Thrombin stimulates production of fibronectin by human proximal tubular epithelial cells via a transforming growth factor-β-dependent mechanism

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Abstract

Background. Tubulointerstitial fibrosis contributes to the progression of many forms of glomerular disease and to end-stage renal failure. Inflammatory mediators generated during glomerular injury may induce tubulointerstitial lesions by stimulating tubular cells. Thrombin has multiple biological functions in addition to its role in haemostasis and has been detected in the urine of patients with glomerular diseases. The present study investigated whether thrombin can modulate the production of fibronectin (FN) in cultured human proximal tubular epithelial cells (PTEC).

Methods. Cultured PTEC were incubated with or without thrombin to examine the effect of thrombin on FN production in PTEC. FN and transforming growth factor-β (TGF-β) levels were measured in culture supernatants by enzyme-linked immunosorbent assay (ELISA). Expression of FN mRNA was analysed by reverse transcriptase–polymerase chain reaction. Effects of thrombin on matrix metabolism were examined by enzyme immunoassay for the detection of secreted matrix metalloproteinase (MMP) and its inhibitors (TIMPs) as well as by zymography.

Results. Thrombin stimulated FN secretion in PTEC. Thrombin also stimulated TGF-β secretion in PTEC in a dose-dependent manner. Expression of FN mRNA by PTEC was augmented by thrombin. The stimulatory effect of thrombin on FN secretion was inhibited by neutralizing antibodies against TGF-β but not by an irrelevant antibody. Thrombin-induced FN secretion was also inhibited by thrombin inhibitors, such as antithrombin III, hirudin and argatroban. Although thrombin stimulated TIMP-1 and -2 secretion by PTEC, the stimulatory effect of thrombin on MMP-2 was not statistically significant. Thrombin did not affect the expression of MMP-2 in zymography studies.

Conclusions. We found that thrombin stimulates FN production in PTEC without causing matrix degradation, an effect that may contribute to the formation of tubulointerstitial fibrosis associated with glomerular disease. The stimulatory effect of thrombin on FN production in PTEC is, at least in part, mediated by TGF-β.

Keywords: fibronectin; human proximal tubular epithelial cell; TGF-β; thrombin; tubulointerstitial fibrosis

Introduction

The progression of glomerular diseases to end-stage renal failure clearly correlates with the degree of renal interstitial fibrosis [1,2]. However, the mechanisms responsible for interstitial changes associated with glomerular disease are not fully understood. Because human tubular epithelial cells are an important source of various cytokines, chemokines, growth factors, adhesion molecules and extracellular matrix components that respond to inflammatory stimuli, they are thought to play an important role in the formation of interstitial lesions [3,4]. In glomerular injury, various cytokines and growth factors can be expressed and excreted into tubular fluid. Here, these agents are bioactive and able to stimulate tubular epithelial cells [5].

Thrombin, which is generated during activation of the coagulation cascade, has multiple biological functions in addition to its role in haemostasis [6]. Intraglomerular fibrin deposition is observed in both human and experimental glomerular diseases. Furthermore, thrombin has been detected in the urine of patients with glomerulonephritis [7], and proximal...
Fibronectin production by proximal tubular epithelial cells

Subjects and methods

Cell culture

Human PTECs in culture were kindly provided by Dr M. R. Daha (Leiden University Medical Center, Leiden, The Netherlands). Primary culture of PTEC was obtained from a donor transplant kidney unsuitable for transplantation, and all experiments were performed using cells from passages 3–5. Methods for PTEC culture have been described in detail elsewhere [9,10]. These cells satisfied criteria determined previously for PTEC, including polygonal or cobblestone-shaped morphology, positive immunofluorescence staining of cytokeratin, epithelial membrane antigen and adenosine-deaminase binding protein [9,10]. The consistent demonstration of cytokeratin expression by these cells indicates exclusion of fibroblast contamination. PTEC were cultured in K-1 media, with a 1:1 mixture of Dulbecco’s modified Eagle’s minimum essential medium (DMEM), and Ham’s nutrient mixture F-12 (both from Gibco Laboratories, Grand Island, NY, USA), supplemented with 2% Nu serum, ITS premix (I, insulin; T, transferrin; S, selenium; both from Becton Dickinson Labware, Bedford, MA, USA), hydrocortisone, tri-iodo thyronine and epidermal growth factor (all from Sigma, St Louis, MO, USA) at 37 °C in 5% CO₂.

FN assay

PTEC were cultured in 12-well plates (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA). When cells were grown to confluence, they were washed twice with Hank’s balanced salt solution (Gibco) and incubated for 24–72 h in the presence or absence of 5.0 U/ml human α-thrombin (Sigma). Culture supernatants were harvested at 24, 48 or 72 h. To examine the dose-dependent effects of thrombin, PTEC were also cultured with various concentrations of thrombin for 72 h. After centrifugation to remove cell debris, these culture supernatants were stored at −70 °C until use. Cells were lysed with 1 N NaOH. The cell lysate was subjected to the Lowry method to determine protein content in each well. To examine whether thrombin inhibitors could inhibit the effects of thrombin on FN secretion by PTEC, cells were incubated with 5.0 U/ml thrombin together with antithrombin III (sigma), 10 U/ml of recombinant hirudine (American Diagnostica, Greenwich, CT, USA) or 1 µM of argatroban for 72 h.

The concentrations of FN in culture supernatants were quantified using a commercially available enzyme-linked immunosorbent assay (ELISA) kit for human FN (ANGIOPHARM, O’Fallon, MO, USA) according to the manufacturer’s instructions. The amount of FN was expressed as microgram per 10 µg of total protein.

To examine whether thrombin-induced FN production in PTEC is mediated by transforming growth factor-β (TGF-β), confluent PTEC grown in 12-well plates were cultured with serum-free medium containing 5.0 U/ml thrombin together with 10 µg/ml neutralizing antibody against human TGF-β (R&D Systems Inc., Minneapolis, MN, USA). After 72 h of incubation, culture supernatants were subjected to FN ELISA.

RNA extraction and semi-quantitative reverse transcriptase-polymerase chain reaction (RT–PCR)

Confluent PTEC, grown in 25-cm² flasks (Becton Dickinson), were cultured with 5.0 U/ml of thrombin for 4–8 h. Total cellular RNA was extracted from PTEC using RNA-Bee (Tel-Test, Friendswood, TX, USA) according to manufacturer’s description. The first-strand cDNA was synthesized from 5 µg of total RNA using a RT–PCR kit (Stratagene, La Jolla, CA, USA) as described previously [11]. The cDNA was amplified by PCR using specific primers for human FN (sense, 5'-TGTCGACTTCCCCCATCTAT-3'; antisense, 5'-ATCC CACTGTACCCTGAG-3', yielding a product of 199 bp) and for β-actin (sense, 5'-CTTATAGGGCAAAACCAGAG-3'; antisense, GTCCCAGCGC4 CCGTTCAG-3', yielding a product of 219 bp). All primers were purchased from Funakoshi (Tokyo, Japan). Each PCR amplification was performed in a total volume of 100 µl containing 2 µl of cDNA solution, 10 µl of reaction buffer (500 mM KCl, 100 mM Tris–HCl pH 8.3, 15 mM MgCl₂ and 0.001% gelatine), 8 µl of 2.5 mmol/l dNTP mix (final concentration was 200 µmol/l of dATP, dCTP, dGTP and dTTP), 5 µl of sense and antisense primer (final concentration was 50 µM each), 69.5 µl of sterile water and 0.5 µl of Tag DNA polymerase (2.5 U). The cDNA was amplified by PCR in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT, USA). Amplification was started with 5 min of incubation at 95 °C, followed by denaturing for 45 s at 94 °C, annealing for 45 s at 55 °C for FN and 66 °C for β-actin, and extension for 45 s at 72 °C. The last extension was performed at 72 °C for 7 min. Amplification was carried out at 28 cycles for FN and at 30 cycles for β-actin. Our pilot experiments confirmed that these cycles were at the exponential phases of amplification. The PCR products were electrophoresed on a 2% agarose gel containing 5 µg/ml of ethidium bromide with size markers (φX174/HincII digest, Toyobo, Tokyo, Japan). Band intensities were determined by densitometric analysis using NIH Image version 1.54 (NIH, Bethesda, MD, USA).

Assay for TGF-β

PTEC, grown to confluence in 12-well plates, were cultured with serum-free medium with or without 5.0 U/ml of thrombin for 24, 48 or 72 h. Culture supernatants and cell lysate were collected as described above.

The concentration of TGF-β in the culture supernatants was measured with commercial sandwich ELISA for human TGF-β₁ (R&D Systems, Inc.). Before assay, HCl (1 N) was added to samples to a 1:10 dilution for 10 min to activate the latent form of TGF-β, and samples were then neutralized with 1.2 N NaOH/0.5 M HEPES. Results are expressed as picograms of TGF-β per 10 µg of total protein.
Assay for matrix metalloproteinase-2 and its inhibitors

To examine the effects of thrombin on matrix metabolism in PTEC, the levels of matrix metalloproteinase-2 (MMP-2) and tissue inhibitor of matrix metalloproteinase-1 and -2 (TIMP-1 and 2) in PTEC culture supernatants were quantified after a 72-h incubation using a commercially available enzyme immunoassay (EIA) kit for MMP-2 and TIMP-1 and -2, respectively, followed according to the manufacturer's protocol (Fuji Yakuhin Inc., Takaoka, Japan).

Zymography

Metalloproteinase expression was also assayed using gelatin zymography as described previously [12]. Confluent PTEC in 75-cm² culture flasks (Becton Dickinson) were incubated with medium alone or medium containing 5 U/ml of thrombin. After 72-h incubation, culture supernatants were harvested. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed on a vertical slab gel containing 7.5% acrylamide and 1 mg/ml gelatin (Difco Laboratories, Detroit, MI, USA). Culture supernatant and pre-stained protein molecular weight standards (Bethesda Research Laboratories, Gaithersburg, MD, USA) were electrophoresed simultaneously to determine molecular weight. After washing with 50 mM Tris–HCl pH 8.0, containing 50 mM NaCl, 10 mM CaCl₂ and 0.05% Brij 35 (Sigma, St Louis, MO, USA), the gels were incubated overnight at 37°C with the same solution. For visualization of the gelatinolytic bands, the gels were stained with 0.1% Coomassie Brilliant Blue. Clear bands on a dark background indicated the presence of proteolytic enzymes.

Statistical analysis

All data are expressed as mean ± 1 SD. Statistical analysis was performed with unpaired t-tests for comparisons of two groups and analysis of variance (ANOVA) for multiple group comparisons. P < 0.05 was considered to be significant.

Results

Time course of FN secretion by PTEC is shown in Figure 1. Secretion of FN was increased in a time-dependent manner in both control and thrombin (5.0 U/ml) stimulated cells. Levels of FN after 24 h were not different between stimulated and unstimulated cells. However, after 48 and 72 h of culture, thrombin stimulated a significantly greater FN secretion compared with controls (48 h: 11.6 ± 4.9 μg/10 μg protein in control and 47.1 ± 21.2 μg/10 μg protein in thrombin-stimulated cells, P < 0.05; 72 h: 15.5 ± 1.6 μg/10 μg protein in control and 63.5 ± 12.7 μg/10 μg protein in thrombin-stimulated cells, P < 0.001).

Figure 2 shows the effects of three doses of thrombin on FN secretion by PTEC. Whereas thrombin at 2.5 and 5.0 U/ml increased FN secretion in PTEC, the 0.5 U/ml dose had no effect.

Figure 3 illustrates the time course of TGF-β secretion by PTEC. Thrombin augmented secretion of TGF-β at 24 h after stimulation. This augmentation of
TGF-β secretion increased further at 48 and 72 h
(24 h: 0.91 ± 0.74 pg/10 μg protein in control and
3.54 ± 1.27 pg/10 μg protein in thrombin-stimulated
cells, P < 0.02; 48 h: 1.49 ± 0.22 pg/10 μg protein in
control and 8.74 ± 2.56 pg/10 μg protein in thrombin-
stimulated cells, P < 0.05; 72 h: 2.59 ± 0.60 pg/10 μg
protein in control and 19.07 ± 5.38 pg/10 μg protein
in thrombin-stimulated cells, P < 0.01). The effects
of three doses of thrombin on TGF-β secretion by PTEC
are shown in Figure 4. Although thrombin at 0.5 U/ml
did not affect TGF-β in PTEC, doses at 2.5 U/ml
causel increased secretion.

Anti-human TGF-β rabbit IgG (10 μg/ml) abolished
thrombin-stimulated FN secretion in PTEC (P < 0.05),
whereas normal rabbit IgG had no effect on thrombin-
stimulated FN secretion (Figure 5).

Thrombin also stimulated mRNA expression of FN
in PTEC. In semi-quantitative RT–PCR, thrombin
augmented FN mRNA expression by 2-fold after 8 h,
but did not affect FN gene expression after 4 h (Figure
6A). Whereas the expression of thrombin-stimulated
FN mRNA was attenuated by anti-human TGF-β
rabbit IgG (Figure 6B), there was no attenuation with
control rabbit IgG (data not shown).

Effects of thrombin inhibitors on FN secretion in
PTEC are shown in Figures 7 and 8. Thrombin-induced
stimulation of FN secretion in PTEC was reduced by
antithrombin III, an endogenous inhibitor of heparin
(Figure 7). The direct inhibitors of thrombin, hirudin
(10 U/ml) and argatroban (1 μM), also inhibited FN
secretion (Figure 8).

The effects of thrombin on the secretion of MMP-2,
TIMP-1 and TIMP-2 are shown in Figure 9. Thrombin
significantly increased the secretion of TIMP-1
(P < 0.01) and -2 (P < 0.001) in PTEC (Figure 9A
and B). However, effects of thrombin on MMP-2
secretion in PTEC were not statistically significant
(Figure 9C). Although culture supernatants of both

Fig. 4. Effect of various concentrations of thrombin on TGF-β
secretion by PTEC. PTEC were incubated with different concentra-
tions of thrombin. After 72 h, medium was collected and subjected
to TGF-β ELISA. Values are means ± SD from three wells, and
representative data from one of two experiments are shown.

Fig. 5. Inhibitory effect of anti-TGF-β antibody on thrombin
stimulated FN secretion in PTEC. PTEC were incubated with
medium alone (control), medium with thrombin (5.0 U/ml), with
thrombin (5.0 U/ml) + anti-human TGF-β rabbit IgG (10 μg/ml) or
with thrombin (5.0 U/ml) + normal rabbit IgG. After 72-h incuba-
tion, FN in the culture supernatants was quantified by ELISA.
Values are means ± SD from three wells. Data shown are repre-
sentative of two experiments.

Fig. 6. Semi-quantitative RT–PCR analysis of FN mRNA expres-
sion by PTEC. (A) Effects of thrombin on FN mRNA expression
by PTEC. PTEC were cultured with or without 5.0 U/ml of thrombin 4
or 8 h and were assayed for the expression of FN and β-actin by RT–
PCR. One representative experiment of the three is shown. (B)
Inhibition of thrombin stimulated FN mRNA expression by anti-
TGF-β antibody. PTEC cultured with medium alone (control),
medium with thrombin (5.0 U/ml) or with thrombin (5.0 U/
ml) + anti-human TGF-β rabbit IgG (10 μg/ml) for 8 h were
subjected to RT–PCR to examine the mRNA expression of FN and
β-actin. One representative experiment of three is shown.
control and thrombin-stimulated PTEC revealed gelatin digestive activity at the 72 kDa size, which is compatible with MMP-2, zymography failed to reveal collagenases of other molecular sizes. Zymography also revealed that expression of MMP-2 was not enhanced by thrombin when compared with controls (Figure 9D).

Discussion

Accumulation of extracellular matrix protein in the glomerulus and tubulointerstitial area is a hallmark of progressive renal disease [1]. It is widely acknowledged that interstitial lesions such as fibrosis have prognostic significance in many forms of glomerulonephritis including IgA nephropathy, the most common form of chronic glomerulonephritis [2]. Although the pathogenetic mechanisms of interstitial lesions associated with glomerular diseases are not fully understood, growth factors and cytokines generated by glomerular inflammation and filtered into the tubular lumen may be involved in the tubulointerstitial damage [4].

It is known that thrombin has various biological effects in addition to its role in haemostasis. For example, thrombin stimulates mitogenesis in various types of cells [6]. In addition, we have reported that thrombin up-regulates mesangial cell collagen synthesis [12,13]. Although effects of thrombin on renal tubular cells have yet to be determined, PTECs are likely to be a potential target because these cells are known to express thrombin receptors. Grandaliano et al. [8] demonstrated in human proximal tubular cells, both in vivo and in vitro, that the main thrombin receptor is the protease-activated receptor-1 (PAR-1). Increased intraglomerular procoagulant activity and fibrin formation have been demonstrated in severe forms of glomerular injury including crescentic glomerulonephritis [14]. In addition, a direct thrombin inhibitor attenuated glomerular damage and caused reduced fibrin deposition in mice with crescentic glomerulonephritis [14]. Moreover, biologically active thrombin has been detected in the urine from patients with mesangial proliferative glomerulonephritis [7]. The data suggest that thrombin is an important mediator that links glomerular inflammation to tubulointerstitial lesion. We therefore hypothesized that thrombin is able to stimulate production of extracellular matrix protein by PTEC.

In the present study, we found that thrombin stimulated the secretion and production of FN, which is an important extracellular matrix component in both the glomerulus and interstitium [15]. This effect seems to be mediated by TGF-β as thrombin-induced up-regulation of FN secretion by PTEC was preceded by increased TGF-α secretion and because thrombin-stimulated mRNA expression of FN was inhibited by anti-TGF-β antibody. Thrombin also stimulated secretion of TIMP-1 and -2. Although thrombin tended to stimulate MMP-2 secretion, MMP expression shown by zymography did not differ between control and thrombin-treated PTEC. Thus, the effect of thrombin on FN production appears to be more dominant than its effect on the balance between TIMP and MMP. This observation is compatible with our previous results that increased synthesis of type IV collagen and TIMP-1 caused by thrombin were not associated with increases in MMP-2 synthesis in human mesangial cells [12]. Taken together, the present findings suggest that thrombin stimulates the production and accumulation of FN in the renal interstitium by increasing production of FN through a TGF-β-dependent mechanism without stimulating matrix protein degradation.

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Fig. 7. Inhibition of thrombin-stimulated FN secretion in PTEC by anti-thrombin III (AT-III). PTEC were incubated with medium alone (control), medium with thrombin (5.0 U/ml) or medium with thrombin (5.0 U/ml) + AT-III (5.0 U/ml) for 72 h and then culture supernatants were subjected to FN ELISA. Values are means ± SD from three wells. Data shown are representative of two experiments.

Fig. 8. Effects of thrombin inhibitors on FN secretion stimulated by thrombin. PTEC were cultured with medium alone (control), medium with thrombin (5.0 U/ml), with thrombin (5.0 U/ml) + hirudine (10 U/ml) or with thrombin (5.0 U/ml) + argatroban (1 μM). After 72 h incubation, amounts of FN in the culture supernatants were quantified by ELISA. Values are means ± SD from three wells. Data shown are representative of two experiments.
was decreased through multiple passages. Thus, the influence of several passages may explain the differences observed in Figures 1 and 2 and in Figures 3 and 4; however, we were not otherwise able to identify the reason these discrepancies.

TGF-β plays a central role in fibrosis in various tissues including kidney [16]. We have reported previously that thrombin stimulates type IV collagen production by mesangial cells through a TGF-β-dependent pathway [13], and that thrombin enhances production of TGF-β by glomerular epithelial cells [11]. Therefore, thrombin may play a central role in the accumulation of extracellular matrix in both the glomerulus and interstitial areas in renal diseases.

A recent report has shown that thrombin stimulates monocyte chemoattractant protein-1 (MCP-1) synthesis in PTEC [8]. MCP-1 is a chemoattractant involved in monocyte recruitment. Thrombin may contribute to the formation of tubulointerstitial lesions by up-regulating PTEC production of MCP-1 as well as FN, and inhibition of thrombin may therefore be essential for the prevention of tubulointerstitial inflammation and fibrosis. For this reason, we also examined the effects of thrombin inhibitors on the thrombin-stimulated FN secretion in PTEC. We found that direct thrombin inhibitors such as hirudin, argatroban and antithrombin III negatively modulated thrombin-stimulated FN secretion. Antithrombin III is widely recognized as an endogenous inhibitor of thrombin, and its antimitogenic effect on mesangial cells was reported by Pahl et al. [17]. Hirudin, originally isolated from the salivary glands of the medical leech Hirudo medicinalis, exerts an inhibitory action on thrombin by forming tight and irreversible bonds to thrombin, and argatroban, a synthetic thrombin inhibitor, interacts with the active site of thrombin [18]. These inhibitors have been used in clinical settings, such as for cardiovascular diseases, with favourable or promising results [18]. Recently Cunningham et al. [14] demonstrated that hirudin ameliorated renal injury in experimental crescentic glomerulonephritis, a pathology in which thrombin is known to play an important role. In another report,
argatroban was shown to induce a change of vascular smooth muscle cells from the contractile to the synthetic phenotype [19]. Further studies will be needed to clarify the contribution of thrombin to the development of human renal diseases and to confirm whether these thrombin inhibitors have therapeutic potential.

Most of the cellular effects induced by thrombin are known to be mediated by G-protein-coupled receptors termed PARs [6]. The present findings did not provide direct evidence for involvement of PARs in the stimulatory effect of thrombin on FN production in PTEC. However, the inhibitory effect of hirudin on thrombin-stimulated FN secretion suggests that stimulation by thrombin is mediated via PARs as others have indicated previously that a hirudin peptide caused impaired binding of thrombin to PAR-1 [20].

In conclusion, the present study indicates that thrombin may contribute to the development of tubulointerstitial fibrosis by stimulating FN production in PTEC. The stimulatory effect of thrombin on FN production is mediated, at least in part, by TGF-β. Our results point to a role for thrombin as a key molecule that may link glomerular injury to tubulointerstitial lesions.

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