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Urokinase-Plasminogen Activator, a New and Independent Prognostic Marker in Breast Cancer[1]

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ABSTRACT

Urokinase plasminogen activator (UK-PA) is a serine protease implicated in cancer invasion and metastasis. In this investigation, patients with breast cancers containing high levels of UK-PA antigen had significantly higher risk of early disease recurrence and shorter overall survival than did patients with low levels of the protein. In univariate analysis, UK-PA was a more powerful discriminator for disease-free interval than axillary node status, tumor size, or estradiol receptor. For overall survival, UK-PA as a prognostic marker, was of similar magnitude to axillary node status but stronger than that of tumor size or estradiol receptor. In multivariate analysis, for both disease-free interval and survival, UK-PA was an independent risk factor, being independent of tumor size, axillary node status, and estradiol receptor. UK-PA appears to be a new and independent prognostic marker in breast cancer.

INTRODUCTION

Considerable indirect evidence from model tumor systems suggests that proteolytic enzymes play a role in cancer invasion and metastasis (for reviews, see Refs. 1 and 2). Proteases implicated in these processes include the collagenases, especially collagenase IV, cathepsin B, and the urokinase form of plasminogen activator (1, 2). If proteases are involved in the spread of human cancers, measurements of their levels in primary tumors might indicate metastatic potential, *i.e.*, proteases might be new prognostic markers in cancer. In a preliminary report we showed that high levels of UK-PA[3] activity in breast carcinoma extracts correlated with a shortened disease-free interval (3). We have now extended this study and show here that high levels of UK-PA immunoreactivity is an independent and significant prognostic marker in patients with breast cancer.

MATERIALS AND METHODS

Breast tumors were homogenised in 50 mM Tris-HCl buffer, pH 7.4, and centrifuged at 2000 × *g* for 10 min. Total PA activity was assayed on the supernatant as previously described by us, except that fibrin was not included in the assay mixture (3, 4). Briefly, the supernatant from the 2000 × *g* centrifugation 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 valyl-leucyl-lysine-*p*-nitroanilide, plasminogen (1 mg/ml), and assay buffer (15 mM Tris-HCl, 30 mM NaCl, and 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. The reaction was carried out at 37°C and the generated plasmin was monitored by measuring the absorbance at 405 nm. Plasminogen-dependent absorbance was calculated as the difference between total and plasminogen-independent absorbance. S-2444 hydrolase was assayed by using a final concentration of 0.3 mM S-2444. S-2444 hydrolase was also assayed by using the supernatant from the 2000 × *g* centrifugation step described above.

To assay UK-PA immunoreactivity, the above supernatant from the 2000 × *g* centrifugation was extracted with one-tenth (v/v) of a solution containing 1 M Tris (pH 8.1), Trasylol (100 μg/ml), 5% Triton-X 100, and 100 mM EDTA. Following extraction, the mixture was centrifuged at 10,000 × *g* for 15 min. UK-PA antigen was assayed in the supernatant by using a previously described enzyme-linked immunosorbent assay (5). Briefly, this assay contained a monoclonal antibody as solid-phase antibody with biotin-labeled rabbit polyclonal antibodies against human PA as second antibody. The third layer consisted of peroxidase conjugated to avidin. Assays were carried out at 5–7 different dilutions of cytosol extract. Recovery of added pro-UK-PA to cytosol extract was greater than 80%. This enzyme-linked immunosorbent assay detects precursor UK-PA, active UK-PA, and UK-PA/inhibitor complexes (6). Investigations of representative samples by sodium dodecyl sulfate polyacrylamide gel electrophoresis and fibrin-agarose zymography (7) showed that the UK-PA in the extracts was exclusively in the M_r 54,000 form, and that the M_r 33,000 degradation product was absent. The cutoff point for UK-PA which gave the best discrimination between patients with good and bad prognosis was found to be 10 ng/mg total protein. ER was assayed by the dextran-coated charcoal method (8). The cutoff point was taken as 200 fmol/g wet weight of tissue.

Primary tumors from 166 patients with breast cancer were evaluated in this retrospective study. The tumor stage, axillary node status, and adjuvant treatment for patients with both high and low levels of UK-PA are shown in Table 1. The median follow-up period for patients with low levels of UK-PA was 35 months (range, 20–60 months) and for patients with high levels it was 33 months (range, 18–61 months). Following diagnosis, routine follow-up of these patients consisted of clinical examination every 3 months for the first 2 years, every 6 months for the next 3 years, and thereafter at 1 year intervals. Locoregional recurrent disease was diagnosed by histological examination of excised tissue. Distant metastases were diagnosed by clinical examination, appropriate X-rays, isotope scans, liver function tests, CA 15-3 determinations, and by biopsy if indicated. Local recurrences were treated either by excision or radio therapy. All the deaths in this study were due to metastatic breast cancer.

RESULTS

Correlation of UK-PA with Recurrence. Table 2 shows the relative risk of disease recurrence for a number of different variables (univariate analysis using proportional hazards general linear model procedure). As can be seen, UK-PA antigen is the strongest predictor of recurrence, being superior to axillary node status, tumor stage, and ER. Furthermore, in multivariate analysis, UK-PA antigen was independent of axillary node status, tumor size, and ER (Table 3). In contrast to UK-PA, neither nodal status, stage, or ER were significantly related to disease recurrence, using multivariate analysis of these 4 parameters (Table 3). Neither total PA activity nor S-2444 hydrolase correlated significantly with disease recurrence.

Correlation of UK-PA with Overall Survival. As with recurrence, high levels of UK-PA antigen was one of the strongest risk factors for shortened overall survival (Table 2). In univar-

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[3] The abbreviations used are: UK-PA, urokinase-type plasminogen activator; PA, plasminogen activator; t-PA, tissue-type PA; ER, estradiol receptor; S-2444, pyroglutamyl-glycyl-arginine-p-nitroanilide.

Table 1 *Tumor characteristics and adjuvant treatment of patients with low levels and high levels of UK-PA*

Stage 1, <2 cm; Stage 2, 2–5 cm; Stage 3, >5 cm; Stage 4, distant metastases.

	Low UK-PA (N = 84)	High UK-PA (N = 82)
Stage		
1	16	12
2	56	52
3	8	5
4	0	10
Unknown	4	3
Nodal status		
–	51	28
+	28	50
Unknown	5	4
ER status		
–	29	44
+	55	38
Adjuvant therapy		
None	30	21
Tamoxifen	44	43
Chemotherapy	10	18

iate analysis, UK-PA as a prognostic marker was of similar magnitude to axillary node status but stronger than either tumor stage or ER. In multivariate analysis, UK-PA was independent of axillary node metastases, tumor stage, and ER (Table 3). Similarly, using multivariate analysis, nodal metastasis was a significant predictor of early death in multivariate analysis. Again, neither total PA activity nor S-2444 hydrolase showed any relationship with survival.

DISCUSSION

Previously, in a preliminary study, we showed that levels of UK-PA activity in breast carcinomas correlated weakly but significantly with both tumor size and axillary node status (3). High levels of UK-PA activity also correlated with a shortened disease-free interval but this association was not independent of stage and axillary node status (3). We have extended these investigations and show here that UK-PA antigen is a new prognostic marker in breast cancer, independent of tumor size, axillary node status, and ER. Similar results were recently obtained by Jänicke *et al.* (9, 10), who also showed that high levels of UK-PA correlated significantly with a shortened time to recurrence and furthermore that the effect of UK-PA was stronger than lymph node or hormone receptor status. Another protease, *i.e.*, cathepsin D, has also been shown to correlate with poor prognosis in breast cancer (11–13).

These results are consistent with the hypothesis that certain proteases are involved in cancer invasion and metastasis. Thus, UK-PA is one of the first examples of a proteolytic enzyme implicated in experimental metastasis which correlates with bad prognosis in a human cancer. If it can be confirmed that proteases are involved in the spread of human cancers these molecules could be targets for agents (inhibitor or antibodies) that might prevent metastasis. Indeed, in one study with the chick embryo model system, antibodies against UK-PA inhibited metastasis of HEp-3 tumor cells (14).

The present study shows that neither S-2444 hydrolase or total plasminogen activator activity correlates with prognosis in present cancer. While S-2444 may be a relatively specific substrate for UK-PA in purified systems, in crude tissue extracts it is likely to be degraded by other proteases in addition to UK-PA. Consistent with this was our finding that UK-PA antigen levels showed no significant correlation with S-2444 hydrolase in the present investigation. Total plasminogen activator is composed of t-PA and UK-PA. Since t-PA correlates with good prognosis in breast cancer (15) and UK-PA with poor prognosis in this and other investigations (3, 9, 10), it is not surprising that measurement of total PA activity has no prognostic value.

In conclusion, UK-PA can now be added to the growing list of biochemical prognostic markers in breast cancers which presently includes estrogen receptors (16), progesterone receptors (16), epidermal growth factor receptors (17), *erbB*-2 gene amplification (18), cathepsin D (11–13), and t-PA (15). We now need to compare a number of these markers in a large study to see which gives the most useful information, especially in patients who have axillary nodes free of metastases. Finally, UK-PA should be investigated as a prognostic marker in other

Table 2 *Relative risk of disease recurrence and death associated with different tumor parameters in patients with breast cancer*

Calculations are based on univariate analysis, using the proportional hazards general linear model procedure. The cutoff point for total PA activity was 0.05 IU/mg protein and 0.1 absorbance unit/mg protein/h for S-2444 hydrolase.

	Disease recurrence			Death		
	Relative risk	95% CI[a]	P	Relative risk	95% CI	P
UK-PA (high *vs.* low)	4.1	1.86–8.85	0.0005	8.76	2.64–29.1	0.0004
Nodal status (positive *vs.* negative)	3.19	1.49–6.82	0.003	7.9	1.06–57.9	0.0008
Tumor stage (3 + 4 *vs.* 1 + 2)	1.99	1.19–3.34	0.042	3.35	1.57–7.2	0.0019
ER (negative *vs.* positive)	2.66	1.42–4.99	0.0019	2.66	1.35–5.26	0.0055
Total PA (high *vs.* low)	1.40	0.6–3.28	0.43	1.94	0.77–4.9	0.166
S-2444 hydrolase (high *vs.* low)	1.19	0.28–5.1	0.807	1.12	0.26–4.85	0.88

[a] CI, confidence interval.

Table 3 *Multivariate analysis of UK-PA, nodal status, stage, and ER as prognostic markers in breast cancer*

	Disease recurrence			Death		
	Relative risk	95% CI[a]	P	Relative risk	95% CI	P
UK-PA (high *vs.* low)	4.14	1.19–14.4	0.0336	11.13	1.22–99.0	0.031
Nodal status (positive *vs.* negative)	1.22		NS	1.51	1.17–2.10	0.001
Stage (3 + 4 *vs.* 1 + 2)	1.025		NS	1.21		NS
ER (negative *vs.* positive)	3.00		NS	2.34		NS

[a] CI, confidence intervals; NS, not significant.

solid cancers as the mechanisms of metastases are likely to be similar in all cancers.

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