Human Urokinase Receptor Concentration in Malignant and Benign Breast Tumors by in Vitro Quantitative Autoradiography: Comparison with Urokinase Levels

Silvana Del Vecchio, Patrizia Stoppelli, Maria V. Carriero, Rosa Fonti, Ornella Massa, Pei Yong Li, Gerardo Botti, Maria Cerra, Giuseppe D'Alito, Giuseppe Esposito, and Marco Salvatore

ABSTRACT

We measured the tissue concentration of human urokinase receptor (uPAR) in 22 breast carcinomas and 9 benign breast lesions using in vitro quantitative autoradiography. Tissue sections were incubated with increasing concentrations of 125I-pro-urokinase in the presence or absence of unlabeled competitor. Breast carcinomas were found to contain 5 times more uPAR than benign breast lesions with respect to their protein content (523 ± 72 versus 108 ± 20 (SE) fmol/mg (P < 0.001)). Simultaneous quantitation of urokinase (uPA) by immunoenzymatic assay on tissue extracts from the same specimens showed that breast carcinomas also contain 19 times more uPA than benign tumors (611 ± 134 versus 32 ± 8 fmol/mg) (P < 0.01). The reliability of quantitative autoradiography measurements was confirmed by uPAR cross-linking assay on membrane fraction from either U937 histiocytic lymphoma cells or breast carcinomas and immunoperoxidase staining with an anti-uPAR antibody on tumor sections. Also, immunoperoxidase staining with an anti-uPA monoclonal antibody showed that uPA is indeed localized on the plasma membrane of epithelial tumor cells in confined areas of breast carcinomas whereas cells from benign breast lesions were devoid of uPA under the same experimental conditions. In conclusion, our findings support the hypothesis that uPAR plays a central role in the acquisition of an invasive phenotype and favor its potential use as a prognostic factor in patients with breast carcinoma.

INTRODUCTION

uPA is one of the two plasminogen activators which convert plasminogen to plasmin, a trypsin-like enzyme of broad specificity. An overexpression of uPA has been reported for many malignant tumors including lung, breast, and colon cancer (1–3). Because uPA can degrade directly or indirectly all components of the extracellular matrix, it has been hypothesized that this enzyme plays an important role in tumor invasion and metastasis. Previous studies have shown that antibodies specific for urokinase can inhibit metastatic dissemination of tumor cells in animal models (4, 5). Furthermore, in vitro invasion assays on cultured human tumor cells demonstrated that uPA activity is essential for the invasive phenotype of these cells (6, 7). Urokinase is secreted as an inactive single chain protein (pro-urokinase or pro-uPA) of 411 amino acids (8). A proteolytic cleavage generates the active, two chain form of uPA (9) which also reacts with plasminogen activator inhibitors (10). The expression of urokinase in cultured tumor cells is modulated by effectors that are also able to promote cell proliferation, including hormones (11), and growth factors, such as epidermal growth factor (12), fibroblast growth factor (13), transforming growth factor β (14), or serum (15). Furthermore, urokinase itself was found to elicit a direct mitogenic effect in certain human tumor cell lines (16) and to activate latent growth factors directly (17) or through a plasmin-mediated mechanism (14). A specific cell surface receptor for uPA (uPAR) has been described on human peripheral blood monocytes and the monocyte-like U937 cells (18, 19). uPA and pro-uPA bind with the same affinity to uPAR (Kd range, 0.1–2 nM) (20). The receptor binding site has been shown to be located in the M, 17,000 ATF in the so-called “growth factor-like” domain of uPA (19, 20). High affinity binding sites for uPA have been described on the surface of several cultured tumor cells (21) and in membranes from human breast carcinoma (22). An autocrine saturation of uPAR receptors occurs in cultured tumor cells overexpressing uPA (23) which may serve to focus the proteolytic activity of uPA at the cell membrane thus enhancing uPA-mediated invasion. Ossowski et al. (24) showed that the mere production of high levels of uPA in the absence of surface receptors was inadequate in endowing a tumor cell with maximal invasive potential. Also, extracellular matrix degradation and invasion by cultured tumor cells have been shown to be dependent on the number and fractional occupancy of surface uPA receptors (25, 26). In agreement with these findings several authors showed an enhancement of plasminogen activation catalyzed by receptor-bound uPA as compared to uPA in solution (27, 28).

To test whether uPAR overexpression is part of the invasive machinery of tumor cells, we determined the intratumoral distribution and concentration of urokinase receptors in histological sections of human breast carcinomas and benign breast lesions using in vitro quantitative autoradiography. This technique was chosen because it preserves tissue anatomy and provides reliable quantitative data. In addition, both the distribution and local concentration of urokinase receptor can be related to the structural components of the tumor, including viable tumor cells, stroma, vascular structures, leading edge of the tumor. Moreover, to determine the uPAR/uPA molar ratio with respect to some biological characteristics of the tumor, such as invasiveness and proliferation, urokinase and pro-urokinase concentration was assessed on tumor extracts obtained from the same specimens analyzed by quantitative autoradiography. The tissue concentrations of uPA and uPAR were then compared with the previously established prognostic factors of breast carcinomas, such as tumor size, lymph node involvement, histology, hormone receptor status, and other factors, such as the rate of proliferation and neovascularization.

MATERIALS AND METHODS

Cell Culture. U937 histiocytic lymphoma cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum at 37°C, 5% CO2. HT1080 fibrosarcoma cells were grown in Dulbecco's modified Eagle's medium-10% fetal bovine serum. 125I-Labeling Procedure and Quality Control. Human recombinant pro-urokinase and the recombinant amino-terminal fragment of human urokinase were gifts of Dr. F. Blasi, Milan, Italy. Pro-uPA was radiiodinated with 125I (Sorin Biomedica, Saluglia, Italy) using the Iodo-Gen method (29). One nmol of protein was reacted with 800 μCi of Na125I and 12 μg of Iodo-Gen (Pierce, Rockford, IL). After 10 min the reaction was stopped by the addition of 1 μmol of N-acetyltyrosine (Sigma Chemical Co, St. Louis, MO). The radiolabeled protein was purified from unbound iodide by Sephadex G-25 (Pharmacia, Uppsala, Sweden) chromatography. The mean percentage of 125I incorporation was 75%. The protein concentration of the radiolabeled product was determined by an enzyme immunoassay (American Diagnostica, Inc., Greenwich, CT) in which recombinant pro-uPA is used as standard. The specific activity of

Received 1/21/93; accepted 4/27/93.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by Associazione Italiana Ricerca Cancro and grants from Consiglio Nazionale delle Ricerche, P.P. A.C.R.O. and P.P. B.T.B.S.

2 To whom requests for reprints should be addressed.

3 The abbreviation used are: uPA, urokinase plasminogen activator; uPAR, urokinase plasminogen activator receptor; ATF, amino-terminal fragment; PBS, phosphate-buffered saline; MoAb, monoclonal antibody.
the product was 14.07 ± 3.18 (SE) μCi/g. The chemical integrity of the radiolabeled pro-uPA was assessed by electrophoresis on a 12.5% sodium dodecyl sulfate-polyacrylamide gel (30). The functional integrity of the radiolabeled protein was assessed by a competitive binding assay on HT1080 fibrosarcoma cells as described previously (23). More than 90% of displacement was obtained in all cases using 0.04 μM cold recombinant pro-uPA and 0.4 μM concentrations of two chain uPA from human kidney cells (Sigma). The same labeling conditions were applied to ATW when used.

Cross-Linking of the uPA Receptor. Extensively ground tumor tissue and U937 cell pellet were homogenized in a hypotonic solution [20 mM Tris-HCl (pH 7.5)-1 mM EDTA-1 mM ethyleneglycol bis(b-aminohexyl ether)-N,N,N',N'-tetraacetic acid-1 mM phenylmethylsulfonyl fluoride-5 μM leupeptin-0.025 TIU/ml aprotinin-25 mM benzamidine]. The lysates were cleared by low speed centrifugation and then subjected to centrifugation at 100,000 x g. The resulting pellet (membrane fraction) was resuspended in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, and stored at −80°C. Membrane fractions from cells or tissue were incubated with 125I-ATF (200,000 cpm) with or without unlabeled competitor and cross-linked with 1 mM disuccinimidyl suberate (Pierce) under conditions described by Nielsen et al. (31). The membranes were then washed twice with an acidic buffer (23) and recovered by centrifugation at 12,000 rpm.

Patients and Tissue Samples. Thirty-one patients undergoing surgery for a breast lump were studied. Twenty-two of them had histologically confirmed breast carcinoma and 9 had benign breast lesions (6 fibroadenomas, 3 fibrocystic disease). These tumors were classified according to the WHO nomenclature (32) and were staged using the tumor-nodes-metastasis system. Nuclear grading was used to define the degree of tumor differentiation (G1, well differentiated; G2, intermediate; G3, anaplasia) according to the classification of Black and Speer (33). The mean age of patients with breast carcinomas was 53.4 ± 10.6 years whereas patients with benign lesions had a mean age of 34 ± 9.8 years. No patient had received previous systemic therapy or preoperative local radiotherapy.

Tumor biopsy specimens were immediately frozen in liquid nitrogen after surgical removal and stored at −80°C until studied. From these specimens, consecutive 8-μm frozen sections were cut in a cryocryomate corresponding to the largest cross-sectional area of the tumor including the leading edge of the lesion. The sections were washed in an acidic buffer (50 mM glycine-100 mM NaCl, pH 3) to remove endogenously bound urokinase. They were then fixed in a solution of 0.25% glutaraldehyde (Sigma) in PBS, pH 7.4, for 20 min and then washed in cold PBS for another 20 min. For histological comparison, some sections were stained with hematoxylin and eosin. Several 5-μm adjacent sections were cut for immunoperoxidase staining.

Tumor specimens were also processed for hormone receptors binding assay and uPA determination.

Saturation Study. Cells were harvested from suspension cultures by centrifugation and washed twice in PBS. The cell pellet was incubated in 20% gelatin (Sigma) and then frozen in liquid nitrogen to form a cell stick (34). Eight-μm frozen sections were then cut in a cryocryomate and processed as the tissue sections.

The tissue sections used to determine the extent of nonspecific binding were preincubated in a humidity chamber (30 min 22°C) with 75 μl of a 20 μM solution of unlabeled urokinase (Sigma) and then washed in cold PBS to remove excess unlabeled ligand. All sections were incubated (30 min 22°C) with PBS containing 2% bovine serum albumin and 10% chicken serum, washed 5 times with cold PBS, and finally incubated in the presence of 0.1 to 4 μM 125I-labeled pro-uPA for 2 h at 22°C. This incubation was performed in the presence and absence of 0.2 μM unlabeled urokinase (Sigma) and was followed by several washes in cold PBS (30 min) and dehydration in 70 and 100% ethanol.

At the end of the saturation assay the chemical integrity of the radiolabeled pro-uPA in solution was checked again by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. No conversion of single chain pro-uPA to two chain uPA was observed.

Quantitative Autoradiography. Quantitative autoradiographic techniques and preparation of autoradiographic standards are described elsewhere (34, 35). Briefly, serial dilution of 125I-labeled human serum albumin were prepared and added to 20% gelatin (Sigma). To determine the amount of radioactivity associated to the standards, aliquots of the gelatin suspension were weighed and counted in a gamma counter. The remainder of each suspension was frozen in liquid nitrogen. Eight-μm sections were cut from each frozen gelatin stick, mounted on glass coverslip, and used as autoradiographic standards. The tissue sections, along with 125I standards, were placed in a film cassette with Kodak SB5 X-ray film. After 2 days of exposure, the film was photographically processed and the resulting images were digitized using an image analysis system (Sistemi Avanzati, Milan, Italy) including a high resolution CCD camera (High Technology Holland) and the Micro Computer Imaging Device (Imaging Research, Inc., Ontario, Canada). The absorbance measurements from autoradiograms were used to reconstruct images in a 512 × 512-pixel matrix. The absorbance readings over the 125I standards were plotted against the respective μCi/g in each standard. A polynomial fitting of these data provided a standard curve. By comparing the autoradiographic images to the images of adjacent sections stained with hematoxylin and eosin, tumor areas were defined and regions of interest were drawn over these areas. The mean absorbance values of the thus selected regions were obtained and the μCi/g of tumor were determined from the standard curve. Using the specific activity of each 125I-pro-uPA preparation, the μCi/g values were converted to pmol of ligand bound/g of tissue.

For each tumor the nonspecific binding curve was subtracted from the total bound activity so that the specific binding curve was generated. In all cases, the specific binding curve reached a plateau corresponding to the maximal amount of 125I-pro-uPA that can specifically bind to tumor or Bmax. The dissociation constant (Ks) of the pro-uPA/uPAR interaction was calculated as the concentration of 125I-pro-uPA that reduces the Smax by 50%.

Tissue Extraction and uPA Determination. In order to quantitate urokinase and pro-urokinase contained in each tumor biopsy specimen, 200–300 mg (wet weight) of tumor were cut in small pieces and pulverized in a Mikro-Dismembrator (Braun, Melsungen, Germany) set to 1 min at maximal power. The resulting powder was suspended in 1:10 (w/v) extraction buffer (36) containing 0.075 M potassium acetate, 0.3 M NaCl, 0.1 M l-arginine, 10 mM EDTA, and 0.25% Triton X-100 (pH 4.2) and homogenized for 5 min in ice cold Potter tubes. Aliquots of crude homogenate were taken for determination of total protein content. Ultra centrifugation was then performed at 100,000 x g for 45 min at 4°C. Supernatants were collected, divided in aliquots, and kept at −80°C.

Determination of total protein content was performed on 20-μl aliquots of crude homogenate by adding 200 μl of a solution of 0.2 N NaOH. After heating to 85°C for 10 min and neutralizing with HCl, samples were assayed for protein content by the method of Bradford (37). A commercially available enzyme-linked immunosorbent assay kit (American Diagnostica) was used for determination of tissue-associated uPA levels. Supernatants were diluted from 1:10 to 1:50 with a dilution buffer and assayed. The results were expressed as pmol/g of wet tissue and fmol/mg of protein.

Estrogen and progesterone receptors were measured using the dextran coated technique according to the European Organization for Research and Treatment of Cancer recommended methods (38).

Immunoperoxidase Staining. Immunoperoxidase staining was performed using MoAb 3936 (American Diagnostica), an IgG2a that binds to soluble and membrane-associated uPAR (39). Tumor sections were processed with a slight modification of previously described standard procedure (40) using 3-amino-9-ethylcarbazole as chromogen. They were given a preliminary wash for 3 min with an acidic buffer to remove endogenously bound uPA and then incubated overnight at 4°C with MoAb 3936 (20 μg/ml). To demonstrate the specificity of uPAR staining, MoAb 3936 was preadsorbed with recombinant native soluble form of uPAR from concentrated conditioned medium of LB6 recombinant native soluble uPAR-producing clone generously provided by Dr. F. Biasi and described previously (41). Under these conditions a strong reduction of the staining was obtained.

The intranuclear localization of uPA was assessed by MoAb 394 (American Diagnostica) which is specific for the B chain of human uPA and recognizes all known forms of human uPA including receptor bound uPA. Tumor sections were incubated overnight at 4°C with MoAb 394 at 20 μg/ml. The intensity of the chromogenic reaction was graded from 0 to 4 plus.

The rate of proliferation of tumor cells was evaluated using the monoclonal antibody Ki67 (Dako, Copenhagen, Denmark) directed against a nuclear antigen present in cells which are in G1, S, G2, or M phases of the cell cycle. Quiescent G0 cells are not recognized by this antibody (42). The sections were examined by light microscopy at × 400. Each section was divided in 4 to 10 regions; a minimum of 100 tumor cells were counted in every region excluding degenerated and necrotic areas. The results were expressed as the mean percentage of positively stained tumor cells in a section.
4). After the incubation, the extracts were treated to obtain binding curves. Note that the binding curves obtained from breast carcinoma were comparable to those obtained from U937 cells.

The number and distribution of intratumoral blood microvessels were assessed by staining endothelial cells for factor VIII using the monoclonal antibody FR/86 (Dako). Each section was divided in 4 to 10 regions. Each region was scanned by light microscopy at low power in order to identify the areas with the highest microvessel density. Individual microvesSELs in each area were counted on a × 400 field. The results were expressed as the mean number of microvessels per field in a section.

Statistics. Student’s t test and simple regression analysis were used as appropriate (43).

RESULTS

Saturation Study on Cultured Tumor Cells. In order to optimize the conditions for the saturation assay followed by quantitative autoradiography on tissue sections, we used U937 histiocytic lymphoma cells bearing a known number of uPA receptors. This cell line has been reported to express 10,000 to 20,000 receptors/cell with a Kd ranging between 0.18 and 1 nm (28, 44). Frozen sections from U937 cell stick to uPAR in extracts of U937 cells and breast carcinoma. Membrane fractions from U937 cells (40 μg; Lanes 5 and 6) and breast carcinoma (120 μg; Lanes 1–4) were incubated in 50 mN 4-(2-hydroxyethyl)-l-piperazineethanesulfonic acid, pH 7.5, with 125I-ATF in the absence (Lanes 1 and 5) and in the presence of 0.1 μM unlabeled pro-uPA (Lanes 2 and 6) or two chain uPA (Lane 3) or ATF (Lane 4). After the incubation, the extracts were treated with disuccinimidyl suberate, dissolved with loading buffer and analyzed by a 12.5% polyacrylamide gel under reducing conditions followed by autoradiography. Arrow, M, 68,000 complex between ATF and uPAR from tumor tissue; right ordinate, position of the molecular weight marker proteins. K, thousands.

Quantitation of Tumor-associated uPAR and uPA. Table 1 summarizes the clinical data, pathological findings, and receptor status of patients with breast cancer. All patients underwent radical surgery except patient 6 who could not undergo axillary node dissection because of impaired left ventricular function.

Frozen sections from tumor biopsy specimens were processed in the saturation study and bound radioactivity was measured by quantitative autoradiography. Fig. 1B shows representative binding curves obtained from Cryostat sections of U937 cells. These binding curves were comparable to those obtained from the study performed on frozen sections of U937 cells. Endogenous uPA levels were also determined by enzyme-linked immunosorbent assay on tumor extracts from the same specimens.

Tables 2 and 3 report the tissue concentration of uPAR determined by quantitative autoradiography and uPA levels measured by immunoenzymatic assay in breast carcinomas and benign lesions, respectively. The results were normalized for both tumor weight (pmol/g) and total protein content (fmol/mg). In breast carcinomas the Bmax
ranged between 89 and 1452 fmol/mg with a mean of 523 ± 72 fmol/mg. In benign breast lesions the B_{max} ranged between 35 and 234 fmol/mg with a mean of 108 ± 20 fmol/mg (Fig. 3). There was a significant statistical difference between the two groups (P < 0.001). On the contrary the average Kd values calculated for the two groups were similar (1.9 ± 0.9 nm for breast carcinomas, 1.2 ± 0.9 nm for benign lesions).

Also, breast carcinomas contained 19 times more uPA than benign lesions (611 ± 134 fmol/mg versus 32 ± 8 fmol/mg) (P < 0.01) as assessed by immunoenzymatic assay on tissue extracts (Fig. 3). Simple regression analysis showed that the tissue concentration of uPAR is significantly correlated to the tissue concentration of uPA (r = 0.62, P < 0.002) in breast carcinomas. The mean molar ratio between uPAR and uPA was 0.85 for breast carcinomas. This ratio was inverted in benign lesions which contained 3 times more uPAR than uPA.

There was no significant difference in uPAR tissue concentration and uPA levels between patients with lymph node involvement and patients without lymph node metastasis. Neither uPAR nor uPA correlated with patient age, tumor size, nuclear grade, or estrogen or progesterone receptor levels.

Analysis of regional distribution of uPAR showed 3 different autoradiographic patterns depending on the type of tissue analyzed. In breast carcinomas the distribution of uPAR was quite heterogeneous. Regions of high concentration were interspersed with areas of low concentration. A comparison of autoradiographic and histological images showed that in most of the cases, uPA receptors were mainly concentrated in areas of high cellularity. These areas were often located at the periphery of the tumor (Fig. 4A), although in a number of instances they were also found scattered throughout the tumor section. In fibroadenomas the uPAR concentration was diffuse and homogeneous (Fig. 4B) and again corresponding to cellular elements in the sections. However, the density of uPARs was significantly lower than in malignant lesions. In fibrocystic breast disease the uPAR distribution showed a positive and heterogeneous pattern of staining (Fig. 4C).

A confirmatory experiment was performed by staining sections of breast carcinomas with an anti-uPAR MoAb. Although the intensity of the chromogenic reaction varied in different tumors, all breast carcinomas showed a positive and heterogeneous pattern of staining (Fig. 4A). The most intense positivities were found on the membrane of epithelial tumor cells protruding into the tumor stroma with an aligned pattern of growth or at the periphery of large clusters of tumor cells. Weak and diffuse staining was also observed in the tumor stroma along with scattered positive fibroblast-like cells. All benign breast lesions were negative for immunoperoxidase staining with anti-uPAR MoAb except for occasional and rare focal positivity of ductal epithelial cells (Fig. 5B).

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yrs)</th>
<th>Histology</th>
<th>Pathological stage</th>
<th>Nuclear grade</th>
<th>Size (cm)</th>
<th>uPAR fmol/mg</th>
<th>uPA fmol/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>59</td>
<td>Lobular</td>
<td>T1N</td>
<td>Mo</td>
<td>2</td>
<td>&gt;5</td>
<td>109</td>
</tr>
<tr>
<td>2</td>
<td>59</td>
<td>Ductal</td>
<td>T1N</td>
<td>Mo</td>
<td>3</td>
<td>3.8</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>60</td>
<td>Lobular</td>
<td>T1N</td>
<td>Mo</td>
<td>1</td>
<td>2.3</td>
<td>119</td>
</tr>
<tr>
<td>4</td>
<td>66</td>
<td>Ductal</td>
<td>T1N</td>
<td>Mo</td>
<td>2</td>
<td>4</td>
<td>262</td>
</tr>
<tr>
<td>5</td>
<td>61</td>
<td>Lobular</td>
<td>T1N</td>
<td>Mo</td>
<td>1</td>
<td>2</td>
<td>87</td>
</tr>
<tr>
<td>6</td>
<td>69</td>
<td>Lobular</td>
<td>T1N</td>
<td>Mo</td>
<td>2</td>
<td>2.2</td>
<td>93</td>
</tr>
<tr>
<td>7</td>
<td>47</td>
<td>Mucinous</td>
<td>T1N</td>
<td>Mo</td>
<td>1</td>
<td>2.5</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>48</td>
<td>Lobular</td>
<td>T1N</td>
<td>Mo</td>
<td>2</td>
<td>&gt;5</td>
<td>12</td>
</tr>
<tr>
<td>9</td>
<td>39</td>
<td>Ductal</td>
<td>T1N</td>
<td>Mo</td>
<td>2</td>
<td>1.3</td>
<td>17</td>
</tr>
<tr>
<td>10</td>
<td>62</td>
<td>Lobular</td>
<td>T1N</td>
<td>Mo</td>
<td>1</td>
<td>2.4</td>
<td>882</td>
</tr>
<tr>
<td>11</td>
<td>52</td>
<td>Ductal</td>
<td>T1N</td>
<td>Mo</td>
<td>1</td>
<td>1.7</td>
<td>98</td>
</tr>
<tr>
<td>12</td>
<td>36</td>
<td>Ductal</td>
<td>T1N</td>
<td>Mo</td>
<td>2</td>
<td>2</td>
<td>28</td>
</tr>
<tr>
<td>13</td>
<td>51</td>
<td>Ductal</td>
<td>T1N</td>
<td>Mo</td>
<td>1</td>
<td>1.5</td>
<td>58</td>
</tr>
<tr>
<td>14</td>
<td>62</td>
<td>Ductal</td>
<td>T1N</td>
<td>Mo</td>
<td>2</td>
<td>4</td>
<td>13</td>
</tr>
<tr>
<td>15</td>
<td>40</td>
<td>Ductal</td>
<td>T1N</td>
<td>Mo</td>
<td>3</td>
<td>3.4</td>
<td>3</td>
</tr>
<tr>
<td>16</td>
<td>56</td>
<td>Ductal</td>
<td>T1N</td>
<td>Mo</td>
<td>3</td>
<td>2.3</td>
<td>7</td>
</tr>
<tr>
<td>17</td>
<td>51</td>
<td>Ductal</td>
<td>T1N</td>
<td>Mo</td>
<td>2</td>
<td>3.4</td>
<td>ND</td>
</tr>
<tr>
<td>18</td>
<td>38</td>
<td>Ductal</td>
<td>T1N</td>
<td>Mo</td>
<td>3</td>
<td>3.2</td>
<td>ND</td>
</tr>
<tr>
<td>19</td>
<td>45</td>
<td>Lobular</td>
<td>T1N</td>
<td>Mo</td>
<td>2</td>
<td>3</td>
<td>75</td>
</tr>
<tr>
<td>20</td>
<td>71</td>
<td>Ductal</td>
<td>T1N</td>
<td>Mo</td>
<td>3</td>
<td>3</td>
<td>40</td>
</tr>
<tr>
<td>21</td>
<td>41</td>
<td>Ductal</td>
<td>T1N</td>
<td>Mo</td>
<td>2</td>
<td>2</td>
<td>212</td>
</tr>
<tr>
<td>22</td>
<td>65</td>
<td>Ductal</td>
<td>T1N</td>
<td>Mo</td>
<td>2</td>
<td>2</td>
<td>212</td>
</tr>
</tbody>
</table>

* fmol/mg of protein in cytosol.

b ER, estrogen receptor; PgR, progesterone receptor; TNM, tumor-nodes-metastasis classification; ND, not determined.
breast lesions were unreactive to MoAb 394 except for rare and dotted positivities in ductal epithelial cells (Fig. 6B).

**Rate of Proliferation and Neovascularization.** Since uPAR is associated to the highly cellularized areas of the tumor we determined the rate of proliferation of tumor cells using the monoclonal antibody Ki67 directed against a nuclear antigen expressed by cells in proliferation. Table 4 reports the percentage of tumor cells that are in a proliferative state as assessed by staining with MoAb Ki67 and the number of microvessels per field assessed by staining endothelial cells for factor VIII. Simple regression analysis showed a significant positive correlation between tumor concentration of uPA determined by enzyme-linked immunosorbent assay (pmol/g) and rate of proliferation of malignant tumor cells \((r = 0.55, P < 0.01)\). However, the percentage of tumor cells in a proliferative state was not correlated to tissue concentration of uPAR determined by quantitative autoradiography. Also, neither uPAR nor uPA appeared to be correlated to the number of microvessels per field.

**DISCUSSION**

The present study reports both the tissue concentration and the distribution of uPAR in 22 breast carcinomas and 9 benign breast lesions by in vitro quantitative autoradiography. The concentration of tumor-associated uPA was also determined by enzyme-linked immunosorbent assay on tumor extracts. We found that the expression of uPAR and uPA is coordinately enhanced in breast carcinomas as compared to benign breast lesions. Malignant tumors contained 5 times more uPAR and 19 times more uPA than benign tumors.

Interestingly, an equimolar ratio was found between the mean content of uPAR and the mean level of uPA in those tumors. If the concentration of uPAR and uPA is expressed in terms of pmol/g of wet tissue, i.e., nmol/liter, we can apply the well known binding equation:

\[
\frac{B}{F} = \frac{B_{\text{max}}}{K_d} - \frac{B}{K_d}
\]

Assuming that the ligand and its specific receptors can freely interact, we can predict 70% of the receptor binding sites be occupied in breast carcinomas whereas the theoretical value for occupancy in benign breast lesions is only 21%. On this basis, breast carcinomas contain 10 times more receptor-bound uPA than benign breast lesions. Although immunostaining with anti-uPA MoAb 394 could not provide quantitative data on the fractional occupancy of uPAR, it confirmed that in breast carcinomas uPA is heavily concentrated on the membranes of tumor cells whereas cells from benign lesions were reproducibly devoid of uPA.

As mentioned before, uPA catalyzed plasminogen activation on the cell surface is much more efficient than that occurring in solution (27, 28). The 10-fold increase in the concentration of receptor-bound uPA, which we estimated in breast carcinomas, may then result in a potent self-amplification of the proteolytic system on the surface of malignant tumor cells. Functionally active plasmin generated on the plasma membrane of tumor cells may allow them to cross basement membranes, capillary walls, and interstitial connective tissues and to spread to other organs or tissues. The increased expression of uPA in malignant breast tumors has already been reported (45). Our data show that uPA and uPAR are coordinately overexpressed in breast carcinomas supporting the hypothesis that receptor-bound uPA is required for the

**Table 3 Tissue concentration of uPAR determined by quantitative autoradiography and uPA levels determined by immunoenzymatic assay in benign breast lesions**

<table>
<thead>
<tr>
<th>uPAR (pmol/g)</th>
<th>uPA (fmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.80</td>
</tr>
<tr>
<td>2</td>
<td>4.74</td>
</tr>
<tr>
<td>3</td>
<td>1.20</td>
</tr>
<tr>
<td>4</td>
<td>2.20</td>
</tr>
<tr>
<td>5</td>
<td>2.58</td>
</tr>
<tr>
<td>6</td>
<td>4.14</td>
</tr>
<tr>
<td>7</td>
<td>2.86</td>
</tr>
<tr>
<td>8</td>
<td>2.84</td>
</tr>
<tr>
<td>9</td>
<td>2.40</td>
</tr>
</tbody>
</table>

* pmol/g of wet tissue.

<table>
<thead>
<tr>
<th>uPAR (pmol/g)</th>
<th>uPA (fmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>172</td>
</tr>
<tr>
<td>2</td>
<td>136</td>
</tr>
<tr>
<td>3</td>
<td>35</td>
</tr>
<tr>
<td>4</td>
<td>73</td>
</tr>
<tr>
<td>5</td>
<td>90</td>
</tr>
<tr>
<td>6</td>
<td>98</td>
</tr>
<tr>
<td>7</td>
<td>67</td>
</tr>
<tr>
<td>8</td>
<td>234</td>
</tr>
<tr>
<td>9</td>
<td>72</td>
</tr>
</tbody>
</table>

* fmol/mg of protein in crude homogenate.

**Fig. 3. Tissue concentration of uPAR determined by quantitative autoradiography and uPA levels measured by immunoenzymatic assay in 22 malignant (M) and 9 benign (B) tumors of the breast. Bars, mean values. Statistical difference: uPAR concentration in malignant versus benign tumors, \(P < 0.001\); uPA concentration in malignant versus benign tumors, \(P < 0.01\).**
metastatic cascade. In agreement with our findings, uPA activity has been reported to be significantly associated with an increased relapse rate in patients with breast carcinoma (45, 46). The lack of correlation between uPA/uPAR expression and lymph node involvement shown in the above mentioned studies as well as in ours suggests that tumor-associated uPA may be mainly involved in the metastatic process occurring directly through blood vessels rather than in lymphatic dissemination. There is indeed experimental evidence implicating that uPA is an essential factor in tumor cell intravasation (47).

uPAR was heterogeneously distributed within breast carcinomas without a clearcut preferential location at the periphery of the tumor. However, when uPAR localization was studied at cellular level by immunoperoxidase staining, the most intense staining was found in malignant cells located in areas of the tumor that, from histological criteria such as pattern of growth or stroma infiltration, appeared to be invasive.

In vitro quantitative autoradiography has been extensively used to measure the binding parameters of hormones and neurotransmitters (48) to receptor sites and to quantitate tumor-associated antigens in histological sections of solid tumors (34). To the best of our knowledge, ours is the first study in which quantitative autoradiography has been used to measure receptors involved in the mechanism of tumor invasion and metastasis. The reliability of autoradiographic measurements was confirmed testing tumor cells bearing a known number of
receptor binding sites, by cross-linking experiments, and by immunoperoxidase staining with several MoAbs. Previous studies on membranes from breast carcinomas reported an uPAR concentration of 250 fmol/mg of protein (22) whereas we found a mean uPAR concentration of 523 fmol/mg of protein. This difference either could be due to a different extraction procedure or reflect the presence of a natural soluble form of uPAR in different compartments of the tumor.

A significant positive correlation was found between tissue concentration of uPA, determined by immunoenzymatic assay on tumor extracts, and rate of proliferation of malignant tumor cells as assessed by immunostaining with MoAb Ki67. This result was not totally unexpected since several growth factors, such as epidermal growth factor, fibroblast growth factor, platelet-derived growth factor, and colony-stimulating factor 1, besides their ability to elicit DNA synthesis, were shown to stimulate the synthesis of urokinase mRNA in fibroblasts, endothelial cells, and cultured human tumor cells (49). Recently, it has been reported that uPA can directly activate latent growth factors (17) thus contributing to an enhanced proliferative activity. In any case, the lack of correlation between uPAR concentration and rate of proliferation of malignant tumor cells in our study suggests that, if a causal relationship exists between uPA activity and proliferation, uPAR is not involved in transducing a mitogenic signal within the cell. Furthermore, the correlation between uPA levels and rate of proliferation indicates that uPA expression in vivo is likely modulated by effectors similar to those which have been shown to regulate uPA production in cultured human tumor cell lines. It is of interest that the tumor with the highest level of uPA in our study also showed overexpression of the oncogene c-erbB-2 which encodes for a protein highly homologous to epidermal growth factor receptor (data not shown).

Immunoperoxidase staining revealed the presence of uPAR and uPA not only in malignant tumor cells but also in fibroblast-like cells scattered in tumor stroma. It is known that normal fibroblasts bear uPAR and if properly stimulated can secrete urokinase (21). Local
secretion of proteolytic enzymes by fibroblasts might be regulated by factors produced by tumor cells themselves. For instance, Davies et al. (50) have described a polypeptide secreted by transformed cells that induces the production of human plasminogen activator in cultured fibroblasts. Furthermore, many authors reported that fibroblasts isolated from human solid tumors or cocultured with human tumor cells have an increased capacity to synthesize and secrete hydrolases (51, 52). On this basis, the fibroblast-like cells, positively stained for uPAR and uPA in our study, can be considered as helper cells contributing to the disruption of matrix structures. Also, they may participate to the very complex cross-talk which occurs between epithelial tumor cells and stromal cells during tumor development.

In conclusion our study showed that both uPAR and uPA are over-expressed in breast carcinomas as compared to benign breast lesions. On the basis of simultaneous quantitative measurements receptor-bound uPA was estimated to be 10 times more concentrated in malignant tumors than in benign lesions. This may result in an enhanced kinetics of plasminogen activation at the surface of malignant tumor cells thus leading to a solid-phase proteolytic cascade the end point of which is the pericellular degradation of extracellular matrix. Further studies are needed to define the prognostic significance of uPAR concentration and the correlation with the probability of disease-free survival in patients with breast cancer. The technique described here appeared to be particularly suitable for such studies providing quantitative data on uPAR concentration in tumors. Finally the approach that we used in this study, namely the simultaneous determination of the receptor concentration and the levels of its specific ligand, could be applied to other systems involved in the mechanism of tumor invasion and metastasis, thus contributing to clarify the complex network of interactions leading to tumor spread.

ACKNOWLEDGMENTS

We wish to thank Dr. F. Blasi for the generous gift of some monoclonal antibodies and cell lines. We are also indebted to Dr. J. C. Reynolds for many
HUMAN UROKINASE RECEPTOR CONCENTRATION

REFERENCES


Human Urokinase Receptor Concentration in Malignant and Benign Breast Tumors by \textit{in Vitro} Quantitative Autoradiography: Comparison with Urokinase Levels

Silvana Del Vecchio, M. Patrizia Stoppelli, Maria V. Carriero, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/53/13/3198

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, use this link http://cancerres.aacrjournals.org/content/53/13/3198.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.