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Immunoassays of urokinase (uPA) and its type-1 inhibitor (PAI-1) in detergent extracts of breast cancer tissue

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Abstract

Two immunoassays for quantitation of the biological markers uPA and PAI-1 were evaluated for their use with detergent extracts of breast cancer tissue. Both assays were based on murine monoclonal capture antibodies and rabbit polyclonal detector antibodies. Horseradish peroxidase-conjugated goat anti-rabbit antibodies enabled measurement of the bound antigen. The detection limit of the uPA assay was 13 pg/ml, with a linear dose-response relationship up to 350 pg/ml. The assay detected free uPA as well as uPA in complex with PAI-1 and/or with its receptor. The detection limit of the PAI-1 assay was 50 pg/ml, with a linear dose–response relationship up to 1500 pg/ml. The assay detected both free PAI-1 and uPA:PAI-1 complex. Both assays were validated for detergent extracts using immunoabsorption and recovery tests. Highly significant associations between tumour tissue uPA and PAI-1 levels and prognosis were verified in a cohort of 164 lymph node-negative primary breast cancer patients. It is concluded that the two immunoassays are well-suited for the quantitation of uPA and PAI-1 in detergent extracts of breast cancer tissues.

Keywords: Urokinase plasminogen activator; Plasminogen activator inhibitor type-1; Breast cancer; Detergent tissue extracts; Quantitation; Prognosis

1. Introduction

In the primary treatment of breast cancer there is a need to distinguish between patients at a high and low risk of recurrence, in order to implement appropriate adjuvant therapy. Although axillary lymph node status is the best clinical discriminant between high and low risk patients, approximately 30% of the node-negative patients experience a recurrence. Therefore, additional measurement of biochemical parameters involved in metastatic spread have been proposed to accurately separate patients with node-negative disease according to their likely prognosis [1].

The urokinase plasminogen activation system is a cascade of proteolytic enzymes and their regulators that are involved in the degradation of extracellular matrix proteins and cellular migration during cancer invasion and metastasis [2]. uPA is a 52 kDa serine proteinase secreted as an inactive precursor, pro-uPA, that binds to a specific cell-surface receptor, uPAR, where formation of active uPA leads to localised potentiation of plasminogen activation. The plasmin thus generated mediates broad spectrum extracellular proteolysis, facilitating cell migration, proliferation and invasion. uPA-activity is rapidly neutralised by the specific high-affinity inhibitors, PAI-1 and PAI-2. The principal inhibitor, PAI-1, is a 52 kDa protein secreted in an active, but conformationally unstable, form, that gradually loses activity and assumes a latent conformation. Active PAI-1 forms a stable complex with active uPA, thereby blocking further plasmin formation. By a process dependent on uPAR and the low density lipoprotein receptor related protein, the cell-surface uPA:PAI-1 complex is internalised and ultimately degraded in lysosomes.

Immunohistochemical and *in situ* hybridisation studies of malignant tumours have demonstrated uPA at invasive foci, while PAI-1 is predominantly expressed in and around tumour vessels [3]. Furthermore, enzyme-linked immunosorbent assay (ELISA) measurements of levels of uPA, PAI-1, PAI-2 and uPAR in tumour tissue extracts has enabled their relationship to patient survival

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to be studied. Several independent investigations have shown that both uPA and PAI-1 are strong prognostic markers of survival of patients with primary breast cancer or other types of cancer [4,5]. High levels of uPA, PAI-1 and uPAR were found to predict a short survival, whereas high levels of PAI-2 and uPA:PAI-1 complexes seemed to predict a longer survival in breast cancer patients [4–6].

In the aforementioned studies, several different ELISAs were employed, resulting in variations in assay standards, antibody combinations and the extraction buffers used [7,8]. However, it has been reported that detergent-extracted breast cancer tissues are optimal for prognostic applications [9,10]. In the present study, we evaluated two commercially available ELISA kits for uPA and PAI-1. The assays are similar and were tested for sensitivity, performance and specificity, including their ability to detect the different forms of uPA and PAI-1. Special attention was paid to their performance with breast cancer tissue extracted with an optimal low-pH detergent buffer.

2. Patients and methods

2.1. Extraction procedure and patients

Breast carcinoma tissue was obtained from primary tumour tissue surgically resected from breast cancer patients. Tissue specimens were snap-frozen and stored in sealed containers at −80 °C until extraction. On the day of extraction, frozen tissue was mechanically pulverised with a dry ice-cooled powder pistol. The minimum tissue sample required was 50 mg; however, for this study 100 mg tissue powder was suspended in 300 μl ice-cold extraction buffer (75 mmol/l K-acetate, 0.3 mol/l NaCl, 0.1 mol/l L-arginine, 10 mmol/l Na_2-ethylene diamine tetra acetic acid (EDTA), 0.25% (v/v) Triton X-100, pH 4.2), which was previously described as optimal for simultaneous uPA and PAI-1 extraction [9–11]. The suspension was centrifuged at 105 000*g* for 1 h at 4 °C, and the resulting particle-free supernatant was aliquoted and stored at −80 °C until use (<4 months). Immediately before the assay was performed, the extracts were thawed rapidly at 37 °C and diluted in ELISA sample diluent.

For the first part of the study, tissue was obtained from patients where follow-up data was not available. For reference purposes, tissue powders from 10 tumours were pooled before extraction. Such extract was designated tumour extract pool and gave ELISA signals for uPA and PAI-1 that were close to the mean value of the individually extracted tissues.

For the last part of the study, 164 patients with primary breast cancer were included. Each patient underwent surgery in Denmark in the period of 1989–1993 for histologically-verified primary breast carcinoma. The surgical procedure included lumpectomy (followed by local radiotherapy) or modified radical mastectomy, and partial axillary lymph node dissection. 27 of the patients received adjuvant endocrine treatment or chemotherapy. All patients entered into the study had stage I disease (i.e. lymph node-negative). The median patient age at the time of surgery was 56 years (range 29–75 years), and the median follow-up time at the time of data analysis was 8.5 years (range 7.3–11.3 years).The anticipated follow-up period for recurrence was intended to be 10 years and for survival until death, and the information was updated annually. During the observation period, there was a total of 48 recurrences and 35 deaths. Recurrence was confirmed by biopsy and/or other relevant diagnostic procedures. The actual cause of death was not available for all of the patients, so recording of survival was based on death from all causes. For further details please refer to Ref. [6].

2.2. Antibodies

Mouse monoclonal antibodies (MAbs) were used for immuno-capture in the ELISAs. In the uPA assay, a combination of the anti-human uPA clones designated 5 and 6 was applied. Clone 5 reacts with an epitope in the carboxy-terminal region of uPA, whereas clone 6 binds an epitope within the amino-terminal sequence of uPA [12,13]. In the PAI-1 ELISA, anti-human PAI-1 clone 1 was used for the capture. This MAb reacts with a non-linear epitope on PAI-1 [14]. A rabbit polyclonal antibody (PAb) raised against recombinant human pro-uPA (Grünenthal, Aachen, Germany) was used for detection in the uPA ELISA, and in the PAI-1 assay a PAb raised against latent human PAI-1, purified from conditioned media of HT-1080 fibrosarcoma cells, was employed. For immunoabsorption specificity tests, an anti-uPA MAb designated clone 14, reacting with the amino-terminal sequence of uPA, was used in the uPA ELISA. A combination of two anti-PAI-1 MAbs, designated clones 6 and 7, directed against residues 110-145 and a non-linear epitope on PAI-1, respectively, was used for immunoabsorption in the PAI-1 ELISA. The control MAb was against the irrelevant 2,4,6-trinitrophenyl (TNP) hapten. MAbs were all of the IgG_1 subclass, and were purified from hybridoma culture fluids by affinity chromatography on protein A-Sepharose. The specific PAbs as well as a non-immune control PAb were purified from rabbit sera using protein A-Sepharose.

2.3. Preparation of complexes

Pro-uPA:uPAR complexes were freshly prepared before use. Final concentrations of 100 pmol/ml (5 μg/ml) pro-uPA (Grünenthal) and 200 pmol/ml (14 μg/ml) recombinant soluble uPAR (i.e. uPAR lacking the glycolipid anchor) were co-incubated at 20 °C for 1 h in

sample diluent (see below). Appropriate dilutions of this complex was made before the assay, thus the preparation could be considered to contain a mixture of the complex and the free components [15].

Purified uPA:PAI-1 complex was prepared as previously described in Ref. [16]. In short, active high-molecular weight uPA (Serono, Aubonne, Switzerland) and activated PAI-1, purified from conditioned media of HT-1080 fibrosarcoma cells, were co-incubated to form the complex. Purification of the uPA:PAI-1 complex was performed by sequential affinity chromatography using immobilised anti-PAI-1 and anti-uPA MAbs. The concentration of the purified complex was determined by protein analysis according to the Bradford method and confirmed by the Lowry method. The purified uPA:PAI-1 complex was stored at −80 °C.

uPAR:uPA:PAI-1 complex was prepared in the same way as the pro-uPA:uPAR complex, only in this case pro-uPA was exchanged with the uPA:PAI-1 complex.

2.4. Assays

Total protein concentrations in the extracts were measured by the Bradford method for protein analysis, using a protein assay kit employing bovine serum albumin as a standard (Bio-Rad, Hercules, CA, USA).

uPA determinations were performed with an ELISA kit (Cat.#OSDI-12) developed and made available by Oncogene Science (Cambridge, MA, USA): immunoplates (Nunc, Roskilde, Denmark) were coated overnight at 20 °C with 100 μl/well of a mixture of 2.5 μg/ml each of anti-uPA clones 5 and 6 in 0.05 mol/l Na_2CO_3, pH 9.6. After washing and blocking of remaining protein binding sites for 30 min at 20 °C in 300 μl/well of 2% bovine serum albumin in PBS, pH 7.4, the plates were dried by lyophilisation, sealed with a desiccant under vacuum, and placed at 4 °C until use. On the day of analysis, all reagents were brought to room temperature except the biological samples to be tested. 100 μl/well pro-uPA calibrator (Grünenthal (concentration determined on the basis of the amino acid analysis)), high-molecular weight uPA, complexes, and biological samples, appropriately diluted in sample diluent (PBS containing 4% bovine serum albumin, pH 7.4), were incubated for 2 h at 37 °C. After washing six times with an automated microplate washer using 300 μl/well of PBS containing 0.1% (v/v) Tween-20, pH 7.4, plates were incubated for 1.5 h at 20 °C with 100 μl/well of 5 μg/ml of purified anti-uPA rabbit PAb or, as a specificity control, non-immune PAb in PBS containing Na-casein. After six more washes, plates were treated for 30 min at 20 °C with 100 μl/well of horseradish peroxidase-conjugated goat anti-rabbit IgG (0.2 μg/ml) in sample diluent. Finally, plates were washed six times and the peroxidase reaction initiated by the addition of 100 μl/well of 0.5 mg/ml *O*-phenylenediamine (Dako, Glostrup, Denmark) dissolved in 0.1 mol/l citric acid, 0.01% H_2O_2, pH 5.0. The reaction was stopped after 45 min at 20 °C in the dark by the addition of 100 μl/well of 2.5 mol/l H_2SO_4, and the absorbance of each well was read at 490 nm (with a 690 nm reference filter) using an automated microplate reader (Ceres-900^{TM}, Bio-Tek Instruments, Winooski, VT, USA). All determinations were performed in duplicate or triplicate and the mean value was used.

PAI-1 determinations were also performed with an ELISA kit (Cat.#OSDI-17) made available by Oncogene Science, with a protocol identical to the above uPA ELISA, with the following exceptions: immunoplates were coated with anti-PAI-1 clone 1; latent PAI-1 calibrator (concentration determined on basis of amino acid analysis) and samples were incubated on the plates for 3 h at 20 °C; anti-PAI-1 PAb was used for detection; and the peroxidase substrate was 1 mg/ml *O*-phenylenediamine.

2.4.1. Immunoabsorption and Western blotting

For immunoabsorption, pooled tissue extract was diluted 1:20 and mixed with MAbs coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia Biotech). This mixture was incubated with end-over-end rotation at 20 °C overnight and subsequently centrifuged at 40×g. The resulting gel-free supernatant was diluted further and assayed in the ELISAs.

For western blotting, samples of 25 μl crude tissue extract, diluted in Laemmli sample buffer, were electrophoresed on a 10% sodium dodecyl sulphate (SDS)-polyacrylamide minigel, and the separated proteins blotted electrophoretically onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). After blocking in skimmed milk powder in tris-buffered saline (TBS) at 4 °C overnight, membranes were sequentially incubated with either 20 μg/m anti-uPA or anti-PAI-1 rabbit PAbs, and then alkaline phosphatase-conjugated monoclonal anti-rabbit IgG diluted 1:1000 (Sigma, St. Louis, MO, USA). Finally, colour was developed with nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) (Boehringer Mannheim, Penzberg, Germany).

2.5. Statistical analyses

Survival curves for the time to recurrence and the time to death were estimated by the Kaplan–Meier method. It was prespecified that uPA and PAI-1 were to be scored first as the actual value (log transformed), and then as indicator variables dichotomised by the median value. The log rank test was used to test for equality of strata. The assumption of proportional hazards was verified graphically. The Cox proportional hazards model was used for the analysis of survival. A *P*-value (*P*) less than 0.05 was considered significant, and the 95% confidence interval (CI) for each relative risk (RR) was calculated.

3. Results

3.1. uPA ELISA

When the uPA ELISA protocol described above was used, there was a linear relationship between absorbance and pro-uPA calibrator concentration up to at least 350 pg/ml (linear correlation coefficient, $R > 0.99$) (Fig. 1a). The limit of detection for the assay was 13 pg/ml, defined as the lowest antigen concentration giving a signal higher than the mean of the sample diluent control plus 3 times the standard deviation of this latter value. When equimolar concentrations of pro-uPA, high-molecular weight active uPA, or a mixture of pro-uPA:uPAR complex and corresponding free components [15] were compared in the assay, virtually identical signal intensities were observed up to at least 350 pg/ml (data not shown). For the uPA:PAI-1 complex and for a mixture of uPAR:uPA:PAI-1 complexes and corresponding free components, a minor signal decrease of approximately 20 and 25%, respectively, was observed (Fig. 1a). When three different batches of pro-uPA calibrator were compared with one another, a maximum difference of 20% in signal was found. The assay did not detect free uPAR or PAI-1 assayed in concentrations up to 700 pg/ml. When the anti-uPA detector PAb was substituted with non-immune rabbit IgG, no signal was observed with pro-uPA.

3.2. uPA ELISA performance with extracts

To investigate the ELISA performance, a pool of extract from 10 ductal breast carcinomas with diverse concentrations of uPA was prepared with a low-pH detergent Camiolo buffer [11]; this pool contained 52 ng/ml uPA. When different dilutions of the pool were assayed in the ELISA, the signal was linearly related to dilution, parallel to the calibrator curve in the uPA ELISA, up to a concentration of at least 1% ($R > 0.99$) (Fig. 1b). The analytical recovery in the ELISA was tested by adding various amounts (25–200 pg/ml) of pro-uPA calibrator to a 0.33% concentration of this tumour extract pool and comparing the signal to the signal for calibrator diluted in pure sample diluent. The recovery of signal from the calibrator in the pool was 85–90% (Fig. 1b, inset). The within-run imprecision of the ELISA was determined by assaying eight independent 1:300 dilutions of the pool on the same ELISA plate; the calculated within-run coefficient of variation was 3.4%. The between-run (total) imprecision was determined by analysing this pool on 9 separate days, which gave a variation coefficient of 6.8%. In a later series using another ELISA batch and another pool (54 ng/ml uPA), the between-run variation-coefficient was 6.9% ($n = 7$). For individual samples, the coefficients of variation were similar to the above results.

3.3. uPA ELISA specificity

When the anti-uPA detector PAb was replaced with non-immune rabbit IgG during the assay of 12 individual tissue extracts (Fig. 1c) or of the tumour extract pool, no signal above background was observed. Furthermore, specific depletion of uPA from the pool was performed by immunoabsorption on a Sepharose 4B immobilised anti-uPA MAb, different from the capture MAbs used in the ELISA. This resulted in an 80% reduction of the signal (Fig. 1c, inset). Western blot analysis of crude extracts with a particularly high signal in the uPA ELISA (>400 ng/ml undiluted) verified the

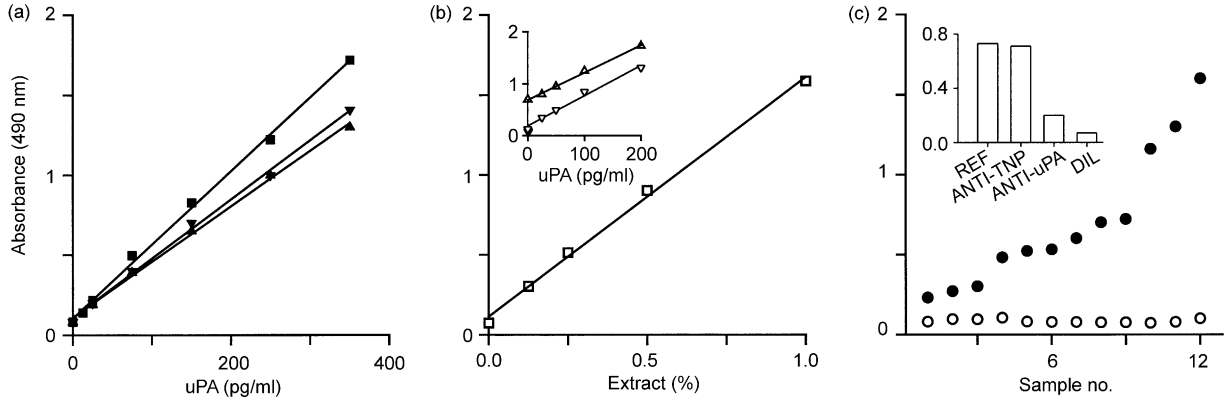

Fig. 1. uPA enzyme-linked immunosorbent assay (ELISA). Ordinates represent assay-well absorbance at 490 nm. (a) Dilution curves of pro-uPA (■), uPA:PAI-1 complex (▼) and uPAR:uPA:PAI-1 complex (▲). Abscissa represents the concentration of the uPA-component in each molecule assayed. (b) Dilution curve of the pooled tumour extracts. Abscissa represents the concentration of the diluted pool. Inset: recovery of signal from internal pro-uPA calibrator in sample diluent (▽) or in 0.33% extract pool (△). (c) Ranking plot of signal obtained from12 individual extracts, using treatment of assay-wells at the detector step with either anti-uPA PAb (●) or non-immune PAb (○). Assay results for each sample are shown in vertical alignment. Inset: direct assay of extract pool before (REF) and after immunoabsorption with either irrelevant anti-TNP MAb (ANTI-TNP) or anti-uPA MAb clone 14 (ANTI-uPA). For comparison, the background signal with sample diluent alone is shown (DIL).

presence of high concentrations of uPA (band at approximately molecular weight (Mr) 50 000), as opposed to extracts selected on the basis of a low ELISA signal (Fig. 2a).

3.4. PAI-1 ELISA

The PAI-1 ELISA had a detection limit of 50 pg/ml with latent PAI-1 calibrator, determined as for the uPA assay, and there was a linear relationship between absorbance and PAI-1 concentration up to at least 1500 pg/ml ($R > 0.99$) (Fig. 3a). When equimolar concentrations of latent PAI-1 and uPA:PAI-1 complexes were compared, a marked signal decrease of approximately 50% was observed with the complex (Fig. 3a). Comparison of two different batches of PAI-1 calibrator with one another resulted in a difference of only 15%. The assay did not detect free uPA assayed in concentrations up to 3000 pg/ml. When the anti-PAI-1 detector PAb was substituted with non-immune PAb, no signal above background level was observed with PAI-1.

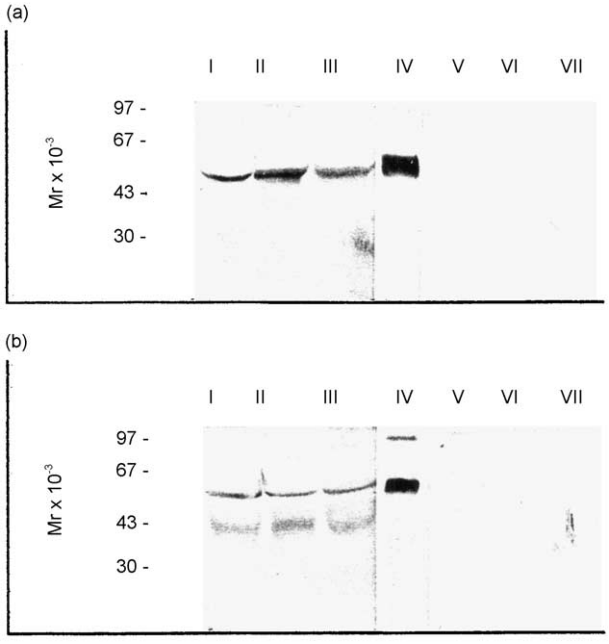

Fig. 2. Western blot. (a) The membrane was probed with anti-uPA PAb to show the immunoreactive material in crude extracts from three tissue samples giving a high signal in uPA ELISA (>400 ng/ml) (lanes I, II and III), 5 ng of purified high-molecular weight active uPA (not calibrator!) (lane IV), and crude extracts from three samples giving a low ELISA signal (<100 ng/ml) (lanes V, VI and VII). (b) The membrane was probed with anti-PAI-1 PAb to show the immunoreactive material in crude extracts from three tissue samples giving a high signal in PAI-1 ELISA (>400 ng/ml) (lanes I, II and III), 5 ng of purified PAI-1 (not calibrator!) (lane IV), and crude extracts from three samples giving a low signal (<100 ng/ml) (lanes V, VI and VII).

3.5. PAI-1 ELISA performance with extracts

Dilutions of a tumour extract pool (24 ng/ml PAI-1) showed a linear relationship with the signal in the PAI-1 ELISA up to at least a 5% concentration ($R > 0.99$) (Fig. 3b). When chosen amounts of PAI-1 calibrator (125–1000 pg/ml) were added to a 2.5% concentration of this pool, the recovery of calibrator in the assay was 83% (Fig. 3b, inset). The within-run variation-coefficient was 6% ($n = 8$) and the between-run variation was 8% ($n = 9$) for this extract pool when diluted 1:40. In a later series using another ELISA batch and another extract pool (21 ng/ml PAI-1), the between-run variation-coefficient was 7.4% ($n = 7$). Similar variation coefficients were observed with the individual extracts.

3.6. PAI-1 ELISA specificity

When the anti-PAI-1 detector PAb was replaced with non-immune PAb during the assay of 12 individual extracts (Fig. 3c) or of the tumour extract pool, no signal above the background level was observed. Specific depletion of PAI-1 from the pool was performed by immunoabsorption on a mixture of immobilised anti-PAI-1 MAbs, different from the capture MAb in the ELISA. The signal in the subsequent assay was extinguished (Fig. 3c, inset). Western blot analysis of crude extracts with a particularly high signal in the ELISA (>400 ng/ml undiluted) verified the presence of high concentrations of PAI-1 (band at approximately Mr 50 000), as opposed to extracts selected on the basis of a low ELISA signal (Fig. 2b).

3.7. uPA and PAI-1 levels in individual detergent extracts

To elucidate the concentration of uPA and PAI-1 measured in detergent extracts with the above ELISAs, we studied a cohort of 164 individually extracted tumour tissue specimens from patients operated upon for lymph node-negative primary breast cancer. For uPA measurements, extracts were diluted 1:300 (0.33%), and for PAI-1 determinations extracts were diluted 1:40 (2.5%). All of the extracts analysed had readily measurable levels of uPA and PAI-1. Each ELISA determination was referred to the total protein concentration in the same extract. Since the extracts were analysed using a series of ELISA kits over several days, we first investigated whether this had any influence on the measured antigen levels. Tests for any association between extract location in the assay series and the measured parameters did not reveal any significant relationship. For uPA, the median value was 5.2 ng/mg protein, range 0.2–61 ng/mg protein, and for PAI-1, the median value was 2.3 ng/mg protein with a range from 0.2 to 80 ng/mg protein. The distributions of

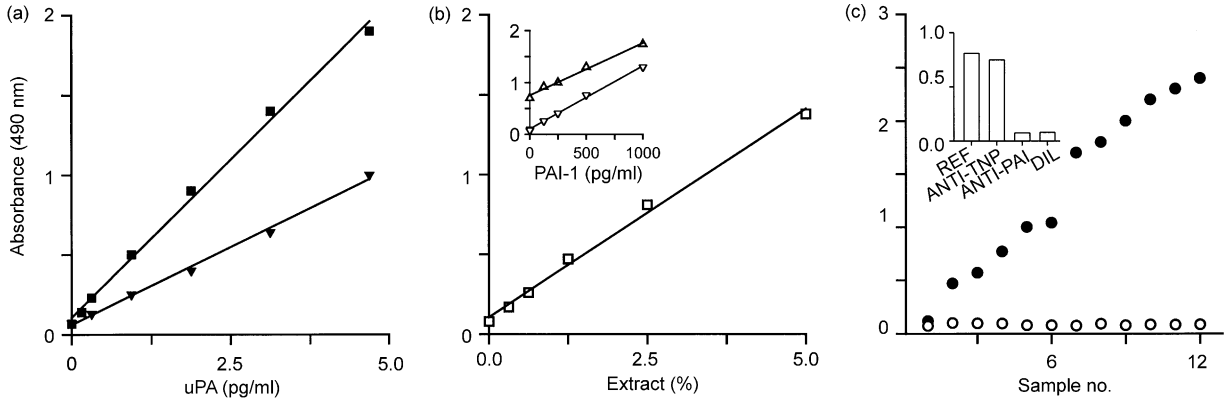

Fig. 3. PAI-1 ELISA. Ordinates represent assay-well absorbance at 490 nm. (a) Dilution curves of latent PAI-1 (■) and uPA:PAI-1 complex (▼). Abscissa represents the concentration of the PAI-1-component in each molecule assayed. (b) Dilution curve of tumour extract pool. Abscissa represents the concentration of the diluted pool. Inset: recovery of the signal from internal PAI-1 calibrator in sample diluent (▽) or in 2.5% extract pool (△). (c) Ranking plot of signal obtained from12 individual extracts, treating assay-wells at the detector step with either anti-PAI-1 PAb (●) or non-immune PAb (○). Assay results for each sample are shown in vertical alignment. Inset: direct assay of extract pool before (REF) and after immunoabsorption with either irrelevant anti-TNP MAb (ANTI-TNP) or anti-PAI-1 MAbs clone 6 and 7 (ANTI-PAI). For comparison, the background signal with sample diluent alone is shown (DIL).

uPA and PAI-1 levels were skewed toward the higher values. When the uPA and PAI-1 levels in each extract were compared (Fig. 4), a close and significant positive correlation was found (Spearman rank correlation coefficient = 0.78; $P < 0.0001$).

3.8. Relationship of uPA and PAI-1 to the prognosis of the breast cancer patients

Ultimately, we performed a retrospective investigation of the associations between tumour tissue uPA and PAI-1 levels and prognosis in the cohort of 164 patients with a median follow-up of 8.5 years. Since the frozen tumour tissue had been stored for several years prior to extraction, 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. Initially, the measured parameters were treated as log-transformed continuous variables. The tumour level of total uPA significantly predicted recurrence-free survival (RFS) ($P = 0.04$) and overall survival (OS) ($P = 0.001$); increasing uPA levels were associated with a shorter survival. Increasing total PAI-1 levels also predicted a shorter RFS and OS (both, $P < 0.0005$).

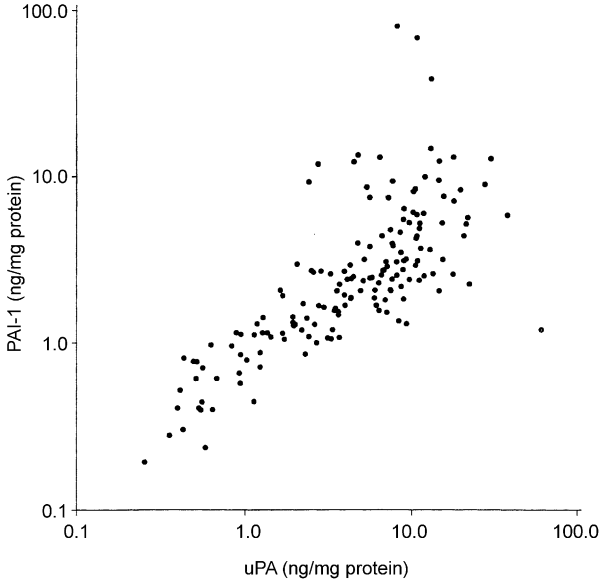

Fig. 4. Scatterplot of uPA level versus PAI-1 level in each of 164 detergent extracts of tumour tissue samples from patients operated upon for lymph node-negative primary breast cancer.

We then used the median value to dichotomise the patient material, in order to further investigate the prognostic significance of the measured parameters. When RFS and OS were compared for patients with uPA levels falling into the two groups, it was observed that the patients with high values tended to have a shorter RFS ($P = 0.07$) (Fig. 5a) and had a significantly shorter OS ($P = 0.001$) (Fig. 5b) than patients with low values. A comparison of patients in the two groups gave an RR of 3.2 (CI 1.5–6.9) for OS. For PAI-1, high levels were significantly associated with a shorter survival (for RFS, $P = 0.005$ (Fig. 6a), and for OS, $P < 0.0001$ (Fig. 6b)), with an RR of 2.3 (CI 1.3–4.1) for RFS and 4.7 (CI 2.0–10.7) for OS.

In a multivariate Cox regression analysis of the survival data including age, tumour size, tumour grade of anaplasia, steroid hormone-receptor status, uPA and PAI-1, the only parameter that independently and significantly ($P < 0.05$) predicted survival was PAI-1. High PAI-1 was associated with a short RFS (RR = 2.3) and short OS (RR = 3.3) (data not shown).

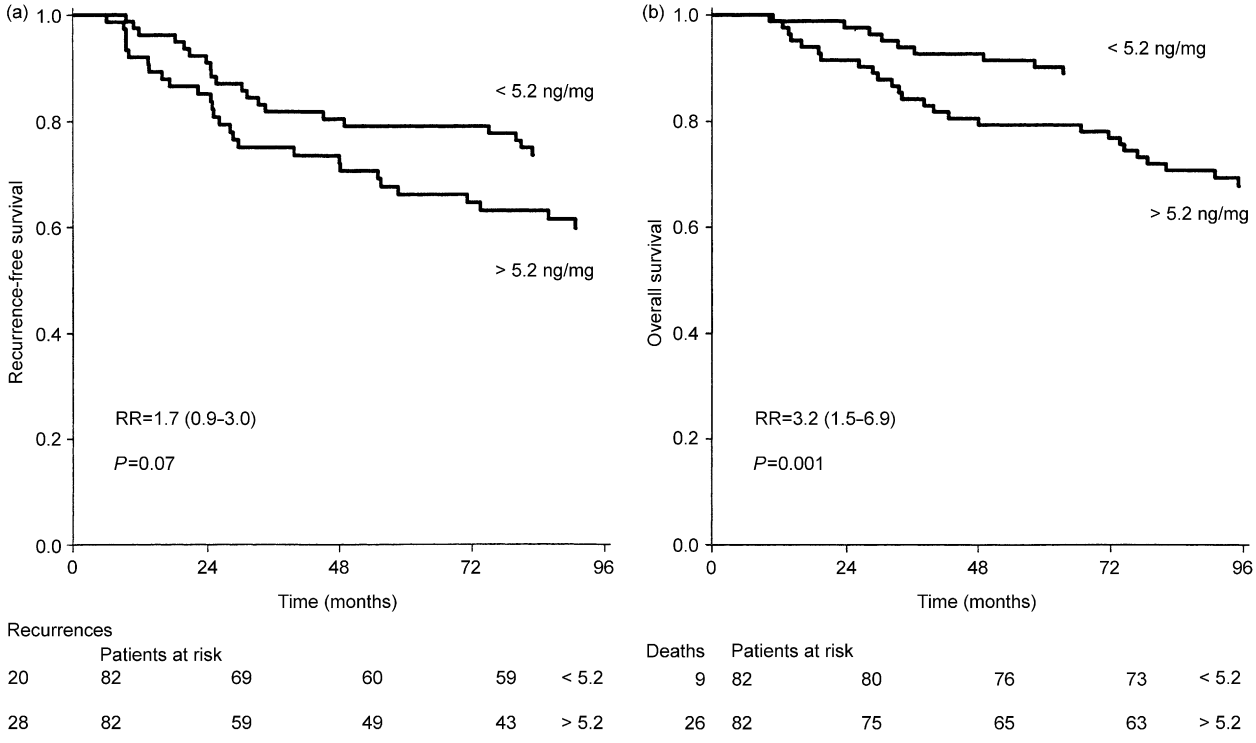

Fig. 5. Survival curves showing the association of tumour tissue uPA levels with RFS (a) and OS (b) of the primary breast cancer patients. Patients were divided into groups above and below the median value. The numbers of events during the period and the numbers of patients at risk after each 2-year interval are indicated. *P* values were calculated by the log-rank test. RR, relative risk.

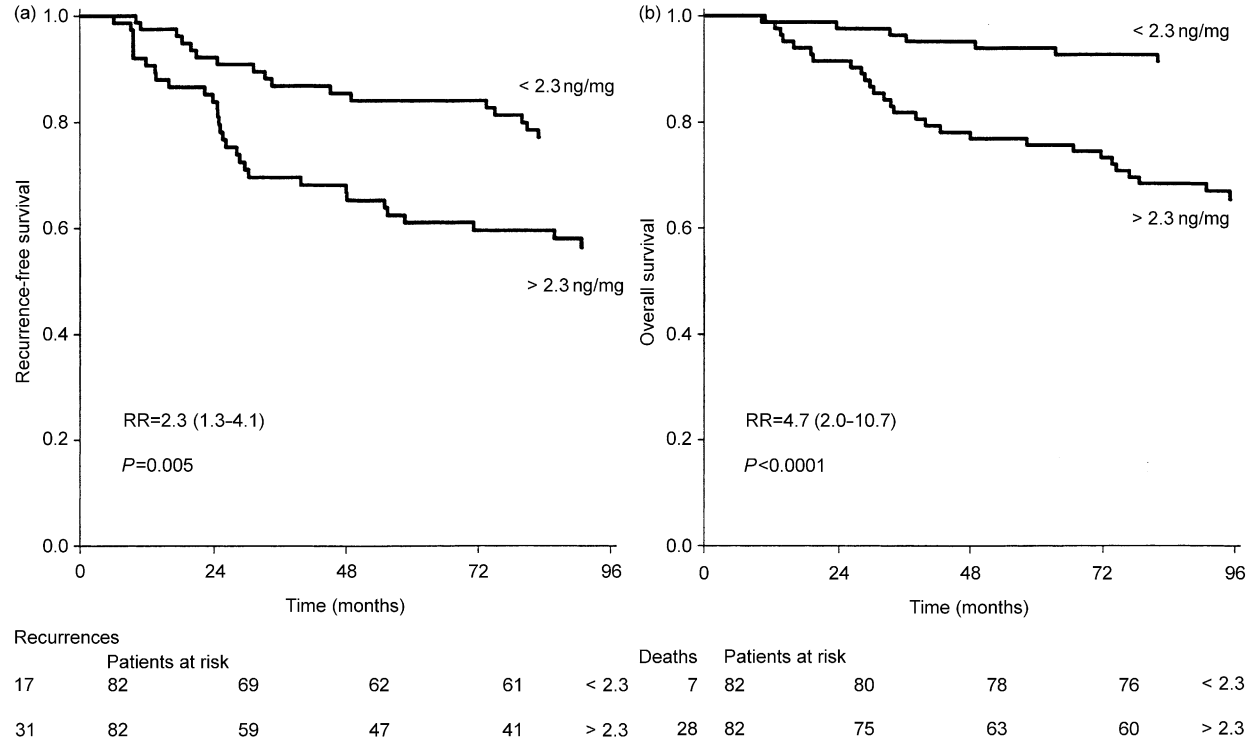

Fig. 6. Survival curves showing the association of tumour tissue PAI-1 with RFS (a) and OS (b) of the primary breast cancer patients. Patients were divided into groups above and below the median value. The numbers of events during the period and the numbers of patients at risk after each 2-year interval are indicated. *P* values were calculated by the log-rank test.

4. Discussion

In the present work, we have evaluated two sensitive and specific ELISAs for uPA and PAI-1 determinations in breast cancer tissue extracts. Based on similar formats employing a combination of monoclonal and polyclonal antibodies, these assays detect free forms as well as complex-bound forms of the respective antigens, and can thus in principle measure the mixture of the various antigen forms represented in a biological sample. For the PAI-1 ELISA, however, it was demonstrated that the uPA:PAI-1 complex was detected at a lower efficiency. This is most probably due to the fact that epitope recognition by a particular PAI-1 MAb can be markedly altered by binding of uPA, or even by the activation of PAI-1 [17–19]. The use of only one MAb for capture in the PAI-1 ELISA may thus be inadequate when significant amounts of complex are present. This problem may be relevant to all available assays of PAI-1 (and uPA) employing MAbs, nevertheless the issue has not yet been adequately addressed. For the uPA ELISA, that employed two capture MAbs, the presence of significant amounts of uPAR did not affect the detection efficiency of uPA. That uPA:uPAR complexes exist in this mixture of molecules has previously been shown by De Witte and colleagues, who used similar experimental conditions to demonstrate a linear dose-response signal in their uPA:uPAR complex ELISA [15].

In a collaborative study with the Receptor and Biomarker Group under the auspices of the European Organization for Research and Treatment of Cancer, we have previously described the successful application of the two ELISAs to tumour cytosols (i.e. extraction with a neutral buffer without detergent) [7,8]. However, it has been reported that detergent-extracted (Triton-X 100) breast cancer tissues are optimal for prognostic applications. Detergent-extracted tissues contain approximately twice as much uPA antigen as the cytosol fractions, and the prognostic power of uPA is stronger in detergent-extracts, this is most pronounced in the subgroup of node-negative patients [9–11]. Therefore, we used tumour tissue extracts made with the low-pH detergent Camiolo buffer, that has been reported to be optimal for simultaneous uPA and PAI-1 extraction. The high performance of the assays was demonstrated by linear dilution curves for such detergent extracts over a wide range, and acceptable recovery of calibrator added to the extracts. These findings indicate that the measurement of uPA and PAI-1 levels was not affected by other unrelated components in the detergent extracts. In addition, assay reproducibility was good, as judged by the low variation coefficients. The specificity of the ELISAs was demonstrated by an absence of signal from extracts when using non-immune IgG at the detector step or pre-assay immunoabsorption, and the presence of genuine antigen in the extracts was verified by western blotting. However, for the PAI-1 blot a second band of a lower molecular weight than the intact PAI-1 was observed in the three samples also showing the intact PAI-1. Whether this band represents a PAI-1 cleavage product, e.g. cleavage by thrombin [20,21], or cross-reaction of the PAb with an unrelated protein cannot be precluded from the present analysis. Altogether, the sensitivity and specificity of the two assays compare favourably with our earlier published results of other assays already in clinical practice, such as the Imubind® ELISA from American Diagnostica [7,8].

The presented ELISAs employ the same calibrators as used by the above Receptor and Biomarker Group for external quality assessment of the diverse assays available [7,8]. The introduction of such uniform common calibrators represents one possible way of allowing direct comparison of absolute antigen levels between different studies and different laboratories, thus promoting the clinical usefulness of these markers [22]. In the present study, the levels of uPA and PA1-1 in individual extracts were all readily measurable and varied widely, and were thus representative of the biological variation to be found in a large set of breast cancer tissues. The reported levels of uPA and PAI-1, and the significant correlation between the molecules in individual extracts, are in agreement with previous studies using different assays for detergent extract application [9,23]. Furthermore, this study demonstrated the prognostic significance of uPA and PAI-1 in primary breast cancer after long-term follow-up, consistent with many previous reports from different laboratories [4,5]: increasing levels of uPA and—in particular—PAI-1 were associated with a shorter survival.

It is noteworthy that these results are valid for the clinically important subgroup of lymph node-negative patients; 70% of these patients will be cured by primary locoregional therapy. Part of the remaining 30% of the patients will need adjuvant systemic treatment to reduce the incidence of recurrent disease. Therefore, markers for accurate prognostic separation of node-negative patients are needed to determine appropriate use of adjuvant therapy [1]. The levels of uPA and of PAI-1 in cancer extracts—as determined by ELISAs—have been shown to be independent, strong prognostic parameters [4,5]. In some studies uPA and PAI-1 could even be combined in order to obtain a reliable prognostic score [24,25], and one prospective study has assessed these parameters as an adequate selection criterion for adjuvant systemic treatment in lymph node-negative breast cancer patients [26]. On the basis of a recent meta-analysis of 18 data-sets [27] uPA and PAI-1 determination now fulfil the criteria for being included into the routine panel of factors assessed during the management of primary breast cancer patients.

The dependence on unfixed tumour tissue for the evaluation of uPA and PAI-1 levels may become an

obstacle for its routine use when the tumour is very small, which will be the case in approximately 10% of all patients. If a simple blood sample from the patient could be used for prognostic purposes, it would be clinically very useful. Release of the protein components of the plasminogen activation system from the tumour tissue [28,29] leads to increased levels in peripheral blood, and the most aggressive tumours appear to release more of these components [12,30,31]. As a consequence, the tissue studies have now been extended to the determination of uPA and PAI-1 in blood. Indeed, recent prognostic studies have shown plasma PAI-1 to be significantly associated with survival for patients with breast and colon cancer [32,33].

In conclusion, the uPA and PAI-1 ELISAs evaluated in the presented study are suitable for the prognostic studies of the levels of these molecules in detergent extracts of breast cancer tissues, and we propose that the suitability of these assays for extracts from other types of cancer tissue and for plasma should be tested.

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