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Urokinase binds to a plasminogen activator inhibitor type-2-like molecule in placental microvillous membranes

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Placental microvillous membranes exhibited saturable binding of urokinase-type plasminogen activator with plateau achieved by 30 min at 4 °C and 10 min at 37 °C. The binding was essentially irreversible. The capacity was about 8 pmol urokinase per mg membrane protein. Half-maximal displacement of ^{125}I-labelled urokinase was achieved with about 1.0 nM unlabelled urokinase when using 75 μg membrane protein/ml. ^{125}I-labelled urokinase did not bind when treated with diisopropylfluorophosphate to block the catalytic activity. Single-chain urokinase (prourokinase), devoid of catalytic activity, did not bind. Catalytically active tissue-type plasminogen activator did compete with ^{125}I-labelled urokinase for binding although less efficiently than urokinase. Binding activity remained in the 100000 × g pellet after treatment of the membranes with 3 M KCl, alkaline stripping at pH 12 or extraction by the detergent Triton X-100. The binding was essentially blocked by antibodies against plasminogen activator inhibitor-type-2 (PAI-2). Sodium dodecyl sulfate polyacrylamide gel electrophoresis of solubilized membranes with bound ^{125}I-labelled urokinase showed that the urokinase-PAI-2 complexes largely migrated in fractions corresponding to a very large M_r although no clearly defined peaks were observed. It is suggested that PAI-2 occurs in a form anchored to syncytiotrophoblast microvilli, possibly to the cytoskeleton.

Introduction

Urokinase-type plasminogen activator appears to play an important role for the degradation of extracellular matrix in a variety of normal and pathological processes including invasive growth of trophoblasts and cancer cells (for review, see Ref. 1). Cultured cytotrophoblasts from term placentas can synthetize and secrete urokinase [2] which may in part account for the previously described capacity of this cell type to degrade extracellular matrix [3]. The human placenta has specific and saturable binding sites for hormones (e.g. insulin), growth factors (e.g. epidermal growth factor), proteins important for transfer of nutrients (e.g. transferrin), and immunoglobulins (IgG). We recently reported that placental membranes also have a common binding site for complexes between α_2-macroglobulin or the homologous pregnancy zone protein and a variety of proteinases [4]. These may help to maintain the balance between proteolytic and antiproteolytic activity on or near the surface of the syncytiotrophoblast.

The present study was initiated to elucidate whether the surface of the syncytiotrophoblast has binding sites for urokinase with the potential of modulating the activity. This might occur in two ways. The binding may leave the catalytic site free to interact with substrate as appears to be the case with urokinase receptors described in several cultured cell lines [1,5]. Alternatively, binding might occur to membrane components interacting with the catalytic site of urokinase and thereby quenching its activity in analogy with the action of plasminogen activator inhibitors type 1 or 2 (PAI-1 or 2) released from various cell types [1].

Materials and Methods

A commercial urokinase preparation (Serono, Switzerland) was purified further by affinity chromatog-

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raphy using a Sepharose-immobilized monoclonal antibody directed against the B-chain [6]. The resulting preparation contained more than 90% 54 kDa urokinase with a minor contamination of low molecular weight urokinase (about 33 kDa).

This urokinase preparation was iodinated using chloramine-T as the oxidizing agent. In brief, about 100 pmol $^{125}I^-$ (Amersham, UK, $8 \cdot 10^6$ Bq in 4 μl) was added to 100 pmol urokinase (20 μl in 0.2 M $NaHPO_4$, pH 8.0) followed by 2.5 μl chloramine-T (1 mg/ml). Incubation with mixing was carried out for 3 min at 20°C followed by the addition of 200 μl 0.1 M Tris, 0.1% Triton X-100, 1% bovine serum albumin (pH 8.1). Incorporation of iodine into protein averaged 50%. Labelled urokinase was separated from $^{125}I^-$ by gel filtration on Sephadex G-50F using the Tris-Triton-albumin buffer (pH 7.4).

Labelled or unlabelled urokinase was inactivated by the addition of diisopropylfluorophosphate to 1 mg/ml followed by incubation at 37°C, pH 8.1, for 60 min. This procedure was repeated once. About 85% of the ^{125}I activity was in 54 kDa urokinase and about 15% in the low molecular weight urokinase.

Prourokinase (single-chain urokinase) was prepared from serum-free conditioned medium of the human fibrosarcoma cell line HT-1080 by immunoaffinity chromatography [6]. Prourokinase was converted to active 54 kDa urokinase by incubation with plasmin [7]. The reaction was stopped by the addition of trasylol to 0.1 mg/ml. Control experiments in the continued presence of trasylol showed no conversion of the prourokinase.

Single-chain and two-chain tissue-type plasminogen activator was prepared as described [8].

The preparation of goat anti-PAI-2 [9] and monoclonal anti-PAI-2 [10] has been described previously. The polyclonal anti-PAI-2 does not crossreact with PAI-1 [11]. Placental microvillous membranes were prepared essentially as described previously [4,12,13]. In brief, the villous tissue from term human placentas was cut in pieces of about 5 g, washed in isotonic $CaCl_2$ and then in phosphate buffered isotonic NaCl (pH 7.2). The buffer was poured off, the tissue minced and an equal volume of 150 mM NaCl was added followed by gentle stirring for 1 h. The slurry was passed through a 56 μm mesh nylon filter and centrifuged at $800 \times g$ for 10 min. The supernatant was centrifuged at $10000 \times g$ for 10 min and the microvillous membranes were pelleted by centrifugation at $100000 \times g$ for 60 min. This membrane vesicle preparation is enriched 14-fold with regard to the plasma membrane marker enzymes alkaline phosphatase and 5'-nucleotidase as compared to placental homogenate and is sparse in enzymes principally associated with intracellular organelles [13]. The pellet was resuspended, placed on top of a discontinuous sucrose gradient (40% w/v overlaid with 25% w/v) and centrifuged for 16 h at $100000 \times g$. This causes a further 2-fold enrichment of 5'-nucleotidase and has been reported free of specific enzyme markers for subcellular contaminants [14]. The pellet was washed twice in 150 mM NaCl, 10 mM Hepes, 1% bovine serum albumin pH 7.4) and the membranes (about 4 mg protein/ml) were stored in that buffer at −50°C. All solutions contained 2 mM phenylmethanesulphonyl fluoride (PMSF) and the procedures were carried out at 4°C.

The binding experiments were carried out as follows unless otherwise stated. Membranes (15 μg protein) were incubated in 200 μl of the above-mentioned buffer plus 5 μg digitonin (Sigma) purified as described previously [15]. The incubations were stopped by passing 150 μl incubate through 0.2 μm Millipore filters (GVWP) soaked in the incubation buffer followed by wash with 2 ml ice-cold buffer. The results are the mean values of triplicate incubations unless otherwise stated. The coefficient of variation between replicates was about 5%, cf. Fig. 2. All experiments were carried out at least four times and representative experiments are shown.

Membrane protein concentrations were measured according to Bradford [16] using bovine serum albumin as standard.

Electrophoresis was carried out in the Laemmli system [17] using 80 mm long, 0.4 mm thick acrylamide/bisacrylamide 30 : 0.8 slab gels (stacking gel 4%, running gel 8–16%) and a sample size of 30 μl. After electrophoresis the gel was dried, cut in 3–4 mm slices and assayed for radioactivity.

Results

Table I shows that both isolated villi and microvillous membranes bound to saturable sites an appreciable fraction of 20 pM ^{125}I-labelled urokinase as compared with other ligands with well established placental receptors. The crude preparation of isolated villi serves only to show that urokinase binding can be demonstrated in a relatively 'unbroken' preparation and further studies

TABLE I

Saturable binding of ^{125}I-labelled urokinase, insulin transferrin and α_2-macroglobulin-trypsin complex to isolated chorionic villi (2.5 mg/ml wet weight) and to microvillous membranes (75 μg protein/ml)

The numbers represent the per cent of the added radioactivity associated with the villi or membranes after subtraction of activity associated with the membranes in the presence of a saturating concentration of unlabelled urokinase (400 nM). The tracer concentrations were about 20 pM. The incubations were carried out for 18 h at 4°C.

Labelled ligand	Per cent saturable binding			
	Urokinase	Insulin	Transferrin	α_2M-trypsin
Chorionic villi	15.3	17.1	–	4.8
Microvillous membranes	23.3	35.4	18.2	21.3

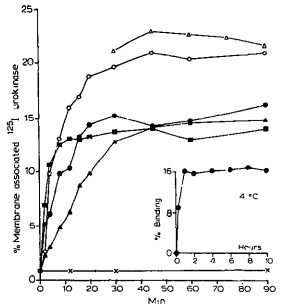

Fig. 1. Time course of ^{125}I-labelled urokinase binding to placental microvillous membranes. The incubations containing 20 pM labelled urokinase and 75 μg membrane protein/ml were carried out at 20 °C (●, ○), 37 °C (■) or 4 °C (▲, △) with (○, △) or without (▲, ●, ■) 25 μg/ml digitonin. Unlabelled (400 nM) urokinase was present in some incubations (× ——— ×) at 20 °C. The inset shows saturable binding at 4 °C at prolonged time.

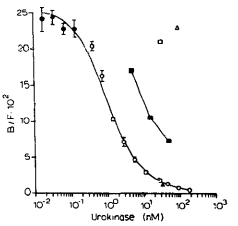

Fig. 2. Concentration dependence of urokinase binding. Microvillous membranes were incubated for 30 min at 4 °C with the ^{125}I-labelled urokinase preparation (●) or with ^{125}I-labelled urokinase plus unlabelled urokinase (○). Some tubes contained tracer plus 100 nM unlabelled prourokinase (△). 33 nM prourokinase treated with plasmin to activate the enzyme (▲). 33 nM one chain tissue-type plasminogen activator (□) or two chain tissue-type plasminogen activator (■) as indicated. Since 1.3% of the urokinase at a concentration of 45 nM was bound to membranes (75 mg protein/l) the binding capacity was calculated as approx. 8 pmol (40 μg urokinase/mg membrane protein.

were carried out on the microvillous membranes derived from the syncytiotrophoblasts.

Fig. 1 shows the time course of ^{125}I-labelled urokinase binding to microvillous membranes. The process was temperature-dependent with a half-time of about 5 min at 20 °C. Nearly the same plateau was achieved at 4 °C, 20 °C and 37 °C by 40 min. Other experiments (not shown) demonstrated that binding at 20 °C was independent of pH in the range 6.8–8.0. The binding was increased by about 50% when the membranes were treated with digitonin (Fig. 1), probably due to permeabilization of the membrane vesicles. Binding to these membranes at 4 °C was stable for at least 8 h (Fig. 1, inset). Dissociation of radioactivity from the membranes after wash or wash plus the addition of unlabelled urokinase (4 °C, 8 h) was negligible (data not shown). The small amount of radioactivity associated with the membranes (i.e. on the filters) in the presence of 400 nM unlabelled urokinase correspond to the radioactivity in trapped buffer using labelled L-glucose as a marker [4]. In other words, all binding of ^{125}I-labelled urokinase could be described as saturable and the amount of radioactivity associated with the filters in the presence of 400 nM unlabelled urokinase was subtracted as a blank value in the following experiments.

Other experiments (not shown) demonstrated that the precipitability in trichloroacetic acid remained unchanged (98%) after incubation of the tracer with membranes, whereas the ability to bind to fresh membranes was progressively reduced. This phenomenon remained unchanged when the incubations were performed with repeatedly washed membranes and is further described below.

Fig. 2 shows that binding of ^{125}I-labelled urokinase was reduced to 50% in the presence of about 1.0 nM unlabelled urokinase. About 15 nM two-chain tissue-type plasminogen activator caused a 50% reduction of the urokinase binding, whereas single-chain tissue-type plasminogen activator had little effect. Single-chain urokinase (prourokinase) did not reduce the binding of ^{125}I-labelled urokinase. Thus, the binding is specific and saturable. The capacity of the membranes is calculated, cf. legend to Fig. 2, as approximately 0.8 nmol urokinase/100 mg membrane protein, equivalent to about 4 nmol placenta.

Table II shows the results using inhibitors with known effects on the catalytic activity of urokinase [18]. Diiso-

TABLE II

Importance of the catalytic activity for binding to microvillous membranes

^{125}I-labelled urokinase was treated with diisopropylfluorophosphate (DFP) or leupeptin before incubation, whereas the other inhibitors were added to the incubate. The incubations (75 μg membrane protein/ml) were carried out for 30 min at 4 °C. Control binding is set at 100%.

Per cent saturable binding				
No treatment	DFP (50 mM)	Leupeptin (1 mM)	Trasylol (0.1 mg/ml)	Soybean trypsin inhibitor (1 mg/ml)
100	5	8	89	104

propylfluorophosphate (DFP) which irreversibly blocks the catalytic site essentially abolished binding to placental membranes. Control experiments (not shown) with cultured human monocytes (U-937) demonstrated that DFP-treated ^{125}I-labelled urokinase displayed full receptor binding activity as described in detail in previous work [5]. Leupeptin, which inhibits urokinase activity [18], severely inhibited the binding to placental membranes (Table II) but not to U-937 receptors (not shown). Trasylol and soybean trypsin inhibitor had no effect in either system in accordance with the lack of effect on urokinase activity [18].

These results show that the placental microvillous membranes do not contain receptors as described in U-937 monocytes. It became essential to see whether the activity measured in the present experiments might be loosely adsorbed to the membranes, particularly since placenta is known to contain both type-1 [19] and type-2 [9,20] plasminogen activator inhibitors. One could envisage this occurring during the preparation with some activity remaining on the membranes in spite of the wash inherent in the preparation procedure.

Attempts to remove the binding activity were negative and the results may be summarized as follows: Repeated simple washings did not reduce the binding activity. Treatment of the membranes with glycine buffer, pH 3.0, or with 3 M KCl did not reduce binding in a following standard incubation. ^{125}I-labelled urokinase prebound at pH 7.4 did not dissociate at pH 3.0 (in contrast to receptor-bound urokinase [1]), whereas binding was essentially abolished when the incubations were carried out at that pH. Alkaline stripping [21] of the membranes of pH 12.0 (pretreatment for 10 min with one volume 5 mM sodium phosphate plus five volumes 2 mM EDTA, 0.2 mM dithiothreitol, 15 mM NaOH), which depletes erythrocyte membranes of extrinsic proteins, had little effect on the binding activity. Finally, the membranes (300 μg protein/ml) were extracted with the nonionic detergent Triton X-100 (1%), a treatment which has been shown to solubilize about half of the placental microvillous membrane protein [22]. The resulting 100 000 × *g* membrane pellet, previously referred to as the cytoskeleton fraction [22], showed in three experiments a 30–40% increase in the binding of ^{125}I-labelled urokinase. Control experiments showed that the Triton X-100 extraction removed more than 90% of the binding sites for α_2-macroglobulin-trypsin complex [4] in the membranes. Thus, the urokinase binding activity is not removed by Triton X-100 and may be associated with the cytoskeleton.

A clue to the nature of the activity is shown in Table III. Polyclonal and monoclonal antibodies raised against PAI-2 markedly inhibited the binding with no effect of preimmune serum. It appears, therefore, that a plasminogen activator inhibitor with immunoreactivity similar to the soluble PAI-2 is anchored to placental microvillous membranes.

Fig. 3 illustrates the sodium dodecyl sulfate polyacrylamid gel electrophoresis pattern of medium and membrane-bound radioactive material. Fig. 3A shows that the tracer in buffer not incubated with membranes contains about 85% of the radioactivity as 54 kDa urokinase, 15% as 33 kDa urokinase. In medium incubated with membranes an additional approximately 90 kDa peak appears. This peak increased progressively when membranes at increasing concentrations were incubated with ^{125}I-labelled urokinase, whereas it was absent when membranes were incubated with DFP-treated ^{125}I-labelled urokinase (data not shown). The 90 kDa peak is markedly inhibited by polyclonal antibodies against PAI-2 and it therefore appears to represent complexes between labelled urokinase and PAI-2. In some experiments the labelled 90 kDa complex was isolated by gel filtration and it was found not to bind to the membranes (data not shown).

Fig. 3B shows the pattern for the membrane-bound material. As expected, there is very little activity in membranes incubated with excess unlabelled urokinase and essentially zero in slices with molecules larger than 60 kDa. In other words, the separation procedure and wash (cf. legend to Fig. 3) was sufficient ot prevent significant trapping of radioactivity from the medium with the membranes. When membranes had been incubated with tracer alone, most of the radioactivity remained in the stacking gel with some smearing into the running gel. In addition, there is a broad peak around 120 kDa. Thus, the bound material is present as a very large complex which is at least partially sodium dodecyl sulfate-resistant and only partially enters the separation gel. Antibodies against PAI-2 markedly reduced the radioactivity in all gel slices containing molecules larger than 60 kDa.

Fig. 3C shows the gel pattern after reduction. Under these conditions about 70% of the radioactivity in the tracer is at 35 kDa and 30% at 20 kDa. A large part of the membrane-bound activity now appears at 20 kDa corresponding to the light chain of urokinase. There is

TABLE III

Effect of antiserum against PAI-2

Membranes (75 μg protein/ml) were preincubated for 4 h at 4°C with goat anti PAI-2 (8401, 0.25 mg IgG/ml; 7901, 1.0 mg IgG/ml), monoclonal anti PAI-2 (0.15 mg IgG/ml) or goat preimmune serum (1.7 mg IgG/ml) and then an equal volume of tracer for 30 min.

Per cent saturable binding				
Control (buffer)	Preimmune serum	Anti PAI-2 (polyclonal) 8401	Anti PAI-2 (polyclonal) 7901	Anti PAI-2 (monoclonal)
16.8	17.0	1.7	1.4	5.8

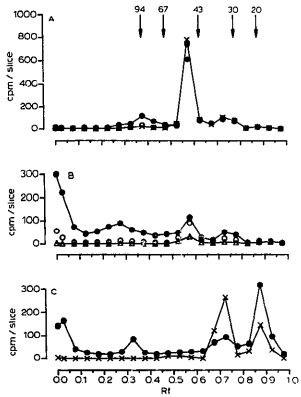

Fig. 3. Electrophoretic pattern of medium and membrane-bound radioactivity. Membranes (75 μg/ml) were incubated with 100 pM ^{125}I-labelled urokinase for 30 min at 4° C. Incubation medium and membranes were separated by filtration on Millipore filters. The medium was diluted 1:4 in sample buffer with 5% sodium dodecyl sulfate, boiled and electrophoresis was carried out using 8–16% polyacrylamide gradient gels (stacking gel 4%). The filter with membranes was carefully washed and immersed in boiling sample buffer. The samples were centrifuged for 15 min at 100000 × *g* before electrophoresis and the radioactivity was recovered quantitatively in the supernatant. Panel A shows radioactivity in the medium: Incubation of tracer with buffer only (× ——— ×), with membranes in buffer plus preimmune serum, 1.7 mg IgG/ml (● ——— ●) and with buffer plus polyclonal anti PAI-2, 8401, 0.025 mg IgG/ml (○ ——— ○). Panel B shows membrane-associated activity: Incubation of tracer with membranes and buffer plus preimmune serum (● ——— ●), membranes and buffer plus 400 nM unlabelled urokinase (△ ——— △) or membranes and buffer plus anti-PAI-2 (○ ——— ○). Panel C shows the electrophoretic pattern after reduction with 20 mM dithioerythritol: Incubation of tracer with buffer alone (× ——— ×) or with membranes and buffer plus preimmune serum (● ——— ●). R_f = 0.0 indicates radioactivity in the stacking gel.

an equivalent reduction in radioactivity of the slices containing the putative binding proteins. Thus, the interchain disulfide bridge of the bound urokinase is, as expected, split by reduction, whereas there is no evidence for disulfide-linked subunits of the putative binding proteins.

Discussion

The urokinase binding activity of the microvillous membranes is at first glance similar to that observed in cells expressing urokinase receptors, e.g., cultured monocytes of the U-937 line. Thus, the time course of association and the apparent half saturation constants are similar [1,23]. However, the placental membrane binding activity is, in contrast to receptor binding activity, dependent on the catalytic activity of urokinase and it is strongly inhibited by antibodies against PAI-2. Binding must therefore occur to sites in the membrane immunologically related to PAI-2, probably to a membrane-anchored form of PAI-2. This conclusion is supported by the observation that two-chain tissue-type plasminogen activator and particularly one-chain tissue-type plasminogen activator compete poorly with two-chain urokinase for binding to the membranes. Similar observations have been reported with purified PAI-2 [24].

The binding capacity of membrane-associated PAI-2 was calculated as around 4 nmol per placenta. This is a minimum number since the microvillous membrane represents only a portion of the syncytiotrophoblast surface [25]. This value should be compared with about 1.4 nmol 48 kDa soluble PAI-2 extracted per placenta [20]. It is therefore likely that membrane-bound PAI-2 represents a significant part of the total placental PAI-2 activity.

The present results show that soluble PAI-2 is released from the microvillous membranes and into the incubation medium. A variety of cell lines, including U-937 [23], produce PAI-2. However, U-937 cells do not contain membrane binding activity inhibitable with antibodies against PAI-2 (Nykjær and Gliemann, unpublished observation). The releasable PAI-2 activity was not readily washed away from the placental membranes which therefore appear to contain a stored pool of this inhibitor different from the pool mediating the urokinase binding.

The microvillous preparation contains the apical part of the syncytiotrophoblast brush-border membrane facing the maternal blood with little contamination of membranes from intracellular organelles [13,14,25]. Most membranes reseal to vesicles and the orientation is reported to be the same as in the intact placenta [25]. Even though it cannot be excluded that some vesicles are inside out it is likely that most of the urokinase binding occurs to the outside of the syncytiotrophoblast microvilli. This conclusion is supported by the finding that ^{125}I-labelled urokinase does bind to isolated chorionic villi. The membrane-associated PAI-2 may be anchored to the cytoskeleton since the activity is not extracted by Triton X-100.

Electrophoresis of sodium dodecyl sulfate-solubilized membranes with bound ^{125}I-labelled urokinase did not indicate a distinct size of the complex. In fact, specific and saturable binding appeared in the entire range from 54 kDA to very large molecules remaining in the stacking gel, cf. Fig. 3B. Even though the samples were centrifuged at 100000 × *g* most of the activity remained in the 4% stacking gel suggesting that the binding

protein(s) is attached to very large molecular structures. A soluble plasminogen activator inhibitor prepared from placental extracts is electrophoretically heterogeneous in non-reduced form but runs as a single 47 kDa band after reduction [26]. In contrast, reduction of the solubilized membrane proteins with ^{125}I-labelled urokinase does not result in an electrophoretically homogeneous band. Molecular characterization of the membrane binding protein(s) must await its purification in an active form. In this respect, low molecular weight urokinase (33 kDa) should be a better probe now it is realized that the microvillous membranes do not contain urokinase receptors.

PAI-2 is important for quenching urokinase activity and the physiological meaning of a membrane-anchored form may be to regulate urokinase activity in the microenvironment. In this connection it is interesting that fibrin depositions are often observed around full-term chorionic villi [27]. It is also possible that PAI-2 in trophoblast membranes can serve as a point of attachment for monocytes with occupied urokinase receptors. Such mechanism might be the reason for the close contact between extravillous trophoblasts and decidual macrophages [28].

In summary, we have demonstrated that placental microvillous membranes contain binding sites dependent on the catalytic site of urokinase. The sites are immunologically related to PAI-2 and may be anchored to the cytoskeleton.

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