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Localization of the Cleavage Sites on Fibronectin Following Digestion by Urokinase

Leslie I. Gold, Agueda Rostagno, Blas Frangione, and Tina Passalaris

Department of Pathology, New York University Medical Center (L.I.G., A.R., B.F.) and Medical Student—Honors Program, New York University Medical School (T.P.), New York, New York 10016

Abstract Urokinase (u-PA) proteolytically cleaves both human plasma (pFn) and cellular (cFn) dimeric fibronectin (M_r 440,000) into four major polypeptides of approximately M_r 210,000, 200,000, 25,000, and 6,000. Amino acid sequence analysis of the polypeptide fragments indicated that the enzymatic cleavage of Fn occurs at two sites: 1) between an arginine/alanine peptide bond located C-terminal to residue 259; this cleavage liberates the N-terminal M_r 25,000 fragment and the M_r 210,000 and M_r 200,000 polypeptides derived from the A and B chains of Fn, respectively; and 2) between an arginine/threonine peptide bond located C-terminal to residue 2,299, thereby yielding an M_r 6,000 dimeric fragment containing the C-terminal interchain disulfide bonds. Predigestion of Fn with u-PA increased the molecule's vulnerability to further attack by the enzymes plasmin and cathepsin D. These data provide further biochemical evidence for the proteolytic cleavage of fibronectin by plasminogen activators and substantiate that u-PA digestion of Fn may be an initial event in the local degradation of the extracellular matrix by malignant cells, possessing elevated levels of these enzymes.

Key words: fibronectin, urokinase, proteolysis, cleavage sites, metastasis

Fibronectin (Fn) is a high molecular weight, multifunctional, dimeric glycoprotein composed of two similar chains, ranging from M_r 210,000 to 250,000 kDa, that are covalently disulfide bonded at the C-terminal of the molecule. The varied chain lengths and differences between the cellular and plasma forms, as well as the differences among species, is due to alternative mRNA splicing in at least three different regions of a common precursor transcribed from a single gene [Hynes, 1990]. Both the plasma and cellular forms of Fn are major components of the extracellular matrix thereby providing a substratum for both the adhesion and migration of cells. Fn mediates the adhesion of cells to substratum due to its binding sites for a receptor on most cell surfaces (integrins) and for collagens, heparins, and proteoglycans, also components of the ECM [for review, Hynes, 1990; Pearlstein et al., 1980].

Tumor cells demonstrate increased proteolytic activity and thus can degrade extracellular matrices, thereby facilitating their metastasis [Mullins and Rohrlich, 1983; Saksela and Rifkin, 1988; Dano et al., 1985; Sas et al., 1986]. In particular, an increase in the plasminogen activators, urokinase (u-PA) and tissue plasminogen activator (t-PA), has been correlated with the metastatic activity of a variety of malignant cells [Saksela and Rifkin, 1988; Hearing et al., 1988; Gaylis et al., 1989; Duffy et al., 1990]. Plasminogen activators are specific serine proteases that convert the zymogen plasminogen to the active enzyme, plasmin, an enzyme of broad specificity. It has been shown that fibronectin is released from the ECM at tumor cell contact sites [Chen et al., 1984] and specific inhibitors of plasminogen activators [Dano and Reich, 1978; Fairbairn et al., 1985], as well as an anti-u-PA monoclonal antibody [Sullivan and Quigley, 1986], obviated ECM degradation and concomitant release of Fn by transformed chicken embryo fibroblasts; another anti-u-PA antibody in-

Abbreviations used: u-PA, urokinase; t-PA, tissue plasminogen activator; Fn, fibronectin; pFn, plasma fibronectin; cFn, cellular fibronectin; kDa, kilodalton; ECM, extracellular matrix; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; BSA, bovine serum albumin; IgG, immunoglobulin gamma; TFA, trifluoracetic acid; HPLC, high pressure liquid chromatography.

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Address reprint requests to Leslie I. Gold, Department of Pathology, New York University Medical School, 400 East 34th Street, New York, NY 10016.

Agueda Rostagno is a fellow of The S.L.E. Foundation, New York, New York.

hibited metastasis of a human tumor cell line [Ossowski and Reich, 1983].

Our previous data has shown that, in addition to the degradation of ECM components by plasmin, requiring u-PA for its activation, plasminogen activators (t-PA and u-PA) can also directly and specifically enzymatically cleave Fn [Quigley et al., 1987; Gold et al., 1989]. Dose/response and kinetic studies employing Fn and u-PA, purified to homogeneity, indicated a specific but limited proteolytic cleavage of Fn by u-PA. These studies also showed that Fn degradation by u-PA ensued in the presence of specific inhibitors of plasmin and was inhibited by antibodies specific for u-PA. Therefore it was proposed that u-PA may be involved independently in the degradation and release of Fn from the ECM. The local degradation of Fn by u-PA at substrate adhesion sites could contribute to the destruction of tissue matrices thereby leading to subsequent invasion and metastasis. With respect to normal physiology, McGuire and Seeds [1990] have shown that there is a plasminogen-independent degradation of Fn at cell contact sites, due to u-PA and t-PA, concomitant with the process of neurite outgrowth.

Since plasminogen, the substrate for plasminogen activators, is cleaved and activated to plasmin at a peptide bond between arginine and valine, we were curious as to the cleavage sites on fibronectin attacked by the plasminogen activator, u-PA. We also proposed that the cleavage of Fn by u-PA may be an initial event that would conformationally alter the molecule so that it would be more vulnerable to proteolysis by additional enzymes [Gold et al., 1989] such as cathepsins and plasmin, also elevated in tumor cells [Dano et al., 1985; Duffy et al., 1991; Rochefort et al., 1990]. The following studies demonstrate the specificity of proteolytic attack by u-PA on Fn by defining the cleavage sites on the Fn molecule and also show that the previous cleavage of Fn by u-PA enhances further proteolytic attack by the additional endogenous enzymes, cathepsin D and plasmin.

METHODS

Materials

Plasma fibronectin (Fn) was purchased from the New York Blood Center and further purified by lysine-affinity and gelatin-affinity chromatography, respectively, as described [Gold et al., 1989]. Partially purified cellular fibronectin isolated from human foreskin fibroblasts was purchased from Accurate Chemical and Scientific Corp. and 500 μg was further purified using 2.0 ml gelatin-Sepharose as described for plasma Fn. Partially purified human urine-derived urokinase (u-PA), a product of American Diagnostica, Inc. (New York, NY) (#128), was further purified by benzamidine-Sepharose affinity chromatography as previously described [Gold et al., 1989]. Both the high molecular weight and low molecular weight forms of u-PA were employed; different lots of u-PA contained variable amounts of both these forms. The Fn and the u-PA were plasminogen free as determined by dot blot analysis [Gold et al., 1989]. Human plasminogen (P5661) and cathepsin D (C3138) were obtained from Sigma Chemical Co (St. Louis, MO). Affinity purified monoclonal antibody to an epitope at the amino-terminal (N-288) and carboxyl-terminal (N-296) regions of Fn were purchased from Mallinckrodt, Inc. (St. Louis, MO).

Proteolytic Reactions

Purified urokinase was incubated separately with human cellular and plasma fibronectin at an enzyme/substrate ratio of 1:20 as previously described [Gold et al., 1989]. This enzyme concentration was chosen because it was previously shown to generate complete cleavage fragments of Fn that were needed for the analytical purposes of this study (i.e., amino acid sequence determination).

Proteolysis of Fn Following Predigestion With u-PA

Plasmin. Plasmin was generated from plasminogen by incubation with urokinase at an enzyme:substrate ratio of 1:5 for one h at 37°C in 0.1 M Tris, pH 8.1, and was added to fibronectin that had been previously digested with urokinase at an enzyme/substrate ratio of 1:4. Aliquots were removed at various time intervals.

Cathepsin D. Plasma Fn, predigested with u-PA, was dialyzed for 2 h against 0.1 M Na acetate, pH 3.5, and incubated with cathepsin D at an enzyme/substrate ratio of 1:200 for four h.

Plasma Fn that was not predigested with u-PA was simultaneously prepared and incubated with either plasmin or cathepsin D and served as a control. Each reaction was terminated by the addition of 5 times concentrated Laemmli gel electrophoresis buffer followed by rapid freezing in methanol and dry ice. All samples were analyzed by SDS-PAGE according to the method of Laemmli.

Western Blot Analysis

Fibronectin fragments, generated by u-PA digestion, were localized within the intact molecule by the use of monoclonal antibodies to different defined epitopes of Fn. The proteins were transferred to a nitrocellulose membrane (BioRad, Richmond, CA) for one h at 300 milliamps in the presence of 10 mM cyclohexylaminopropane sulfonic acid (Sigma) buffer, pH 11.0, containing 10% methanol using a Mini Transblot cell (BioRad). The membrane was blocked with 3% BSA (Sigma), 0.5% Tween 20, in 0.02 M Tris buffered saline (TBS), and incubated for one h with the anti-Fn monoclonal antibody N-288 (to N-terminal; 1:1,000) or N-296 (to C-terminal; 1:1500), followed by alkaline phosphatase labeled anti-mouse IgM (1:1,000; Zymed, San Francisco, CA) or anti-mouse IgG (1:3,000; Promega, Madison, WI), respectively. All antisera were diluted in TBS containing 3% BSA and 0.1% Tween 20. The reaction was developed using the ProtoBlot System (Promega).

Electroblot/Amino Acid Sequence Analysis

Following SDS-PAGE, Fn fragments (approximately 100 pmoles/fragment) obtained by u-PA digestion were transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon, Millipore Corp., Bedford, MA) using the same conditions as described for Western Blot Analysis. The protein bands were visualized by staining with 0.1% Fast Green FCF (BioRad) in 25% methanol and 10% acetic acid for 30 sec, followed by destaining in the same solution without stain for 3 min and distilled deionized water for 5 min or until the stain was faint. The bands that migrated at M_r 210,000 and 200,000 were excised from the membrane, cut into 2–4 mm strips, and subjected to amino acid sequence analysis using a 477A Protein Sequencer (Applied Biosystems, Inc., Culver City, CA).

Isolation of a 3 kDa C-Terminal Fragment of the Fibronectin Monomer

Plasma fibronectin (21 mg) was digested with 1.050 mg of u-PA as described [Gold et al., 1989]and chromatographed through 2.0 ml of benzamidine-Sepharose (Pharmacia, Piscataway, NJ) equilibrated in 0.1 M Tris, pH 8.0, to remove the u-PA. The effluent was partially reduced with a final concentration of 20 mM dithiothreitol (Calbiochem., San Diego, CA) and radioactively alkylated with iodoacetic acid (Sigma) at a final concentration of 45 mM containing 100 μCi of ^{14}C iodoacetic acid (0.7 mCi/mmol, New England Nuclear, Boston, MA): both for one h at room temperature. Following reduction and alkylation, the digested fibronectin was dialyzed against 0.1 M Tris, pH 7.4, concentrated by partial lyophilization to 5.0 ml, and subjected to gel filtration through a 1.6 × 200 cm column containing Sephadex G-50 that had been previously equilibrated with 0.5 M acetic acid and standardized with known molecular weight standards. An aliquot of each fraction was counted in a scintillation counter (Smith-Kline Beckman Co., Fullerton, CA, LS-250) and the peak fractions pooled at the region of elution of the expected M_r 3,000 fragment. The sample (10,000 cpm) was lyophilized, diluted in 1.0 ml of 0.1% trifluoroacetic acid (TFA, Pierce, Rockford, IL), and subjected to reverse phase HPLC using a C18 analytical column (Waters Assoc., Milford, MA). Following a gradient elution with acetonitrile (0–60%; 1.3%/min), each peak was characterized by amino acid analysis (Pico Tag, Waters Assoc.) and 500 pmoles of the prospective fragment of M_r 3,000 was subjected to amino acid sequence analysis (Model 477A, Applied Biosystems, Inc.).

RESULTS

Comparison of Urokinase Digestion of Human Cellular and Plasma Fibronectin

Plasma Fn, derived from the circulation, is incorporated into the ECM [Oh et al., 1981; McDonald et al., 1987]. However, it is the cellular form of Fn that is most proximal to the cell surface and therefore would be most vulnerable to cleavage by u-PA bound to its receptor. Furthermore, because the cellular isoform of Fn contains extra domains due to alternative mRNA splicing, we examined the possibility that a different cleavage pattern than had previously been shown with plasma Fn might occur following u-PA digestion. The digestion of both human cellular (cFn) and plasma fibronectin (pFn) with human u-PA produced identical polypeptide cleavage fragments of M_r 210,000, 200,000, and 25,000 as illustrated by SDS-PAGE in Figure 1 (compare lanes 2 and 3). Different preparations of Fn generally show various proportions of the multimeric and monomeric forms of Fn. This particular preparation of pFn (lane 4) possessed more of the multimeric form of the molecule, observed at the top of the stacking gel, and thus is the most likely reason that there is more of

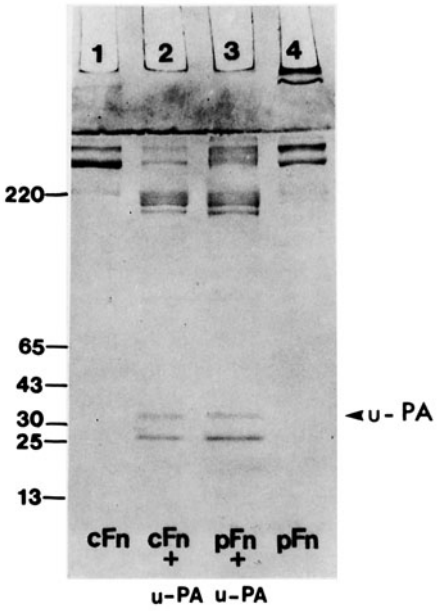

Fig. 1. Analysis of u-PA digestion of plasma and cellular *fibronectin* by SDS-PAGE. *Cellular fibronectin* (cFn; 5.0 μg) and plasma fibronectin (pFn; 5.0 μg) were incubated with u-PA (0.25 μg) for 17 h and the reaction terminated as described in Methods. The samples were analyzed by SDS-PAGE (5–20% acrylamide monomer gradient). **Lanes 1 and 4:** Undigested cFn and pFn; **lanes 2 and 3:** cFn and pFn digested with u-PA. The low molecular weight form of u-PA is shown migrating slightly higher than M_r 30,000. Molecular weight standards are labeled at the left.

the dimeric (M_r 440,000) structure observed for the plasma form after digestion (lane 3). Presumably, if there is more of the multimeric form, it may take longer for the enzyme to access this form for cleavage to occur. A relatively small amount of the monomeric form of fibronectin was observed for both the plasma and cellular forms of Fn prior to digestion.

Localization of Urokinase-Generated Proteolytic Cleavage Fragments in the Fibronectin Molecule by Western Blot Analysis

The fragment of M_r 25,000 and the higher molecular weight fragments generated by digestion of fibronectin with u-PA were compared to the cleavage products obtained using plasmin. Figure 2A illustrates an SDS-PAGE of u-PA (lane 2) and plasmin (lane 3) digestion of Fn. The Western blot in Figure 2B demonstrates that the M_r 25,000 fragment generated by digestion of Fn with u-PA (lane 2) and plasmin (lane 3) can be localized to the amino terminal of the Fn molecule since it reacts with the anti-N-terminal antiserum (N-288). Lane 2 indicates that only a relatively small amount of intact M_r 220,000 monomer remains following digestion with u-PA compared to undigested Fn in lane 1. Evidently the dimeric Fn that remains following cleavage by u-PA has already been cleaved at the N-terminal since the dimer did not react with the anti-N-terminal antiserum (Fig. 2B, lane 2). The M_r 210,000 and M_r 200,000 fragments react only with the anti-C-terminal antiserum (N-296), as shown in Figure 2C. The anti-C-terminal antisera also reacted with a 35 kDa fragment (Fig. 2C, lane 2) that was not resolved in Figure 2A by protein staining; this is a minor cleavage product resulting from the u-PA digestion that can be visualized only because of the sensitivity of the antiserum. This fragment is not sensitive to reduction and thus does not contain the interchain disulfide bonds located at the C-terminal of the Fn molecule (data not shown). Plasmin digestion of Fn produces many more fragments observed in lane 3 of Figure 2A and numerous minor cleavage products that contain the C-terminal epitope (Fig. 2C, lane 3); the cleavage that releases the M_r 25,000 fragment is complete within 17 h, since none of these fragments, except the M_r 25,000 polypeptide, reacted with the anti-N-terminal antiserum (Fig. 2B, lane 3).

Determination of the Urokinase Cleavage Site at the N-Terminal of the Fn Molecule by Amino Acid Sequence Analysis

The substrate for u-PA is plasminogen; activation to plasmin is achieved by cleavage at an arginine/valine peptide bond [Robbins et al., 1967]. Because of the molecular mass of the M_r 25,000 fragment and the arginine specific proteolytic activity of u-PA, we proposed that the N-terminal cleavage site for u-PA in the Fn molecule was between an arginine/alanine peptide bond at residues 259 and 260 [Gold et al., 1989]; this is also the site that is cleaved by plasmin. Following digestion of 25 μg of Fn (113 pmoles) with u-PA, electroblotting of the resulting fragments onto PVDF membranes, and subsequent amino acid sequence analysis of the excised M_r 210,000 and 200,000 bands, we obtained the N-terminal sequence A A V Y Q P Q P H P for both polypeptides. Thus, u-PA cleaved Fn C-terminal to residue 259, thereby releasing the N-terminal M_r 25,000 fragment from both the A and B chains of the molecule. Figure 3 presents a diagram depicting this cleavage site in the Fn molecule. The unequal molecular mass of the two chains of Fn is maintained following this cleav-

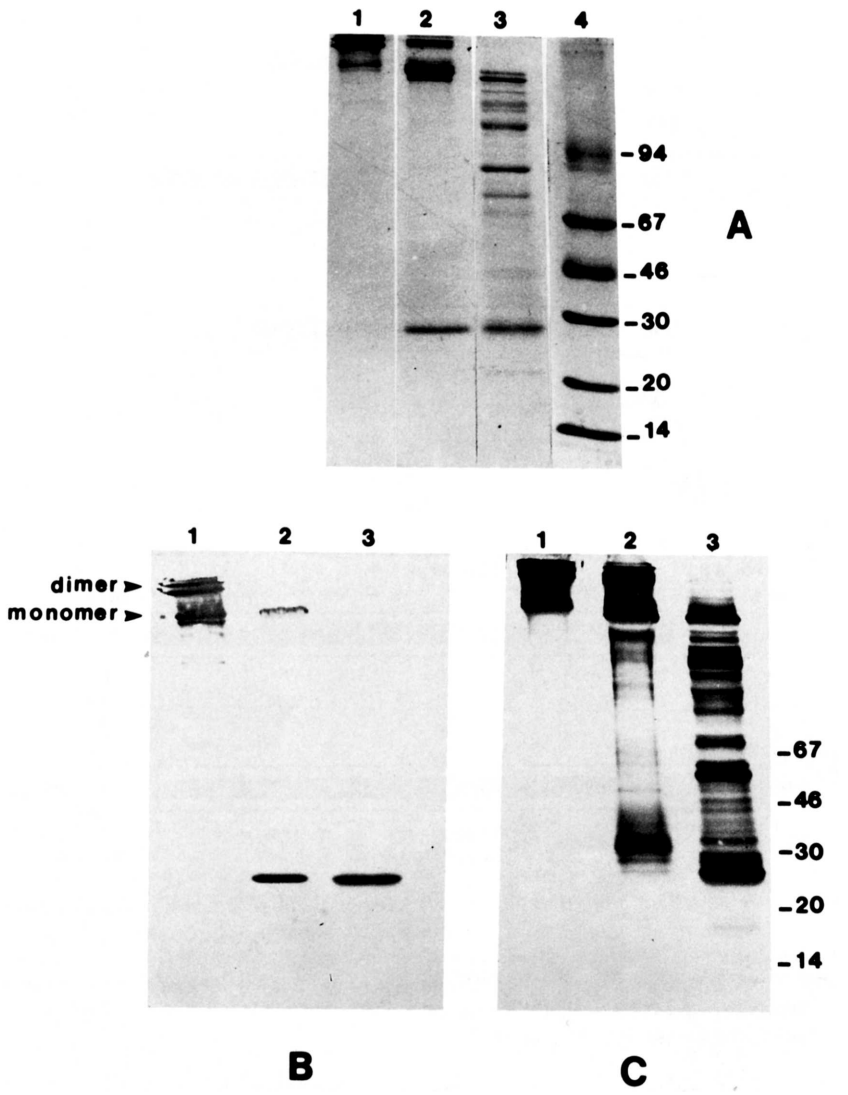

Fig. 2. Localization of urokinase generated cleavage fragments of fibronectin by Western Blot Analysis. Plasma fibronectin (7.5 μg; **lane 1,A–C**) was incubated with u-PA (0.375 μg; **lane 2,A–C**) and with plasmin (1.875 μg; lane **3,A–C**) for 17 h as described in Methods. Plasmin was generated by the addition of u-PA (0.375 μg) to plasminogen (1.875 μg; enzyme:substrate ratio of 1:5), just prior to addition to the Fn. The samples were diluted in sample buffer, subjected to SDS-PAGE (5–20% acrylamide monomer gradient), and transferred to nitrocellulose as described in Methods. Monoclonal antibodies to epitopes at the N-terminal (N-288) and C-terminal (N-296) of Fn were used to localize the fragments in the Fn molecule. The lanes for **Panels A–C** contain the following samples: intact Fn (lane 1), Fn digested with u-PA (lane 2), and Fn digested with plasmin (lane 3). Panel A: SDS-PAGE, molecular weight standards are shown in lane 4. Panel B: Western Blot using the anti-Fn N-terminal antisera (N-288). Panel C: Western blot using the anti-Fn C-terminal antisera (N-296).

age as reflected by the molecular weight heterogeneity of M_r 210,000 and M_r 200,000 fragments.

Purification and Amino Acid Sequence Determination of a 3 kDa Fragment Derived From the C-Terminal of the Fibronectin Molecule

Since the large polypeptides of M_r 210,000 and M_r 200,000 produced by u-PA cleavage of Fn are similar to those observed following chemical reduction of the two interchain disulfide bonds at the C-terminal cystines located at positions 2,306 and 2,310, we also proposed that a second cleavage site may be immediately N-terminal to these disulfide bonds; this would liberate a fragment of M_r 6,000 composed of two M_r 3,000 polypeptide fragments covalently linked by the C-terminal interchain disulfide bonds. Fibronectin (21 mg) was digested by u-PA and

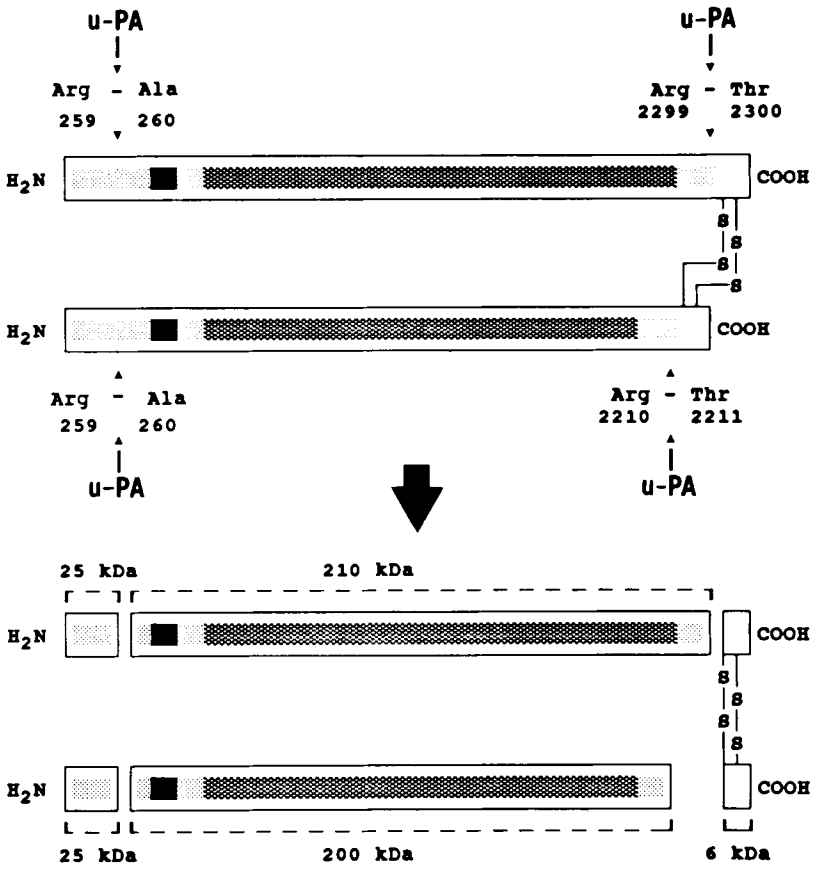

Fig. 3. Diagram of dimeric fibronectin indicating the cleavage sites for urokinase (u-PA). The cleavage sites for u-PA within the Fn molecule were determined following purification of the u-PA generated proteolytic fragments of fibronectin by electroblotting (N-terminal site) and HPLC chromatography (C-terminal site). The purified fragments were subjected to microsequence analysis. The cleavage sites for u-PA in the intact Fn molecule are illustrated by arrowheads in the upper diagram. The fragments obtained by u-PA cleavage are illustrated in the lower diagram. Type I structure □; Type II structure ■; Type III structure ▦.

the enzyme removed by affinity chromatography through benzamidine-Sepharose (data not shown). The expected yield of a fragment of M_r 3,000 from 21 mg of dimeric fibronectin (M_r 440,000) is approximately 286 μg (95 nmoles). Therefore, the fibronectin digested by u-PA was reduced and radioactively alkylated to follow the labeled polypeptide during chromatography. The digested fibronectin was subjected to gel filtration using Sephadex G-50 that was precalibrated with standard proteins, and each fraction was counted in a scintillation counter. The profile of eluted proteins is presented in Figure 4A. A pool of the fractions containing the expected M_r 3,000 fragment was made, corresponding to the approximate elution of unreduced insulin (M_r 5,600), and the sample lyophilized for high pressure liquid chromatography (HPLC). Figure 4B illustrates the elution profile of the radioactively labeled protein. Amino acid analysis of the multiple peaks, eluting from 28–32% acetonitrile, revealed that each peak contained nearly the identical ratio of amino acids. Therefore, the multiple peaks obtained presumably represented various alkylated forms of a similar if not identical protein. The peak fraction, which eluted at 30 min with 30% acetonitrile, was subjected to amino acid sequence analysis and the N-terminal amino acid sequence T N T N V N C P I E C F M P L D V Q A D R E D S R E was obtained; this is the complete sequence to the C-terminal end of the molecule [Garcia-Pardo et al., 1984]. Therefore, the second cleavage site is between the arginine/threonine peptide bond located between residues 2,299 and 2,300 (Fig. 3).

Proteolytic Digestion of Fn by u-PA Enhances Further Enzymatic Degradation

To test the possibility that pre-exposure of Fn to u-PA might render the molecule more vulner-

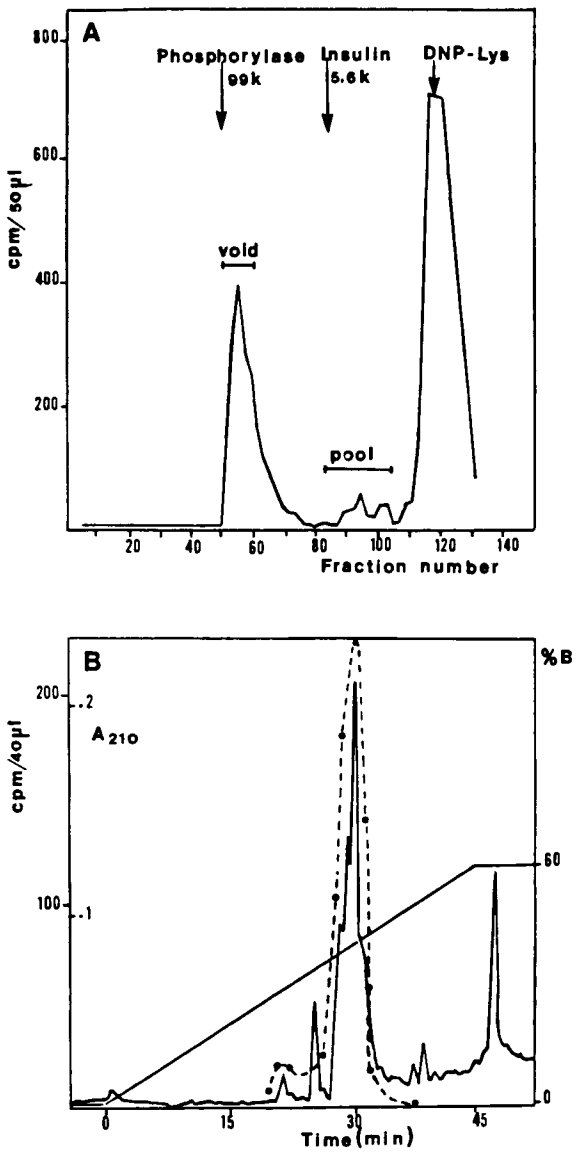

Fig. 4. Purification of a 3 kDa fragment derived from the C-terminal of the fibronectin molecule. Plasma fibronectin (21 mg) was digested with u-PA (1.050 mg) as described in Methods. The protein was reduced with dithiothreitol (20 mM), radioactively alkylated with ^{14}C-iodoacetic acid, and subjected to gel filtration through Sephadex G-50 (A) followed by HPLC (B). A (Sephadex G-50): The column was pre-calibrated with standard proteins as shown by arrows (peak of elution). The fraction volume was 1.4 ml. The profile indicates cpm/50 µl of sample from each fraction. The pooled fraction was chromatographed by reverse phase HPLC. B (HPLC): The sample was applied to an analytical C18 column in 0.1% TFA and eluted with a gradient of 0–60% acetonitrile (%B) at 1.3%/min (dead volume = 7 min). The dotted line indicates the elution profile for cpm and the solid line indicates protein monitored at 210 nm. The peak fraction eluting at 30 min (30% acetonitrile) was subjected to microsequencing (500 pmoles).

able to further enzymatic attack by other endogenous enzymes, a sample of Fn was predigested with u-PA and subsequently treated separately with the endogenous enzymes, plasmin or cathepsin D, for the times indicated in Figure 5. Figure 5A demonstrates that within ten min there was more extensive digestion of fibronectin by plasmin if the sample had been pretreated with u-PA (Fig. 5B) for 17 h, compared to an identical sample of Fn that had been preincubated in buffer alone (Fig. 5A). The high molecular weight form of u-PA is seen in lane B migrating at M_r 52,000 and plasminogen is migrating at approximately M_r 90,000 (arrows). An aliquot (0 time) was taken prior to the addition of plasmin. Whereas cathepsin D digested Fn that had not been preincubated with u-PA (Fig. 5B, lane 3), more extensive digestion was observed in the sample that had been pretreated with u-PA (lane 4). This is based on the observation that a greater number of lower molecular weight fragments were observed in lane 4 (previously digested with u-PA) than in lane 3 (without u-PA digestion).

DISCUSSION

An increase in cellular plasminogen activator proteolytic activity is a marker for malignant transformation both in vitro and in a multitude of human cancers [Mullins and Rohrlich, 1983; Varani et al., 1987; Markus et al., 1983; Kohga et al., 1985], including brain tumors [Sawaya and Highsmith, 1988], lung and breast carcinomas [Sappino et al., 1987], and squamous cell carcinoma [Niedbala and Sartorelli, 1989]. More importantly, PA activity has been specifically associated with the more aggressive phenotypes demonstrating greater metastatic potential [Mullins and Rohrlich, 1983; Gaylis et al., 1989], and in many cases has been correlated with increased extracellular matrix degradation [Dano et al., 1985; Mignatti et al., 1986; Salo et al., 1982; Tryggvason et al., 1987; Jones and De Clerck, 1984]. Both anti-u-PA and anti-t-PA antibodies, and protease inhibitors, have been shown to inhibit metastasis [Hearing et al., 1988; Ossowski and Reich, 1983; Mignatti et al., 1986; Reich et al., 1988] and the release of extracellular matrix components [Varani et al., 1987; Niedbala and Sartorelli, 1989], including Fn [Chen et al., 1984; Fairbairn et al., 1985; Sullivan et al., 1986]. Furthermore, it was shown that Ha-ras NIH3T3 cells transfected with u-PA, that expressed the recombinant protein, transgressed

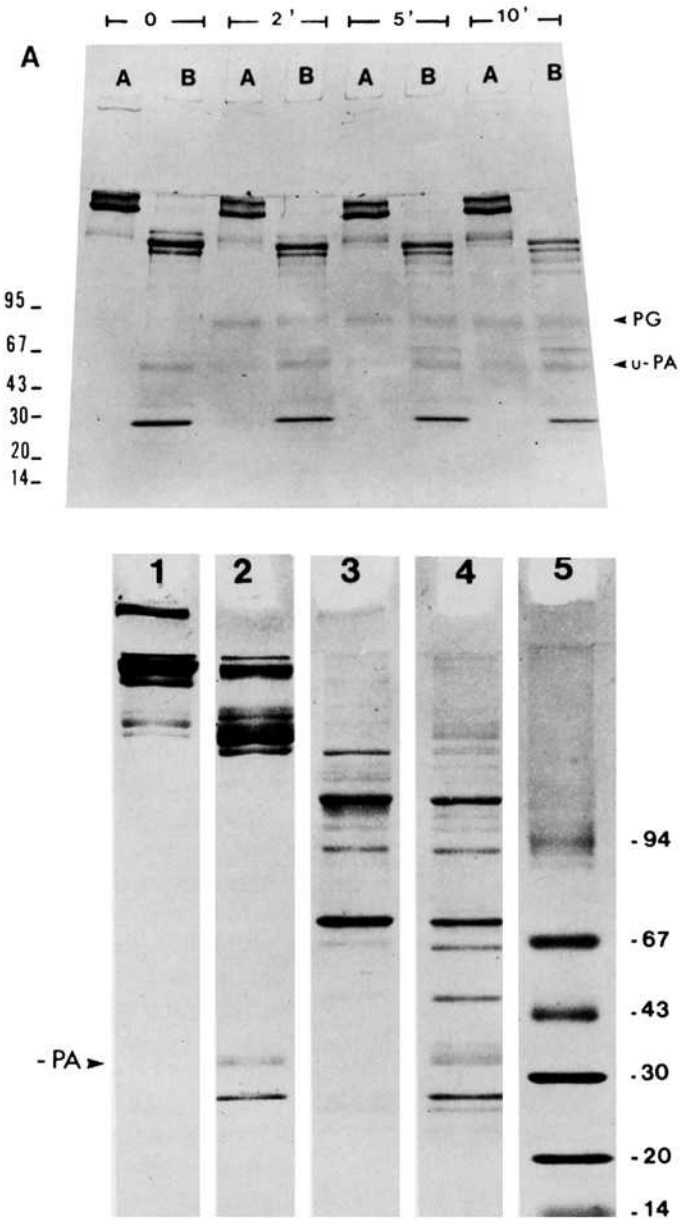

Fig. 5. Proteolytic digestion of fibronectin by urokinase enhances further enzymatic degradation by plasmin and cathepsin D. **A.** Plasmin digestion: Plasma fibronectin (Fn) (7.5 μg) was digested with u-PA (0.375 μg) for 17 h. Subsequently, plasmin was added to the reaction mixture (enzyme:substrate [plasmin:Fn] ratio = 1:4), and the proteolytic reaction terminated with ice-cold sample buffer at the time intervals indicated above the gel, as described in Methods. **Lanes A:** Fn digested with plasmin for the times indicated; **lanes B:** Fn predigested with u-PA and subsequently incubated with plasmin for the times indicated. Molecular weight standards are on the left and plasminogen (PG) and urokinase (u-PA) are marked by arrowheads on the right. Time 0 samples did not receive plasmin. **B.** Cathepsin D digestion: Plasma fibronectin (Fn) (8.5 μg) was predigested with u-PA (0.425 μg) for 17 h. Samples that were to be treated with cathepsin D were dialyzed against 0.1 M Na acetate, pH 3.5, and subsequently digested with cathepsin D for 4 h (0.0425 μg; enzyme:substrate ratio 1:200), as described in Methods. **Lane 1:** Fibronectin (untreated); **lane 2:** fibronectin digested with u-PA for 17 h; **lane 3:** fibronectin digested with cathepsin D for 4 h; **lane 4:** fibronectin pre-digested with u-PA for 17 h prior to the addition of cathepsin D for 4 h. Note: Fn in lane 1 is predominantly a dimeric structure. Molecular weight standards are in **lane 5.**

basement membranes and metastasized to the lung to a much greater extent than their parental counterpart [Axelrod et al., 1989]. Another study demonstrated that the invasive and metastatic properties of a mouse L cell line transfected with u-PA was completely inhibited by anti-u-PA, thus implicating u-PA expression as solely responsible for the invasive phenotype of these cells [Cajot et al., 1989]. These data provide compelling evidence for a causal relationship between the expression of u-PA by tumor cells and their invasive and metastatic potential.

Our studies demonstrate that Fn, a major constituent of extracellular matrices, is cleaved by u-PA at specific sites on the molecule. Although multiple isoforms of Fn exist among species and the cellular form of Fn contains extra domains, both due to alternative mRNA splicing, similar if not identical cleavage products were obtained following digestion of the plasma and cellular forms of the molecule by u-PA (Fig. 1). We have previously shown that similar cleavage fragments were obtained with the avian form of cellular Fn [Quigley et al., 1987]. The two cleavage sites recognized by u-PA are identical to those obtained by limited digestion with trypsin [Garcia-Pardo et al., 1983, 1984] and plasmin [Petersen et al., 1983]. Both are C-terminal to an arginine residue at positions 259 and 2,299 (Fig. 3); these two cleavages yield peptides of M_r 25,000, M_r 210,000, M_r 200,000, and M_r 6,000. The generation of the M_r 210,000 and M_r 200,000 polypeptides is consistent with the size heterogeneity of the A and B chains of Fn. The region of the Fn molecule containing arginine 259 is a region of extended polypeptide sequence, that confers susceptibility to many proteases [Hynes, 1990; Gold et al., 1979].

In addition to increased levels of plasminogen activators and plasminogen, tumor cells elaborate high levels of thiol proteases, such as cathepsins [Sloane et al., 1986; Briozzo et al., 1988] and collagenases [Kuettner et al., 1977; Liotta et al., 1980], that similarly degrade constituents of extracellular matrices. Cathepsin B and D are particularly elevated in carcinoma of the breast [Duffy et al., 1991; Rochefort et al., 1990; Mort et al., 1980; Spyratos et al., 1989]. Furthermore, u-PA and cathepsin D are elevated in malignant breast tumors compared to those that are benign [Foucre et al., 1991]. The concomitant increase in expression of u-PA and cathepsin D in breast carcinomas has been found to be associated with shorter survival time [Duffy et al., 1990; Duffy et al., 1991; Spyratos et al., 1989]. Our studies provide biochemical evidence for the potential for further disruption of matrices by tumor cells possessing elevated levels of both enzymes since we have shown that Fn that has been previously cleaved by u-PA is more vulnerable to further proteolytic attack by the endogenous tumor associated enzymes, plasmin and cathepsin D (Fig. 5A,B); the synergistic effect of these proteases on the degradation of the Fn within the ECM is significant in terms of optimally arming tumor cells for metastatic potential. Additionally, an increase in PAI-1 and PAI-2 was observed in association with the malignant breast phenotype [Foucre et al., 1991]. It is interesting to note that a PAI of M_r 54,000 was also shown to be a substrate for both t-PA and u-PA. The significance of the increase in PAIs in breast carcinoma is unknown; however, the cleavage of PAI by u-PA and t-PA inactivates the molecule [Nielsen et al., 1986]. Thus, in total, aside from plasminogen, the major substrate for plasminogen activators, both PAI and, as we have shown, Fn [Quigley et al., 1987; Gold et al., 1989], are also substrates for these enzymes.

The movement of tumor cells through the extracellular matrix requires release and reformation of cell substratum adhesion sites. The local degradation and release of Fn occurs at these cell substratum contact sites [Chen et al., 1984]. Cell surface bound u-PA has also been localized to cell substratum contact sites; these are regions of closest cell/substratum apposition and are identified as focal contacts [Pollanen et al., 1987; Hebert and Baker, 1988]. In contrast, it has been shown that Fn is confined to more ventral, labile areas referred to as close contacts and the fibronexus [Pollanen et al., 1988; Blasi, 1988]. Whereas it appears that Fn did not co-distribute with u-PA by immunohistolocalization, Fn was present in focal contact membrane preparations [Pollanen et al., 1987]. In one study, the presence of Fn receptors in the focal contacts of cells was dependent on the length of time the cells were in culture and, although Fn receptors were initially localized to the focal contacts, as Fn containing matrix fibers developed, the receptors moved from these contacts along the fibers [Singer et al., 1988]. In this context, the displacement of Fn may be important to the cellular migration process, in general, and the degradation of Fn by u-PA may

be directly related to its dislocation and thus a contributing factor in both physiological and pathological (metastasis) cellular movement.

It has also been shown, by McGuire and Seeds [1990], that the process of neurite outgrowth may be accompanied by extracellular matrix degradation. A plasminogen-independent proteolytic release of Fn from substrate contact sites under neuronal soma and processes was observed. Using neuronal detergent soluble extracts, the authors directly showed that both u-PA and t-PA, associated with both the cell membrane and substratum constituents, proteolytically cleaved radiolabeled Fn incorporated into a polyacrylamide gel, as bands migrating at their respective molecular weights. Thus, these studies clearly indicated that there is a local degradation of Fn by u-PA and t-PA bound to the cell surface. It is interesting to note that the size of the Fn fragments released by the neuronal cells in an intact cell assay were similar to the high molecular weight cleavage products that we have obtained using purified Fn and u-PA in our fluid phase assay.

A receptor for urokinase has been identified on a variety of cell types [Vassalli et al., 1985; Stopelli et al., 1985; Saksela and Rifkin, 1988], including tumor cells [Takahashi et al., 1990], and most of these cells engage in an autocrine mechanism of activity [Stopelli et al., 1986]. Ossowski [1988] has shown that there is a reduction of 25% in the efficiency of invasion if u-PA is not bound to its receptor and that saturation of u-PA receptors on Hela cells resulted in a great enhancement of their invasive activity. To further demonstrate the importance of the occupation of the u-PA receptor in invasive activity, it was recently shown that u-PA can also act in a paracrine manner [Ossowski et al., 1991]. Cells that did not produce u-PA but were transfected with the u-PA receptor were efficient in invasion if they were previously co-cultured with u-PA transfected cells that were lacking the receptor but *secreting* u-PA; neither transfectant alone was efficient in invasion. These results support earlier studies by Quigley [1979], who found that plasma membrane bound u-PA was responsible for the transformation associated morphological effects and decreased adhesion observed in phorbol ester treated chicken embryo fibroblasts. Thus, one can consider that the immobilization of u-PA and plasminogen on the surface of proteolytically active cells represents a mechanism for locally concentrating the enzyme at contact adhesion sites where in situ degradation of Fn occurs. Although we have previously shown that u-PA can partially cleave Fn at an enzyme/substrate ratio of 1:100, a concentration in the range for efficient plasminogen cleavage and for plasmin catalytic activity [Gold et al., 1989], the enzyme/substrate ratio of 1:20 used in the current studies has been found to be more efficient for complete cleavage of Fn into the M_r 210,000, M_r 200,000, M_r 25,000, and M_r 6,000 fragments. However, this enzyme/substrate ratio may be in the realm of a physiological concentration, if one considers that Fn digestion by u-PA is a local event concentrated at the cell surface and occurring over an extended period of time. Furthermore, since it has been shown that there is an increase in both t-PA and u-PA proteolytic activity in tumor cells, for example, as much as 100-fold following viral transformation of chicken embryo fibroblasts and 1,000-fold following the addition of a tumor promoter [Goldfarb and Quigley, 1978; Fairbairn et al., 1985], very high concentrations at tumor cell surfaces may occur. Alternatively, optimal catalytic activity of u-PA requires receptor binding; and furthermore, ECM degradation, including Fn release, apparently is a cell surface event that requires cell contact with substratum. These two facts may explain the necessity of a low enzyme/substrate ratio to obtain complete cleavage of Fn in our studies that were performed in a fluid phase system, under apparently suboptimal conditions for u-PA proteolytic activity.

The requirement for the plasminogen activator, u-PA, in the degradation of extracellular matrices lies not only in the activation of plasminogen to plasmin, an arginine esterase of broad specificity with known catalytic activity for Fn, laminin [Salonen et al., 1984], collagenase [Paranjpe et al., 1980], and other basement membrane and matrix components, but also, as we [Quigley et al., 1987; Gold et al., 1989] and others [McGuire and Seeds, 1990] have shown, in its plasminogen-independent direct cleavage of the Fn molecule. In the present studies, we have defined the amino acid recognition/cleavage sites on Fn for u-PA and have thus provided further biochemical evidence for a direct role for u-PA in the proteolytic breakdown of Fn. This enzyme/substrate interaction may be involved in both the detachment of tumor cells possessing high levels of plasminogen activators, from connective tissue matrices and in their subsequent migration through tissues, thereby con-

tributing to both their invasive and metastatic properties. The direct proteolytic activity of u-PA on Fn may also be involved in other processes requiring cell surface proteolysis of extracellular matrices such as normal cellular migration, tissue remodeling, embryogenesis, differentiation, and inflammation [Mangel, 1990]. These systemic events appear to be regulated by the presence of receptors for u-PA and, as recently shown, by the growth factor TGF-B, which controls levels of plasminogen activator inhibitor (PAI-1) and plasminogen activator mRNA synthesis and protein secretion [Keski-Oja et al., 1988].

REFERENCES

Axelrod JH, Reich R, Miskin R (1989): Expression of human recombinant plasminogen activators enhances invasion and experimental metastasis of H-ras-transformed NIH 3T3 cells. Mol Cell Biol 9:2133–2141.

Blasi F (1988): A surface receptor for urokinase activator: A link between the cytoskeleton and the extracellular matrix. Protoplasma 145:95–98.

Briozzo P, Morisset M, Capony F, Rougeot C, Rochefort H (1988): In vitro degradation of extracellular matrix with M_r 52,000 cathepsin D secreted by breast cancer cells. Cancer Res 48:3688–3692.

Cajot JF, Schleuning WD, Medcalf RL, Bamat J, Testuz J, Liebermann L, Sordat B (1989): Mouse L cells expressing human prourokinase-type plasminogen activator: Effects on extracellular matrix degradation and invasion. J Cell Biol 109:915–925.

Chen WT, Olden K, Bernard BA, Chu FF (1984): Expression of transformation-associated protease(s) that degrade fibronectin at cell contact sites. J Cell Biol 98:1546–1555.

Dano K, Andreasen PA, Grondahl-Hansen J, Kristensen P, Nielsen LS (1985): Plasminogen activators, tissue degradation, and cancer. Adv Cancer Res 44:139–266.

Dano K, Reich E (1978): Serine enzymes released by cultured neoplastic cells. J Exp Med 147:745–755.

Duffy MJ, Brouillet JP, Reilly D, McDermott E, O'Higgins N, Fennelly JJ, Maudelonde T, Rochefort H (1991): Cathepsin D concentration in breast cancer cytolysis: Correlation with biochemical, histological, and clinical findings. Clin Chem 37:101–104.

Duffy MJ, Brouillet JP, Reilly D (1990): Urokinase plasminogen activator and prognosis in breast cancer. Lancet 335: 108.

Fairbairn S, Gilbert R, Ojakian G, Schwimmwer R, Quigley JP (1985): The extracellular matrix of normal chick embryo fibroblasts: Its effect on transformed chick fibroblasts and its proteolytic degradation by the transformants. J Cell Biol 101:1790–1798.

Foucre D, Bouchet C, Hacene K, Pourreau-Schneider N, Gentile A, Martin PM, Desplaces A, Oglobine J (1991): Relationship between cathepsin D, urokinase, and plasminogen activator inhibitors in malignant vs benign breast tumours. Brit J Cancer 64:926–932.

Garcia-Pardo A, Pearlstein E, Frangione B (1984): Primary structure of human plasma fibronectin-characterization of the 6,000 dalton C-terminal fragment containing the interchain disulfide bridges. Biochem Biophys Res Commun 120:1015–1021.

Garcia-Pardo A, Pearlstein E, Frangione B (1983): Primary structure of human plasma fibronectin. J Biol Chem 258: 12670–12674.

Gaylis FD, Keer HN, Wilson MJ, Kwaan HC, Sinha AA, Kozlowski JM (1989): Plasminogen activators in human prostate cancer cell lines and tumors: Correlation with the aggressive phenotype. J Urol 142:193–198.

Gold LG, Schwimmer R, Quigley JP (1989): Human plasma fibronectin as a substrate for human urokinase. Biochem J 262:529–534.

Gold LI, Garcia-Pardo A, Frangione B, Franklin EC (1979): Subtilisin and cyanogen bromide cleavage products of fibronectin that retain gelatin-binding activity. Proc Natl Acad Sci USA 76:4803–4807.

Goldfarb RH, Quigley JP: (1978) Synergistic effect of tumor virus transformation and tumor promoter treatment on the production of plasminogen activator by chick embryo fibroblasts. Cancer Res 38:4601–4607.

Hearing VJ, Law LW, Corti A, Apella E, and Blasi F (1988): Modulation of metastatic potential by cell surface urokinase of murine melanoma cells. Cancer Res 48:1270–1278.

Hebert CA, Baker JB (1988): Linkage of extracellular plasminogen activator to the fibroblast cytoskeleton: Colocalization of cell surface urokinase with vinculin. J Cell Biol 106:1241–1247.

Hynes RO (1990): Structure of fibronectins. In Rich A (ed): "Fibronectins." New York: Springer-Verlag, pp 113–175.

Jones PA, De Clerck Y (1984): Extracellular matrix destruction by invasive tumor cells. Cancer Metastases Res 3:177–191.

Keski-Oja J, Blasi F, Leof EB, Moses HL (1988): Regulation of the synthesis and activity of urokinase plasminogen activator in A549 human lung carcinoma cells by transforming growth factor-B. J Cell Biol 106:451–459.

Kohga S, Shashikumar R, Weaver RM, and Markus G (1985): Localization of plasminogen activators in human colon cancer by immunoperoxidase staining. Cancer Res 45: 1787–1796.

Kuettner KE, Soble L, Croxen RL, Marczynska B, Hitti J, Harper E (1977): Tumor cell collagenase and its inhibition by a cartilage derived protease inhibitor. Science 196:653–654.

Liotta LA, Tryggvason K, Garbisa S, Hart I, Folts CM, Shafie S (1980): Metastatic potential correlates with enzymatic degradation of basement membrane collagen. Nature (Lond) 284:67–68.

Mangel WF (1990): Better reception for urokinase. Nature (Lond) 344:488–489.

Markus G, Camiolo S, Kohga S, Madcja J, Mittelman A (1983): Plasminogen activator secretion of human tumors in short term organ culture including a comparison of primary and metastatic tumors. Cancer Res 43:5517–5525.

McDonald JA, Quade BJ, Broekelmann TJ, LaChance R, Forsman K, Hasegawa E, Akiyama S (1987): Fibronectin's cell-adhesive domain and amino-terminal matrix assembly domain participate in its assembly into fibroblast pericellular matrix. J Biol Chem 92:2957–2967.

McGuire PG, Seeds NW (1990): Degradation of underlying extracellular matrix by sensory neurons during neurite outgrowth. Neuron 4:633–642.

Mignatti P, Robbins E, Rifkin DB (1986): Tumor invasion through the human amniotic membrane: Requirement for a protease cascade. Cell 47:487–498.

Mort JS, Recklies AD, Poole RA (1980): Characterization of a thiol proteinase secreted by malignant breast tumours. Biochim Biophys Acta 614:134–143.

Mullins DE, Rohrlich S (1983): The role of proteinases in cellular invasiveness. Biochim Biophys Acta 695:177–214.

Niedbala MJ, Sartorelli AC (1989): Regulation by epidermal growth factor of human squamous cell carcinoma: Plasminogen activator-mediated proteolysis of extracellular matrix. Cancer Res 49:3302–3309.

Nielsen LS, Andreasen PA, Grendahl-Hansen J, Skriver L, and Dano K (1986): Plasminogen activators catalyse conversion of an inhibitor from fibrosarcoma cells to an inactive form with a lower apparent molecular mass. FEBS Lett 196:269–272.

Oh EG, Pierschbacher M, Ruoslahti E (1981): Deposition of plasma fibronectin in tissues. Proc Natl Acad Sci USA 78:3218–3221.

Ossowski L, Clunie G, Masucci MT, Blasi F (1991): In vivo paracrine interaction between urokinase and its receptor: Effect on tumor cell invasion. J Cell Biol 115:1107–1112.

Ossowski L (1988): In vivo invasion of modified chorioallantoic membrane by tumor cells: The role of cell-surface bound urokinase. J Cell Biol 107:2437–2445.

Ossowski L and Reich E (1983): Antibodies to plasminogen activator inhibit human tumor metastasis. Cell 35:611–619.

Paranjpe M, Engel L, Young N, Liotta LA (1980): Activation of human breast carcinoma collagenase through plasminogen activator. Life Sci 26:1223–1231.

Pearlstein E, Gold L, Garcia-Pardo A (1980): Fibronectin: A review of its structure and biological activity. Mol Cell Biochem 29:103–128.

Petersen TE, Thogersen HC, Skorstengaard K, Vibe-Pedersen K, Shal P, Sottrup-Jensen L, Magnusson S (1983): Partial primary structure of bovine plasma fibronectin: Three types of internal homology. Proc Natl Acad Sci USA 80:137–141.

Pollanen J, Hedman K, Nielsen LS, Dano K, Vaheri A (1988): Ultrastructural localization of plasma membrane-associated urokinase-type plasminogen activator at focal contacts. J Cell Biol 106:87–95.

Pollanen J, Saksela O, Salonen EM, Andreasen P, Nielsen L, Dano K, Vaheri A (1987): Distinct localizations of urokinase-type plasminogen activator and its type 1 inhibitor under cultured human fibroblast and sarcoma cells. J Cell Biol 104:1085–1096.

Quigley JP, Gold LI, Schwimmer R, Sullivan LM (1987): Limited cleavage of cellular fibronectin by plasminogen activator purified from transformed cells. Proc Natl Acad Sci USA 84:2776–2780.

Quigley JP (1979): Phorbol ester-induced morphological changes in transformed chick fibroblasts: Evidence for a direct involvement of plasminogen activator. Cell 17:131–141.

Reich R, Thompson EW, Iwamoto Y, Martin G, Deason JR, Fuller GC, *Miskin R (1988)*: Effects of inhibitors of plasminogen activator, serine proteinases, and collagenase IV on the invasion of basement membranes by metastatic cells. Cancer Res 48:3307–3312.

Robbins KC, Summaria L, Hsieh B, Shah RJ (1967): The peptide chains of human plasmin. Mechanism of activation of human plasminogen to plasmin. J Biol Chem 242:2333–2342.

Rochefort H, Capony F, Garcia M (1990): Cathepsin D: A protease involved in breast cancer metastasis. Cancer Metastasis Rev 9:321–331.

Saksela O, Rifkin DB (1988): Cell associated plasminogen activation: Regulation and physiological functions. Ann Rev Cell Biol 4:93–126.

Salo T, Liotta LA, Keski-Oja J, Turpeenniemi-Hujanen T (1982): Secretion of basement membrane collagen degrading enzyme and plasminogen activator by transformed cells: Role in metastasis. Int J Cancer 30:669–673.

Salonen E-M, Zitting A, Vaheri A (1984): Laminin interacts with plasminogen and its tissue-type activator. Fed Eur Biochem Soc Lett 172:29–32.

Sappino AP, Busso N, Belin D, Vassalli JD (1987): Increase of urokinase-type plasminogen activator gene expression in human lung and breast carcinomas. Cancer Res 47: 4043–4046.

Sas DF, McCarthy JB, Furcht LT (1986): Clearing and release of basement membrane proteins from substrates by metastatic tumor cell variants. Cancer Res 46:3802–3089.

Sawaya R, Highsmith R (1988): Plasminogen activator activity and molecular weight patterns in human brain tumors. J Neurosurg 68:73–79.

Singer II, Scott S, Kawaka DW, Kazazis DM, Gailit J, Ruoslahti E (1988): Cell surface distribution of fibronectin and vitronectin receptors depends on substrate composition and extracellular matrix accumulation. J Cell Biol 106:2171–2182.

Sloane BF, Rozhin J, Johnson K, Taylor H, Crissman D, Honn KV (1986): Cathepsin B: Association with plasma membrane in metastatic tumors. Proc Natl Acad Sci USA 83:2483–2487.

Spyratos F, Maudelonde T, Brouilett JP (1989): Cathepsin D: An independent prognostic factor for metastasis of breast cancer. Lancet ii:1115–1118.

Stoppelli MP, Tacchetti C, Cubellis V, Corti A, Hearing VJ, Cassani G, Appella E, Blasi F (1986): Autocrine saturation of pro-urokinase receptors on human 431 cells. Cell 45: 675–684.

Stoppelli MP, Corti A, Soffientini A, Cassani G, Blasi F, Assoian RK (1985): Differentiation-enhanced binding of the amino-terminal fragment of human urokinase plasminogen activator to a specific receptor on U937 monocytes. Proc Natl Acad Sci USA 82:4939–4943.

Sullivan LM, Quigley JP (1986): An anticatalytic monoclonal antibody to avian plasminogen activator: Its effect on behavior of RSV-transformed chick fibroblasts. Cell 45:905–915.

Takahashi K, Kazuho I, Takashi G, Masaaki T (1990): Local function of the urokinase receptor at the adhesion contact sites of metastatic tumor cells. Thromb Res Supp X:55–61.

Tryggvason K, Hoyhtya M, Salo T (1987): Proteolytic degradation of extracellular matrix in tumor invasion. Biochim Biophys Acta 906:191–217.

Varani J, McKeever PE, Fligiel SEG, Sitrin RG (1987): Plasminogen activator production by human tumor cells: Effect on tumor cell-extracellular matrix interactions. Int J Cancer 40:772–777.

Vassalli JD, Baccino D, Belin D (1985): A cellular binding site for the Mr 55,000 form of the human plasminogen activator, urokinase. J Cell Biol 100:86–92.