Human plasma fibronectin as a substrate for human urokinase

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An early event in malignant transformation is the increased expression of proteases, such as plasminogen activator, which can degrade surrounding extracellular matrices, thereby conferring an advantage for tumour cell invasion and metastasis. The present studies provide evidence that plasma fibronectin (Fn), which is a component of the extracellular matrix, is a direct substrate for the plasminogen activator urokinase (UK). Human plasma Fn was incubated with human UK under plasminogen-free conditions. Fn cleavage was both time- and dose-dependent and was evident within 30 min. The proteolytic digestion was limited and complete within 12 h at an enzyme/substrate ratio of 1:20. Analysis of the final proteolytic digestion products demonstrated the disappearance of the native dimeric 440 kDa structure of Fn with the concomitant appearance of three proteolytic fragments of 210, 200 and 25 kDa. Since two large fragments of similar size to the 220 kDa monomeric chains of Fn were obtained following proteolysis, it is proposed that UK cleaves Fn at two sites, one towards the *N*-terminal and one close to the *C*-terminal, but *N*-terminal to its interchain disulphide bonds. These studies suggest that the local proteolytic digestion and release of Fn from the extracellular matrix by tumour cells possessing high levels of UK may involve the direct proteolytic breakdown of Fn by UK.

INTRODUCTION

Major significant phenotypic differences between normal and tumour cell counterparts are the rounded morphology of the latter, associated with a concomitant loss of cellular adhesion, and their ability to exhibit increased migration. The extracellular matrix glycoprotein fibronectin (Fn) mediates the adhesion and spreading of fibroblasts on collagenous matrices [1,2]. Both the loss of cell surface Fn following transformation [3,4] and the ability of exogenously added Fn to restore these cells to a normal morphology [5] implies that the loss of Fn and cellular adhesion to the substratum may contribute to the ability of tumour cells to migrate and invade the surrounding tissues. Another characteristic of malignant cells is their increased proteolytic activity which is also associated with their ability to degrade and thus invade the surrounding extracellular matrix [6]. Increased intracellular and secreted levels of plasminogen activators is an early event associated with oncogenic transformation [7,8] in a variety of tumour cell types [9–11]. Immunochemical staining for the plasminogen activator urokinase (UK) illustrates a predominance of staining in the region of tumour cell invasion of surrounding tissue [12,13].

The destruction of extracellular matrices by tumour cells was shown to be plasminogen-dependent [14], and since plasmin is a protease of broad specificity, it was thought that the involvement of plasminogen activator in extracellular matrix degradation was related to the generation of plasmin from plasminogen. However, we recently provided biochemical evidence for avian cell surface Fn as a direct substrate for avian plasminogen activators [15]. The present studies have extended this finding to show that although the sites for proteolytic

attack are not identical, Fn isolated from human plasma undergoes limited proteolysis by human UK. These results provide supporting evidence for a causative relationship between the observation of high levels of plasminogen activators associated with the malignant phenotype and the observed decrease in cellular adhesion.

MATERIALS AND METHODS

Purification of Fn, UK and plasminogen

Human plasma Fn was obtained from the New York Blood Center and solubilized with sterile water at a concentration of 5 mg/ml at 37 °C. In order to ensure a plasminogen-free preparation, the Fn was subjected to lysine–Sephacrose 4B (Pharmacia) chromatography [16] and the Fn effluent purified by gelatin–Sephacrose 4B chromatography (Pharmacia) equilibrated with 0.1 M-Tris, pH 7.2. The peak protein fractions were eluted with 4.0 M-urea in 0.1 M-Tris, pH 7.2, pooled, and dialysed against 0.1 M-Tris, pH 8.1. Samples were frozen at –70 °C until use. In one experiment Fn was iodinated by the Iodogen method (Pierce). The trichloroacetic acid-precipitable radioactivity represented 99.9% recovery and specific radioactivity was 5.0×10^6 c.p.m./μg of Fn.

Partially purified human UK from urine, donated by Dr. Anne Ball, Department of Health and Human Services, NIH, Bethesda, MD, U.S.A. was purified to homogeneity by affinity chromatography on benzamidine linked to Sepharose 6B (Pharmacia) [17]. A fibrinolytic profile for the column was obtained using purified human plasminogen in a ¹²⁵I-fibrin plate assay as previously described [18]. The fibrinolytic activity of the final pooled fractions of UK indicated that 30 munits (180 pg) of UK released 50% of the total counts. SDS/polyacrylamide-

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gel electrophoresis (SDS/PAGE) analysis indicated that the preparation was more than 99% pure, consisting of more than 95% of the high molecular mass form (50–52 kDa) and less than 5% of the 30–33 kDa low molecular mass form. The UK was stored at -70°C in the buffer used for elution and was dialysed against 0.1 M-Tris, pH 8.1, overnight, prior to experimental use. Human plasminogen was prepared from human serum by affinity chromatography using lysine-Sepharose [16].

Preparation of plasminogen-free anti-UK antisera

To remove any traces of plasminogen or plasmin from the anti-(human urinary UK) (American Diagnostica, Inc.), goat IgG, one vial of IgG (5.0 mg/ml) was incubated with 0.5 ml of equilibrated lysine-Sepharose 4B [19] for 2 h with rotation at 4°C . The mixture was centrifuged at 1000 rev./min for 5 min to remove the Sepharose particles and the supernatant IgG was used to inhibit UK digestion of Fn. Inhibition of the proteolytic activity of UK was previously determined to be 1.0 mg of IgG per 0.6 μg of UK.

Proteolytic reaction

Purified human UK was incubated with purified plasma Fn in 0.1 ml of 0.1 M-Tris, pH 8.1, at 37°C for various lengths of time and at enzyme/substrate ratios indicated in individual experiments. The proteolytic digestion was terminated by adding 0.25 μl of 5 $\times$ conc. gel electrophoresis sample buffer [19], followed by quick-freezing in methanol and dry ice. By using the fibrinolytic plate assay, it was determined that 20 units of Trasylol/ml completely inhibited plasmin activity. Trasylol (Aprotinin; Sigma) was added prior to the addition of UK in designated reaction mixtures. All experiments were analysed by SDS/PAGE according to the method of Laemmli [19]. Plasmin was generated from human plasminogen (Sigma) for 1 h in the presence of human UK at a ratio of 5:1. Benzamidine, an inhibitor of UK, was purchased from Sigma.

RESULTS

Concentration-dependent digestion of Fn by UK

Fn, as purified from plasma, exists mainly as 440 kDa dimer; however, variable amounts of both aggregated multimeric and the 220 and 210 kDa monomeric forms are present in most preparations (Fig. 1, lane 1). Cleavage of Fn by UK results in the progressive breakdown of the 440 kDa dimeric structure with a concomitant increase in the 220 kDa monomer. As the enzyme concentration increases, the 220 kDa monomer disappears and the appearance of 210 and 200 kDa polypeptides can be observed (Fig. 1, lanes 2–5). Very little 440 kDa dimer remains at a substrate/enzyme ratio of 50:1 and none remains at 10:1 (the darker band above the 440 kDa represents multimeric forms). The 220 kDa monomer is almost absent at 25:1 substrate/enzyme ratio (lane 4) and completely disappears at 10:1 (lane 5). Also, there is evidence of lower molecular mass material migrating near the gel front, detectable at a 100:1 substrate/enzyme ratio and increasing in amount with increasing UK concentration. Two minor polypeptide products were observed below the 220 and 210 kDa monomers in the undigested Fn preparation (lane 1) but they did not

Fig. 1. Concentration-dependent digestion of Fn by UK analysed by SDS/PAGE

Fn was incubated with increasing concentrations of UK as described, and analysed by SDS/PAGE using 6% acrylamide monomer. The reaction was terminated after 17 h as described. Lane 1, Fn alone, 44 μg ; lane 2, Fn and 44 μg of UK; lane 3, Fn and 0.88 μg of UK; lane 4, Fn and 1.76 μg of UK; lane 5, Fn and 4.4 μg of UK. The faint band observed only in lane 5, at approx. 50 kDa, is UK (4.4 μg).

increase upon incubation with increasing concentrations of UK.

Time-dependent digestion of Fn by UK

UK digestion of plasma Fn at a substrate/enzyme ratio of 20:1 for increased time periods from 30 min up to 24 h progressively generated decreasing amounts of the dimer, with a concomitant increase in the appearance of the 220, 210 and 200 kDa monomeric forms, and a 25 kDa fragment (Fig. 2). Digestion of Fn is evident within 30 min by the slight disappearance of the 440 kDa dimer, a small increase in the amount of the 220 and 210 kDa dimers, and the distinct appearance of the 25 kDa fragment (lane 2). The appearance of the latter fragment is particularly apparent when compared with a reaction time of zero (lane 1) and a 24 h incubation without UK (lane 9). The 220 kDa monomer is present in trace amounts at 8 h (lane 6) but has completely disappeared by 12 h (lane 7). The loss of the 220 kDa fragment is accompanied by the appearance of a broad band at 210 kDa and a sharp band at 200 kDa. The digestion of Fn by UK is limited, since by 24 h no further breakdown of the molecule was observed (compare lane 7 with lane 8). A 24 h incubation of fibronectin alone (lane 9) showed no change in the pattern observed at zero time incubation, indicating that active proteolytic enzymes are not contaminating the Fn preparation. The

Fig. 2. Time-dependent proteolytic digestion of Fn by UK analysed by SDS/PAGE

Fn (15 μ g) was incubated with 0.75 μ g of UK for various lengths of time. The samples were applied to a 5–10% acrylamide gradient gel. The lanes are labelled for incubation time in hours. Lane 1 illustrates a sample of Fn (15 μ g) to which 0.75 μ g of UK was added and the reaction immediately terminated as described. Lane 9 represents a sample of Fn (15 μ g) that was incubated for 24 h in the absence of UK.

high (52 kDa) and low (33 kDa) molecular mass forms of UK are marked with arrows.

Comparison of UK and plasmin digestion of Fn

Although the presence of 4.0 M-urea during gelatin affinity chromatography should prevent plasminogen from binding to Fn [20], if trace amounts of plasminogen contaminated the plasma Fn preparation, the exogenously added UK would then activate the plasminogen to plasmin which subsequently could give rise to the observed cleavages. To rule out this possibility, lysine-Sepharose affinity chromatography [16], a procedure which removes plasminogen from plasma, was employed in the Fn purification, prior to conventional gelatin affinity chromatography. Furthermore, dot-blot analysis indicated the absence of any plasminogen in either the Fn (20 μ g) or UK (20 μ g) preparations [level of detection was 2.5 ng using a monoclonal antibody to plasminogen (American Diagnostica)] (results not shown).

Trasylol, an inhibitor of plasmin which does not inhibit UK, was also tested in an Fn digestion experiment. Analysis of the resulting gel is shown in Fig. 3. The digestion of Fn by UK in the presence of Trasylol revealed the identical cleavage pattern obtained with UK and Fn alone, i.e. loss of the 440 kDa dimer and appearance of the 25 kDa fragment (compare lane 2 with lane 3). Addition of exogenous plasminogen, to mimic possible contamination, resulted in the generation of plasmin which cleaved Fn into a number of major fragments (lane 4). The latter digestion was inhibited by Trasylol and only the cleavage pattern typical of UK-mediated digestion was evident (lane 5). The Trasylol concentration employed in these studies (20 units/ml) completely inhibited plasmin activity in a fibrin plate assay (results not shown), thus indicating that the proteolytic cleavage

Fig. 3. Analysis of UK- and plasmin-mediated digestion of Fn by SDS/PAGE

Fn (7.5 μ g) was incubated with UK (0.375 μ g) for 17 h (volume 0.03 ml) and the reaction terminated by the addition of 0.01 ml of 5 $\times$ sample buffer. The samples were analysed by SDS/PAGE using a gradient of 5–20% acrylamide monomer and a BioRad Mini Gel System. Lane 1, Fn alone; lane 2, Fn and UK; lane 3, Fn, UK and 20 units of the plasmin inhibitor, Trasylol; lane 4, Fn, UK and 1.87 μ g of plasminogen; lane 5, Fn, UK, 1.87 μ g of plasminogen and 20 units of Trasylol; lane 6, Fn, UK and 2 mM-benzamidine.

products observed in lanes 2, 3 and 5 result directly from UK digestion alone. The digestion of Fn by UK was completely inhibited by the addition of 2 mM-benzamidine (lane 6), an inhibitor of UK. This also indicates that the digestion of Fn is specific for UK and that no further proteolysis could have occurred following the addition of the gel buffer to the samples for SDS/PAGE analysis. To further prove that the proteolytic cleavage of Fn by UK was specific, goat anti-(human UK) IgG was incubated with UK prior to exposure to 125 I-labelled Fn. The UK-specific IgG completely inhibited the enzymic digestion of Fn (Fig. 4, lane 3). The scatter in the autoradiograph did not permit resolution of the individual 220, 210 and 200 kDa forms.

Analysis of the subunit structure of UK-mediated cleavage products of Fn

The Fn dimer is joined together at the C-terminal by disulphide bonds at $\frac{1}{2}$ cystine residues located at positions 2306 and 2310 (out of 2325 total residues) [21]. Since UK digestion of Fn produced final cleavage products of molecular masses similar to the monomeric forms (210 and 200 kDa), the cleavage site(s) would presumably be close to the N-terminal unless proteolysis occurred toward the middle of the molecule which would cause the resulting cleavage products to be composed of both the A and B chains held together by the interchain disulphide bonds. Similarly, if the 25 kDa fragment is derived from the terminal, reduction of the interchain disulphide bonds would yield two fragments, one from each of the monomers. To examine this possibility, UK-mediated cleav-

Fig. 4. Inhibition of UK activity by anti-UK IgG

UK (0.22 μ g) was incubated with 376 μ g of either lysine-affinity-purified goat anti-UK IgG (American Diagnostica) or a non-specific goat IgG (control), for 1 h at room temperature prior to addition to the enzymic reaction mixture. The anti-UK specific IgG was previously shown to neutralize UK activity at the above antigen/antibody ratio. The previously incubated IgG and UK was added to 4.375 μ g of unlabelled Fn and 48 ng of 125 I-labelled Fn (240000 c.p.m.) and incubated for 17 h. The samples were applied to a gradient gel of 5–20% acrylamide monomer and the gel was dried by vacuum and exposed to XAR-2 film (Kodak) for 6 h. Lane 1, Fn; lane 2, Fn and UK; lane 3, Fn, UK and anti-UK IgG.

age by Fn was analysed under reducing conditions (in the presence of β -mercaptoethanol) and compared with non-reducing gels. The results of such comparison (Fig. 5) indicate that while the untreated 440 kDa fibronectin dimer (lane 1) is reduced to a broad 220 kDa monomer (lane 3), the major cleavage products are basically unchanged upon reduction (lane 2 versus lane 4). The reduced 25 kDa fragment actually migrated as a slightly higher molecular mass polypeptide compared with the unreduced form. This is probably due to the presence of intrachain disulphide bonds which, following reduction, linearize the polypeptide, slightly retarding its migration through the gel.

DISCUSSION

Tumour cells can degrade extracellular matrices [22,23], causing the specific release of Fn [24]. The direct correlation of the observed increase in levels of UK and plasminogen activator in tumour cells, with the proteolytic digestion of specific components of the extracellular matrix, especially Fn, and the loss or decrease of Fn from the surfaces of tumour cells, has

Fig. 5. Analysis of UK-mediated digestion of Fn by SDS/PAGE with and without reduction

Fn (15 μ g) was incubated with (lanes, 2 and 4) and without (lanes 1 and 3) UK (0.75 μ g) for 17 h at 37 $^{\circ}$ C, the reaction terminated, and the samples applied to a gradient gel of 5–20% acrylamide monomer. The samples were analysed under both non-reducing (lanes 1 and 2) and reducing (lanes 3 and 4) conditions using a final concentration of 5% β -mercaptoethanol.

been difficult to prove. The ability of tumour cells to invade and metastasize as a result of their increased proteolytic activity has largely been attributed to plasmin and collagenase activity [23]. Thus, the role for plasminogen activators in matrix degradation and metastasis has been assumed to be a result of plasminogen zymogen activation. Initial evidence for a possible direct involvement of plasminogen activators in matrix degradation was obtained from the observation that specific inhibitors of the proteolytic activity of plasminogen activator could inhibit matrix degradation and release of Fn [7,25]. It has also been shown that a monoclonal antibody to avian plasminogen activator obviated both matrix degradation and transformation-associated morphology of Rous sarcoma virus-transformed chicken embryo fibroblasts, in the absence of plasminogen [25], and that an anti-UK antibody directly inhibited metastasis of a human tumour cell type [26]. More recently, Quigley *et al.* [15] provided biochemical evidence for the direct involvement of avian plasminogen activator in the proteolytic degradation and release of Fn from the extracellular matrix. Similarly, the present studies demonstrate that plasma Fn, which has been shown to be incorporated into the extracellular matrix [27], is degraded by UK under plasminogen-free conditions and in the presence of Trasylol, an enzymic inhibitor of plasmin.

Although in both the present study and that of

Quigley *et al.* [15], limited proteolysis by UK produced two major large polypeptide chains similar in molecular mass to the monomeric A and B chain of Fn, two different major cleavage products were also obtained. Digestion of human plasma Fn by UK yielded a 25 kDa fragment that was not generated using avian cellular Fn as a substrate [15] for both human UK and avian plasminogen activator. Likewise, a 100 kDa fragment of Fn was a cleavage product found only in the avian system. The observed discrepancies in fragment generation probably reflect both inherent differences in enzyme specificity and/or differences in the primary sequence between the cellular and plasma forms of Fn, or the species sources [28].

The amino acid sequence of the entire human plasma Fn molecule has been deduced from the cDNA [29]. Thus, based on the size of the fragments generated from the Fn molecule, the C-terminal location of the interchain disulphide bonds within the molecule, and the known cleavage specificity of UK for an Arg-Val peptide bond in plasminogen, one could predict the possible cleavage sites resulting from the proteolytic digestion of Fn by UK. Since the 25 kDa fragment did not yield two fragments upon reduction (Fig. 5, lane 4), it must be derived from the N-terminal of the molecule. A possible cleavage site, therefore, may be between Arg and Ala at residues 260 and 261 respectively. Digestion of Fn by plasmin generated by UK activation of plasminogen produced a 25 kDa fragment which migrated identically with the fragment generated by UK digestion alone (Fig. 3, compare lane 4 with lanes 2, 3 and 5). It has previously been shown that plasmin cleaves between residues 260 and 261 [29,30]. If UK cleaves the peptide bond between Arg-Ala within the Fn molecule, this would establish a new substrate specificity for UK proteolytic activity, and possibly a new amino acid sequence recognition site for this enzyme.

A second cleavage site is placed at the C-terminal, N-terminal to the interchain disulphide bonds. This is deduced because large polypeptides of 210 and 200 kDa were produced by UK cleavage, which is similar to that observed following chemical reduction of the interchain disulphide bonds in the intact molecule (Fig. 5, lane 3).

It appears that the formation of the 25 kDa fragment and the 220 and 210 kDa fragments occur simultaneously since they both are evident, to the same quantity, within 30 min of digestion (Fig. 2, lane 2). However, it is difficult to establish whether the 25 kDa fragment is derived from one or both of the A and B chains. It is also unknown at this time whether the 220 kDa fragment observed within the first 8 h of digestion (Fig. 2, lanes 2-6) is further cleaved to the 210 or 200 kDa fragment, or if the 200 kDa fragment is cleaved initially, but remains resistant to further proteolytic digestion. These questions may be answered through chain separation and amino acid sequence determination of the UK-mediated cleavage fragments.

The digestion of Fn by UK is efficient only at a relatively high enzyme/substrate ratio compared with plasmin. However, if one is to consider the recent observation that plasminogen-independent (but not plasminogen-dependent) matrix degradation requires cell surface contact with substratum [24], that there is a high concentration of UK at cell substrate contact sites [31], that UK is bound to specific receptors on the surfaces of cells that secrete UK [32] and that there is a local loss of

Fn at these contact sites [33,34], then local degradation of Fn due to UK activity at focal contacts could directly contribute to loss of cellular adhesion leading to eventual detachment from the substratum, and subsequent invasion and metastasis. One could envisage that UK produces an initial cleavage site in the Fn molecule, thereby reducing Fn to its monomeric form. The release of partially degraded Fn monomers could enhance the molecule's vulnerability to further proteolytic attack by other endogenous enzymes causing extensive disruption of the extracellular matrix. These studies suggest that limited proteolytic digestion of Fn by UK at cell substratum contact sites by tumour cells producing high levels of UK may be an initial event leading to tumour cell detachment from connective tissue extracellular matrices.

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