Multiple Forms of Plasminogen Activator in Human Breast Tumors

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ABSTRACT

Total plasminogen activator (PA) activity, tissue-type PA (t-PA) activity, urokinase-like PA activity, and immunoreactive t-PA were measured in benign breast tumors (fibroadenomas), primary breast carcinomas, axillary node metastases, and chest wall recurrences. Total PA activity did not differ significantly in the different types of tumors. However, benign tumors contained predominantly t-PA activity. Urokinase-like PA activity was significantly higher in the malignant tumors compared with the benign group. Both t-PA activity and immunoreactive t-PA were significantly lower in chest wall recurrences compared with primary carcinomas. The ratio of t-PA to urokinase activity was significantly decreased between stages 1 and 3 in the primary tumors. Also, immunoreactive t-PA levels were significantly lower in stages 2 and 3 compared with stage 1. No correlation was found between PA (either total or its different forms) and tumor grade, histological type, or the presence or absence of axillary node metastases.

INTRODUCTION

PAs2 are serine proteases which catalyze the cleavage of the inactive plasminogen to the active protease plasmin. Considerable evidence suggests that the PA/plasmin system plays a role in tumorigenesis and metastasis (for recent reviews see Refs. 1 and 2). Two main forms of PA have been described, t-PA and UK-PA. These forms differ from each other in both biochemical and immunological properties. Which of the 2 forms of PA is the more important in cancer is presently unknown. Recently, Osowski and Reich (3) showed that antibodies to UK-PA inhibited metastasis in experimental systems, suggesting that this form of PA may be important in metastasis. On the other hand, DePetro et al. (4) suggested that the t-PA form was responsible for morphological cell transformation.

Both forms of PA are found in tumors. In most malignant samples UK-PA appears to be the dominant form, but in some, t-PA predominates (see Ref. 1). Using an assay carried out without the addition of fibrin, Evers et al. (5) concluded that UK-PA was the main form of PA in human breast carcinomas. These workers omitted fibrin from the PA assay on the basis that fibrin augmentation of the t-PA activity might overestimate the amount of this form present (6). However, it is also possible that omission of fibrin may have the opposite effect and that t-PA activity present may be undetected or underestimated. For this reason we reinvestigated PA in breast tumors using an assay that includes fibrin degradation products. Specific antibodies to both t-PA and UK-PA were used in the assay to quench activity.

Finally, t-PA antigen was measured by an immunoradiometric assay.

MATERIALS AND METHODS

Materials. Fibrinogen was obtained from human plasma and digested with cyanogen bromide as described by Verheijen et al. (7). Plasminogen was purified from human plasma according to the method of Deutsch and Mertz (8). The plasminogen was only used if it contained no detectable plasmin. t-PA was purified from human melanoma cell culture medium (9). Urokinase was obtained commercially from Green Cross Co., Japan. Activities of both t-PA and UK-PA were produced in rabbits. The IgG fractions were isolated by affinity chromatography on Protein A-Sepharose (7). Non-immune rabbit IgG was prepared in a similar manner. The IgG preparations were diluted in assay buffer (see below) to a concentration of 1.0 mg/ml and were stored at -20°C. H-D-Val-Leu-Lys-p-Nitroanilide-2-HCl (S-2251) was obtained from Kabi-Vitrum (Stockholm, Sweden). Breast tumors were stored, homogenized, and examined histologically as described previously by Duffy et al. (10).

PA Assays. All PA assays were carried out on supernatants obtained after centrifugation at 2000 x g for 10 min. Activity assays were carried out in a Titrertek Multiscan spectrophotometer using 8- x 12-well flat-bottomed polystyrene microtitration plates (Flow Laboratories, McLean, VA). The procedure used was as described by Ranby et al. (11), with minor modifications. Briefly, freshly thawed tumor extract was diluted in 0.01 M Tris-HCl, pH 7.4, containing 0.1% Triton X-100. Aliquots of 100 µl were mixed with 100 µl of a reaction mixture composed of 0.6 mM S-2251, plasminogen (1.0 mg/ml), fibrinogen digested with cyanogen bromide, and assay buffer (15.0 mM Tris-HCl, 30 mM NaCl, 0.1% Triton X-100, pH 8.8) in the wells of a microtiter plate. Controls had either plasminogen or tumor extract omitted from the reaction mixture. All extracts were assayed using at least two dilutions in duplicate. The assay was carried out at 37°C, and the generated plasmin was monitored by measuring the absorbance at 405 nm. The absorbance was measured at Time 0 and at intervals for up to 6 h. Background absorbance was corrected for by subtracting the absorbance at 492 nm, which was typically less than 0.03 absorbance units. Plasminogen-dependent absorbance was calculated as the difference between total and plasminogen-independent absorbance. A standard curve of UK was included in each microtiter plate. UK was dissolved in 0.01 M Tris-HCl, pH 7.4, containing bovine serum albumin (2.0 mg/ml) and 0.1% Triton X-100, and serial dilutions ranging in activity from 0.4-0.003 IU/ml were assayed. Plasminogen-dependent activities of the tumor samples were calculated from the standard curve and were expressed as IU UK per mg protein.

Quenching by Specific Antibodies. Antisera to t-PA of UK, prepared as described, were diluted to a concentration of 100 µg/ml in assay buffer. Aliquots were then incubated with an equal volume of tumor extract (at a final concentration of 0.01-0.4 IU UK/ml) in duplicate at 4°C for 14 h. Controls included extracts incubated with non-immune IgG (100 µg/ml) or with assay buffer alone. Following incubation, aliquots of 100 µl were assayed for activity as described. A standard curve of UK and t-PA was performed in each experiment.

The activity quenched by the antibodies was calculated as a percentage of that observed in non-immune IgG. Also, residual activity in...
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The presence of anti-t-PA was calculated in terms of units/ml from the UK standards curve and in the presence of anti-UK, from the t-PA standard curve.

Immunological Quantitation of t-PA. The t-PA antigen present in the tumor extracts was determined with the immunoradiometric assay described by Rijken et al. (12). Antigen concentration was expressed as ng t-PA per mg protein.

Protein Determination. Protein was assayed using kits supplied by Bio-Rad Ltd.

Statistics. Non-parametric statistics were used throughout. For comparing differences between 2 different groups, the Mann-Whitney U-test was used.

RESULTS

PA Assay. The PA activity in breast tumors was linear with both dilution of extract and time squared. Interfering background absorbance (e.g., due to tumor extract) was negligible. PA-independent activity was very low (<5%) in all except 3 extracts. These 3 extracts were omitted from the study. The proportion of activity quenched by anti-t-PA and anti-UK antibodies always added up to approximately 100%, indicating that t-PA and UK-PA were the only forms of PA present in the breast extracts.

Correlation Between the Different Forms of PA. No correlation was obtained between UK activity and either t-PA activity \( r = 0.004 \) or immunoreactive t-PA levels \( r = 0.088 \). However, a reasonably good correlation was obtained between t-PA activity and immunoreactive t-PA levels \( r = 0.605 \). The failure to obtain a better correlation between these 2 measurements may be due to the presence of inactive t-PA activity in some extracts. This inactive PA would be expected to react in the immunoassay but not in the activity assay. Inactive t-PA activity could be due to the presence of inhibitors or degraded t-PA.

PA Levels in Benign and Malignant Tumors. Median PA values in benign tumors (fibroadenomas), primary breast carcinomas, axillary node metastases, and chest wall recurrences are shown in Table 1. No statistically significant difference in total PA activity was seen between the 4 groups. When the total PA activity was differentiated into the t-PA and UK forms, the UK activity showed a decreasing trend as the stage increased (Table 2). However, only the difference between stages 1 and 3 was significant \( P < 0.05 \).

Relationship Between PA and Tumor Stage. The median PA levels in different stages of primary carcinomas are shown in Table 2. Total PA activity did not differ significantly between any of the stages. However, activities of both t-PA and UK tended to decrease with increasing stage. The differences were not statistically significant.

Immunoreactive PA levels were significantly higher in stage 1 compared with either stage 2 \( P < 0.0178 \) or stage 3 \( P < 0.005 \). However, median levels between stages 2 and 3 did not differ significantly. Again, the different results obtained with t-PA activity and immunoassays may relate to the fact that larger numbers of samples were assayed using the latter approach compared with the former (see Table 2). The ratio of t-PA to UK activity showed a decreasing trend as the stage increased (Table 2). However, only the difference between stages 1 and 3 was significant \( P < 0.05 \).

Relationship Between PA and Other Histological Parameters. Neither total PA, t-PA, UK-PA activities, nor immunoreactive t-PA showed any significant relationship with histology type, histology grade, or the presence or absence of axillary node metastases.

DISCUSSION

In this investigation PA activity was measured by a kinetic assay in the presence of fibrin. The kinetic assay was more suitable than a fixed time point assay due to the wide variation of PA activity in different tumors. The presence of fibrin was necessary to measure all of the t-PA activity. The amounts of t-PA and UK were quantitated using quenching antibodies against...
both of these forms. We are also one of the first to use an immunoassay to measure t-PA in tumor extracts.

Using the PA assay described above, we show that no difference in total PA activity exists between benign and malignant breast tumors. This is in contrast to a number of reports describing higher levels of PA in carcinomas compared with benign specimens (13, 14). However, since we found most (approximately 90%) of the PA in the benign samples to be t-PA, the inclusion of fibrin by us should explain this discrepancy. Our findings however, agree with those of Tissot et al. (14), who also used a fibrin-based assay. Since the presence of fibrin prevents the underestimation of t-PA, perhaps other tumors should be re-investigated using this component in the assay.

Differentiation of PA into its t-PA and UK forms is likely to provide more meaningful information than just measurement of total activity. In this investigation, we show that t-PA is the dominant form in benign tumors, with UK only contributing approximately 10% of total PA activity. However all the malignant groups (primary carcinomas, axillary node metastases, and recurrences) contained significantly higher UK activity compared with the benign samples. Others have also noted an increase in the UK form of PA following malignant transformation of breast tissue.

To our knowledge there has been no report to date on a significant relationship between PA levels and stage in breast cancer. In this study, a trend toward decreasing immunoreactive t-PA, t-PA activity, UK-PA activity, and t-PA/UK-PA ratio was observed as the tumor stage increased. This trend reached significance when t-PA/UK ratio of stage 1 was compared with stage 3. The decrease in immunoreactive t-PA between stage 1 and either 2 or 3 was also significant. It should be stated that a greater number of samples were assayed for t-PA antigen than t-PA activity.

Our results also show that t-PA levels (both activity and antigen) are significantly decreased in chest wall recurrences compared with primary carcinomas. This finding has not been reported previously.

Do our results tell us anything about the role of PA in breast cancer? As mentioned in the "Introduction," much evidence exists that suggests a role for PA in cancer and metastasis. However controversy still exists (1, 2) and the precise role of PA is not clear. Our results suggest that some of the confusion may arise from the failure to separate PA into its different forms. The finding that t-PA levels were not higher in malignant tumors compared to the benign specimens, the trend toward t-PA levels decreasing as stage of primary carcinomas increased, and the significantly lower levels of t-PA in recurrences compared with the primary cancer makes it unlikely that this form of PA plays a role in metastasis. On the other hand, the increased levels of UK in malignant tumors compared with the benign group is consistent with but does not prove the hypothesis that this form of PA could play a role in metastasis. However, the trend toward decreasing UK activity as tumor stage increases would also seem to make this possibility unlikely. To further clarify the role of PA in breast cancer, we are monitoring these patients, and we hope to show in the future whether any link exists between levels of the different forms of PA and the course of the disease.

REFERENCES

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