Tissue Distribution of Soluble and Receptor-Bound Urokinase in Human Breast Cancer Using a Panel of Monoclonal Antibodies

Maria V. Carrierio, Paola Franco, Silvana Del Vecchio, Ornella Massa, Gerardo Botti, Giuseppe D’Aiuto, M. Patrizia Stoppelli, and Marco Salvatore

Istituto Nazionale Tumori, Via M. Seminola, 80131 Napoli [M. V. C., S. D. V., G. B., G. D., M. S.]; Istituto Internazionale di Genetica e Biofisica, 80125 Napoli [P. F., O. M., M. P. S.]; and Medicina Nucleare, Università degli Studi “Federico II,” 80131 Napoli [S. D. V., M. S.], Italy

ABSTRACT

Current evidence regarding the regulation of urokinase-dependent extracellular proteolysis indicates that plasminogen activation is a surface-associated process. We have compared the histological localization of urokinase plasminogen activator (uPA) and urokinase plasminogen activator receptor (uPAR) in breast cancer sections using a panel of monoclonal antibodies. First, the ability of six different anti-uPA monoclonal antibodies to recognize pro-uPA, uPA, and in vitro-formed complexes of uPA with either soluble uPAR or with plasminogen activator inhibitor type 1 was compared. Then the reactivity of the anti-uPAR antibodies was tested, and the occurrence of an uPA receptor of about Mr 55,000 in samples from breast carcinoma was assessed by immunoprecipitating the uPA receptor from an in vitro 125I-labeled tumor extract. Immunocytochemical data from adjacent sections of 10 tumor specimens showed that antibodies recognizing free and bound uPA mostly stain the cytoplasm and the membrane of epithelial tumor cells in confined areas of the tumor and some fibroblast-like stromal cells. Acid pretreatment of tumor sections, which removes receptor-bound uPA, causes a strong reduction of the immunocytochemical reactivity of epithelial tumor cells, whereas staining of fibroblast-like cells is not considerably affected. Consistent with these results, epithelial tumor cells were mostly unreactive to anti-uPA antibodies unless pretreated with acidic buffer, whereas fibroblast-like stromal cells showed a faint but acid-resistant staining with all anti-uPARs. In conclusion, these results show that occupied uPA receptors are definitely present on the membrane of epithelial tumor cells and suggest the occurrence of uPA-uPAR-dependent proteolytic activity on the surface of breast cancer cells.

INTRODUCTION

Cancer invasion and metastasis result from a series of interdependent processes requiring, as an intermediate step, the active degradation of pericellular matrix and the disruption of basement membranes. Several proteolytic enzyme systems are reported to be involved in the metastatic spread of tumor cells, and one of the most extensively studied is the urokinase (uPA) pathway of plasminogen activation (1, 2). Elevated levels of uPA have been shown to be associated with many malignant tumors including carcinomas of the colon (3), lung (4), prostate (5), and breast (6), and the specific modulation of urokinase-dependent proteolytic activity markedly affects the invasive and metastatic potential of tumor cells (7–9). Consistent with this view is the prognostic value of uPA shown by several investigators in patients with breast and colon cancer (10–12).

Proteolytic activation of plasminogen by urokinase yields the active and broad-spectrum serine protease, plasmin, which in turn is able to degrade fibronectin, vitronectin, laminin, and to activate latent collagenases (1, 2). uPA is secreted as an inactive single-chain protein (pro-uPA) which is proteolytically converted into the active two-chain form, urokinase (2). A large body of evidence shows that both uPA and plasminogen can be bound to the cell surface via specific receptors (13–15) and that plasminogen activation occurring at the cell surface is much more efficient than in solution, resulting in a 16-fold increase in the rate of plasmin generation (16, 17). Recent findings show that the interaction of two-chain uPA with its specific receptor is also able to mediate the internalization and degradation of uPA/PAI-1 complexes, thus down-regulating active uPA (18). The evidence that the catalytic domain of urokinase is not involved in receptor binding has been obtained by independent approaches which assigned the high affinity receptor binding activity to the growth factor-like domain of uPA (19–21). However, recent work has highlighted the role of the serine protease domain in the interaction of uPA and uPA/PAI-1 complexes with the α2-macroglobulin receptor/low density lipoprotein receptor which mediates their internalization and degradation in human monocytes (22) and Hep G2 cells (23).

The urokinase receptor (uPAR) is a Mr 55,000–60,000 highly glycosylated protein anchored to the plasma membrane via a phosphatidylinositol moiety and encoded by a cDNA of 1.4 kilobases expressed in a LB6 mouse cell line (24, 25). Receptors for uPA have been described on a number of different cell types including macrophage-monoocyte-like U937 cells, endothelial cells, fibroblasts, polymorphonuclear leukocytes, and several tumor cell lines (reviewed in Ref. 26). An autocrine and/or paracrine mechanism for secretion and binding of uPA to uPAR has been postulated (27–29). However, regardless of the site of synthesis of plasminogen and pro-urokinase, existing evidence shows that the ability of a tumor cell to invade and metastasize ultimately depends on the presence of uPAR (29–32). Consistent with these results, a number of reports well document the overexpression of uPAR in malignant tumors such as breast (33), ovarian (34), lung (35), and colon cancer (36). Interestingly, soluble uPAR has been detected in the ascitic fluid of patients with highly invasive ovarian carcinomas (37).

The localization of uPA and uPAR in tumor specimens has been further analyzed by several investigators with immunocytochemical techniques. High levels of uPA were found in malignant epithelial cells of human breast and colon cancer as well as in Lewis lung carcinoma (10, 38, 39). However, in situ hybridization studies on colon adenocarcinomas showed the occurrence of uPA mRNA exclusively in fibroblast-like stromal cells, whereas epithelial tumor cells expressed uPAR mRNA at invasive foci and appeared to be devoid of uPA mRNA (36). In breast carcinomas, anti-uPAR antibodies mostly stained periluminal macrophage-like cells, whereas epithelial tumor cells were positively stained only in a small fraction of the cases examined (10). In vitro quantitative autoradiography with radiolabeled pro-uPA, we have previously shown that the amount of detectable uPAR in carcinomas of the breast is about 5-fold higher than in benign breast lesions. Simultaneous quantitation of urokinase by immunoenzymatic assay on tissue extracts from the same speci-
mens showed that breast carcinomas also contain 19 times more uPA than benign tumors (41).

In this study, we used immunocytochemical methods to investigate the tumor compartments where the surface-associated and soluble urokinase-dependent proteolysis may possibly occur in vivo. We reasoned that the antibody recognition of uPA may be prevented by the interaction with uPAR or with the inhibitors (PAI-1, PAI-2, nextrin, etc.) or by the ratio of activated versus single-chain uPA. Similarly, anti-uPAR antibodies may not be able to recognize saturated uPARs on the surface of tumor cells. Therefore, we first characterized the reactivity of a panel of different monoclonal antibodies against uPA, either free or complexed to uPAR or PAI-1. Anti-uPAR monoclonal and polyclonal antibodies were also tested for their ability to recognize uPA-uPAR complexes. Then we analyzed the distribution of uPA and uPAR in breast tumors with the pretreated polyclonal and monoclonal antibodies. Finally, we assessed the localization of receptor-bound uPA by analyzing the staining pattern of tumor sections before and after acidic treatment.

**MATERIALS AND METHODS**

**Materials.** MoAbs 377 and 394 directed against A and B chain of human uPA, respectively, were kindly provided by American Diagnostica (Greenwich, CT). The anti-uPA B chain clone 2 MoAb and the anti-uPA A chain clones 6 and 12 were a gift from Dr. P. A. Andreassen, University of Aarhus, Aarhus, Denmark (42). Anti-kirinle 5B4 antibody is described by Noll et al. (43). Monoclonal anti-uPAR antibody 3936 and rabbit polyclonal antibody 399 (American Diagnostica) are directed against the soluble and membrane-associated uPAR (44). The anti-uPAR R2 MoAb (45) was kindly provided by Dr. F. Blasi (Milan, Italy) and used in some experiments. The ELISA kit for quantitation of uPA and urinary urokinase were from American Diagnostica. Soluble truncated human uPAR completely retaining the ability of binding uPA was purified either from the conditioned medium of the CHO hamster cell line or from mouse L6E cells (46) and was a gift from Dr. N. Pedersen (Copenhagen, Denmark) and Dr. F. Blasi (Milan, Italy). Biotinylated goat anti-mouse, anti-rabbit immunoglobulins and streptavidin-conjugated peroxidase were purchased from Dako (Copenhagen, Denmark). Protein G-Sepharose, blue-Sepharose, and Sephadex G-25 were obtained from Pharmacia (Uppsala, Sweden). Plasminogen activator inhibitor type 1 was a gift from Dr. Wun (Monsanto, MO). Recombinant single-chain pro-uPA was a gift from Farmitalia, Carlo Erba (Milan, Italy). All of the reagents for cell culture were purchased from GIBCO (Gaithersburg, MD). Na125I was from Sorin Bio medic (Saluggia, Italy) and Iodo-Gen was from Pierce (Rockford, IL). En lightening was from NEN (Cologno Monzese, Italy).

**Cell Cultures.** HT1080 human fibrosarcoma cell line, mouse L6E producing SuPAR, and A431-P1 human carcinoma cell line overexpressing uPA were used to test the reactivity of the antibodies and to set up the optimal experimental conditions for immunoperoxidase staining on tumor sections. Cell lines were grown in DMEM supplemented with 10% FBS, 100 international unit/ml penicillin, and 50 μg/ml streptomycin. When used for immunocytochemistry, cell lines were grown on glass slides, fixed for 10 min in PBS containing 4% parafomaldehyde and 1% Triton X-100, and incubated with the anti-uPA or anti-uPAR antibodies (see "Immunohistochemical Staining").

**Immunoperoxidase Staining of Cell Lines and Detection of Surface-associated Immunocomplexes.** Conditioned medium for preabsorption of anti-uPAR antibodies was prepared as follows: wild-type and SuPAR-transfected mouse L6E cells were incubated in serum-free medium for 12 h. The medium was then cleared by centrifugation and concentrated 10-fold by lyophilization. The protein content of the conditioned medium was determined according to the method of Bradford (47).

35S-labeling of A431-P1 Cell Line and Immunoprecipitation of prouPA. To metabolically label the A431-P1 cell line, cells were seeded at a density of 2 × 10^5/10 cm dish and grown for 24 h in 10 ml DMEM with 10% FBS. After 24 h, the culture medium was removed and substituted with methionine-free DMEM containing 5% dialyzed FBS. After 8 h, the starvation medium was further substituted with 2.5 ml of methionine-free DMEM containing 400 μCi of [35S]methione, and labeling was allowed to proceed for 16-18 h. Immunoprecipitation was performed according to a previously described procedure (28). For each sample, 100 μl of conditioned medium were incubated with 10 μg/ml of the selected antibody in buffer A [0.1 M Tris-HCl (pH 8.1)-0.3 M NaCl-0.1% bovine serum albumin-0.1 Tween-20] overnight at 4°C. The immunocomplexes were recovered with protein G-Sepharose and then analyzed by 12.5% polyacrylamide gel electrophoresis under reducing conditions followed by autoradiography (48). The 35S-containing polyacrylamide gels were subjected to fluorography with Enlightening before drying and exposure. The same procedure has been used for 125I-labeled purified proteins.

**Radioiodination, Formation of 125I-uPA-PAI-1 and 125I-SuPAR-pro-uPA Complexes.** Two-chain urinary urokinase and soluble uPA receptor were radioiodinated with Na125I using Iodo-Gen as described previously (41). The radiolabeled proteins were purified from unbound iodide by Sephadex G-25 chromatography, and the resulting specific activity was approximately 30 μCi/μg. The radiolabeled two-chain urinary uPA (35 ng corresponding to 106 cpm) was allowed to react with 72 ng of PAI-1 for 1 h at room temperature.

Radioiodabeled SuPAR (7 × 105 cpm) was incubated with 20 μl of a 10-fold concentrated unlabeled conditioned medium from A431-P1 cells, allowed to react for 1 h at room temperature, and cross-linked with 1 mM disuccinimidyl suberate for 15 min at 4°C. The resulting complexed proteins were finally immunoprecipitated in a total volume of 50 μl of buffer A containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.5) with 10 μg/ml anti-uPA and anti-uPAR antibodies as described above.

**Binding of Antibodies to Cultured Cells.** HT1080 cells were seeded at a density of 2 × 105 cells/well in a 24-well plate and incubated overnight in DMEM-10% FBS. Then the culture medium was removed, and the cells were treated or not treated with an acidic buffer containing 30 mM glycine-HCl-0.1 mM NaCl (pH 3) at 22°C for 3 min, and extensively washed with PBS (28). Further incubation was performed for 3 h at 4°C with 8 × 105 cpm of 125I-labeled anti-uPA MoAbs (specific activity, 4 × 105 cpm/μg), in the presence or in the absence of 0.2 μM unlabeled MoAb. Parallel experiments included incubation of acid-treated or untreated cells with 10 μg/ml unlabeled anti-uPA antibodies and detection of surface-associated immunocomplexes with 125I-labeled protein A. After extensive washing, cell-bound radioactivity was assessed in a gamma counter, and the results were expressed as a mean of values obtained for triplicate samples.

**Tissue Preparation.** Tumor biopsy specimens were obtained from patients undergoing surgery for a breast lump. Ten histologically confirmed breast carcinomas and three benign breast lesions were preliminarily analyzed using the whole panel of MoAbs. The immunocytochemical analysis was then extended to an additional 25 breast carcinomas and 9 benign breast lesions using two MoAbs, i.e., anti-uPA 394 and anti-uPAR 3936. Tumors were classified according to WHO nomenclature (49) and included a total of 23 ductal and 12 lobular carcinomas, whereas benign breast lesions consisted of 7 fibroadenomas and 5 fibrocystic diseases. The specimens were immediately frozen in liquid nitrogen, stored at −80°C, and then used for immunocytochemistry, measurements of uPA concentration in tissue extracts, and preparation of radiolabeled tumor extracts.

125I-labeling of Tumor Extracts and Immunoprecipitation of uPAR. One hundred mg of breast carcinoma samples were homogenized in a hypotonic solution [20 mM Tris-HCl (pH 7.5)-1 mM EDTA-1 mM EGTA-1 mM phenylmethylsulfonyl fluoride-5 mM leupeptin-25 mM aprotinin-25 mM benzamidine]. The lysates were then cleared at 800 × g for 10 min, and the particulate cell fraction was recovered by centrifugation at 100,000 × g for 45 min. The pellet was solubilized in 0.1 M Tris-HCl (pH 7.5)-0.1% Triton X-100, preabsorbed with a 1:1 suspension of protein G-Sepharose and blue-Sepharose, and assayed for protein content according to the method of Bradford (47). Fifteen μg of preabsorbed membrane proteins were radiiodinated with 300 μCi of Na125I and immunoprecipitated with anti-uPAR antibodies as described above. A mixture of 15 μg of membranes and 1.4 μg of purified SuPAR from CHO was radiiodinated as a control. The radiiodinated products were diluted in buffer A to an activity of 400 × 105 cpm in 200 μl and incubated overnight at 4°C with 10 μg/ml of anti-uPA antibody. Twenty-five μl of a 1:1 suspension of protein G-Sepharose was added to the samples and incubated at 4°C for 2 h under gentle shaking. After extensive washing, the immunoprecipitated samples were resuspended in 40 μl of Laemmli buffer and loaded onto a 12.5% SDS-PAGE under reducing conditions followed by autoradiography.
Immunohistochemical Staining. Five μm-thick serial frozen sections corresponding to the largest cross-sectional area of the tumor were cut, placed on clean glass slides, air dried, and used for immunohistochemical staining.

The immunostaining was performed by the streptavidin-biotin method as described previously with minor modifications (50). Briefly, sections were incubated with monoclonal or polyclonal antibodies (10 and 5 μg/ml, respectively) overnight at 4°C. After the incubation with the primary antibody and several washes in PBS (pH 7.4) 9.3 μg/mL of biotinylated goat anti-mouse immunoglobulins or 1:200 diluted biotinylated goat anti-rabbit immunoglobulins were applied to tumor sections at 22°C for 60 min. Sections were washed again in PBS and then incubated with streptavidin-biotinylated horseradish peroxidase complex for an additional 30 min according to the manufacturer’s procedure. The peroxidase-dependent staining was developed by a filtered, saturated solution of diaminobenzidine in 0.05 M Tris-HCl (pH 7.4) containing 3% H₂O₂. The slides were finally counterstained with hematoxylin, dehydrated, and mounted. To test the effect of receptor-bound uPA on the intensity of the staining, adjacent sections were pretreated or not treated with an acidic buffer under the conditions described for HT1080 cells in “binding of antibodies to cultured cells” and processed as described above. Negative controls were performed either without the primary antibody or by preabsorbing anti-uPA and anti-uPAR antibodies with recombinant pro-uPA and SuPAR, respectively.

The intensity of immunostaining with anti-uPA and anti-uPAR antibodies was graded from 1 to 3 corresponding to faint, moderate, or intense staining. The absence of chromogenic reaction allocated tumor specimens in class 0.

To examine the antibody reactivity against receptor-bound uPA, we took advantage of the purified SuPAR obtained by inserting a nonsense codon toward the carboxy-terminal end immediately upstream of the region required for membrane attachment of the receptor via the glycolipid anchor (see “Materials and Methods”). Incubation of serum-free conditioned medium from A431-P1 cells with 125I-SuPAR followed by cross-linking resulted in the formation of 125I-SuPAR complexes. A further incubation of this mixture with the anti-uPA MoAbs showed that all of the anti-A and anti-B chain MoAbs were able to react with receptor-bound pro-uPA (Fig. 1C). In this particular experiment, the different ability of anti-uPA MoAbs to recognize 125I-SuPAR-pro-uPA complexes accounts for the extent of the coimmunoprecipitated 125I-SuPAR. Fig. 1C shows that the amount of cross-linked complexes and coimmunoprecipitated 125I-SuPAR detected by MoAb 377 is definitely high as compared with clone 2, clone 6, 5B4, and, in particular, with 394. A conformational change of the epitope for 377 may be responsible for its increased in vitro immunoreactivity toward pro-uPA bound to uPAR versus free pro-uPA (Fig. 1, A and C).

The reactivity of these antibodies against receptor-bound pro-uPA, two-chain uPA, uPA-uPAR, and uPA-PAI-1 complexes, and cross-linked complexes and coimmunoprecipitated 125I-SuPAR detected by MoAb 377 is definitely high as compared with clone 2, clone 6, 5B4, and, in particular, with 394. A conformational change of the epitope for 377 may be responsible for its increased in vitro immunoreactivity toward pro-uPA bound to uPAR versus free pro-uPA (Fig. 1, A and C). Binding of PAI-1 to 125I-urinary uPA did not prevent recognition by any of the antibodies, including the anti-B chain MoAbs, but it slightly reduced the reactivity of 394 (Fig. 1D). In conclusion, these data suggest that binding of uPA to its receptor does not dramatically affect the reactivity of any of the anti-uPA MoAbs in vitro; however, antibody recognition of uPA bound to the cell membrane may be somehow affected by the complex protein interactions occurring in that particular context.

To further test the ability of anti-uPA MoAbs to recognize receptor-bound uPA on the surface of tumor cells, a competitive binding assay with 125I-labeled anti-uPA MoAbs was performed on acid-treated and untreated HT1080 cells bearing saturated uPARs. The extent of specific binding was determined by incubating the cells with and without an excess of unlabeled antibody and then measuring the amount of cell-associated radioactivity. Table 1 summarizes the results of these
to HT1O8O cells is greatly decreased by acid pretreatment of the cells, showing that the antibodies are mostly reacting with membrane-bound uPA.

In conclusion, this set of experiments indicates that all of the antibodies tested do recognize uPA either free or complexed with SuPAR and PAI-1, although to a different extent; the variable amount of immunoprecipitated antigen may depend on the relative affinity of the antibody. Furthermore, all MoAbs are reactive to receptor-bound pro-uPA on the surface of cultured tumor cells.

Testing Anti-uPAR Antibodies. To test the reactivity of the anti-uPAR antibodies, purified SuPAR from CHO cells was labeled with Na125I and further incubated with recombinant pro-uPA to allow formation of the 125I-SuPAR-recombinant pro-uPA complexes. Then anti-uPAR monoclonal antibody 3936 or rabbit polyclonal antibody 399 were incubated with the cross-linked 125I-SuPAR-recombinant pro-uPA under the conditions described in “Materials and Methods.”

As shown in Fig. 2 (left), all of the tested antibodies were able to immunoprecipitate 125I-SuPAR from CHO cells; however, when SuPAR was prebound to pro-uPA, only the polyclonal antibody was able to immunoprecipitate a consistent amount of it, whereas MoAb 3936 barely detected it (Fig. 2, right). The higher amount of 125I-SuPAR detected by 399 as compared with 3936 may be due to immunoprecipitation of both free and noncovalently complexed 125I-SuPAR (Fig. 2, right). Under the same conditions, MoAb R2 (see “Materials and Methods”) was able to recognize both free and complexed SuPAR (not shown).

Similarly to anti-uPA MoAbs, anti-uPAR 3936 and 399 antibodies were allowed to bind to HT1O8O cells pretreated and not treated with acidic buffer. The immunocomplexes associated with the cell surface were then detected with 125I-protein A. Table 1 shows that the extent of specific binding to uPAR is increased by acid pretreatment, whereas binding of anti-uPAs is decreased by the same treatment (see also previous paragraph). These results indicate that saturation of uPARs causes a reduction of the reactivity of anti-uPAR antibodies and therefore suggest the use of an acidic pretreatment of the tissue section before the immunostaining procedures.

**Table 1 Binding of anti-uPA and anti-uPAR antibodies to untreated and acid-pretreated HT1O8O cells**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Untreated* (cpm)</th>
<th>Acid pretreated* (cpm)</th>
<th>Binding to acid extractable uPA† (cpm)</th>
<th>Acid-dependent increase of binding to uPAR‡ (cpm)</th>
<th>Untreated excess cold* (cpm)</th>
<th>Acid pretreated excess cold* (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>125I-394</td>
<td>11932</td>
<td>7690</td>
<td>4242</td>
<td>2302</td>
<td>795</td>
<td>1094</td>
</tr>
<tr>
<td>125I-377</td>
<td>6129</td>
<td>2909</td>
<td>3220</td>
<td>795</td>
<td>814</td>
<td>806</td>
</tr>
<tr>
<td>125I clone 2</td>
<td>4559</td>
<td>2396</td>
<td>2163</td>
<td>956</td>
<td>814</td>
<td>806</td>
</tr>
<tr>
<td>125I-clone 6</td>
<td>6189</td>
<td>2652</td>
<td>3537</td>
<td>955</td>
<td>814</td>
<td>806</td>
</tr>
<tr>
<td>125I-594</td>
<td>5780</td>
<td>2853</td>
<td>2927</td>
<td>1512</td>
<td>1318</td>
<td>1318</td>
</tr>
<tr>
<td>396†</td>
<td>4164</td>
<td>5187</td>
<td>1023</td>
<td>1023</td>
<td>1023</td>
<td>1023</td>
</tr>
<tr>
<td>399†</td>
<td>7357</td>
<td>8086</td>
<td>729</td>
<td>938</td>
<td>1046</td>
<td>1046</td>
</tr>
<tr>
<td>No antibody*</td>
<td>506</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a* Cell-bound total radioactivity.

*b* Radioactivity associated with acid-pretreated cells.

*c* Acid-dependent decrease of cell-bound radioactivity (a-b).

*d* Acid-dependent increase of cell-bound radioactivity (b-a).

*e* Binding of 125I-MoAb to untreated and acid-pretreated cells in the presence of 0.2 μM cold MoAb.

*f* 10 μg/ml of unlabeled antibody detected by 125I-protein A.

*g* Binding of 125I-protein A to acid-pretreated cells in the absence of antibody.

Tissue Distribution and Quantitation of uPA. Table 2 summarizes clinical data and pathological findings of all patients with breast cancer. We have performed immunocytochemical analysis of frozen tissues. As expected, specific binding of all anti-uPA antibodies to HT1O8O cells is greatly decreased by acid pretreatment of the cells, showing that the antibodies are mostly reacting with membrane-bound uPA.

![Fig. 2. Reactivity of the anti-uPAR antibodies to SuPAR and SuPAR-pro-uPA complexes.](image)

**Fig. 2** Reactivity of the anti-uPAR antibodies to SuPAR and SuPAR-pro-uPA complexes. *Left,* 125I-SuPAR was immunoprecipitated with or without 10 μg/ml of 3936 and 399 antibodies and subjected to 12.5% SDS-PAGE under reducing conditions. *Right,* preformed 125I-SuPAR-pro-uPA complexes (see “Materials and Methods”) were immunoprecipitated and analyzed under the same conditions as described for the left. Arrows, 125I-SuPAR-pro-uPA cross-linked complexes (about M, 100,000) and free 125I-SuPAR (about M, 55,000).
sections from 10 human breast carcinomas (patients 1–10) and 3 benign breast lesions using the whole panel of MoAbs described above. Table 3 lists the grading of the immunohistochemical staining observed with anti-uPA and anti-uPAR antibodies in breast carcinomas along with the tissue concentration of uPA as determined by immunoenzymatic assay on tissue extracts from the same tumors. Anti-uPAR antibodies reacted to all of the tumors examined to a similar extent, whereas anti-uPA antibodies produced a comparable staining of each specimen except for tumors 2 and 6. In these samples, the very low level of uPA could only be detected by a subset of antibodies. Control immunostaining of A431 and HT1080 cells showed a weak general reactivity of these cell lines to both anti-uPA and anti-uPAR antibodies as compared with malignant tumor sections (not shown).

With regard to the pattern of immunostaining, all of the anti-uPA antibodies, except 377, stained most of the epithelial tumor cells in sections from antigen-positive tumors. At higher magnification, positive epithelial tumor cells showed a homogeneous and diffuse staining of cytoplasm and a prominent and heterogeneous staining of plasma cell membranes in confined areas of the tumor (Fig. 4).

In addition to epithelial tumor cells, anti-uPA antibodies and, in particular, the anti-A chain frequently recognized fibroblast-like cells (Fig. 4 d and g) whereas 394 was poorly reactive toward the stromal components (Fig. 4e). On the contrary, anti-uPA antibody 377 exclusively stains both the fibroblast-like stromal cells and the extracellular matrix (Fig. 4h). Furthermore, clone 12 MoAb displays a faint reaction with both cellular and stromal component of the tumor section (Fig. 4f) probably due to its low affinity toward uPA (Fig. 1). The in vitro characterization of anti-uPA antibodies suggests that they may also detect receptor-bound uPA. Since it is known that a short acid pretreatment allows dissociation of the surface-bound uPA from A431 carcinoma cells without significantly affecting their viability, we decided to pretreat the sections in a similar way (see “Materials and Methods” and Ref. 28). Acid treatment of the sections caused a great reduction of the staining of epithelial cells, whereas the staining of the fibroblast-like cells was mostly resistant to this treatment (Fig. 4, b and e). Direct evidence for the presence of acid-extractable membrane-bound uPA was provided by immunoenzymatic assay performed on the neutralized acidic wash of malignant tumor sections. In agreement with our previous data on the higher content of uPA in breast carcinomas (41) with respect to benign lesions, the amount of acid-extractable uPA in sections from 3 breast carcinomas (patients 2, 5, and 7) was an average of 17-fold higher than that released by acidic treatment of sections from two benign lesions. These findings suggest that most of the positivity from epithelial tumor cells is due to receptor-bound uPA, whereas uPA associated with fibroblast-like cells is indeed mainly intracellular.

The immunocytochemical study was then extended to an additional 25 specimens of breast carcinoma (patients 11–35) and 9 benign lesions using MoAb 394 only. Considering the whole series of cases...
studied with MoAb 394, 71% (25 of 35) of breast carcinomas showed positive immunostaining of epithelial tumor cells. The intensity of staining was graded as faint (grade 1), moderate (grade 2), and intense (grade 3) in 8, 11, and 6 breast carcinomas, respectively. Ten specimens were allocated in class 0 for the total absence of chromogenic reaction. A positive, significant correlation \((P < 0.001)\) was found between immunohistochemical reactivity of anti-uPA antibodies and tissue concentration of uPA as determined by ELISA on tissue extracts from the same specimens, supporting the reliability of the immunohistochemical findings (Fig. 5). No significant correlation was found between uPA expression as determined by immunostaining or ELISA with anti-uPA antibodies and tumor size, nuclear grade, or lymph node status. All benign lesions tested either with 394 or with the whole panel of the anti-uPA MoAbs were negative, showing only a rare and spotted chromogenic reaction in ductal epithelial cells (data not shown). Tissue extracts from those specimens contained 15 times less uPA than carcinomas as assessed by immunoenzymatic assay.

**Tissue Distribution of uPA Receptor.** The results obtained in vitro by testing anti-uPAR antibodies suggest that the occupancy of the uPAR may partially prevent its recognition by anti-uPAR anti-
Cryptic Urokinase Receptors in Breast Cancer

In agreement with our prediction, the reactivity of MoAb 3936 to the epithelial tumor cells dramatically increased after acidic treatment, again suggesting that most of the uPA receptors were occupied with uPA (Fig. 6, a and b). On the contrary, the reactivity of anti-uPA antibodies decreased after treatment with the same buffer (Fig. 4, a, b, d, and e). However, it has to be noted that uncovering of the receptors did not result in a significant change in the reactivity of the fibroblast-like stromal cells. Polyclonal antibody 399 (Fig. 6 c and d) and MoAb R2 which could recognize in vitro 125I-SuPAR-pro-uPA complexes also showed an acid-enhanced staining. This finding suggests that the accessibility of uPAR on the tumor section may be limited by its spatial relationships with other membrane proteins either interacting or simply surrounding the uPA receptor.

Simultaneous immunostaining with two different anti-uPAR antibodies was then performed on acid-pretreated sections from 10 breast carcinomas (Table 2, patients 1–10) and the results are reported in Table 3. The intensity of the general chromogenic reaction observed in all specimens with the two antibodies was in good agreement. A heterogeneous pattern of staining was found in each individual tumor with both 3936 and 399 antibodies, the strongest immunoreactivity being associated with epithelial tumor cells protruding into the tumor stroma with an aligned pattern of growth and/or at border of neoplastic nodules (Fig. 6, b and d). Epithelial tumor cells showed more pronounced staining of plasma cell membranes beside intracytoplasmic positivities. Limited uPAR positivity, slightly enhanced by acidic treatment, was also observed in a few scattered fibroblast-like cells and in the peritumoral stroma around neoplastic nodules. Both

Fig. 5. Correlation between uPA concentration in tumor extracts and intensity of immunostaining with anti-uPA MoAb 394. Samples from 33 cases of breast carcinoma were subjected to immunoperoxidase staining and to quantitation of uPA content (fmol/mg) by ELISA. Staining intensities were graded as faint (1), moderate (2), or intense (3). No chromogenic reaction allocated the tumor specimen in class 0. Statistical significance, P < 0.001.

Fig. 6. Representative patterns of immunostaining with anti-uPAR antibodies in frozen sections of breast carcinoma. Serial frozen sections were subjected to immunohistochemical staining with MoAb 3936 (a, b) and polyclonal antibody 399 (c, d). The reactivity of both antibodies to epithelial tumor cells dramatically increases in acid-treated tumor sections (b and d), whereas staining of fibroblast-like stromal cells is not greatly affected by acid pretreatment. Counterstaining with hematoxylin, X200.
antibodies were mostly unreactive to all benign lesions, showing only a few and weak positivities in normal ductal cells. Preabsorption of 3936 and 399 antibodies with SuPAR purified from SuPAR overexpressing LB6 or concentrated conditioned medium from the same cell line caused a strong reduction of both epithelial and extra-
ct, stromal immunostaining.

We also extended the immunohistochemical analysis with anti-
SuPAR MoAb 3936 to 25 additional breast carcinomas (Table 2, patients 11—35) and 9 benign lesions. The staining patterns observed do confirm the results described for the first set of cases (data not shown). A faint reactivity was graded as 1 (observed in 6 specimens), moderate as 2 (12 specimens), and intense as 3 (17 specimens).

Immunoperoxidase grading with anti-uPAR MoAb 3936 in a total of 35 cases was positively correlated to tumor size ($P < 0.05$) and nuclear grade ($P < 0.01$). However, the level of uPA receptor was not significantly different in patients with or without lymph node metastases. A comparison between the reactivity of the sections to the anti-uPA and anti-uPAR antibodies showed a positive correlation between the grading with the two sets of antibodies ($P < 0.05$), suggesting the occurrence of a coordinate expression of uPA and uPAR in breast carcinomas (41).

DISCUSSION

This article describes the distribution of uPA and its receptor (uPAR) in sections of human breast carcinoma using a panel of monoclonal and polyclonal antibodies. We show that: (a) epithelial tumor cells exhibit a weak staining with anti-uPA antibodies and a markedly heterogeneous staining with anti-uPA MoAbs; (b) acid pretreatment of the sections increases the reactivity of anti-uPAR antibodies and decreases the reactivity of anti-uPA MoAbs to tumor cells; and (c) fibroblast-like stromal cells display a moderate and acid-resistant staining with both anti-uPAR and anti-uPA antibodies. These findings, taken together, indicate that saturated uPA receptors are definitely present on the membrane of epithelial tumor cells, thus suggesting the occurrence of a surface-associated urokinase-dependant proteolysis in breast carcinoma. Although we do not provide any evidence about the site of uPA synthesis, our data support the hypothesis of a direct involvement of bound uPA in tumor invasion and metastasis.

Cryptic uPA receptors have been originally described by Bajpai and Baker (27) in human foreskin fibroblasts and subsequently in A431 human carcinoma cells (28). The latter study has also shown that an autocrine saturation of uPA receptors occurs in cultured tumor cells expressing uPA and that saturated uPA receptors become accessible by treating the cells with a buffer at pH 3 for a short time. This finding led to the hypothesis that the synthesis of uPA and uPA receptor by the same cell may provide a pathway for the activation of the metastatic potential of malignant cells (28). The occupancy of uPARs in breast cancer has also been predicted on the basis of the results obtained in a recent study, which has revealed an equimolar ratio between uPA and uPAR in malignant tissues (41). Considering the kinetics of receptor-ligand interaction, an average 70% fractional occupancy of uPAR could be estimated in breast carcinomas, whereas the theoretical value for occupancy in benign breast lesions was only 21%. In agreement with these observations, a dramatic improvement of uPAR immunostaining was obtained in acid-pretreated sections, particularly at the level of epithelial tumor cells growing in a packed pattern. Conversely, acid treatment of adjacent tumor sections caused a strong reduction of the immunostaining with anti-uPA MoAbs. The presence of uPA in acidic washes of carcinoma sections confirmed the occurrence of acid-extractable membrane-bound uPA in those tumors. Unexpectedly, an acid-enhanced staining was also observed for anti-
uPAR antibodies, namely 399 and R2, capable of recognizing both free and complexed uPAR in vitro. However, it has to be mentioned that the functional assembly of plasminogen activation system on the cell surface implies the occurrence of multiple interactions involving the single components of the system and a number of other molecules which mediate cell attachment and adhesion (53). Immunofluorescence and ultrastructural studies have shown that uPA is localized at the focal contact sites of HT1080 fibrosarcoma cells as well as at areas of cell-to-cell contacts and cell extensions (54). At these sites, uPA is bound to its receptor (55), and both are reported to codistribute with other structural protein components of the cellular contact sites, such as vitronectin and vitronectin receptor (a,b, integrin), serving an anchoring function (56, 57). Interaction of receptor-bound uPA-PAI-1 complexes with a,macroglobulin receptor (22, 23) may further reduce the accessibility of saturated uPARs on tumor cells. Therefore, the complex network of interactions occurring at the cell surface may prevent the binding of antibodies yet able to fully recognize complexed uPAR in vitro.

The simultaneous use of a panel of MoAbs directed against different domains of the same molecule has several advantages. First, it allows a comparison of the staining patterns, thereby highlighting their similarities and increasing the reliability of the results obtained. Also, antibodies directed against epitopes involved in protein-protein interaction may distinguish between free and complexed antigens on tissue sections. In our case, all antibodies were able to immunopre-
cipitate pro-uPA and two-chain uPA with a comparable affinity; however, they exhibited a slightly different ability to bind uPA complexed either with SuPAR or with PAI-1. These differences may even be greater on tumor sections because of the above-mentioned multiple interactions and the high affinity binding of uPA to different classes of PAIs. For instance, MoAb 377 does react with its specific antigen in the soluble phase and on the surface of cultured tumor cells similarly to all of the tested anti-uPA MoAbs. However, when used on tumor sections, it exclusively stains both the fibroblast-like stromal cells and the stroma itself. This clear-cut reactivity of 377 may be partially explained by its unaffected reactivity toward uPA-PAI-1 complexes and its increased affinity toward uPA-SuPAR complexes which may be present at the stromal level (37). Furthermore, MoAb 377 is directed against the amino-terminal domain of uPA, which contains low affinity binding sites for components of cell-matrix junctions such as laminin-nitrogen (58), heparin and related sulfated polysanions (59). Although MoAb 377 does not prevent the binding of uPA to heparin in vitro (59), the involvement of uPA epitope for 377 in other cell-matrix interactions cannot be excluded.

An active role of tumor stroma in malignant invasion and metastasis has been recently advocated based on observations indicating the expression of several proteases (60, 61) and protease inhibitors (62, 63) by stromal cells in different types of cancer. Our study showed a moderate immunostaining of fibroblast-like cells both with anti-uPAR and anti-uPA antibodies, especially those directed against the A chain of uPA. The substantial acid independence of this pattern suggests that, in fibroblast-like cells, the fractional occupancy of uPAR is low and uPA is mainly intracytoplasmic. Normal fibroblasts have been reported to bear uPARs and to secrete uPA if properly stimulated (61). Although the nature of this stimulation remains unclear, it is possible that fibroblast-like cells contribute to the disruption of matrix structures with a paracrine mechanism.

In a recent study on breast carcinoma sections, anti-uPAR antibodies displayed immunoreactivity to epithelial tumor cells only in 8 of 51 cases examined, whereas they definitely reacted in all cases with peritumoral macrophages (40). Although we did not perform double staining for uPAR and macrophage-specific antigens, anti-uPAR antibodies in our study mainly reacted with the majority of cells at the
border of tumor nodules in acid-untreated sections, thus resembling the staining pattern described by Pyke et al. (40). Monocyte and monocytelike U937 cell line were the first cells shown to possess specific binding sites for active uPA and pro-uPA (13, 19). Nykjaer et al. (44) reported that the development of macrophage properties in cultured human monocytes is accompanied by a decrease in affinity and a large increase in the binding capacity of uPAR. Although the contribution of uPARs associated with peritumoral macrophages remains to be defined, our findings in acid-treated sections showed that uPAR expression also occurs in epithelial tumor cells in virtually all cases of breast carcinoma.

Finally, our approach based on previously characterized antibodies binding to free and complexed antigens may provide useful insight into the concerted action of those molecules leading to uPA-dependent proteolysis.

ACKNOWLEDGMENTS

We wish to thank Dr. F. Blasi and Dr. N. Pedersen for the generous gift of anti-uPAR monoclonal antibodies and cell lines. We are also indebted to Dr. P. A. Andreassen for providing anti-uPA antibodies. The authors acknowledge Dr. M. Rambaldi for his help with the photomicrographs and the expert technical assistance of M. Terracciano.

REFERENCES


Tissue Distribution of Soluble and Receptor-Bound Urokinase in Human Breast Cancer Using a Panel of Monoclonal Antibodies

Maria V. Carriero, Paola Franco, Silvana Del Vecchio, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/54/20/5445

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.