Soluble Receptor for Urokinase Plasminogen Activator in Both Full-Length and a Cleaved Form Is Present in High Concentration in Cystic Fluid from Ovarian Cancer

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ABSTRACT

We assayed the levels of soluble urokinase plasminogen activator receptor (su-PAR) with an ELISA in various body fluids from 77 patients with benign or malignant ovarian tumors, histologically classified as follows: benign; possibly malignant (borderline); and well, intermediate, and poorly differentiated malignant. The concentration of su-PAR in fluid from malignant cysts was extremely high, ~10-fold higher than the concentration in ascitic fluid and ~100-fold higher than that in blood. Also, the concentration in malignant cysts was ~10-fold higher than the concentration in benign cysts. Such high concentrations of su-PAR were found not only in truly malignant but also in possibly malignant cysts. Thus, we suggest that the concentration of su-PAR in cystic fluid can serve as a marker, allowing early diagnosis of malignant ovarian cysts. The concentration of su-PAR in fluid aspirated transvaginally with ultrasonographic guidance can be used to discriminate possibly and truly malignant concentrations of su-PAR in fluid aspirated transvaginally with ultrasonographic guidance can be used to discriminate possibly and truly malignant cysts from benign cysts without surgery.

The high concentrations of su-PAR in ovarian cystic fluids allowed us to characterize the molecular forms. Cross-linking of a radiolabeled ligand to the receptor demonstrated that at least a fraction of su-PAR was able to bind the ligand. A cleaved form of the receptor containing domains 2 and 3 was detected with Western blotting. The cleaved receptor, which is devoid of the ligand-binding domain 1, has not previously been demonstrated in body fluids; it has only been demonstrated on cell surfaces. su-PAR domains 2 and 3 were found in cystic fluids from both malignant and benign ovarian tumors.

INTRODUCTION

u-PA and its cell surface receptor (u-PAR) play a major role in pericellular proteolysis, and the content of these two proteins is reportedly high in a number of malignant human tumors (1). Both u-PA and its zymogen, pro-u-PA, bind with high affinity (0.1–1 nm) to u-PAR, and binding of plasminogen to adjacent sites on the cell surface accelerates formation of plasmin, a serine protease with broad specificity. Initiation of this proteolytic cascade eventually results in degradation of the extracellular matrix structure. In addition, u-PAR is a receptor for the adhesive protein vitronectin and is able to bind both ligands simultaneously (2–5).

High-affinity cell surface receptors for u-PA were first demonstrated on monocytic cells (6, 7) but have since been found in a variety of normal and neoplastic cell lines and in colon cancer (8, 9), breast cancer (1, 10, 11), lung cancer (12), and ovarian cancer (13). su-PAR is present in low concentrations in plasma from healthy individuals (14), and its concentration is slightly elevated in plasma from patients with colorectal cancer (15). Furthermore, su-PAR has been demonstrated in ascitic fluid from patients with ovarian cancer (16).

u-PAR is a highly glycosylated protein with $M_\text{r} \approx 55,000–60,000$ (17). The receptor is anchored to the cell membrane by a COOH-terminal glycosylipid anchor (18). u-PAR consists of three homologous repeats, of which the NH₂-terminal domain 1 is involved in both u-PA (19, 20) and vitronectin (5) binding. u-PA can, either directly or through the activation of plasminogen, mediate a specific cleavage of u-PA, releasing the ligand-binding domain 1 (21, 22). The cleaved receptor, u-PAR (2+3), was first demonstrated on cultured U937 monocyte-like cells (21) but has subsequently been found on the surface of several other cell lines of neoplastic origin, i.e., in HT 1080 fibrosarcoma cells (23), in MDA-MB-231 tumors xenografted into nude mice, and in extracts of primary Lewis lung tumors (24).

Ovarian cancer is the most severe gynecological malignancy. Due to a prolonged asymptomatic interval, the disease usually presents in advanced stages, with extensive spread of the tumor in the abdominal cavity. In these stages, the 5-year survival rate is only ~25% and has not improved much despite the use of extensive surgery and modern chemotherapy (25). On the contrary, early stages of ovarian cancer, which are limited to the ovaries, are associated with good prognosis. Therefore, detection of the tumor in early stages would be a most significant step toward reducing mortality and morbidity. However, no method for detection that combines high sensitivity with high specificity has, as yet, been presented. The aim of this study was to evaluate whether concentrations of su-PAR in peripheral blood, peritoneal/ascitic fluid, or cystic fluid can be used as markers in the early detection of malignant and premalignant ovarian cysts. We found the cystic fluid concentrations of su-PAR to be ~10-fold higher in malignant cysts than in benign cysts, and the difference was sufficient for discrimination between them on that basis to be highly accurate. Furthermore, molecular characterization of su-PAR in cystic fluids identified a cleaved form, su-PAR (2+3), in addition to intact su-PAR.

MATERIALS AND METHODS

Blood and Fluid Samples. A total of 77 patients, ages 20–80 years, who were admitted for surgery of ovarian tumors were included in the study. Peripheral blood samples were drawn from the cubital vein the day before surgery, and serum was prepared. Peritoneal fluid (<100 ml) or ascitic fluid (>100 ml) was aspirated perioperatively, immediately after the abdominal cavity was opened, to avoid contamination with blood. Regardless of its volume, this aspirate is subsequently referred to as peritoneal/ascitic fluid. Tumor blood was aspirated from veins on the surface of the tumor, and the serum was prepared. Cystic fluid was aspirated after removal of the tumor. All blood and fluid samples were immediately centrifuged at 2000 × g for 20 min, and the supernatant was frozen at −20°C until assay. All tumors were epithelial, serous, or mucinous, and they were histopathologically classified as follows: benign; possibly malignant (borderline); and well, intermediate, and poorly differentiated cancers. Truly malignant tumors, i.e., well, intermediate, and poorly differentiated cancer, were further classified with respect to the clinical stage of the disease (Fédération Internationale des Gynécologues et Obstétristes; Ref. 25) as follows: stage I, growth restricted to the ovaries; stage II, growth restricted to the pelvic cavity; stage III, growth restricted to the abdominal cavity; and stage IV, growth outside the abdominal cavity. The...
sampling procedure had been approved by the Institutional Review Board for studies on human subjects at the Lund University Hospital.

Enzymes. The ATF of human u-PA was a kind of gift from Dr. G. Lassani (Le Petit, Italy) and was labeled with 125I as described previously (17). N,N'-disuccinimidyl suberate was from Pierce Chemical Co. (Rockford, IL). Active human u-PA was purchased from Seropon (Aubonne, Switzerland) and inactivated by DFP treatment (17).

Antibodies. The rabbit polyclonal antibodies (11) and the mouse monoclonal antibodies against human u-PAR have been described previously (11, 20, 26). The monoclonal antibodies R3, R5, and R9 recognize different epitopes on domain 1 of u-PAR, and the monoclonal antibodies R2 and R4 recognizes different epitopes in domains 2 or 3.

ELISA for su-PAR. A recombinant soluble variant of u-PAR, su-PAR, lacking the glycolipid anchor (26), was used as a reference in both ELISA and Western blotting. The concentration of su-PAR in all samples was determined by ELISA (14). Fifteen-μl fractions were collected. The ATF of u-PA was iodinated, and the 125I-labeled ATF was used for chemical cross-linking to the purified su-PAR using N,N'-disuccinimidyl suberate. The cross-linked products were analyzed by SDS-PAGE and visualized by autoradiography. Reagents for SDS-PAGE were from Bio-Rad (Richmond, VA). Nolabeled DFP-treated u-PA was used as a competitor in the cross-linking assay. A detergent-phase extract from U937 cells was analyzed in parallel as a positive control (17).

Immunoblotting. For Western blotting analysis, su-PAR was immunoaffinity-purified from nine ovarian cystic fluids. The samples were diluted 1:1 with a buffer [0.15 M PBS (pH 7.4)-0.1% CHAPS] and purified on a column with the monoclonal anti-u-PAR antibody R2 coupled to protein G-Sepharose (2.24 mg/ml R2 in Sepharose; Ref. 26). Fractions containing su-PAR were identified by ELISA.

The purified su-PAR samples were electrophoresed on an 12% SDS-polyacrylamide gel under nonreducing conditions (27) and then electroblotted onto nitrocellulose membranes (Hybond-C Extra; Amersham International, Amersham, England) using the semidyblot system (Ancon, Copenhagen, Denmark). For detection of su-PAR (2+3), the sheets were incubated with the monoclonal anti-u-PAR antibody R2 coupled to protein G-Sepharose (2.24 mg/ml R2 in Sepharose; Ref. 26). Fractions containing su-PAR were identified by ELISA.

The purified su-PAR samples were then end-over mixed overnight at 4°C. The Sepharose beads were spun down for 5 min at 5000 rpm, and su-PAR was eluted from the anti-su-PAR-Sepharose 5 min at 4°C under acid conditions, essentially as described previously (14). Fifteen-μl fractions were collected. The ATF of u-PA was iodinated, and the 125I-labeled ATF was used for chemical cross-linking to the purified su-PAR using N,N'-disuccinimidyl suberate. The cross-linked products were analyzed by SDS-PAGE and visualized by autoradiography. Reagents for SDS-PAGE were from Bio-Rad (Richmond, VA). Nonlabeled DFP-treated u-PA was used as a competitor in the cross-linking assay. A detergent-phase extract from U937 cells was analyzed in parallel as a positive control (17).

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Elisa (see "Materials and Methods"). Histological differentiation of the tumors was classified as follows: benign (1); possibly malignant (borderline; 2); and well (3), moderately (4), and poorly (5) differentiated malignant. Column 3: numbers of samples analyzed in each group per fluid category; bars, SE. Concentrations of su-PAR were higher in truly malignant tumors than in benign tumors when analyzed in cystic fluid (P < 0.0001; note different scales). The concentrations in tumor blood were not significantly different from those in peripheral blood or when samples from malignant tumors were analyzed separately. Concentrations of su-PAR were higher in truly malignant tumors than in benign tumors when analyzed in cystic fluid (P < 0.0001), peritoneal/ascitic fluid (P = 0.001), tumor blood (P = 0.02), and peripheral blood (P = 0.01), and peripheral blood (P = 0.001).
analyzed. Sensitivity of the test was 91%, specificity was 91%. The positive predictive value was 91% and the negative predictive value was 91%.

The su-PAR from cystic fluids had to be immunoenriched (see “Materials and Methods”) prior to analysis by Western blotting. Without this step, the Western blotting was not possible, despite the high su-PAR concentration in the samples. Cystic fluids from nine different ovarian tumors (four malignant and five benign) were analyzed. The selection of the samples for analysis was dependent on the viscosity of the samples. Several cystic fluids contain mucous to a degree, which makes pipetting extremely difficult. The nine cystic fluids analyzed were, thus, the least viscous of the cystic fluids analyzed in ELISA. The monoclonal anti-u-PAR antibody R4 was used for detection of cleaved receptor, su-PAR (2+3), as well as full-length receptor, su-PAR (Fig. 4A). The antibodies R3 and R9 detected only full-length su-PAR (Fig. 4B). Varying amounts of su-PAR and su-PAR (2+3) were detected in all samples, and in addition, the ratio between su-PAR and su-PAR (2+3) varied between samples. Thus, cystic fluids from ovarian tumors, benign as well as malignant, contain su-PAR both the full-length and the cleaved form.

**DISCUSSION**

We have previously reported the presence of su-PAR in plasma from healthy individuals (14). The mechanism by which the glycolipid-anchored u-PAR is released from the cell surface is not known, but phospholipase has been suggested (28). Our present finding of 1–2 ng/ml su-PAR in peripheral and tumor blood from patients with benign ovarian cysts was in the same range as that we previously
reported for healthy individuals. Blood concentrations of su-PAR were higher in patients with malignant as compared to benign tumors. Because, however, tumor blood concentrations were not significantly higher than peripheral blood concentrations, it is possible that su-PAR enters the circulation via other routes in the malignant cases, e.g., resorption from the peritoneal/ascitic fluid.

We found significant amounts of su-PAR in peritoneal/ascitic fluid, which is in agreement with a previous study (16). Generally, these concentrations were ~10-fold higher than corresponding blood concentration. Peritoneal/ascitic fluid concentrations of su-PAR were higher in patients with malignant than with benign tumors. The difference was, however, not great, possibly due to dilution in larger volumes of ascitic fluid. A comparable difference was found between peritoneal fluid samples and ascitic fluid samples in patients with truly malignant tumors, contributions from peritoneal metastases and malignant cells in the ascites presumably counteract the effect of the dilution.

Patients with malignant ovarian tumors had cystic fluid concentrations of su-PAR that were ~100-fold higher than corresponding blood concentrations and ~10-fold higher than corresponding peritoneal/ascites fluid concentrations. Similarly, we have previously found the concentration of u-PA to be higher in cystic fluid than in peritoneal/ascitic fluid and higher in peritoneal-ascitic fluid than in blood (29). There are several possible explanations for high levels of su-PAR in cystic fluid. The rate of resorption may be low because the fluid and its components are trapped in a compartment that is lined by an epithelium that does not normally function to resorb fluid or constituents. In addition, the rate of degradation of su-PAR in the cystic fluid may be low. Alternatively, the glycosyl phosphatidyl inositol anchoring of receptors to the cell membrane may be inadequate in malignant cysts, thereby allowing a greater fraction of u-PAR to appear in a soluble form. However, such high concentrations of su-PAR could not be obtained without a higher expression of u-PAR in ovarian cancer. We have, in fact, previously demonstrated increased number of receptor sites in cell membranes prepared from malignant as compared to benign ovarian tumors (13).

Cystic fluid levels of su-PAR were very high in malignant as compared to benign ovarian tumors. Remarkably reduced levels of su-PAR were, however, found in cystic fluids of malignant tumors with poorly differentiated histology. This may result from reduced u-PAR gene expression, slower release of u-PAR from the cell surface, or proteolytic degradation of su-PAR into small fragments that are not recognized by the antibodies of our ELISA. This observation needs further elucidation.

Most cases of ovarian cancer have inherent poor prognosis due to detection in late stages. If ovarian cancer is detected in early stages, however, prognosis is excellent. Thus, it is imperative to detect patients in the early asymptomatic interval, when the disease is limited to the ovaries. Blood concentrations of several biochemical markers, most notably CA125, have been tested for screening purposes but have not been found to be reliable enough (30). Ultrasonography has been used for the same purpose, but mass screening has not yet been shown to reduce mortality and is not generally recommended (31). However, screening is, in a way, already practiced, because ultrasonography is used extensively in gynecological praxis and also in asymptomatic women coming for a regular check-up. Such examinations generate an increasing number of ovarian cysts, and many of these patients have surgery unnecessarily because no test can satisfactorily discriminate possibly and early malignant cysts from benign cysts. We found cystic fluid concentrations of su-PAR to be ~10-fold higher in patients with malignant as compared to benign tumors. This was also true for possibly malignant and early-stage malignant cysts. Because ovarian cysts can easily be aspirated with ultrasonographic guidance and fluid obtained for analysis, we suggest that the concentration of su-PAR can be used as a marker of malignancy in cysts, which have not yet developed the ultrasonographic criteria for cancer. Cystic fluid concentration of su-PAR does recommend itself in this respect because the levels in early-stage malignant tumors were as high as those in advanced-stage tumors. This is crucial because survival benefits will result only from detection of early stages. In contrast, the blood levels of su-PAR, as well as those of other markers studied in the past, do not meet this requirement.

The very high concentrations of su-PAR in malignant cystic fluids allowed molecular characterization after immunopurification. At least a fraction of the receptor pool was not occupied by u-PA because it bound radiolabeled ATF (Fig. 3). Furthermore, Western blotting revealed that a significant fraction of su-PAR in cystic fluids was present in the cleaved form, su-PAR (2+3). Cleaved u-PAR has been
identified on the cell surface of cultured neoplastic cells (21, 23, 32), xenografted tumors, and primary tumors of Lewis lung (24) but has not previously been demonstrated in human body fluids. We have recently shown that u-PA cleaves u-PAR with high efficiency on the cell surface but only slowly in solution (22). It is, therefore, likely that su-PAR (2+3) found in the cystic fluid originates from cell-bound u-PAR (2+3), which has detached from the cell surface as a result of enzymatic cleavage.

Cleavage of u-PAR between domain 1 and 2 on the cell surface has been proposed as an alternative regulatory mechanism for plasminogen activation. However, cleavage of u-PAR is also indicative of u-PA activity because the amount of u-PAR (2+3) decreases when cells are grown in the presence of an anticalytic antibody to u-PA (21, 22, 24). Thus, the content of u-PAR (2+3) in cancer tissue has been suggested to be a stronger prognostic parameter than the levels in benign tumors (33). We have recently shown that u-PA cleaves u-PAR with high efficiency on the cell surface of malignant tumors (33).

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REFERENCES


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