Increase of Urokinase-type Plasminogen Activator Gene Expression in Human Lung and Breast Carcinomas

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ABSTRACT

To assess the postulated correlation between plasminogen activators (PAs) and malignancy, we determined the mRNA content for urokinase-type (u-PA) and tissue-type (t-PA) enzymes in a prospective series of 29 primary lung and 27 primary breast carcinomas. Dot blots of total RNAs were hybridized with appropriate cRNA probes under conditions that allow quantitative measurement of the mRNA level for each PA. Most tumors (43 of 56) had a u-PA mRNA content higher than the mean + 1 SD of nonmalignant tissue counterparts. A large, 4- to 20-fold, increase in u-PA mRNA content was demonstrated in 14 of 29 lung carcinomas and in 10 of 27 breast carcinomas.

A statistically significant correlation (Fisher's test, P = 0.007) was found between elevated u-PA mRNA content in lung carcinomas and the presence of regional lymph node metastases. These results are consistent with a role for u-PA in tumor invasiveness and metastatic propensity and may have important prognostic and therapeutic implications.

INTRODUCTION

Lung and breast cancer patients often have regional lymphatic metastases at the time of diagnosis and will subsequently die of disseminated disease (1, 2). Metastasis, the major obstacle to a control of common solid tumors, is generally considered to be a dynamic multistep phenomenon, involving complex interactions between malignant cells and the host (3-5). Degradation of the extracellular matrix is thought to be an early and crucial feature of the metastatic process (4-6).

Neoplastic cells have been shown to express various proteolytic enzymes, which, in animal models, give them invasive properties and favor their dissemination to distant sites (6-8). Two such enzymes, the serine proteases u-PA* and t-PA have been extensively studied in rodent and human malignancies (for reviews see Refs. 9-11). Abundant experimental evidence supports u-PA participation in tumor invasion and metastasis formation: oncogenic viruses and tumor promoters induce synthesis and release of PAs in a variety of cell lines and primary cell cultures (12, 13); in an invading and metastasizing murine lung tumor, u-PA has been localized immunohistochemically in areas of invasive growth and adjacent tissue degradation (14); antibodies which inhibit human u-PA have been shown to interfere with the metastatic propensity of a human tumor cell line transplanted into chick embryos (15). Several enzymatic and immunometric assays performed on human tissues have demonstrated increased amounts of u-PA in tumors, when compared to their nonneoplastic counterparts (for review see Refs. 11, 16-20). Although a correlation between overall fibrinolytic activity and metastasis has been observed in some studies (7, 8, 21), there is no general agreement on this matter (17-20). These discrepancies can perhaps be attributed to the limitations of current biochemical assays, when addressing enzymatic cascades that can be tightly controlled by the local and systemic production of specific protease inhibitors (22, 23).

Materials and Methods

Tissue Sampling. Tumors were collected immediately after surgical removal. Portions free of visible necrosis, fat, or connective tissue were dissected, washed in buffered saline, immersed in liquid nitrogen, and kept at -80°C until analysis. Gynecomastia samples and nonneoplastic tissues were similarly processed. One to 4 of 27 tumors (38%) had metastasized into extrapulmonary lymph nodes.

Histopathological Assessment. According to the WHO classification (24), 16 lung tumors were classified as adenocarcinoma, 11 as epidermoid, and two as large-cell undifferentiated carcinomas. Four to 15 extrapulmonary lymph nodes ( hilar, intrathoracic-bronchial, and mediastinal) obtained with lobectomy or pneumonectomy specimens were analyzed. Eleven of 29 tumors (38%) had metastasized into extrapulmonary lymph nodes.

All breast tumors were invasive intraductal carcinomas. Four to 32 axillary lymph nodes obtained with tumorectomy or modified mastectomy specimens were examined. Axillary sampling was not performed in one patient. Nineteen of 26 patients (73%) had axillary lymph node metastases; 16 of 27 tumors (59%) were considered to be steroid receptor positive, as determined by the dextran-coated charcoal separation method (25).

RNA Extraction. Total cellular RNA was extracted as previously described (26). Briefly, 0.5 g of tissue was homogenized with a Polytron in 0.1 M Tris-HCl (pH 7.4), 0.1 M β-mercaptoethanol, and 4 M guanidium thiocyanate. After addition of solid CsCl (0.4 g/ml), the homogenate was layered onto 1 ml of a 5.7 M CsCl, 0.2 M EDTA (pH 7.0) cushion and centrifuged at 35,000 rpm for 20 h at 20°C. The pelleted RNAs were dissolved in 300 μl of 10 mM Tris-HCl (pH 8.1), 5 mM EDTA, 0.1% sodium dodecyl sulfate, extracted twice with phenol/chloroform, ethanol precipitated, and resuspended in water.

Hybridizations. pSP64-hUK, containing the 610-basepair EcoRl-Pstl fragment isolated from pUKH-8 (27); pSP65-hTA, containing the 614-basepair BglII-EcoRI fragment isolated from pW349F (28); and pSP65-α-A-C, containing the 320-basepair Avall-BglII fragment corresponding to the coding region of rat α-smooth-muscle actin, which hybridizes to all actin mRNAs, were transcribed in vitro using SP6 RNA polymerase (29), with 100 μCi of (32P) labeled GTP (400 Ci/mmole). For Northern-blot hybridizations, RNAs were denatured with glyoxal, electrophoresed in 1.2% agarose gels, and transferred overnight onto Pall
Biodyne A nylon membranes (30). Prehybridization, hybridization, and posthybridization procedures and autoradiography were performed as described (26). After a first hybridization, the probe was eluted from the filters by boiling for 5 min in 20 mM Tris-HCl (pH 8.1); filters were exposed to X-ray films to document complete removal of the first probe and subsequently subjected to further hybridizations.

For dot blot hybridizations (31), RNAs were denatured with formaldehyde, serially diluted with 15×SSC [standard saline citrate (0.15 M sodium chloride:0.015 M sodium citrate, pH 7.4)] into a 96-well microtest plate and applied to nitrocellulose using a Schleicher and Schuell minifold apparatus. Filters were baked, prehybridized, washed, and autoradiographed for a visual evaluation of relative mRNA levels as described (26, 31). Quantitation was obtained by soft-laser densitometric analysis.

With regard to PAs, the HEP-3 (32) and Bowes (28) cell lines produce exclusively u-PA and t-PA, respectively. Thus, analysis of RNA from these cells in parallel with the tumor samples allowed assessment of the specificity of the probes and an absolute measurement of the number of specific PA mRNA molecules in a given sample. From the specific activities and the lengths of the probes we calculated that there are approximately 2.5 pg of u-PA mRNA/μg of total HEP-3 RNA and 2.5 pg of t-PA mRNA/μg of Bowes melanoma RNA.

Total actin mRNA was detected by using pSP65-α-A-C.

Statistical Analysis. Contingency tables were analyzed with Fisher's exact test. Data on u-PA and t-PA mRNA levels in tumors were transformed by log10 before statistical calculation because of their positively skewed distribution. We used Student's t test to compare u-PA mRNA content with the regional lymph node status. Significance was assessed (26).

Fig. 1. Northern blot analysis. All lanes were loaded with 12 μg of RNA. Filters were hybridized with u-PA, t-PA, and α-A-C actin (ΔP) labeled cRNA probes (for breast RNAs, a single filter was subsequently hybridized to the different probes, whereas for lung RNAs three different filters were hybridized in parallel). T, tumor; N, nonmalignant tissue sample from the same patient; numbers, samples also shown on Fig. 2.

RESULTS

All malignant and nonmalignant tissues examined contained single u-PA and t-PA mRNA transcripts as seen by Northern blot hybridization (Fig. 1). The estimated sizes of the 2.5-kilobase u-PA mRNA and the 2.6-kilobase t-PA mRNA transcripts correspond to the sizes reported for other tissues (26, 28, 32). An increase in u-PA mRNA was observed in the majority of tumors. This increase is specific since t-PA does not follow the same pattern and since the actin mRNA content is similar in malignant and in nonmalignant tissues (Fig. 1). Quantitative dot blot hybridization was used to measure u-PA and t-PA mRNA levels in tumors and in nonmalignant tissues and representative examples are shown in Fig. 2.

u-PA, u-PA mRNA levels were significantly higher in carcinomas than in nonneoplastic tissues (Table 1). Of the 56 tumors analyzed, 43 (77%) had an elevated u-PA mRNA content, when

Table 1. u-PA mRNA content of the tumors according to regional lymph node status

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<td>LN—</td>
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</tr>
<tr>
<td>LN+</td>
<td>28.2 (11)</td>
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* Numbers in parentheses, number of samples examined.
* LN—, all examined lymph nodes were free of metastasis; LN+, presence of lymph node metastases.
* Axillary sampling was not performed in one patient.

Fig. 2. Quantitative dot blot analysis of PA mRNA levels. Serial dilutions (2-fold) of total RNA (10, 5, 2.5, and 1.25 μg) were applied to the filters. After hybridization with u-PA (left) or t-PA (right) cRNA probes, filters were exposed for 15 h at −80°C. T, tumor; N, nonmalignant tissue sample from the same patient; F, fibroadenoma; G, gynecomastia; hep, total cellular RNA extracted from the u-PA producing HEP-3 carcinoma cell line (2.5–0.3 μg/dot) (30); mel, total cellular RNA extracted from the t-PA producing Bowes' melanoma cell line (2.5–0.3 μg/dot) (26).

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Fig. 3. PA mRNA levels in tumors and nonmalignant tissues. Values, obtained by densitometric analysis of dot blots as illustrated in Fig. 2, are expressed as densitometric units/10 μg of total RNA. 80 ng of total RNA from the u-PA producing HEP-3 cell line and from the t-PA Bowes’ melanoma cell line give a value of 1 on these relative densitometric scales. F, fibroadenoma; G, gynecomastia; means and medians (± SD) are presented.
compared to the mean + 1 SD of the corresponding nonmalignant tissues. Wide variations were found among individual carcinomas as opposed to the narrow range in nonneoplastic tissues (Figs. 2 and 3).

The median u-PA mRNA content in lung carcinomas was 6-fold higher than in nonmalignant bronchial tissues and 14 of 29 tumors showed a 4- to 20-fold increase in u-PA mRNA content (Fig. 3). The median u-PA mRNA content in breast carcinomas was 4-fold higher than in nonmalignant breast tissues and 10 of 27 breast carcinomas showed a 4- to 15-fold increase in u-PA mRNA content (Fig. 3).

t-PA. Median t-PA mRNA contents in lung carcinomas and in nonmalignant tissues were equivalent (Fig. 3), although nine tumors exhibited increased t-PA mRNA levels, seven with a concomitant increase in u-PA mRNA (see for example tumor 2 in Fig. 2). For breast carcinomas, t-PA mRNA levels were comparable to those in nonmalignant tissues (Fig. 3), with the exception of two tumors, which had elevated t-PA and normal u-PA mRNA levels. We also analyzed one fibroadenoma and two gynecomas. All three specimens displayed t-PA and u-PA mRNA contents comparable to those of nonmalignant breast tissues (Figs. 2 and 3).

Statistical analysis showed a significant relationship between elevated u-PA mRNA content and lymph node involvement for lung carcinomas (t test, P = 0.016). No significant relationship was found for breast carcinomas (Table 1). When the tumors showing a greater than 4-fold increase in u-PA mRNA content were compared to the remaining tumors in a contingency table, high u-PA mRNA content in lung carcinomas was significantly associated with the presence of regional lymphatic metastases (Fisher's exact test, P = 0.007) (Table 2).

In contrast, we found no significant association between size, histological type, and steroid receptor content of carcinomas, and the u-PA or t-PA mRNA levels.

### DISCUSSION

Elevated PA activity has been associated with malignant human tumors in a number of studies, although conflicting results have been reported (for review see Ref. 11). Here we have used a method that evaluates the synthetic potential of a given tissue for u-PA and t-PA.

Our analysis shows that u-PA mRNA content is significantly increased in primary lung and breast carcinomas, when compared to their nonmalignant counterparts. Although nine lung and two breast carcinomas had increased levels of t-PA mRNA, a high t-PA mRNA level without concomitant increased u-PA mRNA was observed in only four tumors. In contrast, u-PA mRNA content was elevated in 43 of the 56 tumors analyzed. These results agree qualitatively with u-PA protein estimations obtained by others (17–20), which showed that lung and breast carcinomas are frequently associated with the production of u-PA, but not t-PA.

The quantitative comparisons of u-PA mRNA content between malignant and nonmalignant tissues reported here differ somewhat from previous estimations of u-PA at the protein level: the mean increase of u-PA enzymatic content in lung tumors over nonmalignant lung tissues was reported to be only 3-fold, with a minority of tumors (6 of 37; i.e., 16%), showing an increase greater than 4-fold (17). In our series, lung carcinomas displayed a mean 6-fold increase in u-PA mRNA levels over their nonmalignant counterparts and 14 of 29 (48%) tumors were associated with a greater than 4-fold increase in u-PA mRNA content. Similarly, the mean PA content in 33 primary breast carcinomas was reported to be only 2-fold higher than in normal tissues, with less than 10% of the tumors having a greater than 4-fold increase (18). The mean increase in u-PA mRNA levels of breast carcinomas reported here was 3-fold and 10 of 27 (37%) tumors had a greater than 4-fold increase in u-PA mRNA content. Differences in methodological approaches can account for these apparent contradictions. For instance the rate of activation of the proenzyme form of u-PA (33), the presence of protease inhibitors (22, 23, 35) as well as the interaction of u-PA with a specific cellular binding site (36), will all influence enzymatic measurements. Analysis of mRNA content avoids potential artefacts in the estimation of u-PA production based on its enzymatic activity and since there is no evidence for translational control of PA expression in somatic cells, mRNA determinations might not only provide complementary information about u-PA synthetic potential in different tissues, but might also be instrumental in uncovering interesting regulatory features of PA control. For instance, we do not know yet if increased u-PA expression is due to enhanced transcriptional activity or to reduced mRNA turnover. In addition, while both protein and RNA determinations do not discriminate the relative contribution of cellular subpopulations within a given tumor, measurements of the concentration of individual mRNA species are not affected by the inherent variations of extracellular components to the total tumor mass.

We found a significant correlation between u-PA mRNA content in lung carcinomas and the presence of regional lymph node metastases. In any case, this is to our knowledge the first report establishing an association between elevated u-PA synthetic potential and lymph node metastases in a human tumor; this finding supports the hypothesis that u-PA catalyzed proteolysis plays a part in the metastatic propensity of malignant cells, by facilitating cell migration via degradation of the extracellular matrix. Although previous studies failed to correlate overall fibrinolytic activity and tumor aggressiveness in vivo (for review see Refs. 10, 11, and 17), this discrepancy might be due to the uncertainties discussed above in estimating u-PA production by enzymatic assays.

The lack of correlation between u-PA gene expression and regional lymphatic spread in breast carcinomas at the time of analysis doesn't preclude participation of this enzyme in the metastatic process of these tumors. Indeed regulatory mechanisms are expected to affect the expression of PA genes in breast tissues: steroid hormones are known to regulate mammary production of PAs (37) and were recently found in two mammary cell lines, including one derived from a carcinoma, to differentially modulate u-PA and t-PA gene expression (26, 38). In addition, PA production in breast tissues might be influenced by cytokines involved in growth control, such as EGF and basic fibroblast growth factor. Aggressive breast carcinomas have been shown to express EGF receptors (39) and an EGF-induced increase in u-PA mRNA content has been demonstrated in a tumor cell line expressing EGF receptors.

### Table 2: Distribution of lung tumors according to u-PA mRNA content and lymph node status

<table>
<thead>
<tr>
<th>U-PA mRNA content</th>
<th>LN+</th>
<th>LN-</th>
</tr>
</thead>
<tbody>
<tr>
<td>u-PA T &gt; 4 x N‡</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>u-PA T T &lt; 4 x N</td>
<td>2</td>
<td>13</td>
</tr>
</tbody>
</table>

*LN+ and LN−, see Table 1, legend.

‡ u-PA T > 4 x N, carcinomas associated with a more than 4-fold increase in u-PA mRNA, as compared to the mean of nonmalignant tissues.
(40). Similarly, a protein extracted from ovine pituitaries and structurally related to basic fibroblast growth factor has been shown to enhance PA activity in breast tumor cell lines (41). In breast carcinomas, expression of the PA genes is thus likely to depend on the hormonal environment and to vary during tumor progression. Furthermore, the preponderance of patients with axillary lymph node metastases in our series suggests that the samples were collected at a later stage of tumor evolution.

Although our results need to be confirmed on larger numbers of primary carcinomas and to be compared to survival data, they may have important prognostic and therapeutic implications: surgical excision of lung carcinomas is performed with a curative intent, but more than 30% of the operated patients have regional lymph node metastases (1). The majority of such patients will die of metastatic disease. u-PA mRNA content determination might help identify patients at risk to relapse and therefore select candidates for adjuvant treatments.

ACKNOWLEDGMENTS

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