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The Complex between Urokinase and Its Type-1 Inhibitor in Primary Breast Cancer: Relation to Survival

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ABSTRACT

We examined the relationship between tumor tissue level of the complex formed of urokinase (uPA) and its type-1 inhibitor (PAI-1) and survival of breast cancer patients. The study included 342 axillary lymph node-negative and -positive primary breast cancer patients with a median follow-up of 67 months. Using a newly established ELISA, the levels of preformed uPA·PAI-1 complex were measured in tumor tissue extracts and analyzed with respect to total uPA, total PAI-1, and clinicopathological parameters, including survival. uPA·PAI-1 complex comprised a minor, variable fraction of both total uPA and PAI-1 levels. The complex levels were higher in node-negative tumors than in node-positive tumors and higher in small and low-grade tumors (all, $P \leq 0.002$). The tumor levels of complex, uPA, and PAI-1 were all associated with survival; high complex levels predicted longer recurrence-free survival ($P = 0.03$) and overall survival [OS ($P = 0.005$)], whereas high uPA or PAI-1 levels significantly predicted shorter survival. In multivariate Cox analysis, the only parameters that independently predicted survival were total PAI-1 level and lymph node status for recurrence-free survival and OS and, additionally, steroid hormone receptor status and grade for OS. This is the first demonstration of a relationship between uPA·PAI-1 complex tumor level and patient survival. However, total PAI-1 level showed superior prognostic power. Additional studies are needed to understand the relationship of these parameters to cancer biology and to assess the clinical utility of the uPA·PAI-1 complex.

INTRODUCTION

In the primary treatment of breast cancer, there is a need to distinguish between patients at high and low risk of recurrence to permit focused use of adjuvant therapy. Axillary lymph node status is recognized as the best clinical discriminant between high- and low-risk patients (1, 2). However, because 30% of the patients with node-negative disease experience a recurrence, additional measurements of biochemical parameters involved in metastatic spread have been proposed for accurate prognostic separation (3).

A central role of uPA[2] in cancer invasion and metastasis is well established (4, 5). This M_r 52,000 serine proteinase is secreted as an inactive precursor, pro-uPA, that binds to a specific cell surface receptor, uPAR, where formation of active uPA leads to localized potentiation of plasminogen activation (6). The plasmin thus generated mediates broad spectrum proteolysis, facilitating cell migration, proliferation, and invasion (4). uPA activity, whether in solution (7) or receptor-bound (8, 9), is rapidly neutralized by its specific high-affinity inhibitors, PAI-1 and PAI-2. PAI-1, the principal physiological uPA inhibitor, is a M_r 52,000 protein secreted in an active but conformationally unstable form that gradually loses activity unless it is stabilized by binding to the extracellular matrix protein vitronectin (10, 11). Active PAI-1 forms an equimolar, SDS-stable, M_r 100,000, enzymatically inactive complex with active uPA, whereas the inactive forms of these two components cannot form a complex (12, 13). By a process dependent on uPAR and the low-density lipoprotein receptor-related protein (α_2-macroglobulin receptor), cell surface uPA·PAI-1 complex is internalized and ultimately degraded in lysosomes (5, 14).

ELISA measurements of levels of uPA, PAI-1, PAI-2, and uPAR in tumor tissue extracts have enabled several studies of their relationship to cancer patient survival (5, 15). High levels of uPA and uPAR were found to predict short survival, consistent with the proposed role of the protease in facilitating matrix degradation and the proposed role of the receptor in promoting uPA activity on cell surfaces. Surprisingly, high levels of PAI-1 have also been reproducibly found to be predictive of short survival, despite the known ability of PAI-1 to inhibit uPA activity. On the other hand, high levels of PAI-2 seem to predict longer survival in breast cancer patients.

ELISAs used in the prognostic studies were developed to measure the total amount of each molecule, including proforms, active, inactive, and complex-bound forms. However, to further investigate the relationship of tumor tissue uPA activity and its PAI-1 inhibition to patient survival, we decided to measure the level of uPA·PAI-1 complex because this parameter selectively represents that minute fraction of uPA (16, 17) that has been activated from proenzyme and subsequently inactivated by active PAI-1. For this purpose, we have recently developed a kinetic uPA·PAI-1 complex ELISA for extracts of patient tumor tissue (18). We now report the results of an exploratory retrospective study of the complex levels in 342 primary breast tumors to test for associations with clinicopathological parameters, RFS, and OS of the patients, including the important subgroup of axillary lymph node-negative patients.

PATIENTS AND METHODS

Patients. This study included 342 patients who underwent surgery in Denmark in the period 1989–1993 for histologically verified primary breast cancer. A total of 10,918 patients were registered by the DBCG during the period. Patients were entered into the present study consecutively, provided that unfixed, frozen tumor tissue was accessible and that they were included in the treatment protocol DBCG-82 or DBCG-89 (19). The number of patients to be included in the study was chosen to permit detection of a significant difference in survival between patients when they were divided into at least two groups. The surgical procedure included breast-conserving lumpectomy (followed by local radiotherapy) or modified radical mastectomy, and partial axillary lymph node dissection. None of the patients entered into the study had evident distant metastases. Complete clinicopathological data were registered for the patients (Table 1), of whom 164 were free of axillary lymph node metastases, and 178 were lymph node positive at the time of primary surgery. Lymph node-negative patients with a tumor larger than 5 cm in diameter (pathologists' estimate), patients in the DBCG-82 protocol with skin or thoracic wall invasion, premenopausal patients in the DBCG-89 protocol with a ductal carcinoma showing grade II or III cell anaplasia (20), and all lymph node-positive patients received adjuvant therapy. The median patient age at time of surgery was 56 years (range, 29–75 years), and the median follow-up

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[2] The abbreviations used are: uPA, urokinase; PAI, plasminogen activator inhibitor; uPAR, urokinase receptor; RFS, recurrence-free survival; OS, overall survival; CI, 95% confidence interval; RR, relative risk; DBCG, Danish Breast Cancer Cooperative Group.

Table 1 *Characterization of patients studied and the other patients at time of primary operation*

Parameter	Studied patients		Other patients	
	n	%	*n*	%
Age (yrs)	342		10,576	
≤39	21	6	544	5
40–49	86	25	2,166	20
50–59	96	28	2,326	22
60–69	105	31	2,447	23
70–79	34	10	2,077	20
≥80	0	0	1,016	10
Menopausal status	342		10,573	
Premenopausal	110	32	2,890	27
Postmenopausal[a]	232	68	7,683	73
Tumor size (cm)	328		9,676	
≤2	100	31	5,217	55
2.1–5	194	59	3,845	39
≥5	34	10	614	6
Histological type	338		10,030	
Ductal carcinoma	280	83	8,093	81
Lobular carcinoma	40	12	1,168	12
Other types	18	5	769	7
Tumor grade	277		7,719	
I	82	30	2,796	36
II	123	44	3,475	45
III	72	26	1,448	19
Receptor status	332		8,125	
Receptor positive[b]	261	79	6,553	81
Receptor negative	71	21	1,572	19
Positive lymph nodes	342		10,197	
0	164	48	5,911	58
1–3	95	28	2,570	25
≥3	83	24	1,716	17

[a] If menostasia ≥ 5 years.
[b] If estrogen and/or progesterone analysis was positive (*i.e.*, ≥10 fmol/mg cytosol protein by biochemistry or ≥10% stained cells by immunohistochemistry).

time at the time of data analysis was 67 months (range, 53–101 months). The anticipated follow-up period was intended to be 10 years, as described previously (19, 21), and the information was updated annually. During the observation period, there were 130 recurrences and 113 deaths. Recurrence was confirmed by biopsy and/or other relevant diagnostic procedures and defined as the appearance of new breast cancer lesions after primary surgery. The actual cause of death was not available for the patients, therefore recording of survival was based on death from all causes. Tumor tissue samples were obtained in accordance with the Helsinki Declaration.

Tissues. After routine histopathological procedures, including steroid hormone (estrogen and/or progesterone) receptor analysis (22), the remaining tissue was stored in sealed containers at −80°C. On the day of extraction, frozen tissue was mechanically pulverized with a dry ice-cooled powder pistol. Tissue powder (100 mg) was suspended in 300 μl of ice-cold extraction buffer [75 mM potassium acetate, 0.3 M NaCl, 0.1 M L-arginine, 10 mM Na_2-EDTA, and 2.5 ml/liter Triton X-100 (pH 4.2)], which was previously described as optimal for extraction of uPA·PAI-1 complex, total uPA, and PAI-1 (18, 23–25). Moreover, this low-pH buffer prevented *ex vivo* formation of complex from free uPA and PAI-1 in the tissue extract without destabilizing preformed uPA·PAI-1 complex (18). The suspension was centrifuged at 105,000 × *g* for 1 h at 4°C, and the resulting particle-free supernatant was stored aliquoted at −80°C until use (<4 months). Immediately before assay, the extracts were thawed rapidly at 37°C and diluted.

Assays. All assays were performed without knowledge of the clinicopathological data and patient outcome.

Total protein concentrations in the extracts were measured by the Bradford method for protein analysis (26), using a protein assay kit using BSA as a standard (Bio-Rad, Hercules, CA). For reference purposes, a pool of tumor tissue extracts was made, comprising equal volumes from 17 randomly chosen tumor tissues. The pool had a protein content close to the mean value found for extracts from all of the patients, and the interassay variation for protein measurement of this pool was 8.3% ($n = 9$ assays).

Total uPA concentrations and total PAI-1 concentrations in extracts were determined using ELISA kits (Oncogene Science Diagnostics, Cambridge, MA) as described previously (27, 28). The uPA assay has a detection limit of 25 pg/ml and detects the uPA antigen with equal efficiency, regardless of whether it is present as pro-uPA, uPA, uPA·PAI-1, or uPA·uPAR complexes with closely similar efficiency. The interassay variation for the reference pool was 9% ($n = 9$ assays). The PAI-1 ELISA has a detection limit of 100 pg/ml and measures the PAI-1 antigen with equal efficiency whether present as the latent or the active form. However, the efficiency of measurement of PAI-1 in complex with plasminogen activators is approximately 50%.[3] The interassay variation was 3.9% ($n = 9$ assays).

The concentration of uPA·PAI-1 complex in extracts was determined using a new kinetic ELISA that meets strict criteria of specificity and sensitivity (18). In short, this sandwich ELISA consists of a coating with two murine monoclonal antihuman PAI-1 capture antibodies. After blocking the remaining protein binding sites, standard dilutions of purified, stable uPA·PAI-1 complex and diluted extracts were incubated. The complex standard was prepared from purified high-molecular weight active human uPA (Serono, Aubonne, Switzerland) and activated human PAI-1, purified from conditioned media of dexamethasone-treated HT-1080 fibrosarcoma cells (18). The standard was measured with a detection limit of 8 pg/ml, and neither free uPA nor free PAI-1 was detected by the assay. To block *ex vivo* formation of the uPA·PAI-1 complex from free uPA and PAI-1 in the diluted extracts during incubation of the assay plates, *p*-nitrophenyl guanidinobenzoate (an inactivator of uPA; Sigma, St. Louis, MO) was added to the dilution buffer (18). A combination of three biotinylated monoclonal antihuman uPA antibodies was used as the detection reagent, and the data were collected as kinetic measurements of bound streptavidin alkaline phosphatase activity (Dako, Glostrup, Denmark). The interassay variation of the extract pool for this study was 13% ($n = 9$ assays).

Western blotting of the uPA·PAI-1 complex was carried out as described previously (29). Briefly, 35 μl of pooled crude tissue extract were diluted 3:1 in Laemmli sample buffer, the sample was electrophoresed on a 10% SDS-polyacrylamide minigel, and the separated proteins were blotted electrophoretically onto a polyvinylidene difluoride membrane. After blocking, the membrane was sequentially incubated with rabbit polyclonal antihuman PAI-1 antibodies and alkaline phosphatase-conjugated monoclonal antirabbit antibody (Dako). Finally, color was developed with the phosphatase substrate solution nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate.

Statistical Analyses. Spearman rank correlation coefficients (r_s) were calculated for matched pairs of sample measurements of total uPA, total PAI-1, and uPA·PAI-1 complex. Tests of hypotheses on the location parameter for these variables were done using Wilcoxon's rank-sum test (the Kruskal-Wallis test was used for comparisons of more than two groups). Survival curves for the time to recurrence and the time to death were estimated by the Kaplan-Meier method. It was prespecified that total uPA, total PAI-1, and uPA·PAI-1 complex were to be scored first as the actual value (log transformed) and then as indicator variables for each quartile. The ratios of uPA·PAI-1 complex to total uPA and PAI-1, respectively, were considered in a similar manner. The log-rank test was used to test for equality of strata. The Cox proportional hazards model was used for analysis of survival. The assumption of proportional hazards was verified graphically. Reduced models were obtained by backward selection in a monotonic fashion; the exclusion level was 0.05. $P < 0.05$ was considered significant, and the 95% CI for each RR was calculated.

RESULTS

Characteristics of Patients. The cohort of 342 primary breast cancer patients included 164 lymph node-negative patients and 178 lymph node-positive patients, with a median follow-up of 67 months. In addition to the traditional clinicopathological data shown in Table 1, primary tumor tissue detergent extracts were analyzed for total protein concentration, total uPA, total PAI-1, and uPA·PAI-1 complex level. For comparison, Table 1 also shows the clinicopathological data for the other breast cancer patients ($n = 10,576$) who were operated on during the study period. It appears that the study patients in general presented characteristics that were representative of all of the patients

[3] A. N. Pedersen, unpublished results.

operated on during the study period. Minor exceptions were related to patient age (no patients >75 years old were included in any protocol) and tumor size (the availability of frozen tissue was a prerequisite for this study). The characteristics of the study group and other breast cancer patients appeared comparable with regard to histological type, tumor grade, and steroid hormone receptor or lymph node status (Table 1).

Levels of uPA·PAI-1 Complex, Total uPA, and Total PAI-1. One tissue specimen was lost during handling, and thus only 341 extracts were analyzed by ELISA. All of the extracts analyzed had measurable levels of uPA·PAI-1 complex, total uPA, and PAI-1. Each ELISA determination was referred to the protein concentration in the same extract. Because the frozen tumor tissue had been stored for several years before the extraction procedure, we first investigated whether the storage time had any influence on the measured antigen levels. Tests for any association between tissue storage time and the measured parameters did not reveal any significant relationship. Also, the presence of intact tumor tissue uPA·PAI-1 complex in extracts stored for several months was demonstrated by Western blots (Fig. 1, *inset*).

The complex level among the extracts varied from 0.22–5.3 ng/mg protein, with a median value of 0.75 ng/mg protein. Fig. 1 shows the percentiles plot for the level of complex found in the individual extracts, and it is evident that the distribution is skewed toward the higher values. For total uPA, the median value was 5.2 ng/mg protein (range, 0.17–61 ng/mg protein), and for total PAI-1, the median value was 2.4 ng/mg protein (range, 0.19–80 ng/mg protein). The distributions of uPA and PAI-1 levels were also skewed toward the higher values. uPA·PAI-1 complex was found to represent a minor, variable fraction of total uPA ($r_s = -0.24$; $P < 0.0001$; Fig. 2*A*) as well as total PAI-1 ($r_s = -0.21$; $P < 0.0001$; Fig. 2*B*). In contrast, there was a close and positive association between total uPA and PAI-1 ($r_s = 0.78$; $P < 0.0001$; data not shown). We then determined the ratio of uPA·PAI-1 complex to total uPA and total PAI-1, respectively, for each extract by dividing the complex value by the total value. The median value for the complex:total uPA ratios was 0.13, and the median value for the complex:total PAI-1 ratios was 0.27. Because the complex was only a minor fraction of the total antigen levels and represented a narrow range as compared with the total antigen levels, there was a very close association between the ratios and the corresponding total antigen levels (both, $r_s = 0.9$; $P < 0.0001$).

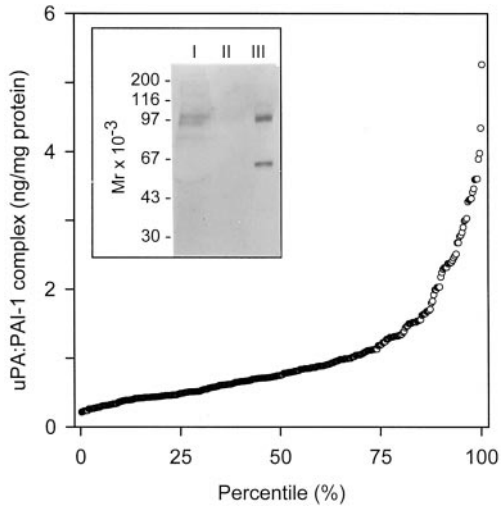

Fig. 1. Percentiles plot for uPA·PAI-1 complex levels in 341 tumor tissue extracts. *Inset*, Western blot using anti-PAI-1 antibodies to show the immunoreactivity of 35 μl of crude extract from a pool of 10 samples with high complex levels (*lane I*) or from a pool with low complex levels (*lane II*). For comparison, a mixture of 1 ng of purified uPA·PAI-1 complex and 1 ng of free PAI-1 is shown (*lane III*), and the molecular weights of marker proteins are indicated.

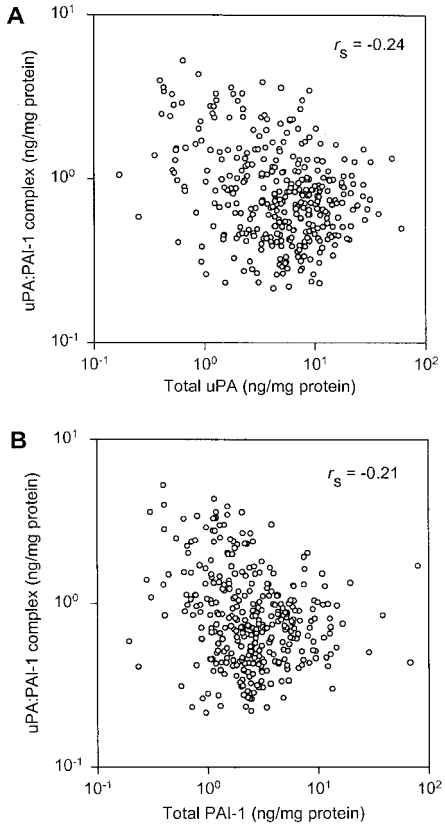

Fig. 2. Double logarithmic scatterplot of total uPA level (*A*) and total PAI-1 level (*B*) *versus* uPA·PAI-1 complex level from 341 tumor tissue extracts. r_s, the Spearman's rank correlation coefficient.

Table 2 *Wilcoxon rank-sum/Kruskal-Wallis comparison of studied parameters*

	P		
Parameter	Total uPA	Total PAI-1	uPA·PAI-1 complex
Age[a]	0.88	0.59	0.31
Menopausal status	0.89	0.42	0.03
Tumor size	0.009	0.08	<0.0001
Tumor grade	<0.0001	<0.0001	0.002
Receptor status	<0.0001	<0.0001	0.32
Lymph node status	0.53	0.48	<0.0001

[a] Premenopausal, below/above 40 years; postmenopausal, 10-year intervals.

Table 2 shows the comparison of the measured biochemical and clinicopathological parameters. It is noteworthy that for uPA·PAI-1 complex, the levels were significantly higher in lymph node-negative tumors than in lymph node-positive tumors ($P < 0.0001$), and complex levels were also higher in small tumors ($P < 0.0001$) and in those with a low grade of anaplasia ($P = 0.002$). Conversely, total uPA or PAI-1 levels were higher in large tumors and those with a high grade of anaplasia, whereas no significant association was found between nodal status and uPA or PAI-1 levels (Table 2). To further illustrate the association between complex and nodal status, Table 3 shows the frequencies of lymph node involvement with the complex values divided into quartile groups. Although a significant relationship (χ^2 test for trend, $P < 0.0001$) between the parameters could be demonstrated at the extremes, the relationship broke down in the intermediate complex concentration range.

Table 3 *Relation between lymph node status and uPA·PAI-1 complex level*

Complex	1st[a]	2nd[a]	3rd[a]	4th[a]	Total
Node negative	27[b]	38	40	59	164
Node positive	58	47	45	27	177

[a] Quartile of the values determined.
[b] Number of patients.

Relation of uPA·PAI-1 Complex, Total uPA, Total PAI-1, and Ratios to Survival. Initially, the measured parameters were treated as log-transformed continuous variables. The tumor level of total uPA significantly predicted RFS ($P = 0.0003$) and OS [$P = 0.0003$ (Table 4)]; increasing uPA levels were associated with shorter survival. Comparing patients at the 75th percentile value with those at the 25th percentile value, the RR was 1.2 (CI, 1.1–1.3) for RFS and 1.2 (CI, 1.1–1.4) for OS. Increasing total PAI-1 levels also predicted shorter RFS and OS ($P < 0.0001$ for both). When comparing patients at the 75th and 25th percentile values, the RR for RFS was 1.4 (CI, 1.3–1.6), and the RR for OS was 1.6 (CI, 1.4–1.8). Conversely, increasing levels of uPA·PAI-1 complex predicted longer RFS and OS. However, the scoring of the complex by its log-transformed value did not adequately fit a linear model in the analysis; consequently, this approach was not pursued further. However, scoring of the ratio of complex to total uPA or PAI-1 fitted the data well; both ratios highly significantly predicted survival ($P < 0.0001$ for both), with increasing ratios associated with longer survival. For example, for the complex:total PAI-1 ratio, the RR was 0.5 (CI, 0.4–0.6) for RFS and 0.4 (CI, 0.3–0.5) for OS when comparing patients at the 75th and 25th percentile values. Clinicopathological parameters of prognostic significance in the univariate Cox model were large tumor size, high tumor grade of anaplasia, and positive lymph node status; each of which predicted shorter survival, whereas positive steroid hormone receptor status predicted longer survival (Table 4).

We then used the quartiles to divide the patient material to further investigate the prognostic significance of the measured parameters. When RFS and OS were compared for patients with uPA·PAI-1 complex levels falling into four groups, it was observed that the patients with high complex values had a significantly longer RFS ($P = 0.03$; Fig. 3*A*) and OS ($P = 0.005$) than patients with lower values. A comparison of patients in the fourth and first quartiles gave a RR of 0.5 (CI, 0.3–0.8) for RFS and a RR of 0.4 (CI, 0.2–0.8) for OS. For total uPA, high values were associated with a shorter survival [RFS, $P = 0.007$ (Fig. 3*B*); OS, $P = 0.003$, which was also the case for total PAI-1 [for both RFS (Fig. 3*C*) and OS, $P < 0.0001$]. Moreover, divided by the quartiles, the complex:total uPA and complex:total PAI-1 ratios clearly separated the four patient groups with regard to prognosis. For example, for the ratio to uPA, the RR was 0.4 for RFS (Fig. 4*A*) for patients in the fourth quartile *versus* those in the first quartile, and for the ratio to PAI-1, the RR was 0.3 for RFS (Fig. 4*B*).

As a consequence of the above-mentioned findings for uPA·PAI-1 complex, we analyzed the data set to find the optimal cutoff point between good and poor prognosis. The maximum of the partial likelihood at given complex values revealed a value between 1.3 and 1.4 ng/mg protein to be the best cutoff value for separating the patients into two groups (Fig. 5, *inset*): (*a*) those with long-term RFS; and (*b*) those with short-term RFS (Fig. 5). A comparison of patients with a tumor tissue uPA·PAI-1 complex value above the optimal cutoff value with those with a value below the optimal cutoff value gave a RR of 0.4 for RFS.

Multivariate Analysis. Multivariate Cox regression analyses of the survival data were performed to compare the statistical power of the studied parameters. Analyses were done with respect to all studied parameters [except for tumor grade of anaplasia (see below)] for those patients for whom complete data were available. uPA·PAI-1 complex, total uPA, total PAI-1, and the ratios were stratified by quartiles. When treating these parameters as continuous variables, results similar to those below were obtained (data not shown).

Firstly, all studied parameters were included in the model. In this analysis, the only parameters that independently and significantly predicted survival were total PAI-1 and lymph node status for RFS

Table 4 *Univariate Cox survival analysis for studied parameters of all patients*

	RFS			OS		
Parameter	*P*	RR	CI	*P*	RR	CI
Age[a]	0.84			0.99		
Menopausal status	0.62			0.54		
Tumor size (cm)	0.014			0.002		
⩽2 *vs.* 2.1–5		0.7	0.5–1.1		0.5	0.3–0.9
>5 *vs.* 2.1–5		1.6	1.0–2.4		1.5	1.0–2.5
Tumor grade[b]	0.0009	2.3	1.4–3.8	<0.0001	3.8	2.0–7.2
Receptor status	0.006	0.6	0.4–0.9	<0.0001	0.4	0.3–0.6
Lymph node status[c]	<0.0001	2.7	1.8–3.9	<0.0001	4.0	2.6–6.2
Total uPA[d]	0.0003	1.2[e]	1.1–1.3[e]	0.0003	1.2[e]	1.1–1.4[e]
Total uPA[f]	0.003			0.003		
2nd *vs.* 1st quartile		1.7	1.0–2.9		1.7	0.9–3.1
3rd *vs.* 1st quartile		1.8	1.1–3.2		1.6	0.9–3.0
4th *vs.* 1st quartile		2.5	1.5–4.3		2.8	1.6–4.9
Total PAI-1[d]	<0.0001	1.4[e]	1.3–1.6[e]	<0.0001	1.6[e]	1.4–1.8[e]
Total PAI-1[f]	<0.0001			<0.0001		
2nd *vs.* 1st quartile		1.7	1.0–3.1		1.4	0.7–2.7
3rd *vs.* 1st quartile		2.2	1.2–3.8		1.8	1.0–3.4
4th *vs.* 1st quartile		3.9	2.3–6.7		4.5	2.5–7.9
uPA·PAI-1 complex[f]	0.03			0.005		
2nd *vs.* 1st quartile		1.0	0.6–1.5		0.8	0.5–1.3
3rd *vs.* 1st quartile		1.1	0.7–1.7		1.2	0.7–1.9
4th *vs.* 1st quartile		0.5	0.3–0.8		0.4	0.2–0.8
Complex:total uPA ratio[d]	<0.0001	0.8[e]	0.8–0.9[e]	<0.0001	0.8[e]	0.7–0.9[e]
Complex:total PAI-1 ratio[d]	<0.0001	0.5[e]	0.4–0.6[e]	<0.0001	0.4[e]	0.3–0.5[e]

[a] Premenopausal, below/above 40 years; postmenopausal, 10-year intervals.
[b] >grade I *vs.* grade I (ductal carcinomas only).
[c] Node positive *vs.* node negative.
[d] Continuous variable.
[e] Comparison of patients at the 75th and 25th percentile values.
[f] Quartiles.

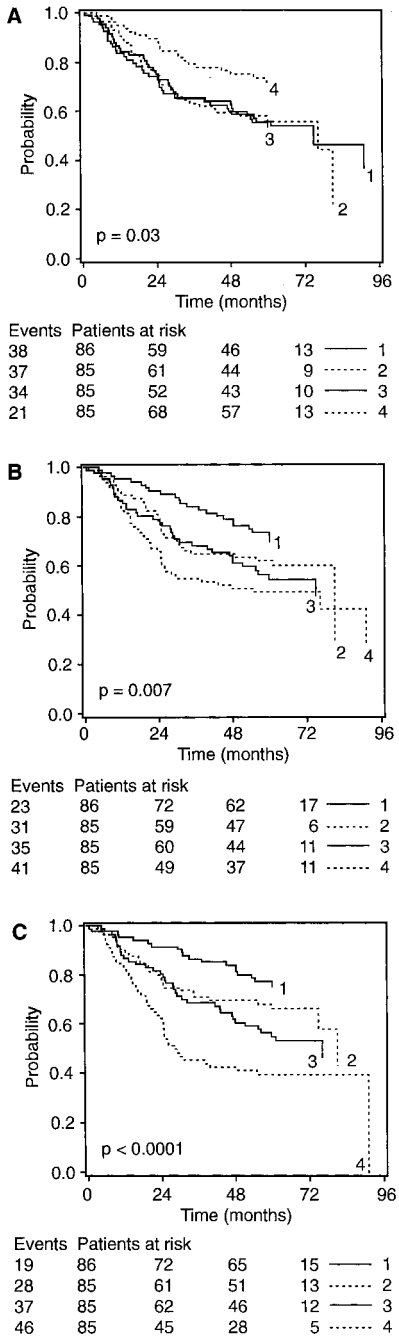

Fig. 3. Survival curves showing the association of tumor tissue uPA·PAI-1 complex level (*A*), total uPA level (*B*), and total PAI-1 level (*C*) with RFS. Patients were divided into those with a value within the first quartile (*1*; for complex, <0.49 ng/mg protein; for uPA, <2.4 ng/mg protein; for PAI-1, <1.4 ng/mg protein), the second quartile (*2*; for complex, <0.75 ng/mg protein; for uPA, <5.2 ng/mg protein; for PAI-1, < 2.4 ng/mg protein), the third quartile (*3*; for complex, <1.2 ng/mg protein; for uPA, <9.2 ng/mg protein; for PAI-1, <4.3 ng/mg protein), or the fourth quartile (*4*). The numbers of events during the period and the numbers of patients at risk after each 24-month interval are indicated. *P*s were calculated by the log-rank test.

and OS ($P < 0.0001$ for all) and additionally steroid receptor status for OS (Table 5). High PAI-1 was strongly associated with both short RFS with a RR of 3.8 (CI, 2.2–6.4) and short OS with a RR of 3.5 (CI, 1.9–6.3) when comparing patients in the fourth quartile with those in the first quartile. If tumor grade of anaplasia, which was only available for the ductal carcinomas, was included in the model, grade II or III *versus* grade I gave a RR of 2.4 (CI, 1.3–4.7) for OS ($P = 0.007$), and the level of significance for independent prediction of RFS was 0.06. The results for the other, previously included parameters did not change appreciably (data not shown).

Secondly, we investigated the prognostic value of uPA·PAI-1 complex and the ratios as compared with the clinicopathological para-

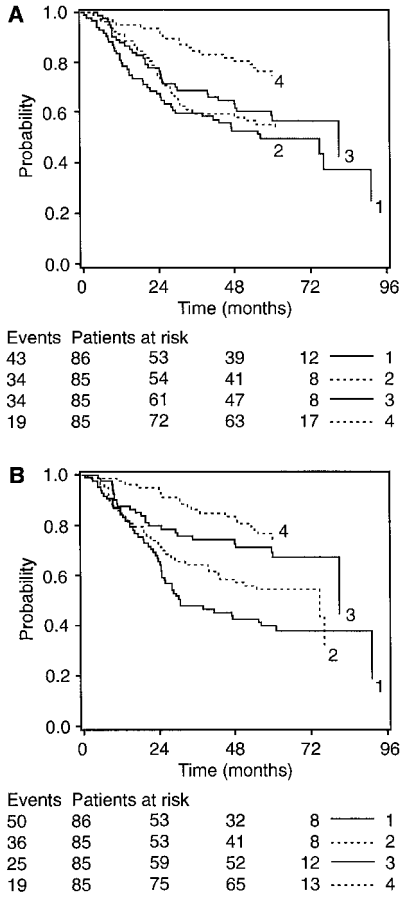

Fig. 4. Survival curves showing the association of tumor tissue level of the complex: total uPA ratio (*A*) and the complex:total PAI-1 ratio (*B*) with RFS. Patients were divided into those with a value within the first quartile (*1*; for complex:uPA, < 0.06; for complex:PAI-1, < 0.14), the second quartile (*2*; for complex:uPA, <0.13; for complex: PAI-1, < 0.27), the third quartile (3; for complex:uPA, <0.4; for complex:PAI-1, < 0.69), and the fourth quartile (*4*). The numbers of events during the period and the numbers of patients at risk after each 24-month interval are indicated.

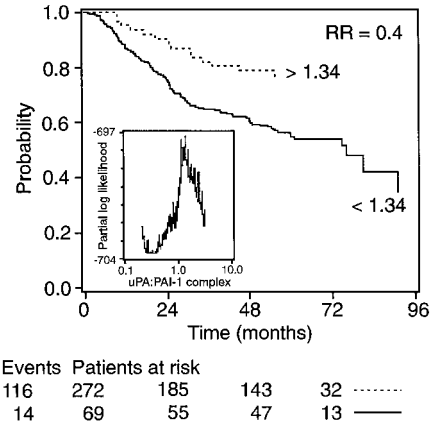

Fig. 5. Survival curves showing the association of tumor tissue uPA·PAI-1 complex level with RFS. Patients were divided into those with a value above (——) and those with a value below (- - - -) the optimal cutoff value (1.34 ng/mg protein). The numbers of events during the period and the numbers of patients at risk after each 24-month interval are indicated. *Inset*, uPA· PAI-1 complex value range *versus* partial likelihood score for RFS.

Table 5 *Multivariate Cox survival analysis for studied parameters of all patients*

Parameter	RFS			OS		
	P	RR	CI	*P*	RR	CI
Age[a]	0.71			0.88		
Menopausal status	0.28			0.17		
Tumor size[b]	0.16			0.12		
Receptor status	0.11			0.002	0.5	0.3–0.8
Lymph node status[c]	<0.0001	2.6	1.8–3.8	<0.0001	4.4	2.8–6.8
Total uPA[d]	0.99			0.95		
Total PAI-1[d]	<0.0001			<0.0001		
2nd *vs.* 1st quartile		1.6	0.9–2.8		1.3	0.7–2.5
3rd *vs.* 1st quartile		1.9	1.1–3.4		1.5	0.8–2.8
4th *vs.* 1st quartile		3.8	2.2–6.4		3.5	1.9–6.3
uPA·PAI-1 complex[d]	0.46			0.13		
Complex:total uPA[d]	0.82			0.85		
Complex:total PAI-1[d]	0.61			0.56		

[a] Premenopausal, below and above 40 years; postmenopausal, in 10-year intervals.
[b] ≤2 cm *vs.* 2.1–5 cm *vs.* >5 cm.
[c] Node positive *vs.* node negative.
[d] Quartiles.

meters, *i.e.*, total uPA and PAI-1 were omitted from the model. In this model, the only parameters that independently and significantly predicted RFS and OS were the complex:total PAI-1 ratio and lymph node status ($P < 0.0001$ for both; data not shown). It was not surprising that the complex:PAI-1 ratio became an independent prognostic value in this model, considering the very close association between the ratios and the corresponding total antigen levels, as described above. If, in addition, the ratios were omitted from the model, the level of significance for uPA·PAI-1 complex to independently predict RFS was 0.06. The results for the remaining parameters did not change appreciably (data not shown).

Subgroup Analysis. A separate exploratory analysis of the clinically important node-negative patient subgroup was performed, with the understanding that the uPA·PAI-1 complex levels were significantly higher in lymph node-negative tumors than in node-positive tumors. In the node-negative group of 164 patients, there were only 39 recurrences and 25 deaths during the observation period. Thus, in univariate analysis using the uPA·PAI-1 complex, total uPA, and PAI-1 quartiles to divide this patient material, only PAI-1 showed a significant association with survival (data not shown). For PAI-1, a comparison of patients in the fourth and first quartiles gave a RR of 6.1 (CI, 2.2–17) for RFS ($P = 0.0002$) and a RR of 8.4 (CI, 2.5–29) for OS ($P < 0.0001$). In the fourth quartile, there were 41 patients among whom 17 recurrences and 17 deaths occurred (data not shown). None of the clinicopathological parameters were of prognostic significance. Multivariate analysis of the node-negative patient group confirmed PAI-1 to be the only parameter that independently and significantly predicted survival (data not shown).

DISCUSSION

During the past decade, a series of publications from different laboratories have consistently reported the prognostic significance of uPA and PAI-1 levels measured in breast cancer tissue extracts (5, 15). In the present study of 342 breast cancer patients with an extended median follow-up period of 67 months and with representative traditional clinicopathological parameters, we again confirmed the prognostic significance of uPA and PAI-1. However, this is the first report in which uPA·PAI-1 complex was also measured, enabling an assessment of the relationship between all three parameters and clinicopathological parameters and survival. The results of this retrospective study provide evidence for an association between uPA·PAI-1 complex and survival in breast cancer patients. However, in contrast to uPA and PAI-1 levels, high tumor uPA·PAI-1 complex levels were associated with a favorable prognosis.

The present study was based on a newly developed kinetic uPA·PAI-1 complex ELISA that allowed a high level of sensitivity and specificity in the quantitation of this complex in tissue extracts (18). Using a sandwich format of two monoclonal anti-PAI-1 capture antibodies and three anti-uPA detector antibodies, this assay selectively detects the complex but not the free forms of the individual components. Furthermore, free uPA and PAI-1 in relevant pathophysiological concentrations do not interfere with the detection of complex. Thus, in the present study of 341 breast cancer extracts, uPA·PAI-1 complex could be readily detected in all of the samples. Importantly, the assay also assures that only preformed complex is quantified. This is achieved in two steps: (*a*) extraction of the tumor tissue at a low pH prevents *ex vivo* formation of complex from free uPA and PAI-1 in the tissue without destabilizing preformed complex; and (*b*) during incubation of the assay plate at neutral pH, uPA·PAI-1 complex formation from free components in the diluted extracts was blocked by *p*-nitrophenyl guanidinobenzoate, which rapidly inactivates uPA. Moreover, there was no evidence of complex instability during tissue and extract storage because the complex levels did not change with tissue storage time, and complex was preserved as shown by Western blots of tissue extracts. Thus, the data obtained by the uPA·PAI-1 complex ELISA were suitable for further analyses of the prognostic implications of the complex in breast cancer extracts.

The uPA·PAI-1 complex level among the 341 extracts varied moderately (~20-fold) as compared with the concentration variation for total uPA and PAI-1 (>100-fold). Furthermore, the complex was found to represent a minor, variable fraction of total uPA or PAI-1, whereas a close association between uPA and PAI-1 was found. Thus, the complex levels appeared to be largely independent of the total uPA and PAI-1 levels, and the complex levels measured could therefore not merely be equated with consumption of uPA or PAI-1. This finding may reflect separate cellular locations of the components, limited activation of uPA, and a preponderance of latent PAI-1. With regard to tumor dissemination, it is noteworthy that in contrast to total uPA and PAI-1, the complex levels were related to lymph node status; patients in the node-negative group had higher tumor levels of complex than did node-positive patients. However, a considerable overlap in the intermediate complex concentration range suggested that the primary tumor complex level could not be used as a surrogate measure of disseminated disease.

The prognostic value of uPA·PAI-1 complex was assessed by comparing survival for patient groups stratified into quartiles of the corresponding complex levels because scoring of the complex as a continuous variable did not adequately fit a linear model in the analysis. High levels of uPA·PAI-1 complex were associated with a lower probability of developing recurrences and of experiencing an early death. We further observed that the patient group with the highest complex values was most clearly separated, indicating that we could search for an optimal cutoff point between good and poor prognosis. Thus, using uPA·PAI-1 complex as an optimally dichotomized variable, a group with the highest complex levels and comprising 20% of the patients could be identified as having a particularly favorable prognosis. We further analyzed the prognostic value of uPA·PAI-1 complex compared with the value of the total uPA or PAI-1 levels. Interestingly, the uPA·PAI-1 complex levels in the extracts were by themselves of less prognostic value than total uPA or PAI-1. Moreover, in multivariate analyses, which also included classical prognostic parameters, neither uPA·PAI-1 complex nor total uPA appeared to be independent predictors of recurrence and death. Total PAI-1, however, was a highly significant and strong independent prognostic marker, consistent with the many previous reports (30, 31). The strong prognostic power of PAI-1 was also confirmed in the clinically important subgroup of lymph node-negative patients, al-

though only a limited number of events were registered during the observation period.

These observations are consistent with the hypothesis that effective local regulation of active uPA by active PAI-1, *i.e.*, formation of complex, in the tumor may limit tumor invasion and metastasis. However, in the absence of a simple inverse relationship between complex and either uPA or PAI-1, these observations do not offer an explanation why high levels of PAI-1 are so strongly associated with short survival. An alternative explanation of why high levels of uPA·PAI-1 complex are related to longer survival takes into account the published data showing that high levels of uPAR predict poor outcome for breast cancer patients (32, 33): because one pathway of uPA·PAI-1 complex removal is mediated by the internalization of cell surface uPAR and the low-density lipoprotein receptor-related protein [α_2-macroglobulin receptor (5, 14)], it can be speculated that high complex levels reflect low uPAR or low-density lipoprotein receptor-related protein expression, leading to impaired removal of uPA·PAI-1 complex. Thus, lower levels of uPAR expression may be the basis for the relationship between high levels of complex and better prognosis. However, this hypothesis is inconsistent with the proposed role of uPAR in facilitating activation of uPA, which is necessary for the formation of uPA·PAI-1 complex, and also with the fact that high levels of total PAI-1 predict poor outcome.

To reconcile the new finding that high complex levels are related to good patient outcome with the established finding that high PAI-1 levels predict poor outcome, our previous proposal of the two-compartment model (34) may be invoked: in one compartment, uPA promotion of local tumor invasion is attenuated by PAI-1 complexation; whereas in another compartment, high levels of PAI-1 protect the provisional pericellular matrix of nascent capillaries forming behind uPA promoted extensions. This interpretation is consistent with the immunohistochemical localization of PAI-1 in endothelial cells in breast cancer tissue (15) and the absence of tumor-inducible angiogenesis in PAI-1-deficient mice (35).

Because the complex comprised a minor, variable fraction of total uPA and PAI-1, the biological significance of the complex could possibly relate more to the ratio of the complex to total antigen level than to the complex level alone, *i.e.*, a high ratio may indicate an effective role of the PAI-1 in the first compartment of the model above. Therefore, we additionally investigated whether the ratios represented an enhancement of prognostic power. Indeed, a high complex:total uPA or complex:total PAI-1 ratio conferred a strong association with longer breast cancer survival as evaluated by univariate analysis. However, multivariate analysis simultaneously including the ratios and the total antigen levels still showed the total (PAI-1) antigen level to be of superior prognostic power. This could suggest that the role of PAI-1 in the second compartment above, *i.e.*, protection of new capillary growth, is the predominant factor in determining tumor expansion. The above-mentioned findings of the relationship between uPA·PAI-1 complex and survival may be compared with reports showing that the complex between metalloproteinase-9 and tissue inhibitor of metalloproteinases in combination with total plasma metalloproteinase-9 is a prognostic marker in gastrointestinal cancer (36) and that the ratio between complex-bound:free serum prostate-specific antigen and the total antigen level is actually a better diagnostic marker for prostate cancer than total prostate-specific antigen itself (37, 38).

In conclusion, our present findings on uPA·PAI-1 complex add new data to the emerging picture we have of the relationship between the tumor tissue levels of components of the uPA system and patient survival. This report demonstrates for the first time that high levels of uPA·PAI complex in tumor tissue are associated with longer survival of breast cancer patients. The results justify further exploratory studies of the utility of tumor uPA·PAI-1 complex levels, including identification of clinically relevant cutoff points for identification of low- and high-risk patients with breast cancer or other malignancies in focused adjuvant therapy.

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