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Increased Cell-surface Urokinase in Advanced Ovarian Cancer

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Urokinase-type plasminogen activator (uPA), uPA receptors, and cathepsin B were quantitated by using an immunological method, enzyme-linked immunosorbent assay, and amidolytic activity assays in 15 malignant and 10 benign epithelial ovarian tumors. The levels of uPA and uPA receptors, as well as cathepsin B, were found to be higher in membrane preparations obtained from malignant tumors than in those obtained from benign tumors. Acid-treated membranes acquired the ability to bind uPA, indicating that uPA is bound to a specific surface receptor that is not completely saturated. Levels of single-chain uPA (pro-uPA) and high-molecular-weight uPA in membrane preparations were measured by immunoadsorbent-amidolytic assay. The finding of a significant increase in amidolytic activity following activation of uPAs by plasmin suggested that less than half (30–40%) of all membrane immunoreactive uPAs is present in the enzymatically inactive pro-uPA form. In the membranes of malignant tumors, levels of uPA receptor and cathepsin B did not vary with stage of disease. On the other hand, we found that the level of receptor-bound uPA antigen/activity was significantly increased in advanced malignant tumors. Receptor-bound uPA may play an important role in determining invasive potential of tumor cells. Since ovarian cancer cells produce both pro-uPA and cathepsin B, the possibility of activation of tumor cell-derived pro-uPA by cellular protease cathepsin B must be considered.

Key words: Urokinase-type plasminogen activator (uPA) — Single-chain uPA — uPA receptor — Cathepsin B — Ovarian cancer

Urokinase-type plasminogen activator (uPA) binds to a specific cellular receptor, and the receptor-bound uPA retains enzymatic activity. The proenzyme form of uPA (pro-uPA) produced by tumor cells can be activated by plasmin and kallikrein, as well as by cathepsin B and cathepsin L.[1-6] Cathepsins B and L are thought to be enzymes involved in tumor invasion and metastasis.[7-9] Cathepsin B converts inactive procollagenase type IV to its active form.[10] Several studies have demonstrated that cathepsin B has an ability to degrade extracellular matrix and the level of cathepsin B has been correlated with cellular invasion.[7-9] We demonstrated that cathepsin B has the capacity to convert efficiently the soluble or tumor cell receptor-bound pro-uPA to the enzymatically active two-chain uPA (HMW-uPA).[5] Thus, the cellular cysteine proteinase cathepsin B may substitute for the plasma proteinase plasmin in the activation of pro-uPA released by tumor cells. In addition, a role for uPA in regulating tumor cell invasiveness has been proposed on the basis of the findings of generally increased uPA activity in several types of metastatic tumor. Thus, uPA may also play roles in tumor invasion and metastasis.[1, 2]

Recently, we found using cell ELISA that the ovarian cancer cell line, HOC-I, has reproducible levels of expression of surface uPA and cathepsin B,[11] and that cell-associated enzymatically inactive pro-uPA can be converted to active HMW-uPA by plasmin and cathepsin B.[5] Proteolytic enzymes are produced in abundance by malignant cells and have been implicated in the processes of tumor cell invasion and metastasis. Proteolytic activities are often concentrated in the pericellular environment or are cell-surface bound. Here we present evidence for the presence of membrane-associated uPA and cathepsin B, as well as of uPA receptors, on human ovarian carcinoma cells. These were studied in relation to clinical prognostic factors for malignant tumors. We conclude that the levels of receptor-bound uPA antigen/activity are significantly increased in advanced malignant tumors, but that neither uPA receptor nor cathepsin B level exhibits variation in relation to the stage of disease.

Abbreviations: pro-uPA, enzymologically inactive single-chain urokinase-type plasminogen activator; HMW-uPA, high-molecular-weight uPA; LMW-uPA, low-molecular-weight uPA; ATF, amino-terminal fragment of uPA; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; BSA, bovine serum albumin; tPA, tissue-type plasminogen activator; DFP, diisopropylfluorophosphate.

MATERIALS AND METHODS

Antibodies Antibodies used include the uPA-specific monoclonal antibody (moAB) 377, which reacts with the A-chain of pro-uPA/uPA, and moAB 394, which reacts with the B-chain of pro-uPA/uPA (Amercian Diagnos-

tica Co., Greenwich, CT). Peroxidase-conjugated sheep polyclonal antibody raised against human cathepsin B was purchased from The Binding Site Limited (Birmingham, England). Other reagents used were of analytical grade.

Clinical materials Fifteen patients with epithelial ovarian cancer and 10 with benign ovarian tumors undergoing surgery were included in the study. Approval had been obtained from the Institutional Review Board for studies on human subjects at Hamamatsu University Hospital. Samples obtained were frozen (−80°C) until assayed or were used immediately. All patients' diagnoses were confirmed by review of operative and pathology reports. Staging of ovarian cancer with the FIGO classification indicated that four patients had stage I disease, three stage II disease, six stage III disease, and two stage IV disease.

Preparation of cell membranes Preparation of tumor cell membranes was as described.[12] Briefly, the tissue was homogenized in 0.01 *M* Hepes buffer, pH 7.2, containing 0.25 *M* sucrose and 0.5% Triton X-100, with 100 μg tissue/ml. The homogenate was centrifuged at 1,700*g* for 10 min, and then at 12,000*g* for 15 min. The membrane pellet was collected following centrifugation of the supernatant (44,000*g*, 45 min), washed once with 0.01 *M* Hepes buffer, pH 7.2, containing 0.25 *M* sucrose, and finally redissolved in a solution containing 50 m*M* Tris and 0.25 *M* NaCl, pH 7.4. In another set of experiments, membrane preparations were treated initially for 3 min with a solution containing 50 m*M* glycine-HCl, 0.5 *M* NaCl, pH 3.0, to dissociate endogenous receptor-bound uPAs, and the membranes were then collected by centrifugation and neutralized with Hepes buffer in order to determine total uPA-binding sites (maximum uPA occupancy).[4, 11] The cell membrane suspension was frozen in aliquots until assayed. Protein concentration was determined by using standard methods.[13]

Enzyme-linked immunosorbent assay (ELISA) Quantitative determination of pro-uPA/uPA by ELISA: 96-well microtiter plates (Costar, Cambridge, MA) were coated with 100 μl of a solution containing moAB 394 (1.5 μg/ml in 0.05 *M* bicarbonate buffer, pH 9.6) for 16 h at 4°C and then blocked with Tris-buffered saline (TBS)-2% BSA (1 h, 23°C). After addition of the sample (16 h, 4°C), biotinylated moAB 377 (1 μg/ml) was added (1 h, 23°C). The plates were washed, then avidin-peroxidase (0.4 μg/ml, DAKO, Copenhagen, Denmark) was added (1 h, 23°C). Peroxidase activity was detected by using a tetramethylbenzidine substrate solution (Cosmo Bio., Tokyo). The reactions were terminated, and the A450 of each well was measured with an EIA reader (Model 2550, Bio-Rad, Richmond, CA).[11] All samples were assayed in triplicate. Aliquots of various concentrations of HMW-uPA standards were also incubated in triplicate. The uPA-ELISA used detects pro-uPA as well as proteolytically degraded forms of uPA (unpublished results). The lower limit of detection was 50 pg of pro-uPA or uPA/ml.

Microtiter plates were coated for 16 h at 4°C with cell membranes treated with or without 50 m*M* glycine-HCl, 0.5 *M* NaCl, pH 3.0, washed with phosphate-buffered saline (PBS) containing 2% BSA, and then reacted with peroxidase-conjugated polyclonal antibody against cathepsin B (5 μg/ml; reacted for 8 h, at 23°C). Peroxidase-conjugated anti-cathepsin B polyclonal antibody was used to quantitate cathepsin B immunologically. Peroxidase activity was detected using the tetramethylbenzidine substrate solution. The reaction was terminated, and the A450 of each well was measured with an EIA reader as described above. All samples were assayed in triplicate. Aliquots of various concentrations of cathepsin B standards were incubated in triplicate to determine the concentration of cathepsin B in the membrane preparations.

Biotinylation of uPA Diisopropylfluorophosphate-inactivated human high-molecular-weight-uPA (DFP-HMW-uPA) was biotinylated using the methods described by Guesdon *et al.*,[14] with N-hydroxysuccinimidyl biotinamidocaproate (Sigma, St. Louis, MO).

Binding of biotinylated DFP-HMW-uPA Microtiter plates coated with cell membranes were treated with biotinylated DFP-HMW-uPA (5 n*M*) in order to detect free uPA-binding proteins. Specifically bound biotinylated DFP-HMW-uPA was detected with avidin-peroxidase (0.4 μg/ml). Membranes were also treated with 50 m*M* glycine-HCl in 0.5 *M* NaCl (pH 3.0) to remove endogenous uPA. Microtiter plates were coated with acid-treated membranes and then PBS-0.1% BSA containing 5 n*M* biotinylated DFP-HMW-uPA was applied. After two washes with PBS-0.1% BSA, the total amount of uPA-binding proteins (total uPA receptors) was assayed as described above. The dissociation constant was estimated from the concentration of unlabeled HMW-uPA required to yield half-maximal displacement. The value of K_d was estimated from the Scatchard plot.

In addition, microtiter plates coated with cell membranes were treated with biotinylated DFP-HMW-uPA (5 n*M*) in the presence of increasing concentrations of unlabeled ligands (HMW-uPA, low-molecular-weight uPA [LMW-uPA], amino-terminal fragment of uPA [ATF], tissue-type plasminogen activator [tPA]) in order to assess the degree of binding specificity.[12, 15] After washing of the plates, avidin-peroxidase was applied to the membrane extracts, and the binding specificity was determined as described above. All samples were assayed in triplicate.

Immunoadsorbent-amidolytic assay of pro-uPA and HMW-uPA in cell membrane preparations This assay

combined the selectivity of immunoassay with the specificity of enzyme activity assays and exploited both the antigenic and enzymatic properties of the two proteins (pro-uPA and HMW-uPA), as described by Corti *et al.*[16] The total uPAs and HMW-uPA concentrations were obtained by interpolation of the optical densities of wells treated or untreated with plasmin on the dose-response curve of HMW-uPA standards. The concentration of pro-uPA was obtained by subtracting the concentration of HMW-uPA from the value for total uPAs.[16]

Cathepsin B enzymatic activity The cell membrane fraction was assayed for cathepsin B activity at pH 6.2 using carbobenzyloxy-arginyl-arginyl-7-amino-4-methylcoumarin (z-arg-arg-AMC) as a substrate, at a concentration of 5 μg/ml; incubation was conducted for 30 min at 37°C. The intensity of fluorescence due to the release of aminomethylcoumarin from the peptide was quantitated using a spectrophotometer.[17,18] Enzymatically active cathepsin B was used as a standard in this assay.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot SDS-PAGE was carried out with a linear 5–15% polyacrylamide gradient.[19] The gel was electroblotted onto polyvinylidine difluoride (PVDF) membrane (Millipore, Bedford, MA) using a semi-dry electroblotting apparatus (Marysol, Tokyo) at 40 mA/gel (90 min, 23°C).[5] Each sheet was incubated with biotinylated cathepsin B antibody, and the bound antibody was detected by using avidin-peroxidase, and then enzyme substrate, 4-chloro-1-naphthol.[5]

Ligand-blotting analysis Biotinylated DFP-HMW-uPA blot analysis was carried out in order to detect free uPA-binding proteins (free uPA receptors). The suspension of cell membranes was subjected to SDS-PAGE and the gel was electroblotted onto PVDF membranes, using the procedure described by Solberg *et al.*[15] The sheets were incubated with biotinylated DFP-HMW-uPA (4 n*M*) and avidin-peroxidase, and then the enzyme substrate, as described above.

RESULTS

Tumor cells containing uPA or cathepsin B were localized immunohistochemically in human ovarian cancer tissues. uPA and cathepsin B were localized to the cytoplasm and cell membranes in formalin-fixed, paraffin-embedded tissues of adenocarcinoma. Tumor cells were stained homogeneously with the antibodies against uPA and cathepsin B, although differences in staining intensity within tumors were observed. Tumor cells at the leading edge of invasively growing tumor nests showed immunoreactivity to uPA (unpublished data). uPA and cathepsin B have been found in many types of normal cells, including macrophages and fibroblast-like cells. However, in our previous study, we found ovarian cancer cells to be the largest producers of uPA and cathepsin B.

Immunological determination of uPA and cathepsin B ELISAs using different uPA-specific monoclonal antibodies and polyclonal antibody against cathepsin B detected membrane-localized uPAs and cathepsin B (Table I). Study with uPA-ELISA demonstrated reproducible levels of expression of uPA in membrane preparations obtained from benign and malignant ovarian tumors. The uPA concentration was significantly higher in membrane preparations from malignant tumors than in those from benign tumors ($P<0.001$). Notably, the levels of membrane-bound uPA were found to be significantly increased in advanced malignant tumors ($P<0.05$). In addition, our assay demonstrated reproducible levels of expression of cathepsin B in the membrane preparations obtained from malignant ovarian tumors, while benign tumors were found not to contain significant amounts of cathepsin B ($P<0.001$). For malignant tumors, the levels of membrane cathepsin B did not vary with the stage of disease.

uPA binding to cell membrane preparations We focused our studies on ovarian cancer-derived uPA and uPA-binding proteins, since membrane preparations obtained from benign tumors showed extremely low, albeit clearly detectable and reproducible, uPA levels. Binding of biotinylated DFP-HMW-uPA to membranes reached saturation after 30 min at 4°C (data not shown). Binding of biotinylated DFP-HMW-uPA to membranes occurred in a dose-dependent and saturable fashion. Specific uPA binding approached saturation at approximately 2 n*M* (range, 1.5–2.5 n*M*) (Fig. 1).

Table I. Immunological Determination of uPA and Cathepsin B in Benign and Malignant Ovarian Tumors

Ovarian tumors		uPA[a]	Cathepsin B[b]
Benign	(n=10)	0.17±0.18[c]	5.0±2.0
Malignant			
stage I+II	(n=7)	1.79±1.04[d]	51.2±40.9
stage III+IV	(n=8)	4.41±2.19[e]	45.4±50.0

Tumor cell membranes were prepared as described in "Materials and Methods." The membranes were washed with PBS with 0.1% BSA.

a) pro-uPA/uPA antigen levels were measured by ELISA as described in "Materials and Methods." Concentration in ng/mg protein.

b) Enzymatically active human cathepsin B was used as a standard to determine cathepsin B concentrations. Concentration in pmol/mg protein.

c) The values are the mean±SD of results of assays carried out in triplicate for each sample. The concentrations of uPA and cathepsin B were significantly higher in malignant tumors than in benign tumors ($P<0.001$). *e*) $P<0.05$, compared to *d*.

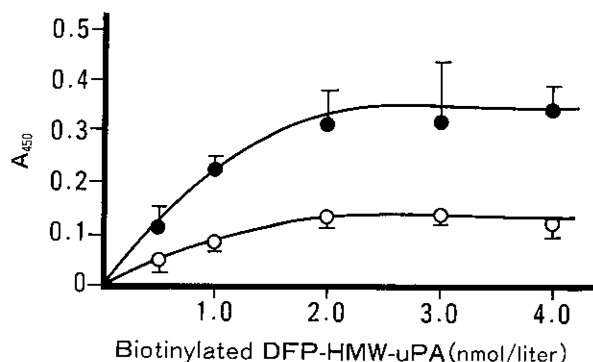

Fig. 1. Specific binding of biotinylated DFP-HMW-uPA to cell membrane preparations. Binding of biotinylated DFP-HMW-uPA to membranes prepared from malignant ovarian tumors (●, n=5, serous cystadenocarcinoma) and benign ovarian tumors (○, n=4, serous cystadenoma). Microtiter plates (96-well) coated with membranes were washed three times with cold PBS containing 0.1% BSA, then incubated with biotinylated DFP-HMW-uPA (60 min, 4°C). The same procedure was used in control experiments, in which biotinylated DFP-HMW-uPA was incubated with membranes in the presence of an excess of cold HMW-uPA (200 n*M*). Assays were carried out in triplicate for each sample. Bar, SD.

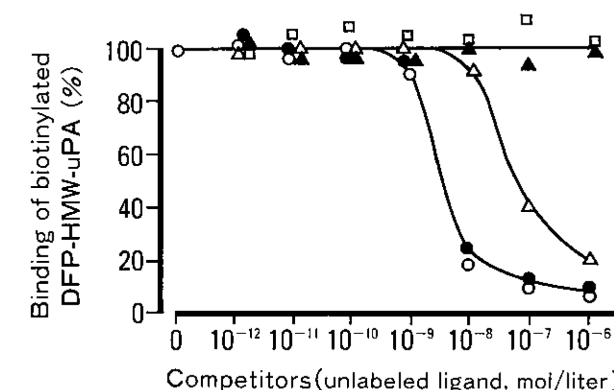

Fig. 2. Inhibition of binding of biotinylated DFP-HMW-uPA to membranes obtained from ovarian cancers in the presence of unlabeled ligands. Membranes were incubated with biotinylated DFP-HMW-uPA with increasing concentrations of parent HMW-uPA (○), pro-uPA (●), LMW-uPA (▲), ATF (△), or tPA (□). Fifty percent displacement of biotinylated DFP-HMW-uPA binding was obtained at 3.0 n*M* for HMW-uPA, 3.0 n*M* for pro-uPA, and 200 n*M* for ATF. Neither LMW-uPA nor tPA competed for binding stites. Assays were carried out in triplicate. The K_d value for this ovarian cancer, derived from the Scatchard plot, indicated specific binding of 1.2×10^{-9} *M*.

Binding of biotinylated DFP-HMW-uPA to membranes was assayed in the presence of increasing concentrations of unlabeled ligands as competitors (Fig. 2). pro-uPA, HMW-uPA, and ATF (amino-terminal fragment of uPA), but not LMW-uPA or tPA, competed for binding sites. Due to contamination with ATF cleaved in the growth factor-like domain at *Lys23-Tyr24* and/or *Lys35-Lys36* in ATF preparations,[20] the affinity of ATF for the uPA receptor is less than those of HMW-uPA and pro-uPA. This type of impaired ATF does not bind to uPA receptors.

The dissociation constant was estimated from the concentration of unlabeled HMW-uPA required to yield half-maximal displacement to be approximately 3×10^{-9} *M* (Fig. 2). In addition, Scatchard plots indicated that the range of K_ds for 15 malignant tumors found to have specific binding was 0.9×10^{-9} to 2.3×10^{-9} *M* with a mean of 1.4×10^{-9} *M*. The amount of uPA bound was in the range of 20–90 f*M*/mg of membrane protein, with a mean value of 42 f*M*/mg. Note that the levels of free uPA-binding sites per milligram of membrane preparation obtained from ovarian cancers were significantly higher for early disease (stages I and II) than for advanced disease (stages III and IV). Cell membranes briefly treated with acid were prepared to determine total uPA-binding sites, which was significantly higher in malignant than in benign tumors. Interestingly, the levels of total uPA-binding sites per milligram of membrane preparation obtained from malignant ovarian tumors did not vary with tumor stage (Table II). The percentage of occupied uPA receptors was significantly increased in

Table II. Amounts of Free and Total uPA-binding Proteins in Malignant and Benign Tumors

Ovarian tumors		uPA-binding sites[a]	
		Total[b]	Free[c]
Benign	(n=10)	32±4[d]	26±5
Malignant			
stage I+II	(n=7)	69±24	52±12[e]
stage III+IV	(n=8)	59±28	33±10[f]

a) The total amount of uPA-binding protein (total uPA receptors), estimated from the Scatchard plot using Fig. 2, was assayed for acid-treated membrane proteins. Concentration in fmol/mg protein. Microtiter plates coated with membrane proteins treated with (*b*) or without (*c*) an acidic buffer were incubated with biotinylated DFP-HMW-uPA (30 min, 4°C). The concentration of occupied uPA receptors was obtained by subtracting that for free uPA receptors from that for total uPA receptors.
d) Mean±SD fmol/mg protein. The levels of free and total uPA-binding sites per milligram of membrane preparations obtained from ovarian cancers were significantly higher than those for benign tumors ($P<0.01$). *f*) $P<0.05$, compared to *e*.

advanced cancer (44% for stages III+IV vs. 25% for stages I+II, $P<0.05$). Membrane preparations obtained from benign tumors had extremely low levels of uPA-binding sites ($P<0.01$, Table II). The binding of pro-uPA to membranes also showed concentration-dependent and saturable binding. In addition, after the membranes had been incubated with unlabeled pro-uPA under conditions resulting in saturable binding (2 n*M*), the membrane-associated uPA was measured using immunological methods. Membranes saturated with pro-uPA also demonstrated highly specific binding (data not shown).

Enzymological determinations of uPA and cathepsin B For quantitative assay of uPA activity in membranes, we applied a chromogenic synthetic substrate, S-2444, to membranes adhering to microtiter plates. Cell membranes alone were capable of degrading S-2444. When membranes were preincubated with plasmin, a considerable elevation in the levels of amidolytic activity was observed (data not shown).

Pro-uPA and HMW-uPA levels in membrane preparations were measured by immunoadsorbent-amidolytic assay (Table III). The finding of a significant increase in amidolytic activity following activation of uPAs by plasmin suggests that less than half (30–40%) of all immunoreactive uPAs among the membranes of ovarian cancers is present in the enzymatically inactive pro-uPA form.

Plasma membrane preparations of malignant and benign ovarian tumors were assayed for cathepsin B activity with the specific synthetic ligand z-arg-arg-AMC. Cathepsin B activity was found to be significantly higher in the membrane preparations obtained from malignant tumors than in those obtained from benign tumors ($P<0.01$) (Table IV). There was no variation in level of cathepsin B activity with stage of disease. When the cysteine proteinase inhibitor E-64 was added to the membranes prior to the addition of z-arg-arg-AMC, no cathepsin B activity was detected.

Identification of uPA-binding proteins and cathepsin B Levels of uPA-binding proteins (uPA receptors) were determined by ligand-blotting analysis for membrane preparations obtained from ovarian tumors. The membrane preparations (detergent phase) were subjected to SDS-PAGE and electroblotted onto PVDF sheets. The sheets were incubated with 4 nM biotinylated DFP-

Table III. Differential Detection of pro-uPA and HMW-uPA in Membrane Protein Preparations

Ovarian tumors		− Plasmin[a]	+ Plasmin[b]
Benign	(n=10)	0.09±0.03[c]	0.21±0.06
Malignant			
stage I+II	(n=7)	0.36±0.08[d]	0.51±0.16[d]
stage III+IV	(n=8)	0.52±0.13[e]	0.84±0.19[e]

Effects of plasmin treatment on uPA, contained in membrane fractions obtained from benign and malignant ovarian tumors and bound to moAB 377 (5 μg/ml)-coated microtiter plates, were examined. The membranes were preincubated without (*a*) or with (*b*) 5 μg/ml plasmin (1 h, 23°C), and were washed twice. Effects of modulation of uPA activity were studied using the chromogenic synthetic substrate S-2444. Assays were routinely carried out in triplicate.
a) Endogenous enzymatically active HMW-uPA activity.
b) Total uPA activity.
c) uPA activity, mean±SD U/mg protein.
e) $P<0.05$, compared to *d*.

Table IV. Cathepsin B Activity in Membrane Fractions Obtained from Benign and Malignant Ovarian Tumors

Ovarian tumors		− E64	+ E64
Benign	(n=10)	0.3±0.2	<0.2
Malignant			
stage I+II	(n=7)	1.7±0.8	<0.2
stage III+IV	(n=8)	2.0±0.6	<0.2

Enzymatic activity of membrane-associated cathepsin B was determined in absorbance units. Intensity of fluorescence was quantitated using a spectrophotometer. + E64, Cysteine proteinase inhibitor E64 was added to the membrane fraction prior to the addition of z-arg-arg-AMC. Assays were routinely carried out in triplicate for each sample. Cathepsin B activity was significantly higher in malignant tumors than in benign tumors ($P<0.01$).

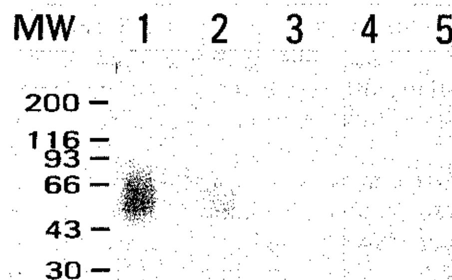

Fig. 3. Ligand-blotting of membrane preparations obtained from ovarian tumors. Triton X-100 detergent phase from ovarian cancer was subjected to 5–15% SDS-PAGE and the gel was electroblotted onto PVDF sheets. The sheets were incubated with biotinylated DFP-HMW-uPA (2 h, 23°C) in the absence (lane 1) or presence of an excess amount of 100 n*M* unlabeled HMW-uPA (lane 3) or pro-uPA (lane 4), or 1 μM ATF (lane 5). The membranes of benign tumors also included uPA receptors, although in lesser amounts than was the case for ovarian cancer in the absence of unlabeled ligands (lane 2).

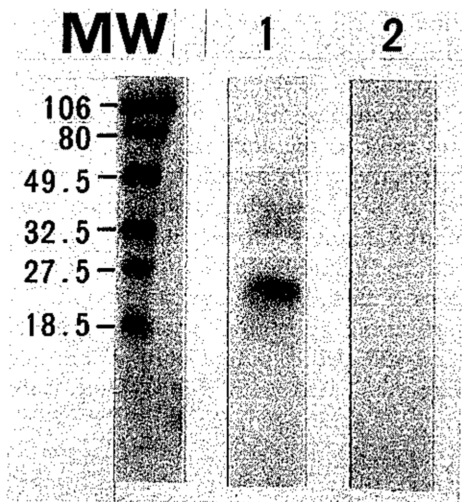

Fig. 4. Western blot analysis with antibody against cathepsin B. Membranes prepared from malignant (lane 1, serous cystadenocarcinoma) or benign ovarian tumor (lane 2, serous cystadenoma) were subjected to 5–15% SDS-PAGE and the gel was electroblotted onto PVDF sheets. The sheets were incubated with biotinylated polyclonal antibody against cathepsin B (8 h, 23°C). 25 kDa, a mature form of cathepsin B; and 30–35 kDa, a precursor form of this proteinase.

HMW-uPA. In the membrane preparations obtained from ovarian cancers, this probe bound to a single polydispersed band with an apparent molecular weight of 50–70 kDa (Fig. 3). This binding was saturable and specific since it was competed for during preincubation of the membranes with 100 n*M* unlabeled HMW-uPA or pro-uPA, or 1 μM ATF. uPA-binding proteins were also detectable in the membrane preparations obtained from benign tumors, although in lower quantities than was the case for ovarian cancer in the absence of unlabeled ligands.

Immunoblot analysis was also carried out to detect cathepsin B. Plasma membrane fractions obtained from ovarian cancer were found to contain a mature 25 kDa form of cathepsin B (major band) and a precursor 30 to 35 kDa form of this proteinase (minor band) (Fig. 4). In contrast, the membrane protein fractions obtained from benign tumors contained no detectable cathepsin B.

DISCUSSION

The finding that anti-catalytic uPA antibody and serine proteinase inhibitors are able to inhibit invasion of the basement membranes by tumor cells of the ovarian cancer cell line, HOC-I, has implicated uPA in the process of invasion of these cells.[11] In addition, in a previous study the cysteine proteinase inhibitor E-64 was found to reduce the invasiveness of the cells tested by some 70%.[11] The findings of these studies have indicated that uPA and cathepsin B have important roles in the processes of tumor cell invasion and metastasis.

The present study has demonstrated that uPA molecules are present in cell membranes in ovarian cancer, and that less than half of the membrane-associated uPA is present in the form of enzymatically inactive pro-uPA. pro-uPA and HMW-uPA are bound to a specific surface receptor that is not completely saturated. The receptor-ligand interaction is rapid, and receptors are saturated at low concentrations of ligands. Binding was specific for HMW-uPA and pro-uPA, since neither LMW-uPA nor tPA interfered with the interaction of HMW-uPA or pro-uPA with the receptors. The ovarian cancer cells have enzymatically active cathepsin B in their plasma membranes. The concentrations of uPA, uPA-binding sites (uPA receptor), and cathepsin B were significantly higher in malignant tumors than in benign tumors. Interestingly, for malignant tumors, the mean levels of total uPA-binding sites and cathepsin B did not vary with tumor stage or size. The major findings of this study, however, were the significant increases in cell-associated uPA antigen/activity and uPA occupancy in advanced ovarian tumors. We speculate that increased cell-surface uPA occupancy contributes to an increase in cell-bound uPA antigen/activity, and that receptor-bound uPA is therefore associated with the invasive potential. We also suspect that increased uPA occupancy on cell surfaces in advanced ovarian tumors may result in an increase in aggressiveness. Our results are in agreement with those of Cohen *et al.*,[21] who reported that decreased cell-surface uPA activity was associated with a decrease in cell-bound plasmin activity, suggesting that receptor-bound uPA plays an important role in the generation of plasmin on the cell surface, and in turn contributes significantly to invasive potential.

Our findings strongly suggest that, since ovarian cancer cells can produce both pro-uPA and cathepsin B, the activation of tumor cell-derived pro-uPA by the cellular proteinase cathepsin B may occur.[5] In the case of ovarian cancer, at least, tumor cell invasiveness is a function of the amount of cell surface receptor-bound uPA present. These findings also suggest that uPA and cathepsin B may have roles in the initial step of the process of tumor cell metastasis.[22–28] Secreted pro-uPA binds immediately to specific receptors for uPA with high affinity.[1, 3, 4] The receptor-bound pro-uPA can be converted to the enzymatically active HMW-uPA by the cell-associated cathepsin B.[5] This latter form of uPA is active, and converts the inert zymogen, plasminogen, into plasmin, which is capable of degrading laminin[29] and type IV collagen,[30, 31] major constituents of the basement membrane.

We previously found in squamous cell carcinoma and adenocarcinoma of the uterus, as well as in adenocarcinoma of the ovary in humans, that uPA was distributed in a heterogenous fashion, but was consistently present in tumor cells in regions of invasive growth.[32] Recently, Grondahl-Hansen *et al.*[33] and Pyke *et al.*[34] have studied the location of uPA in sections of human colon carcinomas. Their results contrast with previously published findings, and indicate that uPA immunoreactivity is present in stromal elements (fibroblast-like cells and endothelial cells) but not in malignant epithelial cells. They speculated that the cancer cells could, in paracrine fashion, induce the resident fibroblast-like cells to produce uPA.

Notwithstanding this discrepancy, quantitation of the uPA content in tumor extracts may have prognostic value. The pro-uPA/uPA content of tissue extracts appears to be an independent predictor of early relapse in breast cancer, the measurement of which enables the differentiation of high- and low-risk patients within certain risk groups.[20, 35, 36] No detailed clinical study designed to determine the impact of these proteinases on ovarian cancer has been performed. Malignant tumors contain and produce increased amounts of proteolytic enzymes, including not only serine proteinases (uPA, plasmin) and cysteine proteinases (cathepsin B, cathepsin L[6]), but also metalloproteinase (collagenase) and aspartylproteinase (cathepsin D). Experiments with *in vitro* and *in vivo* models have shown that a correlation exists between the invasive potential of tumor cells and their capacity to produce these proteinases.[37-39]

The biological and clinical characteristics of these tumor-associated proteinases must be studied to determine whether uPA and cathepsin B contents, as well as the amount of uPA receptor, can be used as clinical parameters (independent predictors of prognosis) for ovarian cancers.

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