Tumor stroma is the predominant uPA-, uPAR-, PAI-1-expressing tissue in human breast cancer: prognostic impact

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Summary. Urokinase-type plasminogen activator (uPA), its receptor (uPAR) and its inhibitor PAI-1, play a key role in tumor invasion and metastasis. uPA and PAI-1 were the first novel tumor biological factors to be validated at the highest level of evidence regarding their clinical utility in breast cancer. Their antigens are determined in tumor tissue extracts by standardized, quality-assured immunometric assays (ELISA). Since the late 1980s, numerous independent studies have demonstrated that patients with low levels of uPA- and PAI-1 in their primary tumor tissue have significantly better survival than patients with high levels of either factor. However, it is unclear whether it is their (relative) levels in the tumor stroma or in the tumor cells themselves that is most relevant to patient outcome. This missing knowledge leads to an uncertainty concerning the management of breast cancer tissue specimens. It is unclear how much tumor stroma is allowed in one tumor tissue specimen for an adequate assessment of the patients' outcome. This is the first study in which tumor cells and stromal tissue of invasive breast carcinomas (n=60) were separated by laser capture microdissection followed by ELISA-based determination of the uPA-, uPAR- and PAI-1-levels. In addition, we have assessed uPA-, uPAR- and PAI-1 distribution in formalin-fixed, paraffin-embedded breast cancer specimens (n=60) by immunohistochemistry.

The uPA-, uPAR- and PAI-1 in tumor stroma only, tumor cells only and not separated tumor tissue did not show any significant differences in protein-levels determined by ELISA. Cox regression analysis showed that patients with high uPA-, high uPAR-, and/or high PAI-1-levels, as compared to patients with low levels of either factor, showed a significantly shorter relapse-free survival and overall survival (p=0.000001). These results suggest that a strong expression of uPA, uPAR and PAI-1 in the tumor stroma, as well as in tumor cells, have the same impact on the clinical behaviour of breast cancer. Conclusion: When using uPA- and PAI-1 levels as prognostic and predictive factors in breast cancer the quantity of tumor stroma in the tumor tissue specimen is not relevant for the assessment of the patients' outcome.

Key words: Breast cancer, Tumor stroma, uPA-system, Prognostic impact

Introduction

Breast cancer is a heterogeneous disease showing great variability of biological and clinical behavior. The determination and location of components of the plasminogen activator system in breast cancer tissue is an important issue to address, since there is substantial evidence that a high concentration of proteolytic factors in primary breast cancer tissue is essential for tumor cell spread, intravasation, access to the systemic circulation, and metastasis. Tumor cells cross host cellular and extra cellular matrix barriers during tumor invasion and metastasis by the attachment to and interaction with components of the basement membrane and the extracellular matrix, and by local proteolysis.

Penetrating tumor cells focus proteolytic activity of the serine protease urokinase-type plasminogen activator (uPA) secreted by tumor cells or surrounding stromal cells to the cell surface through a receptor for uPA (uPAR, CD87), thus facilitating extracellular matrix degradation, cell proliferation, apoptosis, invasion, and metastasis (Jaenicke et al., 1990; Schmitt et al., 1997; Andreasen et al., 2000; Harbeck et al., 1999, 2002, 2004; Binder et al., 2007; Hildenbrand et al., 2008). The proteolytic activity of uPA is regulated by the fast and specific inhibitor plasminogen activator inhibitor type-1 (PAI-1). PAI-1 is able to react with uPAR-bound uPA, resulting in the formation of uPAR-bound uPA-PAI-1 complex (Durand et al., 2004). McMahon and coworkers reported that PAI-1 consistently confers proangiogenic effects, suggesting that PAI-1 may enhance tumor growth and invasion by stimulating angiogenesis (McMahon et al., 2001). Several studies assessing both mRNA and protein levels have found that elevated levels of the uPA-system are associated with both aggressive tumor characteristics and poor prognosis. uPA and PAI-1 were the first novel tumor biological factors to be validated at the highest level of evidence (LOE Ia) regarding their clinical utility in breast cancer (Harbeck et al., 2004). However, it is unclear whether it is their (relative) levels in the stroma or in the tumor cells themselves that is most relevant to patient outcome. This missing knowledge leads to an uncertainty concerning the management of breast cancer tissue specimens. It is unclear how much tumor stroma is allowed in one tumor tissue specimen for a valid application of this measurement, and for an adequate assessment of the patients' outcome. Immunohistochemistry-based studies are especially useful in determining the cellular locations of these proteins and this may help to clarify this point. However, immunhistochemistry is not quantitative. Therefore, in the present study tumor cells and stromal tissue of 60 invasive breast carcinomas were separated by laser capture microdissection, followed by ELISA-based determination of the uPA-, uPAR- and PAI-1-levels. The mean uPA-, uPAR- and PAI-levels in tumor stroma only (stromal tissue without cancer cells), tumor cells only, and not separated tumor tissue were systematically compared and did not show any significant differences. Interestingly, mean values of each factor were highest in tumor stroma only. Moreover, the results from survival analysis suggest that strong expression of uPA, uPAR and PAI-1 in tumor stroma only, as well as in tumor cells only, have the same impact on the clinical behaviour of breast cancer, as compared to results of non-dissected tissue.

Material and methods

Immunohistochemistry

Immunohistochemistry was performed on 2 μm thick paraffin-sections using a mouse anti-uPAR-monoclonal antibody (clone 3B10, IgG2a; American Diagnostica #3936; 1:100 dilution) to uPAR expressed by phorbol ester-stimulated promyeloid U937 cells. Consecutive tissue sections were stained with mAbs to anti-uPA (American Diagnostica, Germany, #3689) and anti-PAI-1 (American Diagnostica, Germany #3785). Immunostaining was based on an alkaline phosphatase-conjugated streptavidin-biotin detection system (Amersham Pharmacia Biotech Inc., Piscataway, NJ), using Fast Red (Roche Diagnostics GmbH) as a chromogen. For both antibodies antigen retrieval was achieved by microwave treatment (3x5 min, 600W). Incubation of the primary antibody was carried out for 60 min at 37°C. Negative controls were performed by substituting non-immune IgG. For recognising macrophages, endothelial cells, fibroblasts, myofibroblasts, smooth muscle cells and lymphocytes, consecutive tissue sections were stained with mAb to CD34, CD68, CD45, smooth muscle antigen (SMA) and to fibroblast antigen (all from DAKO, Hamburg, Germany). The anti-ER (clone 1D5) and anti-PgR (clone PgR636) antibodies (all mouse monoclonal), as well as Hercep Test system for the detection of the HER2/neu expression came from DAKO (Hamburg, Germany). Immunohistochemistry was performed using a standard biotin-streptavidin method with the appropriate antigen retrieval method for each antibody. With the execption of Hercep Test, we used Fast Red (Roche. Mannheim, Germany) as chromogen. Nuclei were counterstained with hematoxylin.

Assessment of staining results

For assessment of staining results ER- and PgR-stained tissue sections were evaluated according to the immunoreactive score (IRS) as described by Remmele and Stegner (Remmele and Stegner et al., 1987). Tumors were considered ER- or PgR-positive when at least 10 % of tumor cell nuclei showed positive immunoreactions. For the determination of HER2/neu protein overexpression, only the membrane staining pattern and intensity of invasive tumor cells were scored. Immunostaining was scored according to the criteria specified by DAKO for the interpretation of the Hercep Test. The score ranged from 0 (negative) to 3+ (strong membrane signal). In three cases the Hercep Test score was 2+. In these cases we performed an additional HER2/neu-fluorescence In situ hybridization analysis (FISH test) as previously reported (Arens et al., 2005).

Expression of uPA, uPAR and PAI-1 was based on the intensity (positive: moderate to strong staining of cytoplasm) and quantity (>10% positive cells) of immunostaining. Cells of one case were considered as positive in a moderate to strong immunoreaction of cytoplasm (> 10% positive cells). A scoring-system (grading) was not used.

Laser capture microdissection

Serial frozen sections (4-8μm) were cut on a

standard cryostat (Leica, Germany) with a clean blade. The unfixed tissue sections were immediately stored at -80°C until use. The frozen sections were stained by toluidine blue (minimal amount of staining to visualize the tissue for microdissection; #19.816-1; Sigma, Germany). After staining and microscopic control of staining quality and tissue preservation, microdissection was performed using a laser capture microdissection microscope (Arcturus) equipped with an infrared laser. The tissue sections were overlaid with optically transparent caps, and tissue/cells were captured by focal melting through laser activation. After visual control of the completeness of dissection, captured tissue/cells were washed (TBS (0.002 M Tris HCL, 0.125 M NaCl, pH 8.5) and destained. Carcinoma cells were completely separated from tumor stroma by microdissection yielding 250-300 mg wet weight of separated carcinoma cells and tumor cell free stromal tissue. Unprocessed (not microdissected) frozen tumor sections were treated (washed and destained) equally yielding also 250-300 mg wet weight tissue (tumor cells and tumor stroma). Between 40 and 80 tissue sections were microdissected for receiving 250-300 mg wet weight tissue (tumor cells only and tumor stroma only) per case. Sixty cases of breast cancer and 10 cases of normal (non-neoplastic) breast tissue were treated by laser capture microdissection.

Tissue extraction and ELISA

Tissue extraction and ELISA was performed as previously reported (Hildenbrand et al., 1998). Briefly, deep frozen specimens of 250-300 mg wet weight (1. carcinoma cells only; 2. tumor cell free stromal tissue; 3. non microdissected tumor tissue) were pulverized by a micro-dismembrator (Satorius, Göttingen, Germany). The resulting powder was suspended in 1.8 ml TBS (0.002 M Tris HCL, 0.125 M NaCl, pH 8.5) and 0.2 ml of the nonionic detergent Triton X 100 10% (Sigma München, Germany) yielding a 1% Triton X 100 final preparation. After gentle stirring for 12 h at 4°C, the suspension was subjected to centrifugation (21000xg for 60 min, 4°C) in order to separate cell debris. The total protein content of the extract was measured by using a conventional biuret-protein reaction assay (bca protein assay kit; Pierce,Il, USA). We performed an uPA-, uPAR- and PAI-1 ELISA using commercially available ELISA-kits (American Diagnostica, Pfungstadt, Germany, Immubind #893, #894, #821) and a conventional ELISA-reader (Thermo Multiscan EX) according to the manufacturers instructions. The measurements were performed in duplicate. uPA- uPAR- and PAI-1 content was determined in the Triton X 100 extract and calculated per mg of tissue protein.

Statistical analysis

Results are expressed as the mean ± standard error of the mean (SEM) and are considered significant at the p<0.05 level (two-tailed). Wilcoxon-Mann-Whitney (rank sum test) was used for statistical analyses. Relapse-free survival (RFS) and Overall survival (OS) probabilities were calculated using the acturial method of Kaplan-Meier (Mantel-Cox log rank test) (Kaplan and Meier, 1958). Cox regressions model was used to test for differences and trends. Multivariate analysis was not performed because of the limited sample size (n=60).

Results

Patients and histomorphological factors

Retrospective analysis of relapse-free survival (RFS) and overall survival (OS) was performed in 60 patients with primary, operable, invasive breast cancer. Patients had been treated for primary breast cancer between 1987-1999 in the Department of Obstetrics and Gynecology, Faculty Clinical Medicine Mannheim, University of Heidelberg. Median age of patients at time of primary surgery was 57±2.3 years (range 31-84 years). Twenty eight patients received modified mastectomie and 32 patients were treated by breast conserving therapy, including radiotherapy. All patients received an axillary lymph node dissection (level-I and -II). Treatment decisions with regard to primary surgery and adjuvant systemic therapy were based primarily on the consensus recommendation at the time. Thirty-nine patients had positive lymph nodes and were treated by adjuvant chemotherapy, the majority received CMF (cyclophosphamide/methotrexate/5-fluorouracil), 10 patients received anthracycline-containing regimes. Fifty patients received adjuvant hormonal therapy by tamoxifen, either alone or in combination with chemotherapy (10 patients). At time of primary therapy, no patient had any clinical or radiological evidence of distant metastases. All patients were examined routinely every 3-6 month during the first 5 years of follow-up and once a year thereafter. Of the 60 patients examined 55 patients showed evidence of disease during follow up (local relapse and/or distant metastasis). All 60 patients died. Fifty-five patients died after previous relapse. Five patients died without evidence of disease. Mean relapse-free survival (RFS) was 25±3.5 month (range 1-94 month). Mean overall survival (OS) was 36±4.1 month (range 1-94 month).

This limited series of breast cancer patients (n=60) has been collected in a quite long time interval (1987-1999), data on prognosis for this cohort of patients are quite unfavorable (mean RFS of 25±3.5 months; mean OS of 36±4.1 months). We can not exclude the possible interference of novel breast cancer treatments on survival data. Nowadays novel therapy strategies have considerably improved prognosis of breast cancer patients.

Tumor sections were stained with haematoxylin and eosin, and graded according to the Elston and Ellis criteria (Elston and Ellis, 1991). Fourty-one tumors were

grade II, 19 were grade III, no tumor was grade I. Fourty-eight carcinomas were of an infiltrating ductal type, 12 were of an infiltrating lobular type. Three tumors were beweeen 18 mm and 20 mm (pT1) in size, 50 tumors were more than 20 mm but not more than 50 mm in size (pT2), 3 tumors were more than 50 mm (pT3) and four tumors showed an ulcerated epidermis (pT4). Further characteristics of patients and tumors are listed in Table 1.

ELISA

This is the first study in which tumor cells and stromal tissue of invasive breast carcinomas were separated by laser capture microdissection followed by ELISA-based determination of the uPA-, uPAR- and PAI-1-levels (Fig. 1). When tumor cells and stroma were not separated by laser microdissection, the mean (±SEM) uPA-level (n=60) was 2.79±0.56 ng/mg (range 0.02-12.5 ng/mg), the mean (±SEM) uPAR-level (n=60) was 4.00±0.45 ng/mg (range 0.05-11.5 ng/mg) and the mean (±SEM) PAI-1-level (n=60) was 12.2±2.4 ng/mg (range 1.3-37.9 ng/mg). When tumor cells and stroma were separated by laser microdissection, the mean (±SEM) uPA-level of tumor cells only (n=60) was 2.78±0.63 ng/mg (range 0.02-10.3 ng/mg), the mean (±SEM) uPAR-level of tumor cells only (n=60) was 4.12±0.53 ng/mg (range 0.05-11.8 ng/mg), and the mean (±SEM) PAI-1-level of tumor cells only (n=60) was 12.6±2.5 ng/mg (range 0.78-39.6 ng/mg). In stromal cells only, the mean (±SEM) of uPA (n=60) was 3.12±0.6 ng/mg (range 0.02-12.9 ng/mg), of uPAR

Table 1. Patients and tumor characteristics.

| All patients	n=60
Age (years) mean±SEM (range)	57±2.3 (31-84)
Tumor size (mean±SEM)(range)	31.1±3.5mm (19-63 mm)
pT1	3
pT2	50
pT3/4	7
Positive nodes (mean±SEM)(range)	3.3±0.5 (1-12)
Removed nodes (mean±SEM)(range)	18.3±2.6 (10-35)
pN0	n=21
pN1-3	n=39
Grade1	n=0
Grade 2	n=41
Grade 3	n=19
ER positive	n=52
ER negative	n=8
PR positive	n=52
PR negative	n=8
Her2neu positive	n=11
Her2neu negative	n=49
L0/V0	n=11
L1 and/or V1	n=49
Ki67 %(mean±SEM)(range)	22.3±2.4% (5%-61%)
uPA (ng/mg) (mean±SEM)(range)	2.79±0.55 (0.02-12.46) n=60
uPAR (ng/mg)(mean±SEM)(range)	4.00±0.45 (0.05-11.5) n=60
PAI-1 (ng/mg) (mean±SEM)(range)	12.2±2.5 (0.78-39.6) n=60
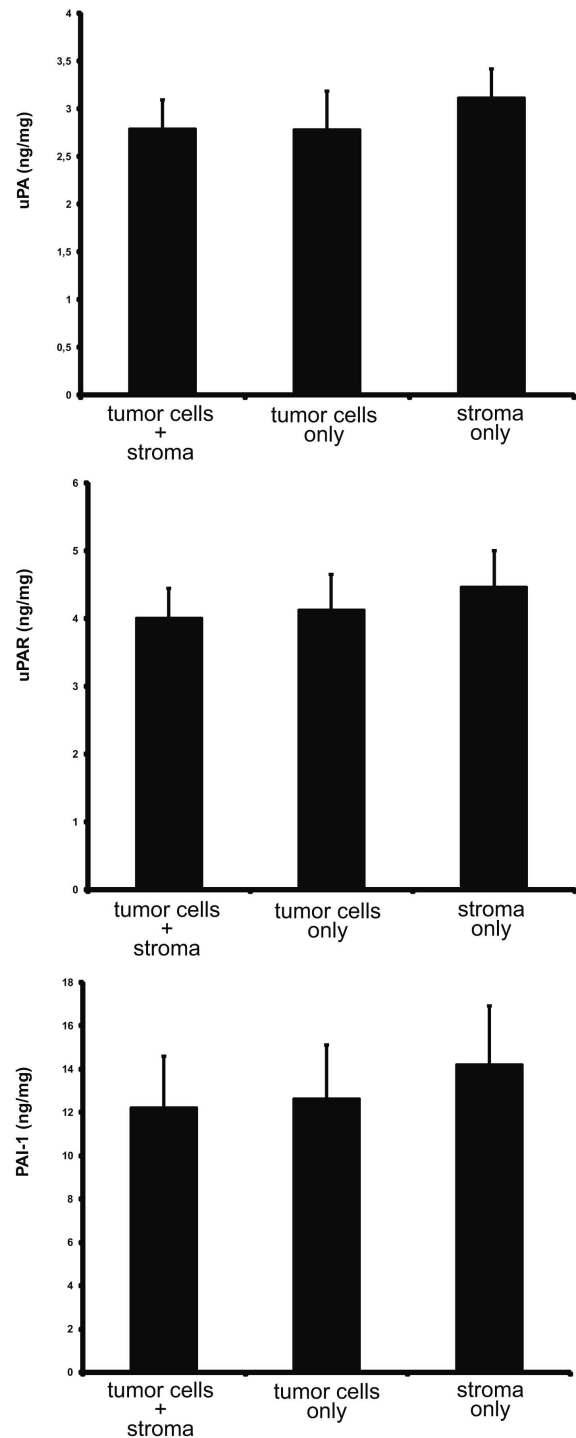
Fig. 1. Tumor cells and stromal tissue of invasive breast carcinomas (n=60) were separated by laser capture microdissection followed by ELISA-based determination of the uPA- **(A)**, uPAR- **(B)** and PAI-1-levels **(C)**. No significant differences between mean values (mean±SEM) of uPA, PAI-1 and uPAR exsist although tumor tissue was separated into tumor stroma only and tumor cells only. Regarding tumor stroma only, there is a statistical trend towards elevated levels of each factor as compared to not separated tumor tissue, or tumor cells only (p<0.05). Mean values of each factor are highest in tumor stroma.

4.46±0.54 ng/mg (range 0.05-12.9 ng/mg), of PAI-1 14.2±2.7 ng/mg (range 0.94-41.6 ng/mg). The uPA-, uPAR- and PAI-1 amounts of non-separated tumor tissue was not significantly different from those in tumor cells only, or from stromal tissue only (p>0.05) (Fig. 1).

A cut-off point of uPA (3.0 ng/mg) and PAI-1 (14 ng/mg) in ELISA was previously defined and validated by different authors (Foekens et al., 2000; Harbeck et al.,

2004). For uPAR (4.0 ng/mg), the arithmetic mean was defined as a cut-off point (Schmalfeldt et al., 1995). According to these cut-off points, the patients were separated into high- and low risk groups, and analyzed by Kaplan-Meier. Figure 2 shows the relationship between uPA (A), uPAR (B) and PAI-1 (C), and both RFS and OS for all patients. The actuarial Kaplan-Meier curves for each factor are identical when separating
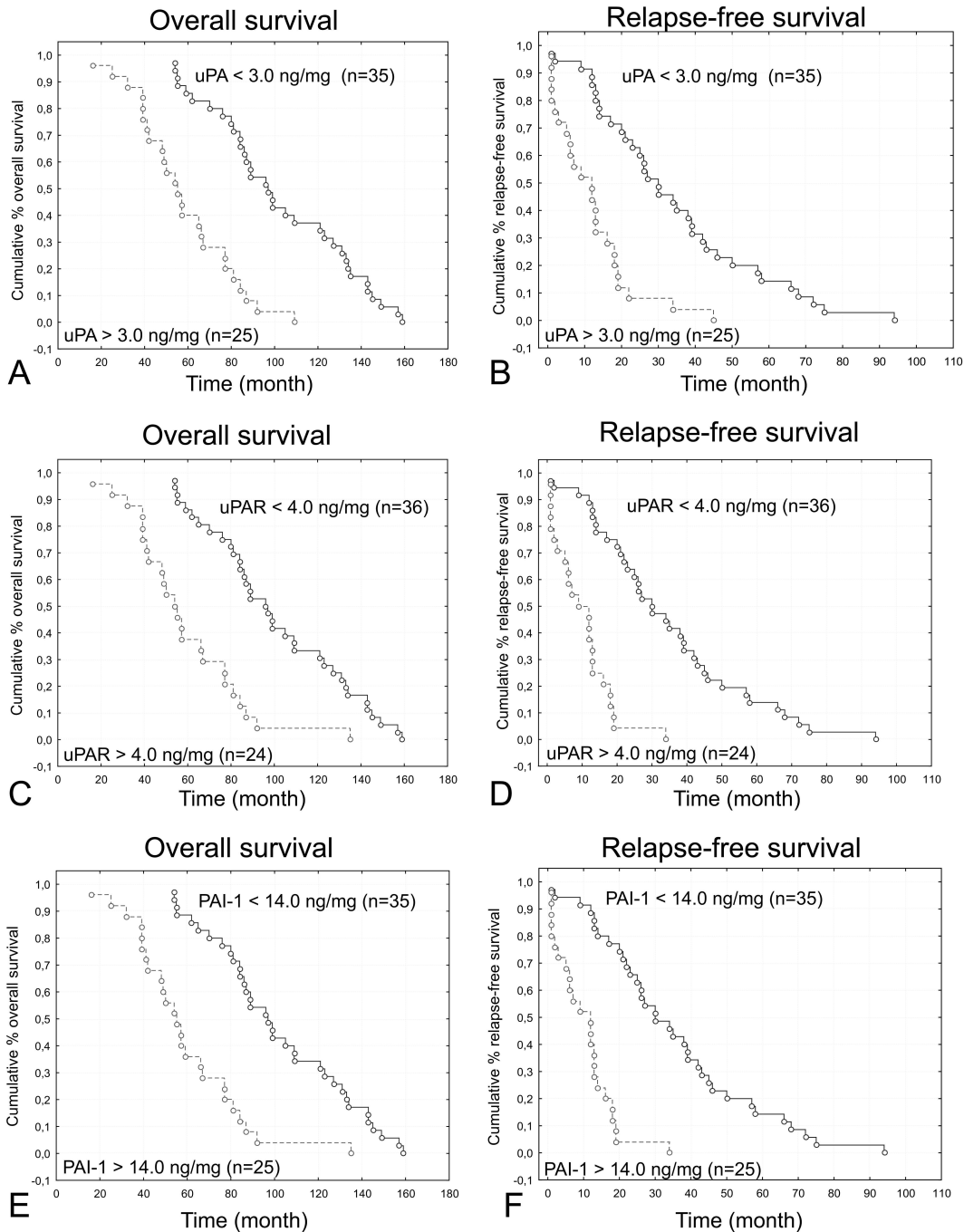

Fig. 2. Relationship between uPA (A+B), uPAR (C+D) and PAI-1 (D+E) and both RFS and OS for all patients (n=60). According to the cut off points (uPA: 3.0 ng/mg; uPAR: 4.0 ng/mg; PAI-1: 14.0 ng/mg) the patients were separated into high- and low risk groups and Kaplan-Meier curves were calculated. After separating tumor tissue into tumor stroma and tumor cells by micro-dissection, determining the three factors by ELISA and grouping according to the cut off values, the high- and low risk groups did not show any differences, and the Kaplan-Meier curves are identical. Patients with high levels of uPA (>3.0 ng/mg), high levels of uPAR (>4.0 ng/mg) and high levels of PAI-1 (>14.0 ng/mg) have a worse prognosis than patients with low levels of the factors (p<0.000001, for each factor; Cox regressions analysis).
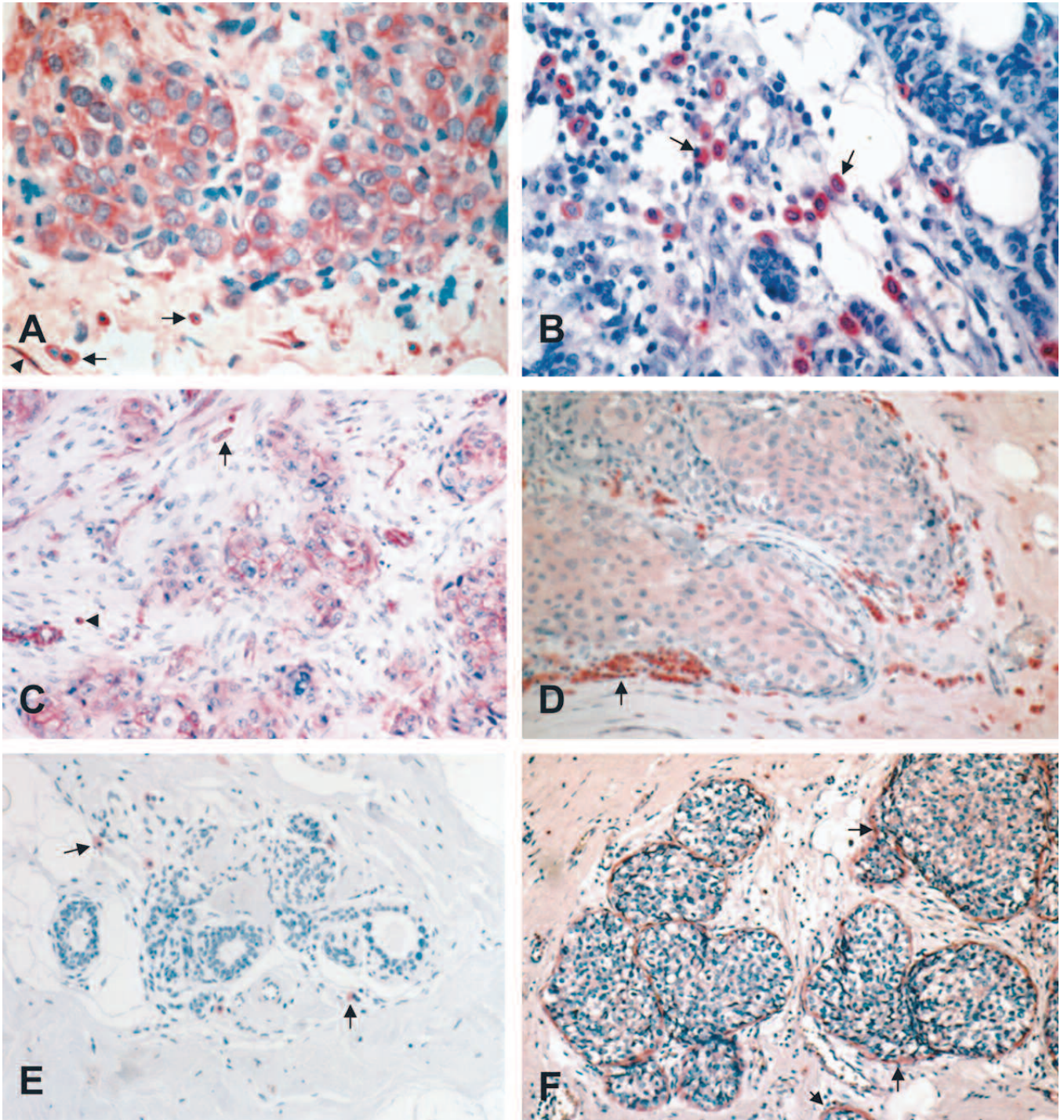
Fig. 3. A. Immunohistochemical staining of an invasive ductal carcinoma using anti-uPA antibody. Cancer cells, fibroblasts (arrowhead) as well as tumor associated macrophages (arrows) are positive. **B.** Immunohistochemical staining of an invasive ductal carcinoma using anti-uPA antibody. Cancer cells do not show any anti-uPA immunoreaction, whereas tumor associated macrophages (arrows) exhibit a strong anti-uPA immunoreaction. **C.** Immunohistochemical staining of an invasive ductal carcinoma using anti-uPAR antibody. Cancer cells express the uPAR antigen. Some cancer cells show a distinct anti-uPAR immunoreaction of their cell membranes. Tumor associated macrophages (arrowhead) as well as fibroblasts (arrow) are also positive. **D.** Immunohistochemical staining of a ductal carcinoma in situ (DCIS) using anti-PAI-1 antibody. Periductal tumor associated macrophages (arrow) exhibit a strong PAI-1 expression, whereas tumor cells show a faint anti-PAI-1 immunoreaction only. **E.** Immunohistochemical immunoreaction of normal (non-neoplastic) breast tissue using anti-PAI-1 antibody. Epithelial cells of the breast gland are negative. Some resident macrophages (arrows) express PAI-1. **F.** Immunohistochemical staining of a ductal carcinoma in situ (DCIS) using anti-uPAR antibody. Myoepithelial cells (arrows) are positive, whereas tumors cells show only a faint anti-uPAR immunoreaction. A, B, x 200; C, F, x 100

tumor tissue into tumor stroma only and tumor cells only by laser capture miscrodissection. As found for non-separated tumor tissue, patients with high levels of uPA (>3.0 ng/mg), high levels of uPAR (> 4.0 ng/mg), and for high levels of PAI-1 (>14.0 ng/mg) have a worse prognosis than patients with a low amount of these factors (p<0.000001, for each factor).

Additionally, 10 cases of normal (non-neoplastic) breast tissue were examined in the same way, yielding the following results: Non separated tissue (mean±SEM): uPA:0.03±0.005 ng/mg; PAI-1: 2.03±0.2 ng/mg; uPAR: 0.04±0.02 ng/mg. Separated stroma only (mean±SEM): uPA:0.04±0.01 ng/mg; PAI-1: 2.23±0.1 ng/mg; uPAR: 0.06±0.03 ng/mg. Separated epithelial cells (of non-neoplastic breast glands) (mean±SEM): uPA:0.03±0.006 ng/mg; PAI-1: 2.1±0.1 ng/mg; uPAR: 0.05±0.02 ng/mg. No significant differences were found.

Correlation of the uPA-system and other traditional prognostic factors

No correlations were found between uPA, uPAR and PAI-1 and tumor size, grade, nodal status, ER- and PR-status, HER2neu-status, or menopausal status. These results are in agreement with other authors, who previously reported that the prognostic impact of uPA and PAI-1 is independent of these other prognostic factors (Harbeck et al., 2004).

Immunohistochemistry

To localize uPA, uPAR and PAI-1 in breast cancer tissue, we studied the 60 cases of invasive breast carcinomas by immunohistochemistry (Fig. 3). Strong uPA and PAI-1 expression was seen in the tumor cells of 59 of 60 cases. uPAR expression in cancer cells was found in 38 of 60 cases. In all of the breast cancer tissues examined fibroblasts/myofibroblasts, as well as macrophages, showed a distinct immunoreaction with mAb to anti-uPA, anti-uPAR, and anti-PAI-1. Likewise, the tissue specimens were screened for reactivity of endothelial cells with the various antibodies: PAI-1 was positive in 50 cases, uPAR in 25 cases and uPA in 55 cases. In 35 cases the invasive breast carcinomas examined were associated with DCIS-components. In all of these cases, intraductal tumor cells expressed uPA and PAI-1, and in 17 cases intraductal tumor cells expressed uPAR.

Additionally, we examined the 10 cases with normal (non-neoplastic) breast tissue. In all cases, macrophages exhibited staining for uPA, uPAR, and PAI-1. Epithelial cells of normal breast glands showed a positive immunoreaction in 4 cases with anti-uPA and anti-PAI-1 antibodies. In the same four cases, some fibroblasts expressed uPA and PAI-1. In one case, uPAR was expressed by epithelial cells and some fibroblasts.

Discussion

Biochemical assays using tumor tissue extracts from breast cancer tissues show that high levels of expression of components of the urokinase system are associated with a poor prognosis (Foekens et al., 2000; Harbeck et al., 2002, 2004). uPA and PAI-1 were the first novel tumor biological factors to be validated at the highest level of evidence (LOE Ia) regarding their clinical utility in breast cancer (Harbeck et al., 2004). However, up to date it is unclear whether it is their (relative) levels in the stroma or in the tumor cells themselves that is most relevant to the patient's outcome. This missing knowledge leads to an uncertainty concerning the standardized management of breast cancer tissue specimens. It is unclear how much tumor stroma is allowed in one tumor tissue specimen for using this method correctly, and for a valid prediction of the individual patient's outcome. This is the first study in which tumor cells and stromal tissue of invasive breast carcinomas were separated by laser capture microdissection, followed by ELISA-based determination of the uPA-, uPAR- and PAI-1-amounts. Our results demonstrate (Fig. 1) that no significant differences between mean values (uPA, PAI-1, uPAR) of tumor stroma only, tumor cells only, or whole tumor tissue extract exist. Regarding tumor stroma only, there was a trend of elevated levels of each factor as compared to non-separated tumor tissue or tumor cells only (Fig. 1). Mean values of each factor are highest specifically in the tumor stroma.

Statistical analysis of patients with a high level of each factor compared to patients with low levels of either factor showed a significant shorter relapse-free survival and overall survival. Kaplan-Meier curves were exactly similar when expressions in tumor stroma only or tumor cells only were analyzed. These results indicate a strong collaboration of tumor stroma and tumor cells and suggest that a strong expression of uPA, uPAR and PAI-1 in tumor stroma only, as well as in tumor cells only, have the same impact on the clinical behaviour of breast cancer.

These results are an important issue for the practical use of tissue sampling. When using uPA- and PAI-1 amounts as prognostic and predictive factors in breast cancer, the quantity of tumor stroma in the tumor tissue specimen is not relevant for the assessment of the patients' outcome. This is important information from this work, since it suggests the analysis of the u-PA-system in breast cancer as one of the most robust tests for predicting outcome at a high level of clinical evidence.

Our ELISA results are confirmed by our immunohistochemical analysis. In all cases uPA, uPAR and PAI-1 were expressed in tumor-associated macrophages and fibroblasts of the tumor stroma. In nearly all (59/60) breast cancer tissues examined, carcinoma cells express uPA and PAI-1. In one case, carcinoma cells do not show any anti-uPA or anti-PAI-1 immunoreaction (immunohistochemical staining was performed several times and in different areas of the tumor), whereas uPAR was expressed by the tumor cells. In this case a strong uPA and PAI-1 expression in

macrophages and fibroblasts was found and uPA-amounts and PAI-1-amounts in isolated tumor cells were 1.82 ng/mg and 7.49 ng/mg, respectively. In this case, one could speculate that uPA and PAI-1 are present in amounts below the detection level of the antibody.

The immunohistochemical localizations of components of the uPA system have previously been reported by other authors (Constantini et al., 1991; Del Vecchio et al., 1993; Bianchi et al., 1994; Christensen et al., 1996; Jahkola et al., 1999; Dublin et al., 2000; Haas et al., 2008). These studies are in agreement with ours with respect to the presence of the uPA-system in carcinoma cells. In contrast to most of the other studies, we found a strong expression of the uPA-system in tumor-associated macrophages and fibroblasts in all invasive breast carcinomas and associated DCIS-components. Stromal expression of components of the uPA-system was also found by Constantini and coworkers (1994), Christensen et al. (1996), Dublin et al. (2000) and Haas et al (2008). They reported that in most cases stromal cells and macrophages express components of the uPA-system. Dublin and coworkers found that fibroblasts were anti-uPA, anti-uPAR and anti-PAI-1 positive. In the present study, we furnish evidence that tumor associated macrophages (TAM) and tumoral fibroblasts strongly express uPA, uPAR and PAI-1 in all cases examined. In normal (non-neoplastic) breast tissue, fibroblasts express uPA and PAI-1 in four out of ten cases, whereas macrophages were positive in all normal breast tissues examined. The increased expression (ELISA and immunohistochemistry) in TAMs and tumoral fibroblasts implies that there is a specific response to tumor growth. The lower levels found in macrophages and fibroblasts of normal breast tissue also indicate that there is more to the tumor-stroma response than is suggested by the concept that tumors are wounds that do not heal (Dvorak, 1986). The differences between invasive tumors and DCIS suggest that loss of the basement membrane and myoepithelial layer are necessary for full expression of these molecules, which is possibly mediated through local cytokines and /or changes in the spectrum of adhesion molecules expressed, and interacting with, the stroma. Furthermore, macrophages are a major component of the lymphoreticular infiltrate of breast cancer, and these cells may promote tumor growth rather than being cytotoxic to tumor cells (Zuk and Walker 1987). Previously, we have isolated different macrophages from normal breast tissue, as well as from breast cancer tissue, and have found that TAMs in breast cancer possess significantly higher levels of uPA, uPAR and PAI-1 compared to resident macrophages of normal breast tissue (Hildenbrand et al., 1998, 1999). Thus, cancer cells may activate TAMs by paracrine and juxtacrine interactions, and this could result in elevated levels of the uPA-system contributing to tumor progression.

The predominant expression of uPA, uPAR and PAI-1 in the tumor stroma reflects the importance of this compartment for the paracrine function of this system, and implies a collaboration between the stroma and cancer cells. Our results suggest that strong expression of uPA, uPAR and PAI-1 in tumor stroma, as well as in tumor cells, have the same impact on the clinical behaviour of breast cancer. A better understanding of stromal contributions to cancer progression will likely increase our awareness of the importance of the combinatorial signals that support and promote tumor growth, invasion and metastasis, and eventually result in the identification of new therapeutics targeting both tumor stroma and tumor cells, and the uPA-system as a paracrine interplay between both.

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