Relationship of Membrane-bound Tissue Type and Urokinase Type Plasminogen Activators in Human Breast Cancers to Estrogen and Epidermal Growth Factor Receptors

G. K. Needham, S. Nicholson, B. Angus, J. R. Farndon, and A. L. Harris

Department of Surgery [G. K. N., S. N., J. R. F.], Cancer Research Unit [A. L. H.], and Department of Pathology [B. A.], University of Newcastle upon Tyne, Royal Victoria Infirmary, Newcastle upon Tyne NE1 4LP, England

ABSTRACT

The total plasminogen activator (PA) activity and the activities of urokinase type (uPA) and tissue type (tPA) plasminogen activators were measured in 43 primary human breast cancer homogenates. The majority of PA activity was found in the 100,000 × g crude membrane pellets (log mean of 490 milli-IU/mg of protein, +1169, −346), and little PA activity was present in the cytosolic supernatant (log mean of 19 milli-IU/mg of protein, +168, −17).

The activities of total PA and of each type of PA were compared to the estrogen receptor (ER) and epidermal growth factor receptor (EGFR) status of the tumors and to their histological grade. Total PA activity and uPA activity were not significantly different in any group of tumors stratified according to receptor status or tumor grade. Tissue type PA levels, however, were significantly lower in ER-negative compared with ER-positive tumors and in EGFR-positive compared with EGFR-negative tumors (P < 0.01 and < 0.05, respectively). The tPA activity was also related to grade, decreasing with worsening differentiation (P = 0.04).

The ER-negative tumors were further stratified into ER-negative and -positive subgroups. Only the ER-negative tumors possessing EGFR had significantly lower tPA levels than the ER-positive tumors (P < 0.01).

Low tPA levels in breast cancers were, therefore, associated with ER negativity combined with EGFR positivity and may be an indication of poorer differentiation and prognosis.

INTRODUCTION

Plasminogen activators are serine proteases which cleave plasminogen to yield plasmin. Plasmin takes part in extracellular proteolysis, and it is involved in numerous processes featuring tissue remodelling and cell migration (1). Additionally, there is evidence that PA is involved in malignancy: antibodies to uPA inhibited metastasis in a chick embryo model (2) and interfered with cellular changes and matrix degeneration associated with the oncogenic transformation of fibroblasts (3); and uPA gene expression was increased in cancers compared with normal tissue (4).

The two types of PA, uPA and tPA, differ in molecular weight, immunological reactivity, and function. They have been shown to be products of separate genes (5), and their synthesis is regulated independently (6, 7). The presence of fibrin is required to achieve the full activity of tPA and tPA binds to fibrin with high affinity (8). The localization of tPA in vessel walls (9) and its secretion by endothelial cells in culture (10) suggested that it had an important role in intravascular fibrinolysis. Urokinase type plasminogen activator is less influenced by the presence of fibrin and is thought to be primarily involved in tissue remodelling and cell migration (1). Both types of PA have been identified within breast cancers and normal tissue (11), and increased uPA activity was found in cancers compared with normal tissues (12, 13).

Regulation of PA synthesis is hormonally influenced in several cancer cell lines. Estradiol stimulated secretion of PA in MCF-7 (14), ZR-75-1 (15), and UCT-Br-1 (16) breast cancer cell lines. Mira-y-Lopez and Ossowski (17) observed hormonal modulation of PA secretion in human breast tumor explants and showed that the presence of both ER and progesterone receptors was necessary for increased PA secretion to occur upon treatment with estradiol. EGF stimulated PA secretion in HeLa (18) and A431 (19) epidermoid cancer cell lines. Estragon receptor concentration was shown to be correlated with PA activity in human breast cancers (20–22), but the relationship between EGFR and PA in human tumors has not previously been investigated. Sainsbury et al. (23) showed that the presence of EGFR in human breast cancers was associated with poor prognosis.

Both types of PA are strongly bound to cells, although they also exist in solution in body fluids. The cell-bound form of PA may be important in the localization of extracellular proteolysis to the immediate vicinity of the cell surface. Natural PA inhibitors are unable to neutralize PA when it is membrane bound (24, 25). A recent animal study suggested that membrane-associated PA was more important than cytosolic PA in determining metastatic potential of tumor cells (26). A receptor has been described which linked uPA to the plasma membrane (27), and we recently reported its presence in human breast cancers (28).

Because of this evidence that the 2 types of PA are separately regulated by hormones and that membrane-associated PA may be of biological significance, we have investigated the occurrence of PA in membrane preparations from 43 human breast cancers and have studied its relationship to both ER and EGFR status and histological grade.

MATERIALS AND METHODS

Materials

Human breast cancers were collected fresh from the operating theater and were stored at −20°C in 0.25 mol/liter of sucrose:1.5 mmol/liter of magnesium chloride:10 mmol/liter of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid in 50% glycerol buffer, pH 7.4. Routine histological examination was performed on adjacent blocks of tumor tissue.

Human thrombin, murine epidermal growth factor (receptor grade), and bovine fibrinogen (96% clottable) were purchased from Sigma. Human plasminogen (KABI Vitrum) was purchased through Flow Laboratories, Irvine, Scotland. Tritiated estradiol and [125I] were obtained from Amersham International, United Kingdom. The neutralizing antibodies against uPA and tPA were polyclonal goat IgG (Biopool AB; Umea, Sweden). The international reference preparation of urokinase was kindly supplied by Dr. Gaffney, National Institute of Biological Standards and Control, Hampstead, London, United Kingdom, and...
this was used to standardize the PA assays. Falcon 24-well plates were used for the PA assay.

Methods

Membrane Preparation

Membranes were prepared as described previously (28). Briefly, tumor was thawed, diced, and homogenized in 10 mmol/liter of Tris:50 mmol/liter of NaCl buffer, pH 7.4, using an Ultra Turrax homogenizer. The homogenate was centrifuged at 100 x g for 45 min, and the supernatant was separated and recentrifuged at 100,000 x g for 45 min. This yielded a membrane pellet which was resuspended in buffer and a cytosolic supernatant. These were stored at -20°C until used in EGFR or PA assays. This method concentrated 5'-nucleotidase activity in the membrane fraction by 5- to 8-fold (data not shown).

Radiolabeling of Proteins. The iodogen method (29) was used to label EGF and fibrinogen with 125 I. One hundred μg of fibrinogen or 10 μg of EGF were incubated with 0.5 to 1 mCi of 125I in a tube coated with iodogen (Pierce) for 12 min. Unreacted iodine was removed by chromatography on G-25 Sephadex, and the 125I-EGF was further purified on a G-50 Sephadex column. Typical specific activities were 80 μCi/μg of EGF and 4 μCi/μg of fibrinogen. No loss of biological activity was observed following iodination.

PA Assay. The labeled fibrin plate method was used (30). Thirty μg of labeled fibrinogen (200,000 cpm) were placed in each well of a 24-well plate and dried at 37°C for 2 days. Fibrinogen was converted to fibrin immediately before the assay by incubating thrombin solution (10 units/ml) in the wells for 2 h. Eighty-five % of the fibrinogen bound to the wells as fibrin, and unbound counts were removed by 3 washes with 0.1 mol/liter of Tris-HCl buffer, pH 8.1.

Samples were placed in the wells with 2 μg of plasminogen and 40 μg of acid-treated bovine serum albumin. The final volume was made up to 400 μl with 0.1 mol/liter of Tris-HCl buffer, pH 8.1. The amount of plasminogen used in this assay was not rate limiting. The plates were incubated at 37°C for 1 h, after which 100 μl aliquots were removed for counting in a gamma counter. Conversion of plasminogen to plasmin occurred at a rate determined by the amount of PA present, and the plasmin degraded the radiofibrin to liberate labeled fibrinopeptides into solution. The number of cpm released was expressed as a percentage of the total number of cpm per well (estimated as the number of cpm released by 1% trypsin treatment of a radiofibrin-coated well).

Each sample was assayed in triplicate. Samples were incubated in the absence of plasminogen to estimate the plasminogen-independent proteolysis, and this was typically less than 3% of the total cpm per well. Proteolysis due to plasmin in the plasminogen preparation was also low (2.5% total cpm per well). Standard curves were prepared using the international reference preparation of urokinase. Detergent was not added because it was intended to measure membrane-bound PA activity.

PA Quenching by Antibodies. To measure tPA and uPA separately, 10-μl membrane samples were preincubated for 1 h at 4°C alone or with 10 μl of anti-uPA or 10 μl of anti-tPA antibodies (1.25 mg/ml). The concentration of antibody used was sufficient to neutralize the activity of at least 10 times the highest expected amount of its appropriate type of PA (Fig. 1). No cross-reaction was observed between uPA and anti-tPA or vice versa. The sum of uPA and tPA activities was appropriate type of PA (Fig. 1). No cross-reaction was observed between uPA and anti-tPA or vice versa. The sum of uPA and tPA activities was always close to the total PA activity measured without antibodies.

Estrogen Receptor Assay. The dextran-coated charcoal method was used (31). The lower limit of positivity was 5 fmol/mg of cytosol protein. Our laboratory participated in the European quality control scheme for ER assays.

EGFR Assay. A 2-point saturation assay was used. Membrane was incubated with labeled EGF for 2 h alone and in the presence of 100-fold excess of unlabeled EGF. The lower limit of positivity was 10 fmol/mg of membrane protein.

Statistics. The PA activity results were log normally distributed, log transformation was performed, and analysis was by unpaired t test.

Protein Assay. This assay was by the method of Lowry et al. (32).

TABLE 1

<table>
<thead>
<tr>
<th></th>
<th>Range</th>
<th>Log mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosol</td>
<td>1-2,730</td>
<td>19</td>
<td>+168, -17</td>
</tr>
<tr>
<td>Membrane</td>
<td>70-11,000</td>
<td>490</td>
<td>+1,169, -346</td>
</tr>
</tbody>
</table>

Fig. 1. Quenching of tPA and uPA using anti-tPA and anti-uPA IgGs. mIU, milli-IU.

Results

Plasminogen Activator Assay. The assay was linear over the range of 0 to 50 milli-IU of uPA, and samples were diluted so that their activity fell within that range.

Localization of PA Activity. The majority of PA activity resided in the membrane fraction (Table 1). The ratio of membrane-bound to cytosolic PA activity ranged from 1 to 300 to 1 [80 ± 102 (SD)]. In 6 cases, the different subtypes of PA were measured in both the cytosol and the membrane. In each case there was an increase in the membrane fraction, but the relative increase for the subtypes differed from patient to patient.

In all 19 cases the subtypes were measured in the membrane fraction, and total activity, in membrane and cytosol. In 8 of 9 cases where urokinase was the major PA, there was more than a 20-fold ratio of membrane to cytosol PA. In only 1 of 10 cases where tPA was predominant was there more than a 20-fold ratio of membrane to cytosol PA (P < 0.01). This suggests that urokinase is more localized to the crude membrane fraction than is tPA.
PA Activity and ER Status. Neither uPA nor total PA activity differed significantly between ER-positive and ER-negative groups of tumors. However, the tPA activity of the ER-positive group was significantly higher than the ER-negative group \( (P < 0.01) \) (Fig. 2), and the uPA activity expressed as a percentage of total PA activity was lower in ER-positive tumors than ER-negative tumors \( (P < 0.05) \). There was no direct correlation between ER concentration and tPA activity.

PA Activity and EGFR Status. In the same tumors, stratified by EGFR status, tPA activity was significantly lower in the EGFR-positive tumors \( (P < 0.05) \) (Fig. 3). There was no significant difference observed in either total PA or uPA activity between EGFR-positive and -negative groups. The uPA activity expressed as a percentage of total PA activity was significantly higher in the EGFR-positive tumors \( (P < 0.05) \). There was no direct correlation between EGFR concentration and tPA activity.

ER and EGFR Status. The distribution according to ER and EGFR status of the tumors is shown in Table 2. Three of 17 (18%) ER-positive tumors also possessed EGFR, while 10 of 26 (38%) ER-negative tumors had EGFR. These proportions were similar to our previous results in 104 tumors which showed a significant inverse relationship between ER and EGFR status (34).

**Table 2** ER and EGFR status of 43 primary breast cancers

<table>
<thead>
<tr>
<th>ER status</th>
<th>+</th>
<th>–</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR status</td>
<td>–</td>
<td>14</td>
</tr>
</tbody>
</table>

**Table 3** Combined ER/EGFR status PA activity and proportion of uPA as percentage of total PA

<table>
<thead>
<tr>
<th>ER/EGFR</th>
<th>n</th>
<th>Total PA*</th>
<th>tPA*</th>
<th>uPA*</th>
<th>% of uPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>3</td>
<td>1949 + 1247</td>
<td>630 + 3351</td>
<td>251 + 473</td>
<td>34 ± 50</td>
</tr>
<tr>
<td>+/–</td>
<td>14</td>
<td>1995 + 5182</td>
<td>1023 + 3697</td>
<td>724 + 2587</td>
<td>45 ± 27</td>
</tr>
<tr>
<td>–/–</td>
<td>16</td>
<td>933 + 2378</td>
<td>363 + 1415</td>
<td>377 + 1746</td>
<td>49 ± 28</td>
</tr>
</tbody>
</table>

* Log of PA activity in milli-IU/mg of protein (mean ± SD).
*\( P = 0.01 \). +/+ versus –/–
*\( P > 0.05 \). +/+ versus –/–
*\( P = 0.01 \). +/+ versus –/–
*\( P = 0.2 \). +/+ versus –/–
*\( P = 0.01 \). –/– versus –/–

Fig. 2. Total activities of 43 breast cancers arranged according to ER status. NS, not significant; mIU, milli-IU.

Fig. 3. Total activities of 43 breast cancers arranged according to EGFR status. NS, not significant; mIU, milli-IU.

Fig. 4. Stratification of tPA activity arranged according to combined ER and EGFR status. mIU, milli-IU.
PA Activity and Combined ER/EGFR Status. Tumors were further stratified into ER+/EGFR+, ER+/EGFR−, ER−/EGFR+, and ER−/EGFR− groups. While there were no significant differences in total PA or uPA activities between any group and another (Table 3), tPA activity was significantly lower in the ER−/EGFR+ group than in the ER+/EGFR− group (P < 0.01) (Fig. 4). The ER−/EGFR− tumors’ tPA activity, however, did not differ significantly from the ER+/EGFR− group. The uPA activity as a percentage of total PA activity was higher in the ER−/EGFR+ group than in both the ER+/EGFR− and ER−/EGFR− groups (P < 0.01).

PA Activity and Tumor Grade. The tPA activity increased with increasing tumor differentiation (P < 0.05 comparing Grade I with Grade III) (Fig. 5). This trend was not observed for total PA or uPA activity.

DISCUSSION

Plasminogen activator is localized on the plasma membrane where it is relatively protected from the action of PA inhibitors so that plasmin production is likely to be limited to the cell surface. Upon secretion, uPA becomes bound in inactive proenzyme form to its receptor on the plasma membrane. It may be activated by plasmin while bound and, when all receptors become saturated, excess uPA is released into solution where it is vulnerable to activation and subsequent inhibition (35). It is known that tPA is also cell bound (36), and recently a tPA receptor was described on endothelial cells (37).

Although many studies have demonstrated the localization of PA to the plasma membrane in cultured cells (24, 38), none has investigated the membrane association of PA in tumor material. There were theoretical advantages in measuring PA in membrane fractions, namely higher PA concentration and, perhaps, absence of interference from PA inhibitors in the samples. Our results suggest that PA and, particularly, uPA were concentrated in the crude membrane fraction of breast cancers. These preparations, while enriched for plasma membrane, were likely to contain other cellular membranes. Previous studies of PA in breast cancers have generally examined cytosols, often extracted with detergents, rather than membrane fractions (11, 12, 20–22, 39, 40). We found that membrane fractions possessed a higher mean PA activity than those in other series in which PA values were given (11, 20, 39). Membrane-associated PA may have more biological importance than cytosolic PA. In a rat mammary cancer model, the crude membrane from lung metastases contained twice as much PA activity as membrane from primary tumors, although the respective cytosols contained similar PA activities (26).

O’Grady et al. (11) showed that tPA was the predominant form of PA in benign breast tumors and that tPA levels were lower in more advanced malignant breast tumors and recurrences. This suggested that lower tPA activity was associated with poorer prognosis tumors. In this series, total PA and uPA activities were not found to be correlated with ER or EGFR status, either alone or combined. However, tPA activity is significantly higher in ER-positive tumors. This finding is in agreement with those of Duffy et al. (22). Estrogen receptor concentration plotted against tPA did not show a significant correlation. This may have been due to the relatively small number of cases studied. Alternatively, it could suggest that there was variable tPA expression independent of ER concentration. Thorsen (21) showed that higher PA activity was found in progesterone receptor-positive cases, irrespective of ER concentration.

The EGFR-positive tumors had lower tPA levels than EGFR-negative tumors, and this would be expected considering the inverse correlation of ER and EGFR in breast cancers (34). The EGFR has been shown to be associated with poorer histological grades of breast cancers (41) and with early tumor recurrence and patient death (23). The lower tPA activity observed in the ER-negative group was due mainly to the reduced tPA content of the ER-negative/EGFR-positive subgroup, since ER-negative tumors without EGFR had tPA activity which was not significantly different from the ER-positive tumors. The histological grade of the tumors was found to correlate with tPA activity but not with either total PA or uPA activity. Previous studies failed to show any such relationship, perhaps due to use of different grading systems or differences in assay technique (11, 39, 40).

Previous authors have suggested that the higher PA activities of ER-positive tumors might account for their supposed better prognoses by preventing the nidation of metastasizing cells (21). We have shown that total PA activities were similar in all groups of tumors stratified according to receptor status, and therefore this does not support their hypothesis. Because of the complexity of PA regulation in the tissues, the difference in expression of both types of PA may still be relevant to biological behavior. While serum PA inhibitors can affect both types of PA, protease nexin I reacts with uPA 500 times more rapidly than with tPA (42). Tumors expressing predominantly tPA may, therefore, be able to produce more local fibrinolysis, although their in vitro PA activity might not differ from a tumor producing mainly uPA. Our results suggest that EGFR may have a negative effect upon the mechanism of tPA secretion while being involved in the stimulation of uPA secretion.

Measurement of tPA levels in breast cancer was suggested as a means of identifying tumors likely to be responsive to endocrine therapy (20, 22). We have shown that the tumor group
with the lowest content of tPA possessed EGFR in addition to being ER negative. Sainsbury et al. (32) showed that these tumors were associated with shorter disease-free interval and survival than other breast tumor types. Low tPA level may, therefore, be of use in identifying poor prognosis tumors and aid in the selection of patients for more aggressive adjuvant treatment. It may be possible to control tumor behavior through modulation of PA expression. One method might be to block uPA binding to its receptor using peptides analogous to its binding domain (35).

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