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ELISA FOR COMPLEXES BETWEEN UROKINASE-TYPE PLASMINOGEN ACTIVATOR AND ITS RECEPTOR IN LUNG CANCER TISSUE EXTRACTS

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A sandwich-type ELISA has been developed for the assessment of complexes between urokinase-type plasminogen activator (uPA) and its receptor (uPAR) in extracts of squamous cell lung carcinomas. The assay is based on a combination of rabbit polyclonal anti-uPA antibodies and a biotinylated mouse anti-uPAR monoclonal antibody (MAb). The detection limit of the assay is approximately 0.5 fmol/ml. A linear dose-response is obtained with up to 40 fmol/ml of uPA:uPAR complexes, while uPA and uPAR separately do not cause any response in the ELISA. A buffer which has been used previously for optimal extraction of uPAR yields the highest amounts of uPA:uPAR complexes. Absorption of tumor extracts with anti-uPA or anti-uPAR MAbs results in a complete disappearance of the ELISA signal, demonstrating the specificity of the ELISA. The recovery of chemically cross-linked uPA:uPAR complexes added to tumor extracts varies between 80% and 105%. The intra- and inter-assay variation coefficients are 5.3% and 9.8%, respectively. Furthermore, a peptide antagonist for uPAR was employed to evaluate *de novo* uPA:uPAR complex formation during tumor tissue extraction and the immunoassay procedure. Our results strongly indicate that *de novo* complex formation is a major factor to consider and that complexes analyzed in the presence of this antagonist represent original uPA:uPAR complexes present prior to tumor tissue processing. The present ELISA appears suitable for studying the potential prognostic impact of uPA:uPAR complexes in lung tumor tissue as well as other types of cancer. *Int. J. Cancer* 72:416–423, 1997.
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The plasminogen activation system represents a complex cascade of proteolytic enzymes which, in concert with other enzyme systems, is involved in the degradation of extracellular matrix proteins during tissue remodeling processes under normal and pathological conditions, including cancer invasion (Danø *et al.*, 1985; Blasi *et al.*, 1987; Mignatti and Rifkin, 1993). The urokinase-type plasminogen activator (uPA) plays a key role in this system in that it activates the zymogen plasminogen into plasmin, which has a broad trypsin-like substrate specificity and the ability to degrade several components of the extracellular matrix (Danø *et al.*, 1985; Mignatti and Rifkin, 1993; Duffy, 1992). uPA itself is synthesized and secreted as an inactive single polypeptide chain pro-enzyme, pro-uPA, which is converted by plasmin and certain other proteases into its active 2-chain counterpart (Petersen *et al.*, 1988; Pöllänen *et al.*, 1991). The overall activity of the plasminogen-activation system is potentiated by the binding of pro-uPA to a specific cellular receptor, uPAR, which localizes proteolysis to the cell surface and strongly enhances activation of surface-bound plasminogen (Danø *et al.*, 1994).

Immunohistochemistry and *in situ* hybridization studies have demonstrated that both uPA and uPAR in adenocarcinoma of colon (Grøndahl-Hansen *et al.*, 1991; Pyke *et al.*, 1991) and breast (Pyke *et al.*, 1993) and in squamous cell carcinoma of the skin (Sappino *et al.*, 1991) are consistently expressed at invasive loci, either by the same cells or by closely located cells. Other investigations have shown that in carcinomas of breast (Grøndahl-Hansen *et al.*, 1993), colon (Verspaget *et al.*, 1995) and bladder (Hasui *et al.*, 1996) uPA can be considered as an independent and significant prognostic marker, high uPA levels being associated with poor prognosis. In addition, elevated levels of uPAR were found to be of prognostic value in patients with squamous cell lung carcinoma and breast cancer (Pedersen *et al.*, 1994; Grøndahl-Hansen *et al.*, 1995). Furthermore, the interplay between uPA and uPAR is an important factor in the invasiveness of malignant cells and suggests that only receptor-bound uPA is active during invasion (Quax *et al.*, 1991; Ossowski, 1988; Ossowski *et al.*, 1991; Ellis *et al.*, 1992). Therefore, it is assumed that the number of complexes between uPA and uPAR may better reflect the activity of the plasminogen-activation system and might represent an even stronger prognostic parameter.

In the present study, we describe the development of an ELISA for the specific detection and quantification of uPA:uPAR complexes in extracts of squamous cell lung carcinoma tissue, using a combination of polyclonal antibodies and monoclonal antibodies (MAbs) previously employed in the ELISA for the separate components (Rosenquist *et al.*, 1993; Rønne *et al.*, 1995). Different buffers were tested for optimal extraction of complexes out of the tumor tissue. The usefulness of a peptide antagonist for uPAR was investigated in order to analyze original uPA:uPAR complexes in tumor tissue extracts.

MATERIAL AND METHODS

Reagents

The following reagents were from the sources indicated: recombinant pro-uPA (Grünenthal, Aachen, Germany), active human uPA (Serono, Aubonne, Switzerland), tissue-type plasminogen activator (tPA) purified from culture fluid of Bowes melanoma cells (Nielsen *et al.*, 1988), soluble variant of uPAR (s-uPAR) purified from Chinese hamster ovary (CHO) cells (Rønne *et al.*, 1995), purified ^{35}S-labeled s-uPAR obtained after *in vivo* labeling of CHO cells (Ploug *et al.*, 1993), protein A Sepharose and CNBr-activated Sepharose (Pharmacia, Uppsala, Sweden), horseradish peroxidase–conjugated streptavidin (Zymed, South San Francisco, CA), *o*-phenylenediamine (Dako, Glostrup, Denmark), BSA fraction V (Boehringer Mannheim, Germany), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and sulfo-N-hydroxysulfosuccinimide (NHS) (Pierce, Rockford, IL). Peptide antagonist (clone 20) for human uPAR and a randomized control peptide (Goodson *et al.*, 1994) were synthesized by Dr. J. Keyte (Department of Biochemistry, University of Nottingham, Nottingham, UK).

Tumor tissue and extraction

Non-small cell lung cancer (NSCLC) tissue was obtained from patients after surgical resection for a primary lung tumor at the Department of Thoracic Surgery, Rigshospitalet, Copenhagen, Denmark and immediately stored at −80°C. Tumor tissue from 6

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randomly selected patients was mechanically pulverized in the frozen state with a pre-cooled powder pistol and then mixed. Next, the pooled material was divided into equal portions and homogenized in each of 3 different ice-cold buffers (buffers A, B and C, see below), using a tumor:buffer ratio (w/v) of 1:3. After centrifugation at 105,000 *g* for 1 hr at 4°C, supernatants were aliquoted and stored at −80°C until further analysis. In the same way, NSCLC tissue samples of 20 other randomly selected patients were each subjected to extraction with buffers A, B and C. To study *de novo* complex formation between uPA and uPAR, additional NSCLC biopsies of 13 patients were obtained from the Department of Pathology, University Hospital Nijmegen, The Netherlands, and frozen in liquid nitrogen immediately after surgery. Each tumor biopsy was pulverized in the frozen state by means of a microdismembrator (B. Braun Biotech, Melsungen, Germany) in the presence of buffer C supplemented with 100 μM of peptide antagonist or control peptide. For the processing of these tumors, a ratio (w/v) of 1:30 was used. After thawing on ice, homogenates were centrifuged at 105,000 *g* for 1 hr at 4°C and the supernatants stored at −80°C until further analysis. Three different buffers, each of which had been used previously for optimal extraction of steroid hormone receptors (buffer A), uPA (buffer B) and uPAR (buffer C), respectively, were tested for their extraction efficiency of uPA:uPAR complexes. The composition of the 3 buffers was: (*i*) 10 mM K_2HPO_4/KH_2PO_4, 1.5 mM K_2-EDTA, 10 mM monothioglycerol, 10% (v/v) glycerol and 10 mM sodium molybdate, pH = 7.5 (Thorpe, 1987); (*ii*) 75 mM potassium acetate, 0.3 M NaCl, 0.1 M L-arginine, 10 mM EDTA and 0.25% (w/v) Triton X-100, pH = 4.2 (Camiolo *et al.*, 1982); (*iii*) 0.1 M Tris/HCl (pH = 8.1), 1% (w/v) Triton X-114, 10 mM EDTA and 10 μg/ml aprotinin (Behrendt *et al.*, 1990). Samples prepared with buffer C should include 0.25% 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate (CHAPS) to avoid phase separation during subsequent steps caused by Triton X-114 at temperatures above 4°C.

Antibodies

Polyclonal rabbit antibodies raised against human uPA were used as catching antibodies in the ELISA (Rosenquist *et al.*, 1993). Biotinylated anti-uPAR clone R2 was used as detecting antibody in the ELISA (Rønne *et al.*, 1991). Mouse MAbs against human uPA and human uPAR were used for immuno-absorption of uPA and uPAR, respectively. Anti-uPA clone 1 and clone 2 specifically recognize epitopes in the A- and B-chains of uPA, respectively (Rosenquist *et al.*, 1993; Kaltoft *et al.*, 1982). Anti-uPA clone 14 binds to the amino-terminal fragment (ATF) of uPA and is supposed to prevent complex formation with uPAR (data not shown). The mouse MAbs directed against human uPAR, clones R3 and R9, are directed against epitopes in domain 1 of uPAR; clone R3 does not recognize uPAR when in complex with uPA (Rønne *et al.*, 1991). Anti-uPAR clones R2 and R4 are directed against different epitopes in either domain 2 or domain 3 of uPAR (Rønne *et al.*, 1991). A MAb against 2,4,6-trinitrophenol (TNP) (Schulman *et al.*, 1978) was used as a control.

Preparation of uPA:uPAR complexes

uPA:uPAR in vitro *complex preparation.* Complexes of pro-uPA with s-uPAR were prepared by mixing both components (100 pmol/ml and 125 pmol/ml final concentrations, respectively) in PBS (1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 0.14 M NaCl, pH = 7.4), containing 1% BSA and 0.1% (v/v) Tween-20, followed by incubation at room temperature for 1 hr. The mixture, referred to as *in vitro* complex preparation, was aliquoted and stored at −80°C. Dilutions of this preparation, containing uPA:uPAR complexes as well as the free components, were used in all experiments for establishment of the ELISA.

uPA:uPAR complex standard. The uPA:uPAR complex standard was prepared by mixing pro-uPA with s-uPAR (1 pmol/ml and 12.5 pmol/ml final concentrations, respectively) in buffer C. The mixture was incubated overnight at 4°C and aliquots were stored at −80°C. The excess of s-uPAR was demonstrated to completely saturate all pro-uPA. The standard curve was obtained by dilution of the uPA:uPAR complex standard in the appropriate assay buffer, the working range corresponding to 0–40 fmol/ml.

uPA:uPAR cross-linked complexes. For preparation of cross-linked uPA:uPAR complexes, equal amounts of pro-uPA and s-uPAR (400 pmol/ml and 500 pmol/ml final concentrations, respectively) diluted in PBS containing 0.1% (v/v) Tween-80 were incubated for 30 min at 4°C, after which cross-linking was initiated by the addition of EDC and sulfo-NHS ester (10 mM, final concentration each). After 20 min incubation at room temperature, ammonium acetate was added to a final concentration of 10 mM, followed by another incubation for 10 min. The incubation mixture was analyzed on a 6–16% gradient SDS-PAGE gel under reducing conditions, showing approximately 25% of uPA and uPAR being cross-linked (results not shown). Cross-linked complexes were further purified by a 2-step immuno-adsorption, using polyclonal anti-uPA and polyclonal anti-uPAR antibodies covalently coupled to CNBr-activated Sepharose according to standard procedures (Behrendt *et al.*, 1990). When dilutions of cross-linked complexes were analyzed in the complex ELISA, a linear dose-response was observed and supplementation of the preparation with increasing amounts of pro-uPA or s-uPAR (up to 400 fmol/ml and 500 fmol/ml final concentrations, respectively) did not evoke a signal increase, indicating the absence of free uPA and/or uPAR (results not shown). Analysis of the cross-linked preparation in the ELISA for uPAR (Rønne *et al.*, 1995) using only biotinylated MAb R3 for detection, gave no signal above background level as compared to the signal evoked by purified s-uPAR alone (data not shown).

ELISA for uPA:uPAR complexes

The ELISA performed was essentially equal to the procedure described for the measurement of uPAR (Rønne *et al.*, 1995). Briefly, microtiter plates were coated overnight at 4°C with purified polyclonal anti-uPA IgG as catching antibody (1.0 μg/ml; 100 μl/well). After washing, subsequent blocking and rewashing the plates, the *in vitro* complex preparation, uPA:uPAR complex standard, cross-linked complexes or samples to be tested, diluted in PBS with 1% BSA and 0.1% (v/v) Tween-20 (100 μl/well), were incubated in the plates for 60 min at 37°C, unless otherwise stated. After washing, plates were incubated for 60 min with biotinylated monoclonal anti-uPAR clone R2 as detection antibody (0.3 μg/ml, 100 μl/well), then washed again prior to incubation for 60 min with peroxidase-conjugated streptavidin diluted 4,000 times in PBS-BSA-Tween (100 μl/well). Finally, plates were washed and the peroxidase reaction initiated by the addition of substrate (0.02% *o*-phenylenediamine, 0.01% H_2O_2, 100 μl/well). The reaction was stopped after 30 min incubation in darkness and the absorbance read at 492 nm (reference wavelength 620 nm) with an automated ELISA reader (AR 2001; Anthos, Salzburg, Austria). All determinations were performed in duplicate.

Protein analysis

The Bradford (1976) method for protein analysis was employed using the Bio-Rad (Richmond, CA) reagent with BSA as a standard.

Statistics

Correlations between variables were evaluated with the Spearman rank order test using the Statistical Analysis System (SAS Institute, Cary, NC). Statistical values of $p < 0.05$ were considered significant.

RESULTS

ELISA for uPA:uPAR complexes

In the complex ELISA there was a linear relationship between absorbance and uPA:uPAR complex standard up to at least 40 fmol/ml (Fig. 1*a*). Fixed dilutions of the *in vitro* complex preparation analyzed in this ELISA also showed a linear dose-response (Fig. 1*a*). When the polyclonal anti-uPA catching antibody was substituted with IgG from a non-immunized rabbit, no signal above background level was observed with the uPA:uPAR *in vitro*

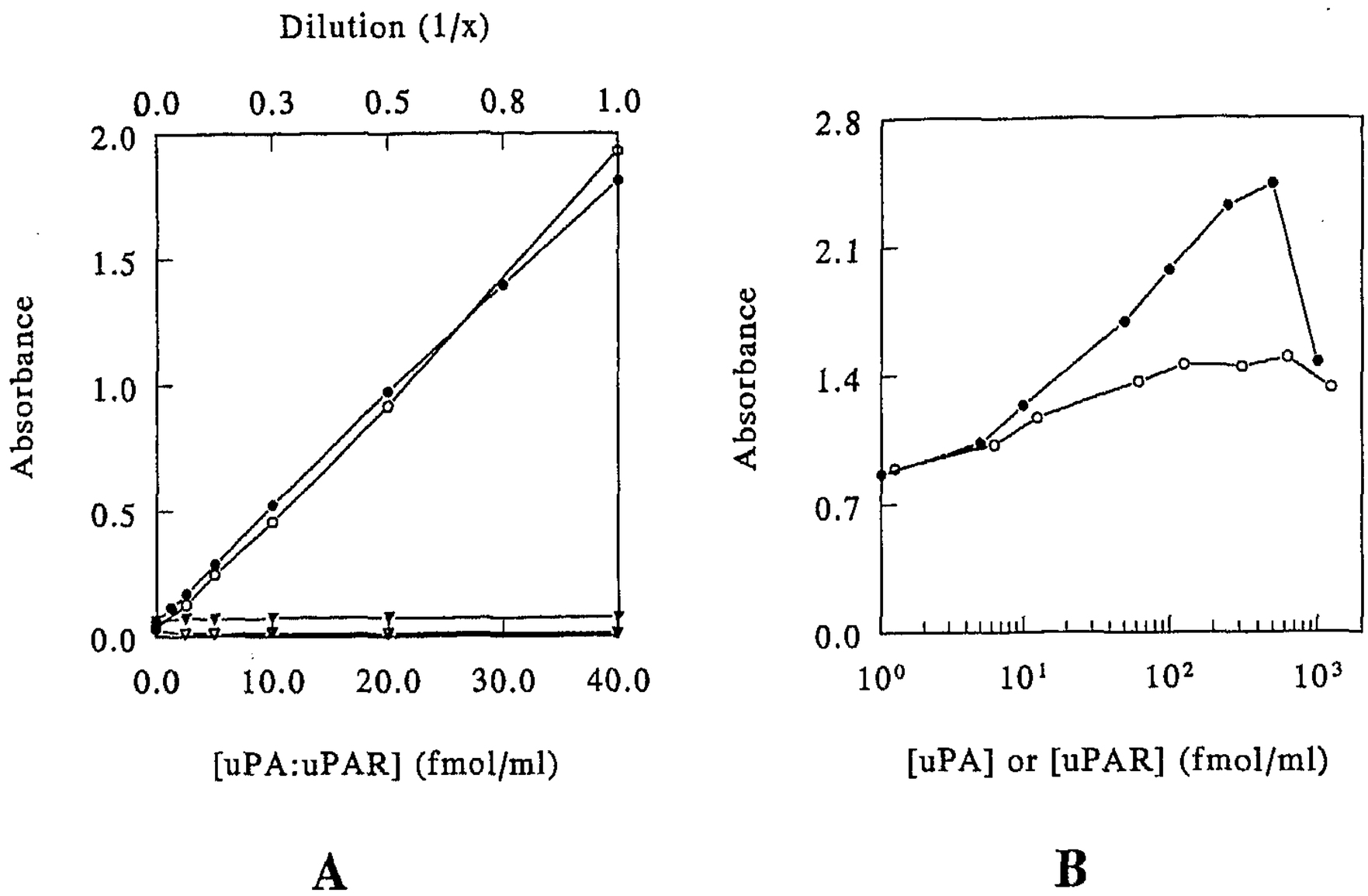


FIGURE 1 – Dose-response curves in the complex ELISA. (*a*) Different concentrations of the uPA:uPAR complex standard (bottom *x* axis) were assayed in the complex ELISA after overnight incubation at 4°C (●–●). Dilutions (1:1,250 to 1:40,000; top *x* axis) of the uPA:uPAR *in vitro* complex preparation (○–○) also were tested in the complex ELISA. As a control, uPA:uPAR *in vitro* complexes were tested when the rabbit polyclonal anti-uPA catching antibody was substituted with non-immune rabbit IgG (▽–▽) and when the biotinylated anti-uPAR antibody clone R2 was substituted with biotinylated anti-TNP MAbs (▼–▼). (*b*) A fixed dilution (1:1,250) of the *in vitro* complex preparation was mixed 1:1 with the indicated (final) concentrations of pro-uPA (●–●) or s-uPAR (○–○) and analyzed in the complex ELISA.

complex preparation (Fig. 1*a*). Similarly, when the biotinylated monoclonal anti-uPAR detection antibody (R2) was substituted with biotinylated anti-TNP, no signal was detected (Fig. 1*a*). Addition of pro-uPA (0–1,000 fmol/ml) or s-uPAR (0–1,250 fmol/ml) to the *in vitro* complex preparation led to an increased signal in the complex ELISA, most probably due to additional complex formation with the free components (Fig. 1*b*). The declining signal obtained with the highest concentration of added pro-uPA indicates competition of uPA with uPA:uPAR complexes for binding to the catching antibodies. The response obtained when adding increasing amounts of s-uPAR indicates complex formation with uPA, leading to exhaustion of free uPA and/or saturation of the solid-phase binding sites (Fig. 1*b*). Background levels were measured when both pro-uPA and active human uPA, s-uPAR or tPA were analyzed individually up to 1,000, 1,250 and 715 fmol/ml final concentrations, respectively (results not shown). When the *in vitro* uPA:uPAR complex preparation diluted in buffer C was absorbed with a mixture of either anti-uPA, clones 1 and 2, or anti-uPAR MAbs, clones R4 and R9, followed by precipitation with protein A Sepharose, a complete disappearance of the signal in the complex ELISA was observed as compared to the response obtained after addition of anti-TNP (Fig. 2*a*). A complete disappearance in signal was also seen in the uPA ELISA after absorption with anti-uPA antibodies, while a partial decrease in signal was obtained after absorption with anti-uPAR IgG (Fig. 2*a*). Similarly, when analyzed in the uPAR ELISA, absorption of the *in vitro* complex preparation with anti-uPA and anti-uPAR resulted in a partial and a complete decrease in signal, respectively (Fig. 2*a*). Comparable results were obtained when the absorption experiment was performed with the *in vitro* complex preparation diluted in buffer A or B (results not shown). Absorption of an extract of the pooled lung tumor tissue prepared with buffer C with anti-uPA IgG (clones 1 and 2) or anti-uPAR IgG (clones R4 and R9) also resulted in an almost complete reduction of the signals (approximately 90% and 75%, respectively) in the complex ELISA (Fig. 2*b*). Comparable results were obtained when the absorption experiment was performed with the pooled lung-tumor sample extracted with buffer A or B (results not shown).

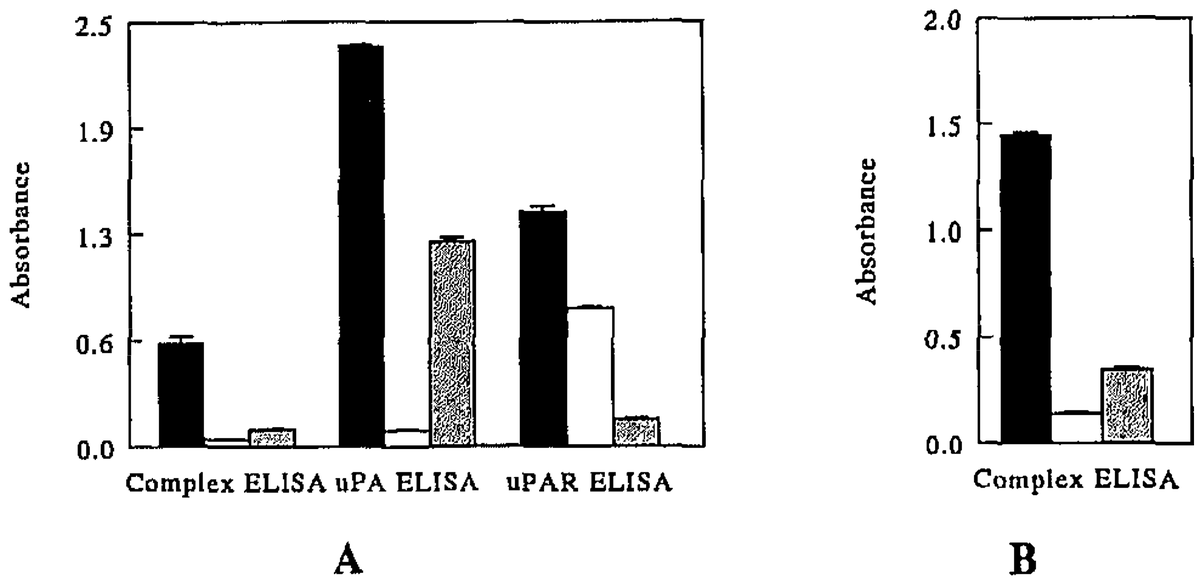


FIGURE 2 – Absorption of the uPA:uPAR *in vitro* complex preparation and of a pooled lung tumor sample extracted with buffer C. (*a*) A 100 times diluted aliquot of the *in vitro* complex preparation in buffer C was incubated for 2 hr at room temperature in the presence of anti-uPA, clones 1 and 2 (white bars); anti-uPAR, clones R4 and R9 (hatched bars); or anti-TNP (black bars). Antibodies were diluted in PBS-BSA-Tween and added in a 1,000-fold molar excess relative to the expected molar concentrations of uPA and uPAR. After incubation, antibody–ligand complexes were precipitated with 25% (v/v) protein A Sepharose in PBS-BSA-Tween and the supernatants analyzed in the complex ELISA, uPA ELISA and uPAR ELISA. Indicated are the mean absorbance values + S.D. of duplicate determinations. (*b*) Non-small cell lung carcinoma tissue of 6 patients was pulverized and the tumor powder pooled and mixed. An aliquot of the resulting sample was used for extraction with buffer C. Equal portions of the extract were then absorbed as described above for the *in vitro* complex preparation and analyzed in the complex ELISA. Indicated are the mean absorbance values + S.D. of duplicate determinations.

Dilutions of extracts from a pool of lung-cancer tissue prepared with buffers A, B and C assayed in the complex ELISA showed a linear dose-response for all three types of extract, the highest signals obtained for extracts prepared in buffer C (Fig. 3). When fixed amounts of chemically cross-linked uPA:uPAR complexes were added to the different extracts, parallelism was found with the dilution curve of the cross-linked complexes (Fig. 4*a, b*), indicating

that the assessment of the complexes is not affected by other components in the biological samples. Recovery of cross-linked complexes in the samples at each standard point varied between 80–90% (extracts prepared with buffer A) and 80–105% (extracts prepared with buffers B and C).

The intra-assay variation of the complex ELISA was determined by duplicate analysis of 10 independent 1:5 dilutions of the pooled lung tumor extract prepared with buffer C in one and the same ELISA plate; the coefficient of variation in the calculated concentrations was 5.3%. The inter-assay variation was determined by analyzing the same pooled extract in a 1:5 dilution on 10 separate days, resulting in a coefficient of variation of 9.6%.

The sensitivity of the complex ELISA was calculated to be 0.5 fmol/ml (0.05 fmol/well), defined as the lowest antigen concentration giving a signal higher than the background value plus 3 times the standard deviation of this latter value. Overall, the coefficient of variation of duplicate measurements was less than 5%.

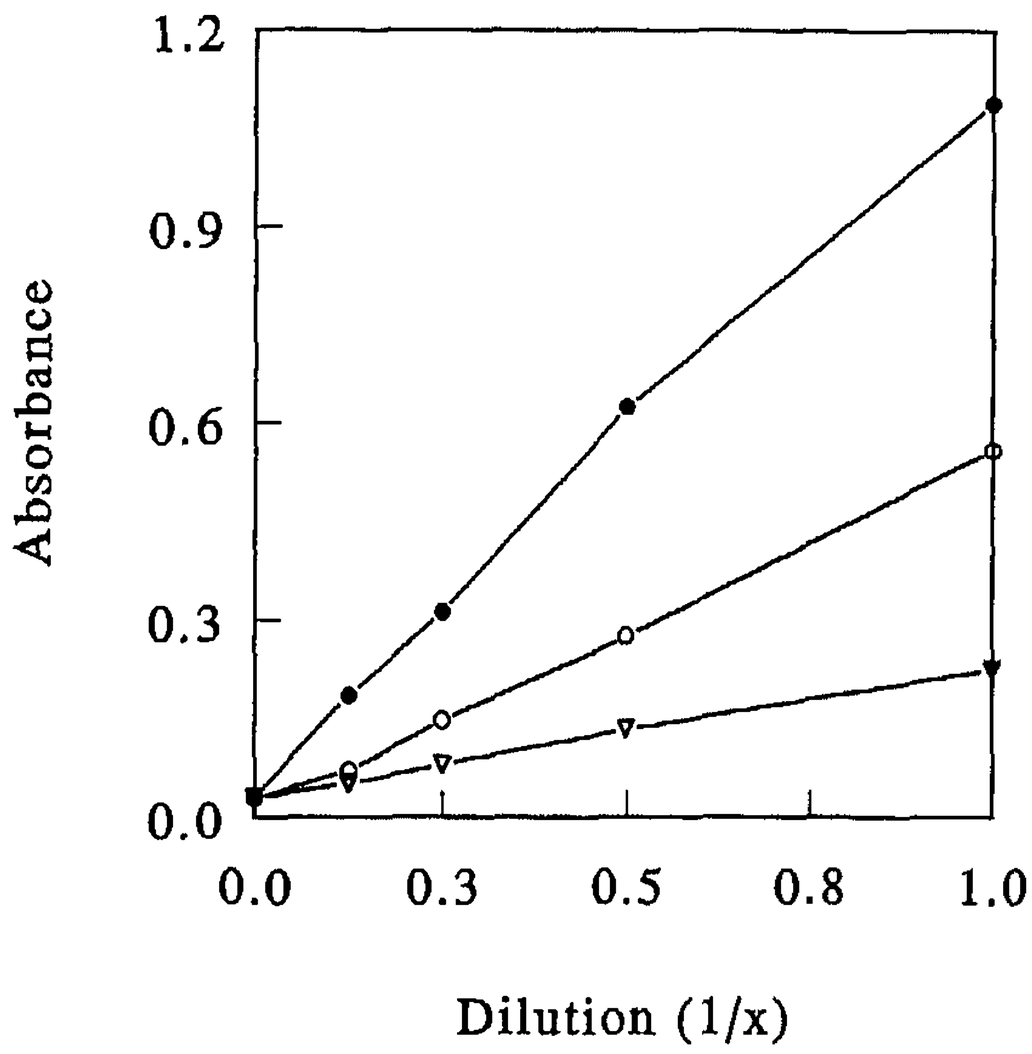


FIGURE 3 – Analysis of different extracts of lung cancer tissue in the complex ELISA. Different dilutions of extracts of pooled lung cancer tissue (starting with 10% extract) prepared with buffer A (○–○), buffer B (▽–▽) and buffer C (●–●).

Tumor tissue extraction with different buffers

Twenty individual NSCLC samples extracted with buffers A, B and C were analyzed in the complex ELISA, as well as in the ELISAs for uPA and uPAR (Table I). Extracts prepared with buffer B contained the highest concentrations of uPA, while extracts prepared with buffer C showed the highest concentrations of uPAR and uPA:uPAR complexes. When the uPA:uPAR complex levels were compared to one another, a highly significant correlation (Spearman correlation coefficient r = 0.78; $p = 0.0001$) was found between extracts prepared with buffers A and C. The levels of uPA:uPAR complexes in extracts prepared with buffer B were shown not to be associated with the levels obtained for extracts in buffer A (r = 0.17; $p = 0.49$) or C (r = 0.38; $p = 0.10$), respectively. Additionally, highly significant correlations were found between the levels of uPA and uPA:uPAR complexes in both extracts prepared with buffers A (r = 0.91; $p = 0.0001$) and C (r = 0.94; $p = 0.0001$). No correlation was found between the uPA and uPA:uPAR complex levels obtained in buffer B (r = 0.29; $p = 0.22$). When comparing the uPAR and uPA:uPAR complex levels, weak but significant correlations were found for extracts prepared with buffers A (r = 0.79; $p = 0.0001$), B (r = 0.66; $p = 0.002$) and C (0.70; $p = 0.0007$).

Complex formation between uPA and uPAR in vitro

To study the ability of uPA and uPAR to form complexes in buffers A, B and C, pro-uPA and radioactively labeled ^{35}S-s-uPAR were mixed with each other in the respective buffers (Fig. 5). Stable uPA:uPAR complexes can be formed in both buffers A and C as shown by the presence of radiolabeled ^{35}S-s-uPAR in the immunoprecipitate with anti-uPA antibodies (Fig. 5, panels I and III, lanes 1 and 2). Complex formation seems to occur in buffer B in the absence of unlabeled s-uPAR (Fig. 5, panel II, lane 1), which most probably should be ascribed to *de novo* complex formation due to dilution of the sample when adding the respective antibodies and, consequently, elevation of the pH to more neutral values (pH = 6.0), allowing antibody–ligand interactions. This was verified by immuno-precipitation in the presence of excess unlabeled s-uPAR, which completely out-competed radioactive s-uPAR in forming complexes with pro-uPA (Fig. 5, panel II, lane 2).

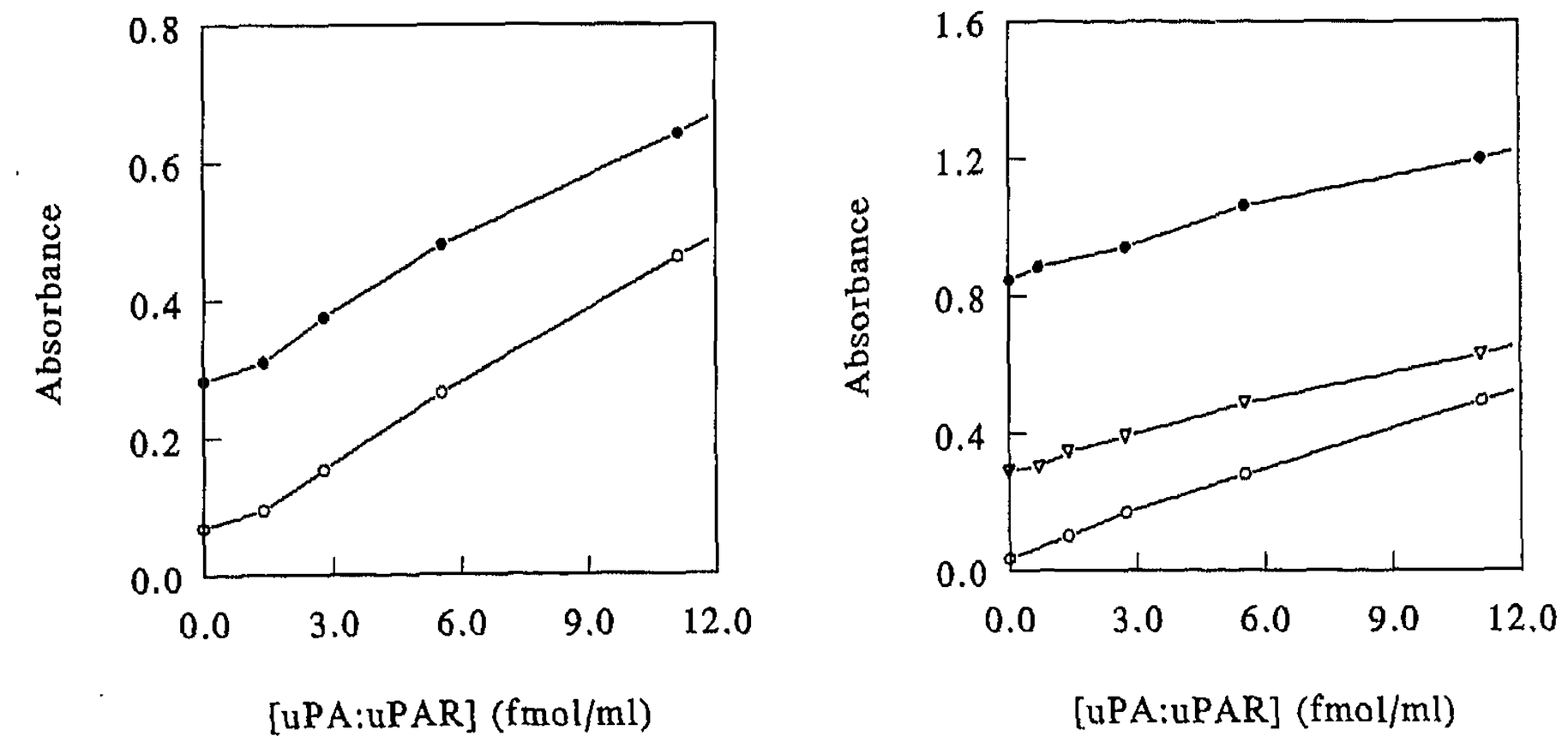


FIGURE 4 – Analysis of cross-linked uPA:uPAR complexes calibrated against the uPA:uPAR complex standard added to different extracts of a pooled lung carcinoma tissue sample. (*a*) Cross-linked complexes in assay dilution buffer (○–○) and in a 10-fold diluted extract of the lung tumor tissue prepared with buffer A (●–●). (*b*) Cross-linked complexes from a different preparation in assay dilution buffer (○–○) and in 10-fold dilutions of extracts prepared with buffer B (▽–▽) and buffer C (●–●).

TABLE I – ANALYSIS OF NSCLC SAMPLES FOR uPA, uPAR AND uPA:uPAR COMPLEXES EXTRACTED WITH 3 DIFFERENT BUFFERS

Buffer	uPA		uPAR		uPA:uPAR	
	fmol/ml	fmol/mg	fmol/ml	fmol/mg	fmol/ml	fmol/mg
A	182.7 (25.0–554.7)	22.2 (3.7–70.8)	538.8 (152.6–1,401.3)	66.5 (22.3–158.3)	54.2 (0–627.9)	7.1 (0–70.9)
B	1,688.0 (436.7–4,355.9)	270.5 (70.0–720.4)	437.2 (192.7–1,195.1)	81.2 (31.0–197.7)	57.9 (0–213.1)	9.5 (0–41.3)
C	560.3 (155.1–1,950.8)	45.0 (11.3–152.9)	787.2 (308.3–1,540.0)	78.6 (19.9–117.3)	154.6 (19.1–1,296.5)	16.1 (1.6–83.8)

Individual lung tumor tissue samples were each extracted with buffers A (n = 18 samples), B and C (n = 20 samples each) and subsequently analyzed for uPA, uPAR and uPA:uPAR complexes in the respective ELISAs. For analysis in the complex ELISA, extracts were diluted 1:10. Incubation of samples was performed for 1 hr at 37°C. Indicated are the median values and ranges (between parentheses) obtained by one single extraction. uPA, uPAR and uPA:uPAR complex levels (expressed in fmol/mg protein) in the different extracts were correlated to one another.

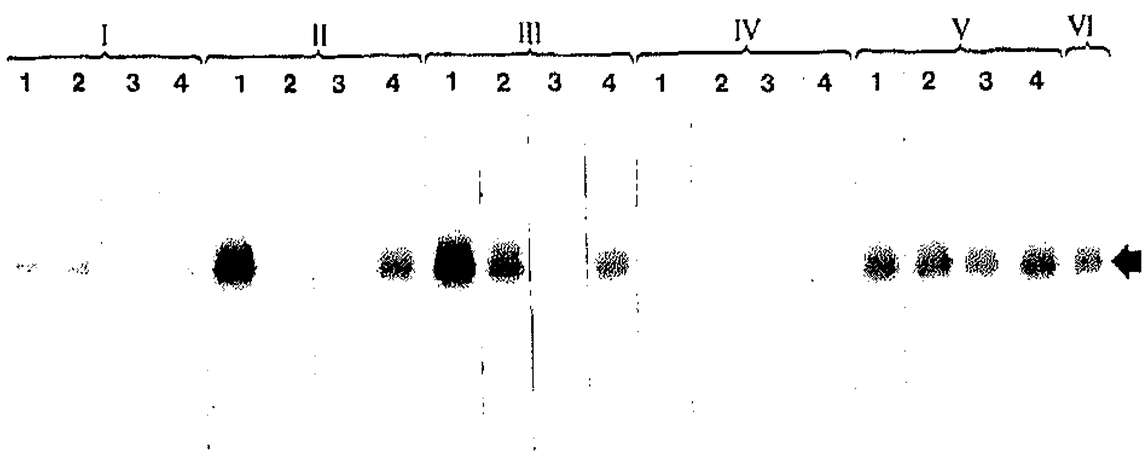


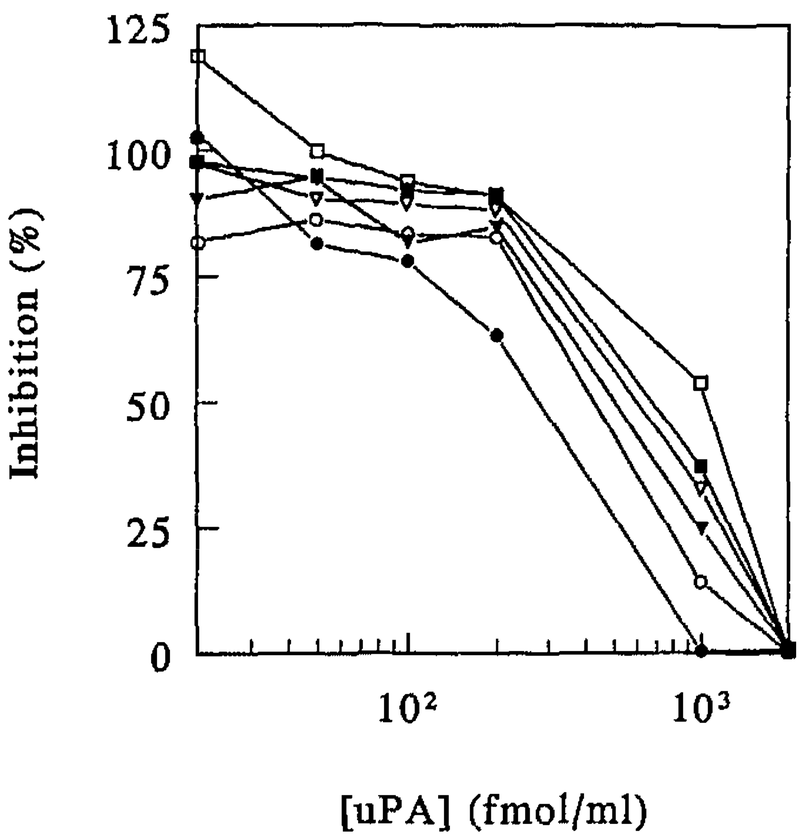
FIGURE 5 – Complex formation in 3 different buffers. Pro-uPA and ^{35}S-labeled s-uPAR, diluted separately in buffers A, B and C, were mixed (30 and 37.5 pmol/ml final concentrations, respectively) and incubated overnight at 4°C. Mixtures were divided into equal aliquots containing 750 fmol/ml of uPA and 937.5 fmol/ml of uPAR, to which either anti-uPA MAbs, clones 1 and 2, clone 14 or irrelevant IgG (anti-TNP) were added in the presence or absence of unlabeled s-uPAR; the antibodies and s-uPAR were diluted in PBS and added in 50- and 100-fold molar excesses, respectively. After overnight incubation at 4°C, immune complexes were incubated for 1 hr at room temperature with 25% (v/v) protein A Sepharose in binding buffer (0.1 M Tris/HCl [pH = 8.1], 300 mM NaCl, 0.1% BSA, 0.1% CHAPS). Pellets obtained after centrifugation, 25 sec at 5,000 *g* and 4°C, were washed twice with binding buffer and twice with binding buffer without BSA. After the last washing step, protein was eluted from the Sepharose pellets by boiling in SDS sample buffer and analyzed by SDS-PAGE on a 6–16% polyacrylamide gel. The presence of a radioactive band (^{35}S-s-uPAR) in the autoradiogram demonstrates the ability of uPA and uPAR to form stable complexes in the respective buffers. Panels I, II and III correspond to complex formation in buffers A, B and C, respectively. Panel IV corresponds to a control incubation of ^{35}S-s-uPAR without pro-uPA added in buffer C; panel V corresponds to a control incubation of uPA and ^{35}S-s-uPAR in buffer C, but binding buffer was added instead of protein A Sepharose. In each panel, lanes 1 and 2 correspond to incubation with anti-uPA, clones 1 and 2, in the absence or presence of unlabeled s-uPAR, respectively; lanes 3 and 4 correspond to incubation with anti-TNP in the presence of unlabeled s-uPAR and incubation with anti-uPA, clone 14, in the absence of unlabeled s-uPAR, respectively. As a reference, ^{35}S-labeled s-uPAR is shown in lane VI. Arrow denotes electrophoretic mobility of standard protein (glutamate dehydrogenase, bovine liver) with an m.w. of approx. 50 kDa.

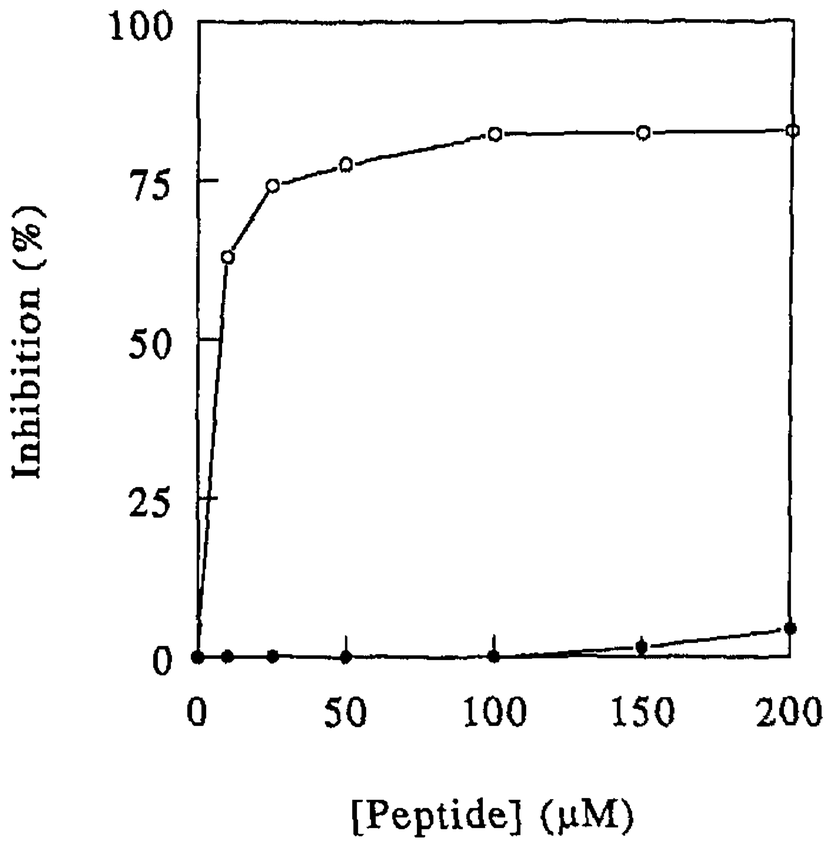
Inhibition of de novo *uPA:uPAR complex formation*

To study the potential formation of uPA:uPAR complexes during tumor tissue extraction and during the sample incubation step in the ELISA, increasing concentrations of the peptide antagonist (Goodson *et al.*, 1994) were tested for inhibitory activity when combining different concentrations of pro-uPA and s-uPAR diluted in buffer C. The selected concentrations of pro-uPA and s-uPAR were comparable to those measured in the 20 lung tumor extracts (Table I). The results shown in Figure 6*a* indicate that when the (final) concentrations of uPA and uPAR are not higher than approximately 200 fmol/ml and 250 fmol/ml, respectively, a concentration of at least 50 μM of peptide antagonist is capable of inhibiting complex formation between uPA and uPAR for more than 80%. Efficient inhibition of complex formation is assured only when the temperature is kept at 4°C. At higher temperatures (*e.g.*, 37°C), blocking of complex formation between uPA and uPAR *in vitro* is far from complete (data not shown). The control peptide did not have any inhibiting effect on *de novo* complex formation (Fig. 6*b*). A concentration of 100 μM of peptide antagonist and control peptide was chosen for further analyses. When different dilutions of the cross-linked uPA:uPAR complexes were analyzed in the presence of 100 μM of peptide antagonist and control peptide, identical signals were obtained in the complex ELISA, indicating no interfering effect of the peptide antagonist with the ELISA reagents (results not shown). When different dilutions of pre-formed *in vitro* complexes in buffer C were tested in the presence of 100 μM of peptide antagonist at 4°C, the peptide antagonist did not show any effect on the signal in the complex ELISA (Fig. 6*c*). These data indicate that, at least *in vitro*, already existing uPA:uPAR complexes do not dissociate in the presence of high concentrations of peptide antagonist. Again, at high temperatures (37°C), the signals obtained are not stable, most likely because of displacement of uPA from the receptor by the peptide antagonist (results not shown). Thirteen NSCLC tissue biopsies were processed in the presence of peptide antagonist and randomized control peptide and subsequently analyzed in the complex ELISA. As shown in Table II, extracts prepared in the presence of peptide antagonist contained lower concentrations of uPA:uPAR complexes when compared to extracts prepared with control peptide. The differences are most probably the result of *de novo* formation of uPA:uPAR complexes in the absence of peptide antagonist, the amount of which appears to vary between 55% and 353% of the amount of complexes measured in the presence of peptide antagonist. The same 13 extracts also were analyzed in the ELISAs for uPA and uPAR, to investigate relations between the levels of uPA:uPAR complexes and the concentrations of the separate components (Table II). All extracts scored uPA and uPAR levels below 200–250 fmol/ml, except for extract 5 (318.1 fmol/ml uPA) and extract 13 (336.1 fmol/ml uPAR). Finally, the concentrations of the different molecules as determined in the 13 NSCLC tissue extracts were correlated to one another. The calculated Spearman correlation coefficients were successively 0.59 ($p = 0.03$) for uPA *vs.* uPAR, 0.75 ($p = 0.003$) for uPA *vs.* uPA:uPAR complex (extraction with control peptide), 0.86 ($p = 0.0002$) for uPA *vs.* uPA:uPAR complex (extraction with peptide antagonist), 0.70 ($p = 0.007$) for uPAR *vs.* uPA:uPAR complex (extraction with control peptide) and 0.67 ($p = 0.01$) for uPA *vs.* uPA:uPAR complex (extraction with peptide antagonist). Moreover, a highly significant correlation was found between uPA:uPAR complex levels measured in the extracts with peptide antagonist and control peptide ($r = 0.86$; $p = 0.0001$).

DISCUSSION

In the present work, we have demonstrated the establishment of a sensitive ELISA for the specific detection of complexes between uPA and uPAR with a combination of polyclonal anti-uPA catching





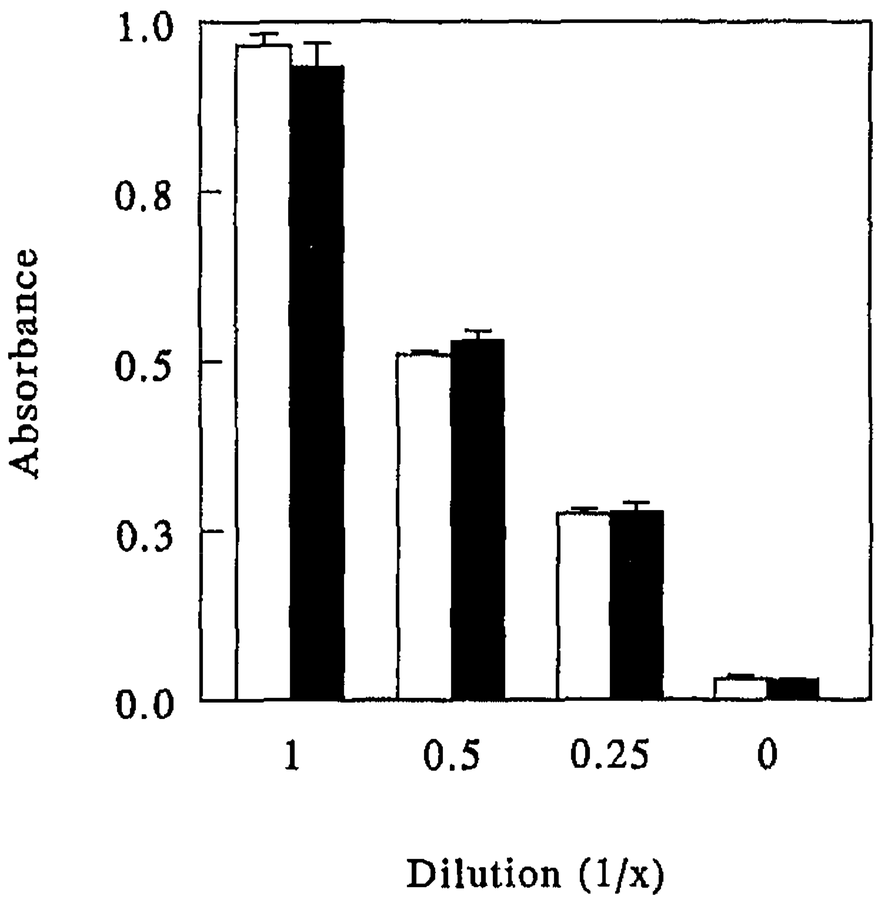


FIGURE 6 – Effect of the peptide antagonist on *de novo* complex formation and on the stability of pre-formed uPA:uPAR complexes *in vitro*. (*a*) Pro-uPA (0, 20, 50, 100, 200, 1,000 and 2,000 fmol/ml final concentrations, as indicated along the *x* axis) diluted in ice-cold buffer C supplemented with increasing concentrations of peptide antagonist or control peptide (0–200 μM final concentrations) and s-uPAR (0, 25, 62.5, 125, 250, 1,250 and 2,500 fmol/ml final concentrations) diluted in ice-cold buffer C without any supplementations were mixed 1:1. After incubation for 1 hr at 4°C, mimicking the centrifugation step when preparing the tumor tissue extracts, the mixtures were diluted 1:10 with PBS-BSA-Tween supplemented with peptide antagonist or control peptide (0–200 μM final concentrations) and analyzed in the complex ELISA (incubation overnight at 4°C). The percentage of inhibiting activity by the peptide antagonist was calculated on the basis of the absorbances and expressed relative to the O.D. signals obtained with control peptide. Peptide concentrations: 10 μM ●–●, 25 μM ○–○ 50 μM ▼–▼, 100 μM ▽–▽, 150 μM ■–■ and 200 μM □–□. (*b*) Pro-uPA (200 fmol/ml final concentration) and s-uPAR (250 fmol/ml final concentration) were incubated with each other in the presence of increasing concentrations of peptide antagonist or control peptide (0–200 μM final concentrations) and assayed in the complex ELISA as described for (*a*). The percentages of inhibiting activity by the peptide antagonist (○–○) as well as that by the control peptide (●–●) were calculated on the basis of the absorbances and expressed relative to the O.D. signals obtained without peptide antagonist or control peptide, respectively. (*c*) Different dilutions (1:5,000 to 1:20,000) of the *in vitro* complex preparation in buffer C supplemented with 100 μM of peptide antagonist (black bars) or control peptide (white bars) were analyzed in the complex ELISA (incubation overnight at 4°C). Indicated are the mean absorbance values + S.D. of duplicate determinations.

antibodies and one anti-uPAR MAb for detection. Thus far, the ELISAs developed for measurement of the constituents of the plasminogen activation system, *i.e.*, uPA and uPAR, detect both the free forms and the respective components in complex with one another, without having the ability to distinguish between them. Moreover, these ELISAs detect each of the various forms with different efficiencies. Therefore, the assay results obtained from these ELISAs at best represent the total amount of uPA or uPAR present. Our study shows the establishment of an ELISA for the specific detection and quantitation of uPA:uPAR complexes in tumor tissue extracts.

The specificity of the complex ELISA is demonstrated by the absorption experiment with the *in vitro* complex preparation as well as the tumor tissue extracts using anti-uPA and anti-uPAR MAbs different from those employed in the assay. After incubation with both types of antibody, no responses in the complex ELISA were observed. Furthermore, pro-uPA, active uPA, uPAR, as well as tPA do not cause any signal in the complex ELISA. The specificity of the ELISA also is expressed by the absence of a response to the *in vitro* complex preparation when the polyclonal anti-uPA catching antibody or the biotinylated anti-uPAR detecting MAb was substituted with polyclonal non-immune IgG or biotinylated irrelevant MAb of the same sub class, respectively. The complex ELISA would not detect complexes of uPA with isolated domain 1 of uPAR because the detection antibody (R2) was directed against domains 2 and 3 of the receptor. When uPAR is cleaved by uPA or plasmin, as shown not only *in vitro* but also *in vivo* (Høyer-Hansen *et al.*, 1992; Solberg *et al.*, 1994), uPA in complex with the ligand-binding domain may be released. However, the apparent ligand-binding affinity of the isolated domain 1 is more than 1,500-fold lower compared to intact uPAR; therefore, complexes between uPA and domain 1 of uPAR are supposed to be very unstable, making it unlikely for these complexes to be present *in vivo* (Ploug *et al.*, 1994).

Of the 3 different buffers studied, buffer C is clearly the most efficient for extraction of uPA:uPAR complexes from lung tumor tissue. This is demonstrated by the high signals obtained in the complex ELISA when assaying the pooled lung tissue sample as well as the 20 individual lung tumor extracts prepared in buffer C. These results are in agreement with the concept that detergent-containing buffers are effective for extraction of uPAR and, thus, uPA:uPAR complexes out of membranes (Nielsen *et al.*, 1988; Behrendt *et al.*, 1990). As found previously and in the present study, extraction with buffer B yields the largest amounts of uPA (Rosenquist *et al.*, 1993), while buffer C is most efficient for uPAR extraction (Rønne *et al.*, 1995). The high concentrations of uPA and uPAR in extracts prepared with buffer B are not attended by high concentrations of uPA:uPAR complexes, which is consistent with the low pH (4.2) of this buffer, causing dissociation of the complexes. Indeed, low pH buffers have been used to release endogenous (pro-) uPA bound to uPAR in order to purify the receptor from cells (Behrendt *et al.*, 1993*a*). The instability of uPA:uPAR complexes at low pH is also clear from the analysis of

TABLE II – ANALYSIS OF 13 DIFFERENT NSCLC SAMPLES FOR uPA, uPAR AND uPA:uPAR

Sample	uPA (fmol/mg)	uPAR (fmol/mg)	uPA:uPAR		%
			Contr. (fmol/mg)	Antag. (fmol/mg)	
1	131.9	197.9	50.6	23.2	118
2	30.8	30.5	8.5	2.6	229
3	32.2	38.9	15.7	3.5	342
4	47.9	152.3	32.2	7.7	320
5	188.4	116.2	26.6	11.0	141
6	45.0	75.8	14.0	3.4	316
7	127.3	73.1	33.6	16.6	103
8	137.5	149.2	55.7	35.8	55
9	67.1	116.4	29.1	10.7	171
10	110.8	84.3	27.6	11.9	133
11	90.0	80.1	22.2	7.9	182
12	35.8	53.0	9.4	2.1	353
13	75.1	191.8	25.8	12.1	113

Thirteen different lung tumor tissue samples were each extracted with buffer C supplemented with peptide antagonist (Antag.) or control peptide (Contr.) and analyzed in the complex ELISA, as well as in the ELISAs for uPA and uPAR. Indicated values are means obtained by one separate extraction and assay of one (uPA and uPAR, 1:15) or 2 (uPA:uPAR complexes, 1:2 and 1:5) independent dilutions. Extracts prepared in the presence of peptide antagonist and control peptide contained comparable concentrations of uPA and uPAR. To assure full antagonistic activity of the peptide, final concentrations of uPA and uPAR in tumor-tissue extracts were kept below 200–250 fmol/ml, because of which the tumor:buffer ratio (w/v) was changed to 1:30 as stated in "Material and Methods". Incubation was performed overnight at 4°C. uPA, uPAR and uPA:uPAR complex levels (expressed in fmol/mg protein) in the different extracts were correlated to one another; % indicates the degree of *de novo* complex formation, calculated as the relative difference between uPA:uPAR complex levels in extracts with peptide antagonist (equated to 0% *de novo* complex formation) and control peptide.

complex formation in buffer B *in vitro* using pro-uPA and radioactively labeled s-uPAR (Fig. 5). As a consequence, the signals obtained in the complex ELISA when assaying lung tumor tissue extracts prepared with buffer B are most probably the result of *de novo* complex formation during the sample incubation step after dilution with a neutral pH buffer in the ELISA. Formation of secondary complexes between uPA and uPAR from different parts of the tumor specimen during tumor tissue processing certainly also could contribute to additional complexes which were not present at the beginning of the extraction procedure. In view of these findings, the response of the clinical samples in the complex ELISA may be considered as a measure of the complex-forming potential in the samples but not directly equated to the amount of complexes present in the tissue specimen at the moment of extraction (see further below).

To study *de novo* complex formation between uPA and uPAR during preparation of the tumor tissue extracts and/or the sample incubation step in the ELISA, we employed a 17-mer peptide which has been shown to act as a potent antagonist for uPAR (Goodson *et al.*, 1994). This peptide was capable of inhibiting formation of complexes between pro-uPA and s-uPAR (in final concentrations of up to 200 and 250 fmol/ml, respectively) *in vitro*, though very high concentrations of peptide antagonist (100 μM) were necessary. Under these conditions, half-maximal inhibition of complex formation was obtained with a peptide concentration just below 10 μM. This value is in apparent contrast to the stated inhibition constant of the synthetic peptide for the uPA:uPAR interaction (IC_{50} approx. 10 nM) (Goodson *et al.*, 1994), which is most probably a reflection of the different assay conditions and the high levels of uPA and uPAR tested in a non-solid phase-dependent system. The peptide antagonist (100 μM) did not have any effect on existing uPA:uPAR complexes *in vitro* when the temperature was kept below 4°C instead of at 37°C, thus opening the possibility of distinguishing between original and *de novo* formed complexes. The instability of the complexes at higher temperatures most likely can be attributed to kinetics-related phenomena.

Assuming an analogous effect of the peptide antagonist *in vivo*, the complexes measured in the 13 NSCLC extracts in the presence of this peptide most likely represent original uPA:uPAR complexes which were present at the beginning of the tumor tissue processing. The way in which the tumor biopsies were processed, which comprises the recommended procedure—*i.e.*, dismembration of the tumor tissue in the presence of extraction buffer C (tumor:buffer ratio [w/v] 1:30) supplemented with peptide antagonist (100 μM, final concentration) at −196°C, thawing the tumor powder and buffer components on ice, centrifugation of the homogenates and analysis of the extracts in the ELISA overnight at 4°C—should ensure full antagonistic potential of the peptide and therefore almost complete blocking of *de novo* complex formation. Measurement of similar levels of uPA:uPAR complexes in 2 different dilutions (1:2 and 1:5) of the tumor extracts prepared with peptide antagonist suggests that the complexes do not dissociate upon dilution to this extent. The large difference between the complex levels detected in the presence of peptide antagonist and control peptide is indicative of a large extent of *de novo* complex formation between uPA and uPAR. Moreover, the degree of *de novo* complex formation differed widely (55–353%) in the 13 individual samples. The actual complex levels were quite low compared to the levels of both uPA and uPAR, suggesting that the (total) levels of uPA and uPAR are determined mainly by the concentrations of the free components. However, the uPA:uPAR complex levels assayed in the presence of peptide antagonist do show a larger degree of variation (factor 17) compared to both the complex levels analyzed in the presence of control peptide and the levels of uPA and/or uPAR (factors 6 and 7). Interestingly, when the levels of uPA and uPAR were correlated to one another, a correlation coefficient of 0.59 ($p = 0.03$) was found, whereas the partial correlation coefficient between the total levels corrected for the confounding complexes was calculated to be 0.14 ($p = 0.67$) (complexes with control peptide) and 0.05 ($p = 0.88$) (complexes with peptide antagonist). The absence of any correlation suggests that the uPA:uPAR complexes are indeed important contributors to the clinical significance of (total) levels of uPA and uPAR as measured in the corresponding ELISAs. Despite statistically significant correlations between the level of either uPA or uPAR and the levels of uPA:uPAR complexes, only a clinical study will prove the surplus value of analysis of complexes over the (total) concentrations of uPA and/or uPAR. The present assay may be considered suitable for studying the potential prognostic impact of uPA:uPAR complexes in lung tumor tissue extracts as well as other types of cancer. Especially for these kinds of studies, it is recommended that the peptide antagonist be employed for analysis of original uPA:uPAR complexes instead of artificially formed *de novo* complexes.

Finally, several studies with invasion model systems, both *in vitro* (Quax *et al.*, 1991; Behrendt *et al.*, 1993*b*) and *in vivo* (Ossowski, 1988; Ossowski *et al.*, 1991; Ossowski and Reich, 1983), indicate that blocking of the binding of uPA to uPAR results in the inhibition of invasion and/or metastasis, which represents a promising new approach for anti-invasive therapy. Previously, an ELISA was developed for studying ligand-binding interactions using an anti-uPAR MAb for catching and an anti-uPA MAb for detection (Behrendt *et al.*, 1993*a*). Furthermore, a microtiter plate–based chromogenic assay has been described to identify substances which interfere with uPA binding to the receptor (Rettenberger *et al.*, 1995). The present ELISA, being completely characterized and above all optimized for quantitative detection of complexes between uPA and uPAR, could be of additional use for screening compounds inhibiting uPA:uPAR interactions.

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