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Intact and cleaved uPAR forms: diagnostic and prognostic value in cancer

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1. ABSTRACT

The cellular receptor for urokinase, uPAR, localizes its ligand, uPA, and thereby the plasminogen activation, to the cell surface. uPA also cleaves uPAR, liberating the ligand-binding domain I, and thereby inactivates the binding potential of uPAR for both uPA and vitronectin. The uPA-catalyzed cleavage of uPAR is fast on the cell surface, when uPA is bound to a neighboring uPAR molecule. uPAR can be shed from the cell surface. However, the soluble form cannot be cleaved by uPA. Glycolipid-anchored and soluble forms of intact, uPAR(I-III), and cleaved receptor, uPAR(II-III) and uPAR(I), have been identified in tissue and body fluids. It is well-established, that the total amount of all uPAR forms is a strong prognostic marker in different types of cancer. Using immunoassays, measuring the individual uPAR forms, has revealed that the cleaved uPAR forms are even stronger prognostic markers and have diagnostic utility. This review will focus on the mechanism of uPAR cleavage and the functional consequences, as well as the clinical applicability of cleaved uPAR forms.

2. INTRODUCTION

The urokinase-type plasminogen activator receptor (uPAR/CD87) is essential for cell surface associated plasminogen activation, mediated by its ligand, urokinase-type plasminogen activator (uPA) (1-4). uPA binds with high affinity to the intact, three domain receptor protein, uPAR(I-III), which is attached to the cell surface by a glycolipid anchor. The proenzyme, plasminogen, is converted into plasmin by uPA-catalyzed cleavage. Plasmin is a key enzyme for degradation of extracellular matrix proteins in a variety of biological processes including cancer invasion, where tissue remodeling and cell migration are essential (5-8). In addition to binding to uPAR(I-III), uPA can cleave uPAR(I-III) in the linker region between domains I and II, liberating domain I and leaving the cleaved form, uPAR(II-III) on the cell surface (9, 10). This cleavage inactivates the ligand binding potential of uPAR (11). Liberated domain I, uPAR(I), has been detected in body fluids and tumor tissue extracts (12-14). Furthermore, the soluble form of uPAR(I-III), suPAR(I-III), as well as the cleaved soluble form,

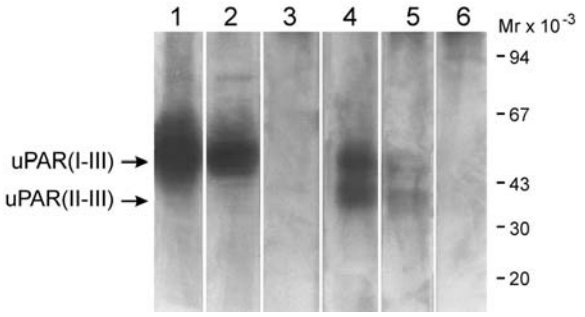

Figure 1. uPAR(II-III) is present in ductal breast carcinoma. Detergent phases from Triton X-114 lysates of $3.75x10^6$ U937 cells (lanes 1 and 4), 25 mg of tumor tissue from a ductal breast carcinoma (lanes 2 and 5), and 25 mg breast tissue from a mamma reduction (lanes 3 and 6) were analyzed by Western blotting employing 20 microg/ml of a mixture of the domain I specific mAbs R3 and R9 (lanes 1-3) and 20 microg/ml of the R4 mAb, reacting with an epitope on uPAR(II-III) (lanes 4-6).

suPAR(II-III), has been identified in body fluids. The generation of these uPAR variants involves shedding from the cell surface by cleavage at or near the glycolipid anchor (8, 13, 15-18). Crystal structures of soluble forms of the human uPAR(I-III) in complex with a peptide antagonist or the amino-terminal fragment of uPA, ATF, have been solved (19-21). There is as yet no crystal structure of glycolipid-anchored uPAR(I-III) nor of a ligand free (s)uPAR(I-III), which has been suggested to be a "latent" form of uPAR (22). There are several reports showing that uPA stabilizes uPAR(I-III) in its "active" conformation, enabling or greatly enhancing binding of vitronectin and integrins (11, 23-25). It follows that inhibition of uPA binding to uPAR(I-III) would also affect binding to other ligands. Blocking the interaction between uPA and uPAR(I-III) has been demonstrated to have pronounced effects on plasminogen activation, primary tumor growth, and dissemination of cancer cells in model systems (26-33).

In tumor tissue, uPAR(I-III) and/or uPAR(II-III) are often located at the invasive front (34, 35). Analyses with immunoassays have demonstrated that high levels of the collective amount of all uPAR forms in tumor tissue as well as in blood are correlated with poor prognosis for patients with different types of cancer (36-41). If uPA cleavage of uPAR(I-III) reflects the amount of active uPA, then measurements of cleaved uPAR forms, compared to the total amount of all uPAR forms, could provide superior prognostic information. This review will concentrate on the different uPAR forms, how they are generated, and what functions the cleavage and the resulting cleaved uPAR forms might have. Finally, an overview of the prognostic and diagnostic findings that have been made in relation to several types of cancers is given.

3. CLEAVAGE OF uPAR(I-III)

A molecular variant of uPAR lacking domain I was first identified on the human histiocytic lymphoma U937 cell line (9). Subsequently, uPAR(II-III) was identified on several neoplastic cell lines of human and murine origin, in xenotransplanted human mammary and ovarian tumors as well as in tumor tissue lysates from ovarian cancer patients (14, 16, 42-45). In tumor tissue lysates from patients with ductal mammary carcinoma, uPAR(II-III) was also present, whereas neither intact nor cleaved uPAR were detected in tissue lysates of mammary reductions (Figure 1). For detection of intact uPAR in Western blotting, a combination of two non-overlapping domain I specific monoclonal antibodies (mAbs), R3 and R9, were used (1, 46). The mAb R4, recognizing an epitope on uPAR(II-III), stained both intact and cleaved uPAR on the Western blot. Using R4 or the mAb R2, reacting with an epitope on domain III, in immunohistochemical staining on ductal breast carcinoma demonstrated uPAR(I-III)/uPAR(II-III) to be located on macrophages immediately surrounding the malignant epithelium and on a few cancer cells (34). The soluble form of uPAR(II-III), suPAR(II-III), has been identified in ovarian cystic fluids (17), in blood from prostate cancer patients (47, 48), in urine from patients with leukemia (13), and in serum from healthy donors stimulated with granulocyte-colony stimulating factor (15).

3.1. uPA-mediated cleavage

The cleavage of uPAR(I-III) on human as well as murine cells is inhibited by culturing the cells in the presence of inhibitory anti-uPA antibodies, indicating that uPA is responsible for the cell surface cleavage of uPAR(I-III) (9, 10, 43). In solution, cleavage of purified uPAR(I-III) in the linker region between domains I and II can be obtained by moderate concentrations (i.e. 10 nM) of uPA as well as low molecular weight (LMW)-uPA, the latter lacking the epidermal growth factor (EGF)-like uPAR(I-III) binding domain (9). Hence, binding of uPA to uPAR(I-III), which requires the EGF-like domain, is not necessary for the cleavage to occur. In contrast to the slow cleavage in solution, uPA-mediated cleavage of uPAR(I-III) on the cell surface is completed within 30 minutes and this acceleration is dependent on binding of uPA to uPAR(I-III), since LMW-uPA added to cells does not result in this fast cleavage (46). Furthermore, the accelerated uPAR cleavage on the cell surface is inhibited by preincubation of the cells with uPA inactivated by binding to diisopropyl fluorophosphate (DFP), whereas preincubation of purified uPAR(I-III) in solution with DFP-uPA has no effect on uPAR cleavage (10**)**. Thus, uPA bound to uPAR(I-III) on the cell surface cleaves a neighboring uPAR(I-III) molecule.

Purified uPAR(II-III), either obtained from purification of U937 cell lysates or by uPA cleavage of purified uPAR(I-III), was a mixture of two forms with different amino-terminal amino acids. This demonstrates that uPA has two cleavage sites in the uPAR linker region, which are located between R^{83} and A^{84} (^{81}SGRAV^{85}) and between R^{89} and S^{90} (^{87}YSRSR^{91}) (10). In plasminogen, the uPA cleavage site is located between R^{560} and V^{561} (^{558}PGRVV^{562}) (49, 50). The optimal sequence for uPA cleavage was experimentally determined to be SGRSA from position P3 to P2´ (51). This implies that one of the target sequences in uPAR is more optimal for uPA

cleavage than that in plasminogen. In analogy with the uPA-catalyzed plasminogen activation, the uPAR(I-III) cleavage is not species specific, since murine uPA (muPA), Chinese hamster uPA, and human uPA all readily cleave human uPAR(I-III), liberating uPAR(I). This has been shown in purified systems, in Chinese hamster ovary cells transfected with human uPAR(I-III), and in human mammary tumors xenotransplanted in nude mice (9, 43, 46). This is in contrast to the high degree of species specificity in the ligand binding between uPA and uPAR(I-III) (8, 52). Interestingly, of the five amino acid residues identified as the "hot spots" for vitronectin binding in uPAR(I-III), two, namely R^{91} and Y^{92}, are very close to one of the uPA cleavage sites (53), whereas the amino acids important for the uPAR(I-III)-uPA binding are more distal (54). As described above, uPAR(I-III) occupied with uPA can be cleaved by uPA bound to a neighboring uPAR(I-III) molecule (10). Whether vitronectin binding to uPAR(I-III) influences subsequent uPA cleavage has not been investigated.

Since uPA is responsible for plasminogen activation on the cell surface, inhibition of uPA activity will prevent plasmin formation. Thus, it could not be excluded that plasmin cleaves uPAR(I-III) from experiments, where U937 cells have been grown in the presence of an inhibitory anti-uPA mAb (9, 10). However, culturing U937 cells in the presence of the plasmin inhibitor Trasylol did not prevent uPAR cleavage, even though plasmin cleaves purified uPAR(I-III) (9). *In vitro*, uPAR(I) obtained by plasmin cleavage of purified suPAR(I-III) does not co-migrate with the liberated domain I purified from cell culture media, since the former has a higher electrophoretic mobility due to intra-domain cleavage (46). This cleavage can be inhibited by preincubation of suPAR(I-III) with the mAb R3, for which the functional epitope is located on E^{33}, L^{61}, and K^{62} (8, 46). R3 is a competitive inhibitor of uPA binding (27). Plasmin cleavage of suPAR in the presence of R3 results in liberated domain I co-migrating with that produced by uPA cleavage (46). *In vivo*, active mouse uPA is required for cleavage of mouse uPAR(I-III), muPAR(I-III), and was only observed in skin extracts from bi-transgenic mice with keratinocyte targeted over-expression of both muPAR(I-III) and catalytically active muPA (4). Plasmin generated from uPA-catalyzed activation of plasminogen was apparently not responsible for this cleavage as equivalent levels of functional muPA-binding muPAR(I-III) was observed in these skin extracts, independently of the background of the bi-transgenic mice, e.g. wild-type, heterozygote, or plasminogen deficient (55).

Both cell culture and *in vivo* experiments point to uPA as the protease responsible for uPAR cleavage (4, 9, 10, 43, 46). However, soluble uPAR lacking at least the lipid moiety of the glycolipid anchor cannot be cleaved by physiological relevant concentrations of uPA (56). This is due to a difference in the conformation of the linker region between domains I and II in uPAR(I-III) and suPAR(I-III) caused by the hydrophobic lipid moiety attached to domain III of uPAR. Conformational alterations as a consequence of removal of the glycolipid anchor have been reported for several other glycolipid-anchored proteins such as Thy-1 glycoprotein (57), Ly-6A.2 (58), carcinoembryonic antigen (59), and CD59 (60). It has been described that antibodies raised against glycolipid-anchored proteins from protozoa and mammalian cells do not bind or bind poorly to the proteins once the lipid moiety has been removed, and antibodies raised to the soluble forms of the proteins reacted poorly with the glycolipid-anchored proteins, and the reactivity was greatly enhanced after removal of the lipid moiety (60). In the case of uPAR, the conformations of the domains are independent of the presence of the glycolipid anchor possibly due to the many disulfide bridges. This is evident from the reactivity of the more than 30 different monoclonal antibodies that we have raised against human and murine uPAR(I-III) and suPAR(I-III) with epitopes located within the domains (1, 27, 47, 61-63). These mAbs detect uPAR and suPAR with similar efficiency in Western blotting. However, the conformation of the linker region between domains I and II is more flexible and an antibody raised to a peptide comprising amino acids 84-94 recognizes uPAR(I-III), but not suPAR(I-III) (56). In order to investigate if the antibody would also react with uPAR(II-III), we cleaved glycolipid-anchored uPAR(I-III) with uPA and subsequently the generated suPAR was cleaved with chymotrypsin to obtain suPAR(II-III). The N-terminal amino acids of uPAR(II-III) are either ^{84}AVTYSRSRYLE^{94} or ^{90}SRYLE^{94} (10). After chymotrypsin cleavage, the N-terminal amino acids are ^{88}SRSRYLE^{94} (64). The peptide antibody reacted with uPAR(II-III), but not with suPAR(II-III), whereas the aforementioned R2 mAb, recognizing an epitope on domain III, reacted with both glycolipid-anchored and soluble variants of uPAR (Figure 2). Interestingly, the amino acid residues 84-90 of the linker region were poorly defined in all the solved crystal structures of suPAR(I-III) (19, 21, 65). uPAR is a heavily glycosylated protein and the glycosylation pattern varies between cell types (42, 66, 67). Thus, the uPA-catalyzed uPAR cleavage could also be dependent on the extent of glycosylation (42). Several other proteases have been shown to cleave uPAR(I-III) and suPAR(I-III) *in vitro*, but whether any of these are functional *in vivo* remains to be determined. If uPA is the only protease capable of cleaving uPAR(I-III) *in vivo*, then all the suPAR(II-III) found in body fluids must be the result of shedding of cell surface uPAR(II-III).

3.2. Functions of cleavage and cleaved uPAR forms

The cleavage of uPAR(I-III) prevents uPA binding and thereby cell surface plasminogen activation. Cleavage also abolishes uPAR-dependent cell adhesion, since vitronectin does not bind uPAR(II-III) (11, 68). uPAR cleavage inhibits internalization of the uPA/PAI-1 (plasminogen activator inhibitor-1) complex, since this event requires the simultaneous binding of the complex to uPAR(I-III) and a member of the low density lipoprotein receptor family (69). There is evidence for a direct binding between domain III of uPAR and the alpha$_2$-macroglobulin receptor/low density lipoprotein receptor-related protein (70, 71), which could be a mechanism enabling the internalization of uPAR(II-III)

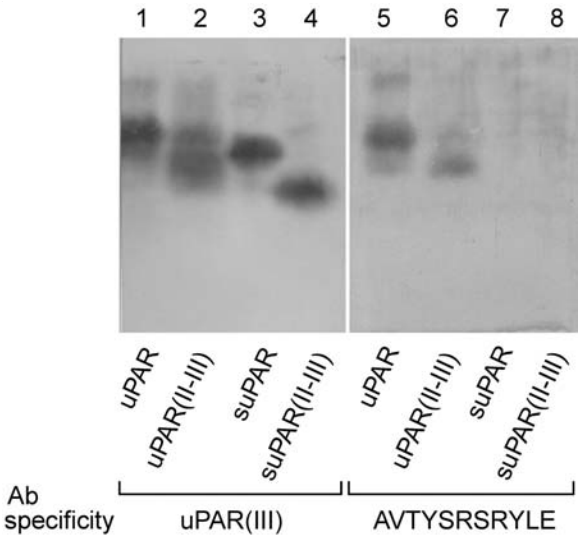

Figure 2. A polyclonal antibody against the linker region between domains I and II reacts with uPAR, but not with suPAR. Purified recombinant uPAR(I-III) was incubated for 20 hours at 37˚C in the absence (lanes 1 and 5) or presence of 10 nM uPA (lanes 2 and 6), while purified recombinant suPAR(I-III) was incubated for 4 hours at 37˚C in the absence (lanes 3 and 7) or presence of 2.5 ng chymotrypsin (lanes 4 and 8) prior to electrophoresis. After separation by non-reducing SDS-PAGE, the samples were subjected to Western blot analysis using the domain III specific mAb R2 (lanes 1-4) and the polyclonal anti-AVTYSRSRYLE (A^{84}-E^{94}) antibody (lanes 5-8). Equal amounts of protein (25 ng) were applied in each lane and 10 microg/ml of antibody was employed in Western blotting.

Whereas the consequences described above of uPAR cleavage are inhibition of function, the cleavage is also required for some biological processes to occur. A recent study demonstrated uPAR cleavage to be a crucial step in the differentiation of fibroblast to myofibroblast (72). Corneal fibroblasts, hepatic stellate cells, and normal lung fibroblasts, grown in media supplemented with fibroblast growth factor-2, maintained a fibroblast morphology and both uPAR(I-III) and uPAR(II-III) were detected in cell lysates by Western blotting. If the same cells were cultured in media containing TGFbeta1, the cells differentiated to myofibroblasts and only uPAR(II-III) was detected in the cell lysates. The transition from fibroblast to myofibroblast was prevented by adding one of the serine proteinase inhibitors, AEBSF or Chymostatin, to the TGFbeta1 containing media, and these inhibitors also prevented uPAR(I-III) cleavage *in vitro*. Transfecting fibroblasts with a non-cleavable uPAR(I-III) impaired their ability to differentiate to myofibroblasts. The non-cleavable mutant harbors the following mutations R83K, Y87C, R89K, and R91K (73) and is not cleaved by uPA (74). Interestingly, cells transfected with this mutant must have lost their ability to adhere to vitronectin, since in another study the relative vitronectin binding of uPAR (R91K) was determined to be 4.5±1.8% in contrast to 100% for wild-type uPAR (53).

Intact recombinant suPAR(I-III) does not induce chemotaxis. However, after cleavage in the linker region the resulting suPAR(II-III) is endowed with chemotactic function (75). suPAR(II-III) containing this chemotactic epitope was shown to be a ligand for the seven-transmembrane receptor, formyl peptide receptor (FPR)-like receptor-1/lipoxin A4 receptor (FPRL1/LXA4R) and to be capable of activating this receptor (76). HEK293 cells transfected with the FPRL1/LXA4R receptor bound $suPAR(II\text{-}III)_{88\text{-}274}$ with an apparent K_d of 83 nM (76). The results shown in Figure 2 indicate that the conformation of the linker region in both intact and cleaved uPAR are different in glycolipid-anchored and soluble forms (56). This might have functional consequences implying that $uPAR(II\text{-}III)_{88\text{-}274}$ does not possess the same effect as the corresponding soluble form. The chemotactic epitope was identified to be ^{88}SRSRY^{92} and the chemotactic effect of suPAR(II-III) can be mimicked *in vitro* by a peptide constituting this sequence, i.e. SRSRY (77). An *in vivo* effect of cleaved suPAR was recently shown for the first time. Administration of the chemotactic peptide SRSRY to mice resulted in migration of mouse CD34-positive hematopoietic stem/progenitor cells from the bone marrow and into the circulation to an extent similar to that observed by administering the widely used mobilization agent granulocyte colony-stimulating factor (78). Interestingly, this peptide is not species specific as it exerts its chemotactic effect on both human and murine cells (15, 78). The corresponding sequence in the linker region in muPAR is PQGRY, but the chemotactic effect of this peptide on human and murine cells has not been tested.

4. MEASURING uPAR FORMS

Due to the central role of uPAR in pericellular proteolysis, it was early proposed that measurement of this molecule might be of value in identifying patients with aggressive tumors and hence poor prognosis. Therefore, once anti-uPAR antibodies were available, enzyme-linked immunosorbent assays (ELISA) for measurements of uPAR in tumor tissue lysates and blood from cancer patients were developed. The different immunoassays quantifying uPAR have recently been extensively reviewed (46). The uPAR forms measured by these assays are dependent on the specificity of the antibodies employed in the individual assay. Since biological samples contain mixtures of uPAR forms, but the standard in the immunoassay is most often suPAR(I-III), the amount should be expressed as molar concentrations. This is unfortunately not always the case. At present all ELISAs developed to quantify uPAR amounts, measure at least two forms. Another confounding factor is that biotinylated antibodies are often used as detection antibodies. Biotinylation of mAbs could change not only their affinity, but also their specificity. These problems are most pronounced when the ratio of biotin to mAb is high and plasma samples are analyzed (79). Biotinylation can be avoided by use of non-biotinylated mAbs and a rabbit anti-mouse polyclonal antibody (pAb) coupled to alkaline phosphatase for detection. Such a kinetic ELISA enables accurate calculation of the uPAR concentration from multiple time-point measurements of the linear color development obtained with a phosphatase

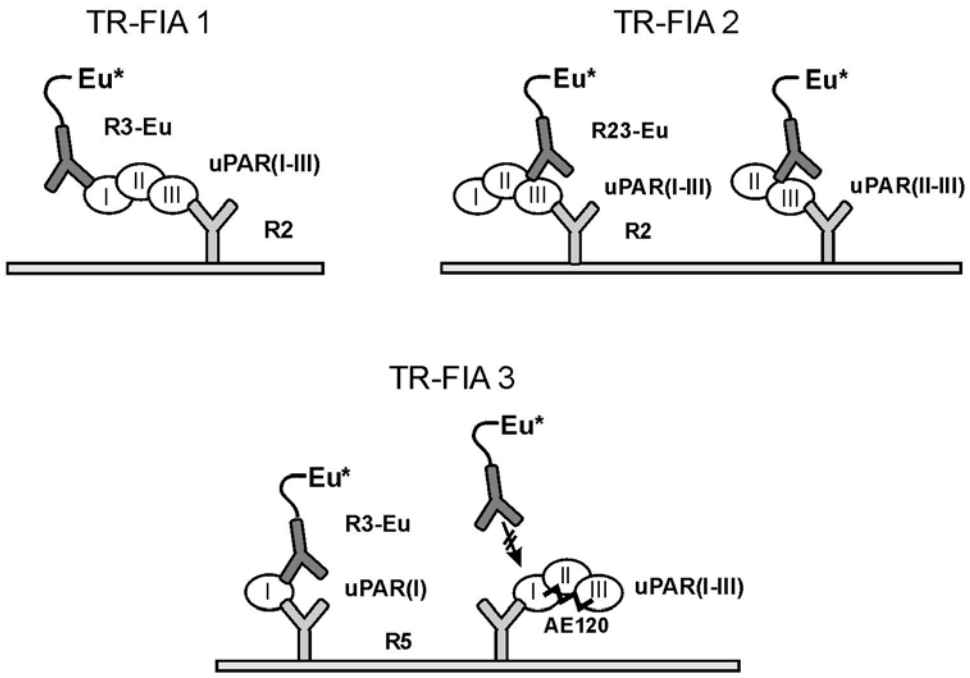

Figure 3. Assay design for the three TR-FIAs. Reproduced from Clinical Chemistry, 2004, Vol. 50:2059-68 with the kind permission of AACC.

substrate system (80). However, this solution does not allow an assay design using mAbs as both catching and detecting antibodies. Serum collections are often used in clinical studies, and when serum samples were analyzed with an ELISA using the R2 mAb for catching and a suPAR pAb for detection, rather high non-specific signals were occasionally observed. These non-specific signals were eliminated by addition of heparin to the assay buffer. The exact mechanism is unknown but the highly charged heparin poly-anion probably binds to the solid phase, thereby blocking the binding of the molecules responsible for the unspecific signal (81).

Two glycolipid-anchored and three soluble forms of uPAR have been identified in human tissue and body fluids. The amounts of these in tissues and circulation will reflect not only the level of cellular expression, but also the activity of uPA and of the enzymes responsible for shedding. Thus, the levels of the cleaved uPAR forms may have a stronger prognostic significance than merely the total uPAR content. To enable studies of the prognostic potential of the individual uPAR forms, we designed specific time-resolved fluorescence immunoassays (TR-FIAs) using different combinations of mAbs. The detection mAb is labeled with Europium. The detection system using time-resolved fluorescence is crucial, since biotinylation of detecting mAbs would cause problems with both sensitivity and specificity, when measuring plasma and possibly also serum samples (79). Three assays are now available (Figure 3): TR-FIA 1 measures non-occupied uPAR(I-III), TR-FIA 2 non-occupied uPAR(I-III) and uPAR(II-III). It follows that the molar concentration of uPAR(II-III) can be calculated by subtracting the molar quantities measured by TR-FIA 1 from those measured by TR-FIA 2. TR-FIA 3 determines the amount of uPAR(I) in a sample. The catching and detecting mAbs in this assay recognize non-overlapping epitopes on domain I. Thus, if only the mAbs defined the assay specificity, both non-occupied uPAR(I-III) and uPAR(I) would be detected. In fact, an assay with that specificity was designed using a chicken anti-suPAR pAb as catching antibody and an anti-uPAR(I) mAb for detection (38). The specific detection of liberated uPAR(I) in TR-FIA 3 was obtained by adding an inhibitor, AE120, that prevents the detecting mAb R3 from binding to uPAR(I-III), but allows R3 binding to the liberated domain I (47). The functional epitope for AE120 comprises amino acids in both domain I (R^{53}, E^{68}) and III (M^{246}, H^{249}, H^{251}, F^{256}) and will therefore only interact with uPAR(I-III) (31). The detection limits for both TR-FIA 1 and 2 were 0.3 pmol/l, while that of TR-FIA 3 was 1.9 pmol/l. The assays have been validated for their use in plasma, serum, and tissue extracts (12, 47, 48). Using these assays we have also determined the levels of the individual suPAR forms in a citrate plasma pool from healthy volunteers and found 42 pmol/l of suPAR(I-III) and 26 pmol/l of uPAR(I), and the calculated suPAR(II-III) content was 39 pmol/l (47).

5. CLINICAL USE OF CLEAVED uPAR DETECTION

High levels of the total amount of uPAR forms specifically measured by different ELISAs predict poor outcome for patients with breast (37, 38, 40), colorectal (41, 82-84), lung (39), and prostate cancer (85). The prognostic value of uPAR is evident both when analyzing tumor tissue extracts and blood. However, there was no correlation between the suPAR levels in preoperatively taken serum samples and cytosolic extracts from primary tumors from 188 patients with breast cancer (40).

To test our hypothesis that cleaved uPAR forms would be stronger prognostic markers than the collective amounts of uPAR, tumor tissue extracts of squamous cell lung carcinomas, in which the total amount of uPAR had previously been quantified, were analyzed using TR-FIA 3. From an original population of 77 patients, tumor tissue extracts from 63 patients were available. The measured amounts of uPAR(I) were found to be significantly associated with poor survival. The prognostic impact of uPAR(I) was stronger compared to that of total uPAR determined previously (12). Intact uPAR (TR-FIA 1) as well as uPAR(I-III) + uPAR(II-III) (TR-FIA 2) were not measured in this study, since the recovery in both assays was very poor when validated in the tumor tissue extracts. The reason for this could be the presence of uPA in these extracts. uPA will form complexes with uPAR(I-III) and such complexes will not be measured by TR-FIA 1 and TR-FIA 2 (12, 47). Similarly, uPA-occupied uPAR(I-III) will not be measured in the ELISA, where a chicken anti-suPAR pAb is the catching antibody and an anti-uPAR(I) mAb, which blocks uPA binding, is used for detection (38). Interestingly, using this ELISA, the levels of the uPAR forms measured in tumor extracts from non-small cell lung cancer patients correlated to prognosis (86).

In prostate cancer, analysis of prostate-specific antigen (PSA) in serum is well-established in the diagnosis and monitoring of prostate cancer patients. However, elevated serum concentrations of PSA are also found in patients with benign prostatic diseases. Even though identification of different forms of PSA has increased the specificity in prostate cancer detection, about two out of three patients need to undergo unnecessary biopsies (48). This illustrates the substantial need for markers that improve the discrimination of patients with cancer from those with benign conditions. In addition to total and free PSA forms, uPAR(I-III), uPAR(I-III) + uPAR(II-III), and uPAR(I) were measured in serum from 224 men with and 166 men without prostate cancer (48). Levels of uPAR(I) and uPAR(II-III) were significantly higher in samples from patients with prostate cancer than in samples from patients with benign disease. In men with moderately increased PSA levels (2-10 microg/l), the combination of the ratio between the two PSA forms and the ratio uPAR(I)/uPAR(I-III) had greater diagnostic efficacy (AUC=0.73), exceeding that of the ratio between the two PSA forms only (AUC=0.68). This suggests that cleaved uPAR forms in serum improve specificity and are complementary to PSA for prostate cancer detection (48).

Ovarian cancer is the most severe gynecological malignancy. Due to sparse symptoms, most patients with ovarian cancer are diagnosed in advanced stages, which is consequently reflected in poor outcome. In contrast, early stage ovarian cancer, i.e. before the tumor has spread in the peritoneal cavity, has excellent curability. Thus, any marker, which could be used for screening of asymptomatic women in groups at risk, would promote early detection and thus increase curability. In order to explore if suPAR would qualify as an early diagnostic marker in ovarian cancer, the levels of the total suPAR forms were measured in different body fluids from ovarian cancer patients (17). The concentration of total suPAR in cystic fluid from ovarian cysts and ascites/peritoneal fluid were compared with the total suPAR concentrations in serum made from peripheral blood and blood aspirated from the surface veins on the tumor. Material was available from 77 patients admitted for surgery of ovarian tumors (17). In this study, the concentrations of total suPAR in body fluids were quite different: in serum the measured concentrations were between 46-98 pmol/l, in ascites/peritoneal fluid between 293-586 pmol/l, and in cystic fluids the concentrations were even higher, i.e. 651- 8468 pmol/l. The concentrations of total suPAR in cystic fluids clearly separated benign and malignant cysts with predictive values above 90%. This suggests that the levels of total suPAR in cystic fluids can be used to discriminate between benign and possible or truly malignant cysts without surgery. Western blotting of immunoprecipitates revealed that the cystic fluids contained both suPAR(I-III) and suPAR(II-III) (17). In another study, tumor tissue, serum, ascites, and urine from ovarian cancer patients were analyzed for their content of the different uPAR forms by immunoprecipitation followed by immunoblotting. Tumor lysates contained uPAR(I-III) and uPAR(II-III), ascites suPAR(I-III) and suPAR(II-III), and urine samples contained all three forms of soluble uPAR (14).

uPAR is expressed on malignant blast cells in acute myeloid leukemia (AML), and elevated levels of total suPAR was found in plasma from leukemia patients (13). In a longitudinal study, in which patients receiving chemotherapy were monitored, it was demonstrated that the total suPAR level in plasma from patients with AML correlated with the number of circulating tumor cells and that these were reduced after chemotherapy. The existence of cleaved uPAR forms in the body fluids from AML patients were analyzed by immunoprecipitation followed by immunoblotting. In plasma, suPAR(II-III) was detected in addition to intact suPAR. suPAR(II-III) was also present in plasma made from bone marrow aspirates. The other cleaved form, uPAR(I), was only identified in urine. Lysates of the leukemic cells contained both intact uPAR and uPAR(II-III). In patients receiving chemotherapy, the amounts of suPAR(II-III) in plasma and of uPAR(I) in urine decreased, indicating that these uPAR forms could be used as response markers (13).

6. CONCLUSIONS AND FUTURE PERSPECTIVES

uPAR is a multifunctional molecule, present in intact and cleaved forms and involved in extracellular proteolysis, cell adhesion, mobility, and cell signaling events (5-8, 87-89). It is located mainly on stromal cells at the invasive front in breast and colorectal cancer and is additionally present in elevated levels in tumor tissue and in blood from cancer patients (46). Cleavage of uPAR is an indication of an active plasminogen activation system. With the design of immunoassays that quantifies the individual uPAR forms, uPAR(I) was found to be a stronger prognostic marker than the total uPAR amount in tumor extracts of non-small cell lung cancer patients (12). The level of uPAR(I) enhances the specificity of prostate cancer detection (48). We have found high levels of cleaved uPAR

forms in blood to be associated with short survival in non-small cell lung cancer, ovarian cancer, and disseminated prostate cancer (C.E. Almasi manuscripts in preparation and unpublished). Thus, the prognostic and diagnostic utility of the cleaved uPAR forms will be investigated in collections of serum/plasma from patients with different forms of cancer. The possibility of measuring the individual forms of uPAR in blood for monitoring therapy response should be investigated. In AML, it has already been shown with semi-quantitative methods, that the levels of suPAR(II-III) in plasma decrease following chemotherapy and this correlates with a decrease in the number of circulating tumor cells (13).

Because of the importance of uPA and uPAR in pericellular proteolysis, they have also been suggested as targets in cancer therapy (26, 30, 33, 90, 91). The first *in vivo* result to support this demonstrated that in a mouse breast cancer model, the volume of the lung metastases was significantly reduced in those mice deficient in uPA (92). Whether this effect is due to inhibition of plasminogen activation and thus reduced degradation of the extracellular matrix only, or whether the lack of cleaved uPAR forms play any role needs to be investigated. The therapeutic potential of blocking the interaction between uPAR(I-III) and uPA has been proven in several model systems (89), whereas the therapeutic potential of blocking uPAR cleavage, if any, is unknown. In order to study these therapeutic options we have developed mAbs raised against murine uPAR, blocking uPA binding (61), as well as anti-catalytic mAbs, raised against murine uPA (93). For therapy experiments in genetically induced mouse cancer models, murine mAbs specific for mouse uPAR and uPA are required. We have shown that anti-muPAR mouse mAbs that inhibit the muPA-muPAR interaction have *in vivo* efficacy and mimics the phenotype of the uPAR deficient mice (61, 94). Treatment of mice with an anti-muPAR mAb, mR1, rescues them from lethality induced by the modified uPA-activatable anthrax toxin (61) and administration of mR1 to tissue-type plasminogen activator (tPA) deficient mice induces fibrin plaques in the liver (94). Additionally, an anti-catalytic anti-muPA mAb has been shown to have *in vivo* efficacy (93). These mAbs will be administered to murine cancer models and the effect on primary tumor growth and metastasis followed. Furthermore, the cleaved uPAR forms will be measured in mice treated with the anti-muPA mAb in order to clarify if uPAR cleavage plays any role in cancer invasion and metastasis.

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Abbreviations: AML: acute myeloid leukemia, ATF: amino-terminal fragment of uPA, DFP: diisopropyl fluorophosphate, EGF: epidermal growth factor, ELISA:

enzyme-linked immunosorbent assay, FPRL1/LXA4R: formyl peptide receptor-like receptor-1/lipoxin A4 receptor, LMW-uPA: low molecular weight uPA, m: murine; mAb: monoclonal antibody, pAb: polyclonal antibody, PAI-1: plasminogen activator inhibitor-1, PSA: prostate-specific antigen, s: soluble, TGFbeta1: transforming growth factor beta 1, tPA: tissue-type plasminogen activator, TR-FIA: time-resolved fluorescence immunoassay, uPA: urokinase-type plasminogen activator, uPAR: uPA receptor, uPAR(I): uPAR domain I, uPAR(I-III): intact uPAR, uPAR(II-III): uPAR domains II + III

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