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

Karin Wahlberg, Gunilla Höyer-Hansen, and Bertil Casslén

Department of Obstetrics and Gynecology, University Hospital, S-221 85 Lund, Sweden [K. W.]; and Finsen Laboratory, Rigshospitalet, DK-2100, Copenhagen, Denmark [G. H.-H.]

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 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 constituting 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 Mr ~55,000–60,000 (17). The receptor is anchored to the cell membrane by a COOH-terminal glycolipid 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-PAR, 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 peroperatively, 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 Gynécologistes et Obstétristes; Ref. 25) as follows: stage I, growth restricted to the ovaries; stage II, growth restricted to the pelvis; stage III, growth restricted to the abdominal cavity; and stage IV, growth outside the abdominal cavity. The
Fig. 1. Concentrations of su-PAR in ovarian cystic fluid, peritoneal ascitic fluid, tumor venous blood, and peripheral venous blood in patients with ovarian tumors. su-PAR concentrations were measured by ELISA (see "Materials and Methods"). Histological differentiation of the tumors was classified as follows: benign (1); possibly malignant (borderline; 2); and well (3), intermediately (4), and poorly (5) differentiated malignant. Columns, numbers of samples analyzed in each group and fluid category; bars, SE. Concentrations of su-PAR in peritoneal-ascitic fluid were higher than those in peripheral and tumor blood \((P < 0.0001)\) but lower than those in cystic fluid \((<0.0001; \text{note different scales})\). 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 \((P = 0.02)\), tumor blood \((P = 0.01)\), and peripheral blood \((P = 0.001)\).

**Enzymes.** The ATF of human u-PA was a kind of gift from Dr. G. Lassani (Le Petit, Italy) and was labeled with \(^{125}\text{I}\) as described previously (17). \(N,N’\)-disuccinimidyl suberate was from Pierce Chemical Co. (Rockford, IL). Active human u-PA was purchased from Serono (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 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 (26). This ELISA is based on a combination of polyclonal and monoclonal antibodies against u-PAR (14). Polyclonal anti-su-PAR antibodies were used for coating, and a mixture of three different monoclonal anti-su-PAR antibodies (R2, R3, and R5) was used for detection (14). The assay detects both free su-PAR and su-PAR in complex with u-PA, as well as cleaved forms of su-PAR.

**Cross-Linking of u-PAR to \(^{125}\text{I}\)-labeled ATF.** For cross-linking analysis, su-PAR was immunoaffinity-purified from nine ovarian cystic fluids. The samples were diluted 1:1 with a buffer \([0.15 \text{ M PBS (pH 7.4)}-0.1\% \text{ CHAPS}\] and purified on a column with the monoclonal anti-u-PAR antibody R2 coupled to protein G-Sepharose \((2.24 \text{ mg/ml R2 in Sepharose}; \text{Ref. 26})\). 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 (Ancos, Copenhagen, Denmark). For detection of su-PAR (2+3), the sheets were incubated with anti-u-PAR R4 \((10 \mu\text{g/ml})\), and intact su-PAR was demonstrated using alkaline phosphatase-conjugated rabbit antimouse immunoglobulin (DAKO,
RESULTS

Quantitation of su-PAR. su-PAR was detected in all samples of peripheral as well as tumor blood. Concentrations in these two sets of samples were not statistically different, but they were significantly correlated \( (r = 0.69, P = 0.0002; \text{Fig. 1}) \). The concentrations of su-PAR in peritoneal-ascitic fluid were \~10-fold higher than those in peripheral blood, and these two concentrations were correlated \( (r = 0.53, P < 0.0001) \). In patients with truly malignant tumors, the concentration of su-PAR was higher \( (P = 0.045) \) in ascitic fluid samples \( (19.4 \pm 2.3 \text{ ng/ml}) \) than in peritoneal fluid samples \( (13.0 \pm 1.6 \text{ ng/ml}) \). Malignant cystic fluid concentrations were \~100-fold higher than the blood concentrations. The level of su-PAR was significantly higher in patients with malignant than as compared to benign cysts in all fluids studied. The most notable difference was found in cystic fluid, with \~10-fold higher concentration in malignant than in benign cysts. Furthermore, the cystic fluid concentrations of su-PAR were elevated to the same extent in possibly malignant as they were in truly malignant tumors (Fig. 1). Cystic fluid levels of su-PAR did not correlate to the clinical stage of the disease (Fig. 2). This was also true for the other fluids studied (data not shown). Unexpectedly, however, cystic fluid levels tended to be lower in tumors with poorly differentiated histology. When cystic fluid levels of su-PAR above 25 ng/ml were evaluated as a test for malignancy (truly and possibly), we found sensitivity and specificity, as well as the positive and negative predictive values, to be above 90%.

Characterization of su-PAR in Cystic Fluid. Chemical cross-linking of su-PAR to \(^{125}\)I-labeled ATF in two samples of immunoenriched cystic fluid from malignant tumors showed that at least a fraction of su-PAR was unoccupied with u-PA because it was able to bind ATF (Fig. 3). The cross-linked receptor was observed as a band at \( M_r 75,000 \), which is in accordance with the sum of su-PAR \( (M_r 55,000-60,000) \) and ATF \( (M_r 17,000) \). Specificity of the binding of \(^{125}\)I-labeled ATF to the receptor was demonstrated by competition with 100 nm DFP-inactivated u-PA.

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

- Glostrup, Denmark), and bands were visualized with alkaline phosphatase conjugate substrate kit (Bio-Rad).

Statistical Methods. Results were given as mean ± SE. The significances of differences between different fluids were calculated with Wilcoxon signed-rank test for paired observations and between different patient groups with Mann-Whitney U test for unpaired observations. All tests were two-sided, and a 5% level of significance was used.

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Fig. 4. su-PAR (2+3) is present in cystic fluids from both benign and malignant ovarian tumors. Cystic fluids from nine different patients with ovarian tumors were immunopurified and analyzed by SDS-PAGE under nonreducing conditions and blotted onto nitrocellulose membranes. The tumors were malignant (Lanes 1–4) or benign (Lanes 5–9). A, for detection of full-length su-PAR and su-PAR (2+3), the filters were probed with anti-u-PAR antibody R4 (10 μg/ml) recognizing an epitope in domains 2 and 3. B, for detection of full-length su-PAR, anti-u-PAR antibodies R3 and R9 (10 μg/ml each), recognizing epitopes in domain 1, were used.

Table 1

<table>
<thead>
<tr>
<th>su-PAR Concentration</th>
<th>Benign (n=5)</th>
<th>Malignant (n=9)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total su-PAR (ng/ml)</td>
<td>125</td>
<td>375</td>
<td>0.001</td>
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</table>

Significant differences were observed in su-PAR concentrations between benign and malignant tumors.

Discussion

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...
been suggested to be a stronger prognostic parameter than the levels of the u-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 suggested to be a stronger prognostic parameter than the levels of either full-length u-PAR or u-PAR (2+3). Our Western blot, results of ovarian cystic fluids from patients with both benign and malignant tumors do indicate that su-PAR (2+3) is present in both cases. However, tissue content of u-PAR (2+3) may very well be higher in malignant than benign tumors because tissue content of u-PAR is higher in the malignant tumors (33).

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