differences, the antisense sequence, the concentration of constructs, the time-points evaluated, and the way the data are reported, often prevent such comparisons.

Inhibition of the uPAR/Vn Interaction

Tumor cells often express reduced levels of adhesion receptors and also often fail to deposit ECM around themselves. The fact that uPAR is upregulated in many tumor cells suggests that the cells may use this alternative adhesion pathway as a response to the altered expression of normal cell adhesion proteins. In glioblastomas and in hepatocellular carcinomas, both Vn and uPAR are present at relatively high levels (De Petro et al. 1998, Gladson and Cheresh 1991, Gladson et al. 1995, Kondoh et al. 1999) and since the interaction between uPAR and Vn induces cytoskeleton rearrangements and increases cell motility (Kjoller and Hall 2001), this interaction may contribute to the highly malignant phenotype of these tumors. Therapeutical antitumor approaches aimed at blocking the uPAR/Vn interaction therefore seems warranted. Development of inhibitors of the uPAR/Vn interaction may possibly start from the uPAR-binding somatomedin B domain of Vn, which is a natural and potent uPAR/Vn-interaction antagonist. As it was shown that uPAR-binding to Vn involves dimerization of uPAR (Sidenius et al. 2002), inhibitors of uPAR-dimerization might also become useful.

Inhibition of the uPAR/Integrin Interaction

As described above, uPAR/integrin interactions affects several cellular properties including cell adhesion, migration, and proliferation, which may potentially be

important in the malignant process of tumor invasion and metastasis. Specific peptide-based inhibitors of the uPAR/integrin interaction have been identified (Wei et al. 1996), and direct evidence supporting a functional role of the uPAR/integrin interaction in tumor progression has come from an *in vivo* bone xenograft model (van der Pluijm et al. 2001). In this study, stably transfected MDA-MB-231 cells that express a peptide which blocks the uPAR/integrin interaction (peptide 25, Wei et al. 1996) showed a significant reduction in tumor progression in bone. Also the continuous systemic administration of peptide 25 resulted in significantly reduced MDA-MB-231 tumor progression when compared to scrambled control peptide. Along the same lines, it will be important to identify uPAR peptides preventing the formation of or dissociating already formed uPAR/integrin complexes (*see* Chapter 23, Degryse).

Combination of uPAR Downregulation with Gene Modulation of Other Molecular Targets

Downregulation of more than one component involved in tumor invasion and metastasis might possibly have a synergistic or additive effect in preventing tumor dissemination. Lakka et al. (2003) reported that intracranial injection of human glioma cells infected with an adenovirus bicistronic construct capable of simultaneously expressing antisense uPAR and matrix metalloproteinase-9 (MMP-9) antisense, showed decreased invasiveness and tumorigenicity in mice. Subcutaneous injections of the bicistronic construct into established tumors caused tumor regression. MMP-9 is involved in the metastasis of various types of cancers, although its inhibition has not led to significant improvements in clinical trials (Klein et al. 2004). Thus, it is hoped that a dual targeted approach, combining MMP-9 and uPAR downregulation, will lead to better efficacy *in vivo*.

Lakka et al. also used the bicistronic plasmid construct targeting both uPAR and MMP-9 simultaneously (Lakka et al. 2005) with total regression of pre-established intracerebral tumor growth in mice. Similarly, Gondi et al. (2004b) showed that RNAi of uPAR and cathepsin B reduced glioma cell invasion and angiogenesis in *in vivo* models. Furthermore, intratumoral injections of these plasmid vectors expressing shRNA for uPAR and cathepsin B resulted in the regression of pre-established intracranial tumors (Sloane et al. 2005).

Injection into SCID mice of an adenovirus construct capable of simultaneously expressing uPAR and MMP-9 antisense has been reported to cause the regression of subcutaneous H1299 tumors (Rao et al. 2005). In addition, lung metastasis was inhibited with A549 cells (Rao et al. 2005). Furthermore, uPAR downregulation might be coupled to overexpression of tumor-inhibiting genes, such as tumor-suppressor genes, for enhanced therapeutic effect. Such an approach was tested by Adachi et al. (2002) by combining uPAR downregulation with p16 tumor suppressor overexpression, to demonstrate a dramatic inhibition of orthotopic and ectopic glioma growth *in vivo*. However, this is the only study that combines

downregulation of uPAR with overexpression of growth-inhibiting genes, and more work needs to be done, although this seems to be a promising and efficacious option: another feasible option would be to overexpress one of the plasminogen activator inhibitors. In most of the studies that combine uPAR downregulation with downregulation of a second important pro-cancer target, the authors fail to report whether the effect was additive or synergistic.

uPAR and Apoptosis

Oncogenic cell transformation is currently viewed as a multistep process in which a series of genetic lesions change cellular physiology leading to the acquisition of new capabilities, such as an enhanced ability to proliferate, migrate, and escape apoptotic cell death (Hanahan and Weinberg 2000). Apoptosis can be viewed as a safe-lock mechanism that could prevent the establishment of a fully transformed phenotype. For instance, it is currently accepted that uncontrolled proliferation could by itself prime the transforming cell to apoptotic cell death (Hood and Cheresh 2002, Pelengaris et al. 2002). This is why the acquired capabilities of resistance to apoptotic cell death and tissue invasion are considered to be obligate steps in tumor progression. Recent findings indicate that a decreased uPAR expression may promote apoptosis. This is the case of SNB19 glioblastoma cells expressing antisense uPAR constructs that are less invasive than parental cells when injected *in vivo* and undergo loss of mitochondrial transmembrane potential, release of cytochrome c, caspase-9 activation, and apoptosis (Yanamandra et al. 2000). Furthermore, glioma cells bearing a reduced uPAR number are more susceptible to tumor necrosis factor-α-related apoptosis than parental cells (Krishnamoorthy et al. 2001). Alfano and colleagues (Alfano et al. 2006) provide a causal link between uPAR signaling and protection from programmed cell death. They show that ligand engagement of uPAR counteracts the pro-apoptotic effect triggered by UV light, cisplatin, and forced detachment from the culture dish. Furthermore, they demonstrate that the expression level of uPAR positively correlates with resistance to anoikis in embryonic kidney epithelial (HEK-293) cell lines. They also show that the uPA/uPAR interaction results in a marked upregulation of the anti-apoptotic factor Bcl-xL, which is required for the uPA-dependent anti-apoptotic activity. In agreement with these observations, targeting the uPAR with inhibitory peptides leads to a reduction of glioma tumor size in mice through inhibition of cell proliferation and increased tumor cell apoptosis (Bu et al. 2004).

Similarly, Besch and co-workers suggest a new function of uPAR acting as a survival factor for melanoma by downregulating p53. They show that uPAR inhibition results in massive cell death via apoptosis. Apoptosis was mediated by p53 and occurred independently of ERK or FAK signaling (Besch et al. 2006). In the emerging picture, the uPA/uPAR system has the ability to support the malignant phenotype through several mechanisms: first, by virtue of its matrix-degrading ability that favors tumor dissemination; second, by stimulating cell motility;

third, by eliciting cell proliferation; and fourth, by protecting cells from apoptosis, thus enhancing tumor survival.

The Next Generation

Delivery of uPAR downregulation constructs, whether plasmid vectors, adenoviral vectors, or synthetic strands, remains to be tested appropriately. With the current state of cancer gene therapy, delivery is a major stumbling block, and various carriers such as liposomes, polymers, and microparticles (Dass et al. 2002) are being evaluated to address this issue. The closest to clinical relevancy in terms of delivery (administration) achieved by the studies listed above was the use of mini-osmotic pumps delivering downregulating agents directly into the brain (Gondi et al. 2004a, Lakka et al. 2005). Anyway, the issue of side-effects of uPAR downregulation on normal tissue and organs were not looked at, even at the cell culture level. Surely, a system as central as uPA–uPAR, which has various physiological functions in the body besides being pro-tumorigenic, needs to be properly respected and monitored.

Little is known about tumor growth and dissemination in mice with targeted distruption of the uPAR gene. uPAR deficiency does not compromise the embryonic development and viability of $uPAR^{-/-}$ mice (Bugge et al. 1995): homozygous uPAR-deficient mice do not display major growth and fertility problems, do not show histological abnormalities in tissues, and do not differ from wild-type mice for spontaneous lysis of experimental pulmonary plasma clot (Dewerchin et al. 1996). This is similar to what is also noted in uPA deficient mice (Carmeliet et al. 1994). Thus, the apparent lack of toxicity from inhibiting this proteolytic system makes it an ideal candidate for targeting as a cancer therapeutic.

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