

Antigen Levels of Urokinase Type Plasminogen Activator and Its Inhibitors in Primary Breast Cancer

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The aim of the study was to monitor urokinase plasminogen activator antigen concentrations and its type 1 (PAI-1) and type 2 (PAI-2) inhibitors in histologically defined forms of primary breast cancer and a comparison with these antigens levels in normal tissue. Another goal was a search for a relationship / or its lack / between the occurrence of the new generation markers of neoplastic disease and a presence /or absence/ of lymph node metastases. U-PA, PAI-1 and PAI-2 antigen levels were determined by ELISA tests in protein extracts of breast cancer tissues. Among the studied breast tumors 32 specimens were ductal carcinomas, 15 specimens were lobular carcinomas and the remaining 13 were other rare histological forms. In comparison to the obtained values of u-PA antigen levels in normal tissue, the values in neoplastic tissues were elevated several times: 11-fold, 6-fold and 15-fold in ductal c., lobular c. and other rare neoplasms. The values of PAI-1 antigen levels were about 20-fold higher for all studied, histologically defined primary breast cancers. The greatest differences of PAI-2 antigen levels growth was observed in histologically defined primary breast cancer forms. It was augmented 10-fold, 40-fold and 20-fold, respectively, for ductal carcinoma, lobular carcinoma and rare forms of neoplasms. In various forms of invasive breast cancer and those without lymph node metastases the content of u-PA, PAI-1 and PAI-2 were also significantly elevated. Among the new generation of independent markers of the neoplastic process, PAI-2 seems to be the most reliable marker for the identification of primary breast cancer.

The goal of the present study was to evaluate a possible combined prognostic value of the three major components of the u-PA system (u-PA, PAI-1 and PAI-2) in patients with defined histopathological forms of primary breast cancer.

Introduction

In clinical breast cancer the enzymatic activity and antigen level of u-PA in the primary tumor is found to be related with the rate of metastasis formation (Foekens *et al.*, 1995a; van Roozendaal *et al.*, 1996; Holst-Hansen *et al.*, 1996), an important finding which was confirmed by measurements of u-PA-antigen levels in several independent studies (Bouchet *et al.*, 1994; Jänicke *et al.*, 1994; Foekens *et al.*, 1992; Spyrtatos *et al.*, 1992).

Recent studies have shown that high levels of u-PA in primary breast tumors are associated with a poor prognosis for patients (Foekens *et al.*, 1992; Jänicke *et al.*; 1991 and 1993; Grøndahl-Hansen *et al.*; 1993; Bouchet *et al.*, 1999). U-PA expression is

not only the result of activation of signalling pathways; u-PA itself can also induce the activation of a signalling pathway via a specific u-PA receptor, localized on the surface of certain cell types (Bu *et al.*, 1994). Thus, u-PA plays at least two roles: it acts either as a proteolytic enzyme, involved in extracellular proteolysis, or as a ligand inducing cell responses such as mitosis and motility (Besser *et al.*, 1996).

The proteolytic activity of u-PA is controlled by two inhibitors: plasminogen activator type 1 (PAI-1) and type 2 (PAI-2), both being members of the serpin family of protease inhibitors (Andreasen *et al.*, 1990). Since PAI-1 is known to block the u-PA activity, it was a surprising finding that high levels of PAI-1 in primary breast tumors were associated

with an increased relapse rate (Foekens *et al.*, 1994; Grøndahl-Hansen *et al.*, 1993; Pappot *et al.*, 1995). Therefore, PAI-1 is also believed to be involved in the control of extracellular matrix degradation, which is a critical step for tissue remodelling, cell migration and tumor invasion (Pepper *et al.*, 1992). PAI-2 is produced mainly in the placental trophoblasts (Kruithof *et al.*, 1995) and is accordingly present in blood during pregnancy (Koh *et al.*, 1992). It is also produced by macrophages (Castellote *et al.*, 1990). It can be detected only rarely in the plasma of men and non – pregnant women (Lecander and Åstedt, 1989). In contrast to PAI-1, a high antigen level of PAI-2 has recently been related to a favourable prognosis in primary breast cancer (Bouchet *et al.*, 1994; Foekens *et al.*, 1995b). Many investigators consider the antigen levels of u-PA and its inhibitors (PAI-1 and PAI-2) in breast cancer tissue to be strong prognostic factors, independent of classical risk parameters (Bouchet *et al.*, 1994; Foekens *et al.*, 1995b; Jänicke *et al.*, 1993; Spyrtos *et al.*, 1992).

In the present study we report that increased antigen levels of u-PA, PAI-1, PAI-2 differ in defined histopathological types of primary breast cancer.

Materials and Methods

Patients

This study was performed on a group of 60 patients with operable primary breast cancer, and without signs of distant metastasis at surgery. No patients were subjected to radio- and chemotherapy before the surgery. Women were from 32 to 86 years old. Tissue specimens from 8 patients with normal mammary gland tissue served as reference group. Breast cancer tissue specimens were obtained at surgery, selected by the pathologist and stored in liquid nitrogen until extraction.

Tissue extract samples

Deep – frozen specimens of about 200–250 mg wet weight were put on ice, cut into small pieces and homogenized at 0 °C in a Potter-Elvehjem homogenizer in 2 ml of Tris-buffered saline (TBS) solution/20 mM tris[(hydroxymetyl)-aminomethane]-HCl pH 8.5, 125 mM NaCl and 1% Triton X-100/. 10% (w/v) suspension samples, were incu-

bated at 4 °C for 12 h under gentle shaking. The suspensions were then subjected to ultracentrifugation (Jänicke *et al.*, 1993) /100 000 × g, 60 min, 0 °C to separate cell debris, nuclei and cell membranes. The supernatant, which contained u-PA, PAI-1 and PAI-2, was divided into aliquots of 50 µl each, and stored at –20 °C until used.

Assay of u-PA, PAI-1, PAI-2

Contents of u-PA, PAI-1 and PAI-2 antigen were measured in the supernatant samples using specific enzyme – linked immunosorbent assay (Biopool TintElize Kits, Umeå, Sweden) according to the manufacturer's instructions. A monoclonal anti-u-PA antibody was raised against pro-u-PA with two forms of u-PA / low and high molecular weight / and u-PA complexes with plasminogen activator inhibitor type 1 or 2. The monoclonal anti-PAI-1 antibody recognized active and inactive forms of PAI-1 and PAI-1 bound to u-PA or t-PA. A monoclonal anti-PAI-2 antibody was raised against two forms of PAI-2 / low (44.6 kDa) and glycosylated high molecular weight PAI-2 (60 kDa)/. The test samples were diluted /1:10 or 1:20 / in PET – buffer from the Biopool Tint Elize Kit [phosphate – buffered saline (PBS) solution pH 7.4, EDTA / Tween 20, containing bovine serum albumin (BSA) 1:1000]. Absorbance was measured at 490 nm on a Bio-Rad model 450 microplate reader. Antigen levels ($\text{ng} \times \text{ml}^{-1}$) were obtained from standard curves. The detection limit was 0.1 $\text{ng} \times \text{ml}^{-1}$ for u-PA, 2.0 $\text{ng} \times \text{ml}^{-1}$ for PAI-1 and 4.0 $\text{ng} \times \text{ml}^{-1}$ for PAI-2. The total protein content of the extract samples was assayed according to Bradford (1976) with bovine serum albumin (BSA) as a standard. The levels of u-PA, PAI-1, PAI-2 antigen were expressed in ng per mg of protein and assays were performed in triplicates.

Reagents

For protein determination a kit supplied by Bio-Rad Ltd, USA was used. Commercially available TintElize Kits / cat.no 111120 for u-PA, cat.no 210220 for PAI-1 and cat.no 220220 for PAI-2 / were obtained from Biopool AB (Umeå, Sweden). All other chemicals and reagents were from Sigma (St. Louis, USA). They were of analytical grade unless stated otherwise.

Statistics

All the final data are presented as the means of the averaged triplicates. Means \pm SD are given for all parameters. To check how normal the distributions of variables were we used the Shapiro- Wilk test, which assesses deviations from normality. Levene's test was employed to assess homoscedasticity. In normally distributed variables data are presented as means \pm SD. Significance of differences was estimated using simple one-way ANOVA and Tukey's honest significant difference test for multiple comparisons. For all the variables which were not normally distributed we presented the data as median; quartile 1; quartile 3 and used Kruskal-Wallis ANOVA and the non-parametric version of Tukey's multiple comparison test to assess the significance of differences among the examined groups.

Results and Discussion

In the present study we investigated the antigen levels of u-PA and its inhibitors (PAI-1 and PAI-2) in defined histopathological forms of primary breast cancer. Age, menopausal status, macroscopic tumor size, lymph node status and histopathological forms, estrogen and progesterone receptor levels (not shown) of carcinoma were known in every case. We present different parameters and characteristics of histopathological types of the investigated carcinomas in Table I. Sixty primary tumors included 32 ductal carcinomas, 15 lobular carcinomas and 13 other rare histopathological forms: 4 mixed ducto-lobular carcinomas, 3 medullar carcinomas, 3 mucinous carcinomas and one papillary, apocryne, metaplastic carcinoma. We analysed the antigen levels of u-PA, PAI-1, PAI-2 carriers of these 13 other rare histopathological forms together due to small numbers of individual forms. The total distribution of u-PA, PAI-1 and PAI-2 in the studied groups of histopathological forms of primary breast cancer patients and in the control subjects with normal mammary gland tissue are presented in Table II. The increase of antigen levels of u-PA and PAI-1 is documented for all studied defined histopathological forms of primary breast cancer in relation to the corresponding values of antigen levels for normal tissue (the control group). In ductal carcinoma and in other rare forms of primary tumors

Table I. Characteristics of patients (pts) and tumors.

Characteristics	Number of pts
Patients:	
Total no.	60
Age (years)	
Mean	59
Range	32–80
Postmenopausal:	
No	17
Yes	43
Tumor size:	
T1 (<2 cm)	11
T2 (>2–5 cm)	46
T3 (>5 cm)	3
Path. axillary lymph nodes:	
<3	32
>3	28
Histopathological type of tumor:	
Ductal	32
Lobular	15
Mixed ducto-lobular	4
Medullary	3
Mucinous	3
Papillary	1
Metaplastic	1
Apocryne	1

the increase of antigen levels of u-PA were 10.9-fold and 14.7-fold, respectively. A smaller elevation of antigen level of u-PA, i.e. 6.3-fold, was found in lobular carcinoma. The increase of antigen levels of PAI-1 in ductal, lobular and other rare forms of tumors were practically the same (17.6-; 20.0- and 18.1-fold, respectively). In the studied forms of primary breast cancer (ductal, lobular and other rare forms of tumors) the antigen levels of PAI-2 were very differentiated: from complete deficiency to high values. In ductal carcinoma and other rare forms of primary tumors the average increase of the value of PAI-2 antigen levels in relation to normal tissues was 9.8-fold and 19.2-fold, respectively. The highest content of antigen level of PAI-2 (46.2-fold) was observed in lobular carcinoma.

In each group of defined histopathological forms of primary breast cancer there were events with or without lymph node metastases (Table III.). Among 32 cases of primary ductal carcinoma 22 specimens were with lymph node metastases (68.8%) and for these cases the mean values of antigen levels of u-PA and PAI-2 tended to be higher than the mean values of these antigens determined for all ductal tumors. For 10 cases of duc-

Table II. Contents of u-PA, PAI-1, PAI-2 (ng × mg protein⁻¹) in the extracts of tissue representing various histopathological forms of primary breast cancer.

Tissue	No.	u-PA		PAI-1		PAI-2	
		Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
Normal Tissue	8	0.2 ± 0.1	0.0–0.4	0.2 ± 0.2	0.0–0.6	0	–
Ductal carcinoma	32	2.0 ± 1.2 p<0.001	0.4–5.7	4.2 ± 2.7 p<0.001	1.5–13.0	9.8 ± 12.9 0.01<p<0.025	0–43.0
Lobular carcinoma	15	1.1 ± 0.8 p<0.001	0.3–2.8	4.8 ± 2.8 p<0.001	1.4–10.6	46.2 ± 25.0 p<0.0005	0–96.6
Other rare forms of carcinoma	13	2.7 ± 1.4 p<0.001	1.0–5.3	4.4 ± 2.6 p<0.001	1.7–9.4	19.2 ± 12.7 p<0.0005	0–82.6

Each value: the mean ± SD for three experiments.

Table III. u-PA, PAI-1 and PAI-1 contents (ng × mg of protein⁻¹) in patients with primary breast cancer.

Tissue	u-PA		PAI-1		PAI-2	
	node positive	node negative	node positive	node negative	node positive	node negative
Ductal carcinoma	2.0 ± 1.2 p<0.001	1.8 ± 1.2 p<0.001	3.9 ± 2.5 p<0.001	4.9 ± 3.1 p<0.001	10.3 ± 13.6 0.01<p<0.025	8.6 ± 11.0 p<0.05
number of cases	22	10	22	10	22	10
Lobular carcinoma	0.4 ± 0.1 p<0.05	1.2 ± 0.8 p<0.001	3.7 ± 0.3 p<0.05	5.0 ± 3.1 p<0.001	48.3 ± 48.3 N. S.	45.9 ± 20.0 p<0.0005
number of cases	2	13	2	13	2	13
Other rare forms of carcinoma	2.3 ± 2.0 p<0.005	2.8 ± 1.2 p<0.001	4.5 ± 1.6 p<0.001	4.4 ± 2.8 p<0.001	2.9 ± 2.1 p<0.025	26.5 ± 20.9 p<0.0005
number of cases	4	9	4	9	4	9

Significance of the increase in antigen content with respect to normal tissue was estimated using the Mann-Whitney test.

tal carcinoma without lymph node metastasis (31.2%) the mean values of u-PA and PAI-2 antigen levels were lower than the mean values of these antigens determined for all studied ductal tumors. The mean value of PAI-1 antigen level for specimens of ductal carcinoma with lymph node metastasis was lower than the mean value for the whole group of these tumors. On the other hand, the mean value of PAI-1 antigen for the ductal cases without lymph node metastasis was higher than the mean value of PAI-1 for the whole group of ductal forms of primary breast cancer. The clinico-pathological criterion – lymph node metastasis or its absence – affected antigen levels of u-PA, PAI-1 and PAI-2 in the primary lobular carcinoma of breast tissue in a different way. Only two speci-

mens (13.3%) of 15 lobular carcinoma carriers were with lymph node metastasis and the mean value of u-PA antigen level was 3-fold lower than u-PA content in tissue without metastasis. However, the mean value of u-PA antigen level for 13 lobular cases without lymph node metastasis (86.7%) was similar to the mean value of u-PA for the whole group of lobular forms of breast tumors. A similar tendency was observed for the mean value of PAI-1 antigen level in lobular carcinomas, although the increase of the mean value of PAI-1 in lobular cases with lymph node metastasis was by about 25% lower than the value of PAI-1 for the whole group of primary lobular carcinomas. The mean value of PAI-1 antigen levels for specimens of lobular tumor without lymph node metas-

tasis was approximately the same as the mean value of PAI-1 for the whole group of lobular forms. The mean value of PAI-2 antigen level for the studied specimens of lobular carcinoma with or without lymph node metastasis was very high and insignificantly different from the high mean value of PAI-2 for the whole group of primary lobular tumors. Mean values of u-PA, PAI-1 and PAI-2 antigen levels for other rare histopathological forms of breast cancer were also analysed in two groups: with or without lymph node metastasis in primary tumors. The results were compared with the mean values of corresponding antigen levels for all other rare forms of breast cancer. For four specimens of rare forms of breast carcinoma with lymph node metastasis (30.8%) the mean values of u-PA and PAI-1 antigen levels were similar to mean values of these antigens, determined for all rare tumors. At the same time, a significant difference was observed for PAI-2. The mean value of PAI-2 for rare cases with lymph node metastasis was 6.6-fold lower than the mean value of this factor /PAI-2/ for all rare forms of primary breast cancer and for rare cases without lymph node metastasis. The mean value of u-PA for nine specimens of rare forms of breast cancer without lymph node metastasis (69.2%) was insignificantly higher than the mean value of u-PA for all analyzed rare forms of this tumor. However, the mean value of PAI-2 for the same cases was 30% higher than the mean value of PAI-2 antigen level for all rare forms of primary cancer. The mean values of PAI-1 antigen levels for both groups (with or without malignancy) of rare forms of breast cancer were similar to the mean value of PAI-1, obtained for all rare histopathological forms of primary breast tumor.

Determination of the tumor antigen levels of the u-PA system components are of predictive value for disease recurrence and the overall survival rate in patients with primary breast cancer. The relationships between u-PA, PAI-1 and PAI-2 factors are statistically significant and have been reported previously (Bouchet *et al.* 1999; Duggan *et al.* 1995; Foekens *et al.*, 1994 and 2000;

Grøndahl-Hansen *et al.*, 1997). This is particularly important for node-negative disease patients who, as a group, have a relatively favorable prognosis.

We observed a similar increase (about 20-fold) of PAI-1 antigen level in ductal carcinoma, lobular carcinoma and rare forms of carcinoma. But it is hard to evaluate its prognostic value in our study. More differentiated antigen levels of u-PA and PAI-2 were obtained for histologically defined forms of primary breast cancer. A significant increase in u-PA level in ductal carcinoma and rare forms of breast cancer (10.9-fold and 14.7-fold, respectively) was connected with a moderate increase in PAI-2 antigen level for these compared cases. Particular attention is paid to lymph node-positiveness in rare forms, where a high u-PA antigen level and a low PAI-2 antigen level were found.

According to Foekens (Foekens *et al.*, 1995b) patients with a high u-PA antigen level who, in addition, contained low tumor levels of PAI-2 experienced a very poor prognosis.

On the other hand, in our study a markedly smaller increase of u-PA antigen level for primary lobular carcinoma was linked with a high level of PAI-2 antigen. The same correlation was observed for node-negative and node-positive subjects. Bouchet (Bouchet *et al.*, 1994) found that high levels of PAI-2 in breast cancers correlated with longer metastasis-free survival in both the overall population and the node-negative subgroup.

Values of u-PA system, being independent prognosis factors, in histologically defined primary breast cancer allow to discriminate patients with primary lobular carcinoma as the group with good prognosis of therapy.

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