Thrombin stimulates fibroblast procollagen production via proteolytic activation of protease-activated receptor 1

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Thrombin is a multifunctional serine protease that has a crucial role in blood coagulation. It is also a potent mesenchymal cell mitogen and chemoattractant and might therefore have an important role in the recruitment and local proliferation of mesenchymal cells at sites of tissue injury. We hypothesized that thrombin might also affect the deposition of connective tissue proteins at these sites by directly stimulating fibroblast procollagen production. To address this hypothesis, the effect of thrombin on procollagen production and gene expression by human foetal lung fibroblasts was assessed over 48 h. Thrombin stimulated procollagen production at concentrations of 1 nM and above, with maximal increases of between 60 % and 117 % at 10 nM thrombin. These effects of thrombin were, at least in part, due to increased steady-state levels of α_1(I) procollagen mRNA. They could furthermore be reproduced with thrombin receptor-activating peptides for the protease-activated receptor 1 (PAR-1) and were completely abolished when thrombin was rendered proteolytically inactive with the specific inhibitors D-Phe-Pro-ArgCH_2Cl and hirudin, indicating that thrombin is mediating these effects via the proteolytic activation of PAR-1. These results suggest that thrombin might influence the deposition of connective tissue proteins during normal wound healing and the development of tissue fibrosis by stimulating fibroblast procollagen production.

INTRODUCTION

Thrombin is a multifunctional serine protease that has a central role in blood coagulation by converting soluble fibrinogen to an insoluble fibrin clot, by promoting platelet aggregation and by converting a number of coagulation factors to their active forms (reviewed in [1,2]). In addition to these procoagulant effects, thrombin also influences a number of other biological responses that might have important roles in tissue repair and wound healing. It is a potent activator of endothelial cells [3–5], acts as a chemoattractant for inflammatory cells [6–8] and is a mitogen and chemoattractant for fibroblasts [9–11] and vascular smooth muscle cells [11–13], so that thrombin might also be involved in the initiation and maintenance of inflammation, as well as the recruitment and proliferation of mesenchymal cells at sites of tissue injury. Most of thrombin's cellular effects are mediated via a specific and widely expressed G-protein-linked receptor, which is activated by proteolytic cleavage of the N-terminal extracellular domain rather than ligand binding [14]. The newly generated N-terminus then acts as a tethered ligand and interacts with the receptor to activate subsequent second messenger events. This receptor was the first of the two currently known receptors activated by thrombin in this unique manner to be fully characterized, and is now commonly referred to as protease-activated receptor 1 (PAR-1).

In addition to being present in acute wounds, thrombin can also be chronically generated at sites of tissue injury, either by the release of the sequestered protease from the provisional matrix [15] or by the continued activation of the coagulation cascade in the presence of an activated endothelium. Thrombin can also remain functional and available for cellular interactions when bound to the extracellular matrix [16] and has been implicated in a number of pathological conditions associated with proliferative responses and excess deposition of matrix proteins, including atherosclerosis [17], restenosis after angioplasty [18], and glomerulonephritis [19]. Thrombin has also been proposed to have a role in the development of vascular remodelling associated with pulmonary hypertension [20], as well as in acute lung injury [21]. More recently, we and others have shown that thrombin levels are increased in the lungs of patients with systemic sclerosis [22,23] and in animal models of this disease [24] and that thrombin is a major fibroblast mitogen present in bronchoalveolar lavage fluid obtained from these patients [22].

The proliferative and chemotactic properties of thrombin have been well characterized. However, the direct effect of thrombin on fibroblast procollagen production has received very little attention [25,26] and the potential mechanism involved has not been addressed to date. The aim of this study was to examine the hypothesis that thrombin has a role in the deposition of connective tissue proteins after tissue injury by directly influencing fibroblast procollagen production. The results obtained show that thrombin is a potent promoter of fibroblast procollagen production and that these effects are mediated via proteolytic activation of the thrombin receptor PAR-1 and increased procollagen mRNA levels. These results represent a significant advance in our understanding of the modulation of fibroblast function by thrombin and suggest that thrombin might influence the deposition of connective tissue proteins during both normal wound healing and the development of tissue fibrosis by stimulating fibroblast procollagen production.

Abbreviations used: DMEM, sterile Dulbecco's modified Eagle's medium; Nbf-Cl, 4-chloro-7-nitrobenzofurazan; PAR-1, protease-activated receptor 1; PPACK, D-Phe-Pro-ArgCH_2Cl; TGF, transforming growth factor; TRAP, thrombin receptor-activating peptide.

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EXPERIMENTAL

Materials

Human foetal lung fibroblasts (HFL1) were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Sterile Dulbecco's modified Eagle's medium (DMEM), tissue culture plates and TRIzol were from Gibco BRL (Paisley, Renfrewshire, U.K.). Thrombin (purified from human plasma, catalogue no. T4393) and hirudin (catalogue no. H0393) were from Sigma (Poole, Dorset, U.K.). D-Phe-Pro-ArgCH$_2$Cl (PPACK) was from Calbiochem-Novabiochem (Beeston, Nottingham, Notts., U.K.). Thrombin receptor-activating peptides (TRAPs) corresponding to the sequence SFLLRNP were generated by Dr. R. P. Mecham (University of Washington Medical School, St. Louis, MO, U.S.A.).

The cDNA probe for type I procollagen α_1(I) (probe Hf677) was kindly provided Dr. M. L. Chu (Thomas Jefferson University, Philadelphia, PA, U.S.A.), and the oligonucleotide for human 28 S rRNA was obtained commercially from Clontech (Palo Alto, CA, U.S.A.).

Fibroblast culture

Human foetal lung fibroblasts were maintained in DMEM supplemented with penicillin (100 i.u./ml), streptomycin (100 i.u./ml) and 10 % (v/v) newborn calf serum in a humidified air/CO_2 (19:1) atmosphere. Cells were routinely passaged every 6 or 7 days and tested for mycoplasma infection; cells used for experiments were between passages 14 and 25.

Determination of procollagen metabolism

Cell culture conditions

Cells were seeded at 10^5 cells/ml in 2.4 cm diameter plates (1 ml per well) and grown to confluence for approx. 5 days. Under these conditions, the cell number at confluence remained constant at approx. $(3.11 \pm 0.06) \times 10^5$ cells per well. On reaching visual confluence, cells were left for 24 h and the medium was replaced with DMEM supplemented with 50 μg/ml ascorbic acid and 0.2 mM proline without serum. After a further 24 h the medium was replaced with identical control medium or medium containing thrombin at various concentrations between 1 and 25 nM. Identical cell cultures were treated in parallel to allow the assessment of cell number at the end of the incubation period.

Sample processing

At the end of each incubation period the cell layer and medium were harvested together as described previously [27]. In brief, the cell layer was scraped into the culture medium and the contents of each well were aspirated. Wells were washed with PBS, which in turn was combined with the initial aspirate. Proteins were precipitated by the addition of ethanol to a final concentration of 67 % (v/v) at 4 °C overnight. Precipitated proteins (ethanol-insoluble fraction) were separated from free amino acids (ethanol-soluble fraction) by filtration through a 0.45 mm pore-size filter (type HV; Millipore, Watford, Herts., U.K.) with a vacuum filtration unit (Millipore). The supernatant was retained and the filter was washed twice with 67 % (v/v) ethanol. Supernatants were evaporated to dryness on a Dri-Block sample concentrator; both filters, with adherent proteins (ethanol-insoluble fraction) and dried supernatant material (ethanol-soluble fraction), were hydrolysed overnight in 6 M HCl at 110 °C. Hydrolysates were mixed with activated charcoal and filtered through an acid-resistant 0.65 mm pore-size filter (type DA; Millipore) before chromatography.

Measurement of hydroxyproline in sample hydrolysates

Hydroxyproline in both ethanol-insoluble and ethanol-soluble fractions was isolated and measured by reverse-phase HPLC after derivatization with 4-chloro-7-nitrobenzofurazan (Nbf-Cl) (Sigma) with a Beckman System Gold (Beckman, High Wycombe, Bucks., U.K.) coupled to an autosampler, as described previously [28]. In brief, an aliquot of each hydrolysate was evaporated to dryness with a centrifugal vacuum concentrator. The dried residue was redissolved in HPLC-grade water buffered with 0.4 M potassium tetraborate (Sigma) and reacted with 36 mM Nbf-Cl in methanol. Samples were protected from light with aluminium foil and incubated at 37 °C for 20 min. The reaction was stopped by the addition of 1.5 M HCl; 167 mM sodium acetate in 26 % (v/v) acetonitrile was then added. Samples were filtered with an HPLC low-dead-volume filter (pore size 0.22 mm, type GV; Millipore) and a 100 μl aliquot was injected on the HPLC column (LichroCART LiChrospher 250 mm × 4 mm, 5 mm particle size, RP-18; BDH/Merck) coupled directly to a precolumn (LiChrosorb, 4 mm × 4 mm, 5 mm particle size, 100 RP-18; BDH/Merck) and then eluted with an acetonitrile gradient [29].

The hydroxyproline content of each sample was determined by comparing peak areas of samples from the chromatogram with those generated from standard solutions, derivatized and separated under identical conditions. Hydroxyproline measured in the ethanol-insoluble fraction was taken as an index of procollagen production, whereas hydroxyproline in the ethanol-soluble fraction represents hydroxyproline derived from procollagen synthesized and subsequently degraded during the culture period. The rate of procollagen synthesis was obtained from the combined values for ethanol-soluble and ethanol-insoluble fractions. Procollagen production and synthesis rates are expressed as nmol of hydroxyproline per well. To correct for the amount of procollagen already present in the cell layer at the onset of the incubation period, zero-time hydroxyproline measurements for both ethanol-insoluble and ethanol-soluble fractions were made on six parallel culture wells and subtracted from all sample values. Zero-time wells were treated in a manner identical with that for other wells, except that at the time of incubation the original medium was removed and replaced with fresh medium into which the cell layer was scraped immediately. The proportion of newly synthesized procollagen degraded was calculated by dividing the amount of hydroyxproline in newly synthesized procollagen degraded by the total amount of hydroxyproline in procollagen.

Thrombin blocking experiments

For thrombin blocking experiments, thrombin, PPACK and hirudin were resuspended in DMEM; thrombin–inhibitor complex formation proceeded for 1 h at 37 °C before addition to cell cultures. PPACK and thrombin were added at final equimolar concentrations of 10 nM, whereas hirudin and thrombin were added at 50 nM and 10 nM respectively.

Northern analysis of procollagen mRNA levels

For Northern analysis of procollagen mRNA levels, cells were grown to confluence in 10 cm diameter tissue culture plates and exposed to 10 nM thrombin for various durations up to 48 h under identical conditions to those used for procollagen metab-

olism experiments. At the end of each incubation period, total RNA was extracted from the cell layer with TRIzol reagent in accordance with the manufacturer's instructions. Total RNA (5 μg) was added to an equal volume of RNA loading buffer (5 Prime-3 Prime, Boulder, CO, U.S.A.) and heated to 65 °C for 10 min, before being loaded on a 1 % (w/v) agarose/formaldehyde gel run at a constant 80 V for 2 h. RNA was transferred to nylon membranes (Hybond N; Amersham International, Little Chalfont, Bucks., U.K.) by Northern transfer and fixed by UV cross-linking.

For assessment of procollagen mRNA levels, membranes were hybridized overnight at 42 °C in standard pre-hybridization solution containing 50 % formamide with a [^{32}P]dCTP-labelled cDNA probe for α_1(I) procollagen (probe Hf677), generated with an oligolabelling kit (Pharmacia Biotech, Piscataway, NJ, U.S.A.). At the end of the hybridization, filters were washed twice at low (2 × SSC/0.1 % SDS for 10 min at room temperature; SSC is 0.15 M NaCl/0.015 M sodium citrate) and once at high stringency (0.1 × SSC/0.1 % SDS for 10 min at 65 °C). Membranes were exposed to a phosphorimage screen (Kodak) for 30 min to 2 h and mRNA levels were quantified by phosphorimage analysis with a Storm 860 Phosphorimage Analyser (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

For normalization, membranes were stripped by being boiled at high stringency and re-probed overnight at 61 °C in a solution of 5 × SSPE [0.15 M NaCl/10 mM sodium phosphate (pH 7.4)/1 mM EDTA]/2 × Denhardts/0.1 % SDS/75 μg/ml salmon-sperm DNA, with a ^{32}P-5′-end-labelled human 28 S oligonucleotide probe (Clontech Laboratories) generated with a T4 kinase labelling kit (Gibco BRL). Membranes were washed at low stringency (2 × SSPE/0.1 % SDS) at room temperature for 1 h, followed by a high-stringency wash (5 × SSPE/0.1 % SDS) at 61 °C for 20 min and exposed to a phosphorimage screen for 20 min for densitometric analysis.

Statistical analysis

All numerical results are presented as means ± S.E.M. from six replicate cultures, unless otherwise indicated. Statistical evaluation was performed with an unpaired Student's *t* test and, where appropriate, linear regression. The mean values of various parameters were taken to be significantly different when the probability of the differences of that magnitude, assuming the null hypothesis to be correct, fell below 5 % (i.e. $P < 0.05$).

RESULTS

Effect of thrombin on fibroblast procollagen synthesis and production

Figure 1 shows the results of a representative experiment from a total of three separate experiments for the effect of increasing concentrations of thrombin on fibroblast procollagen production (Figure 1a) and synthesis (Figure 1b) and on the proportion of newly synthesized procollagen degraded (Figure 1c) at the end of a 48 h incubation period. Thrombin stimulated the production of procollagen at all concentrations of 1 nM and above, with values significantly increased by 20 ± 3 %, 60 ± 1 % and 47 ± 8 % at 1, 10 and 25 nM thrombin respectively (all $P < 0.01$). The trend for procollagen synthesis was similar, with maximal stimulatory effects of 77 ± 1 % at 10 nM thrombin ($P < 0.01$). The amount of newly synthesized procollagen degraded was also increased from 10 nM thrombin and above, with values significantly increased by 73 ± 7 % and 61 ± 4 % (both $P < 0.01$) relative to media controls at 10 and 25 nM thrombin respectively, but when expressed as a proportion of procollagen synthesis, these proportions were unchanged in thrombin-stimulated cells (Figure 1c). For comparison, an optimal concentration of transforming growth factor β_1 (TGFβ_1) (10 pM) stimulated procollagen synthesis and production by 56 ± 6 % and 67 ± 8 % (both $P < 0.01$) above media controls respectively, and decreased the proportion of newly synthesized procollagen degraded from 15 ± 1 % to 10 ± 1 % ($P < 0.01$). At all doses examined, thrombin and TGFβ_1 had no effect on cell number at the end of the 48 h incubation (results not shown).

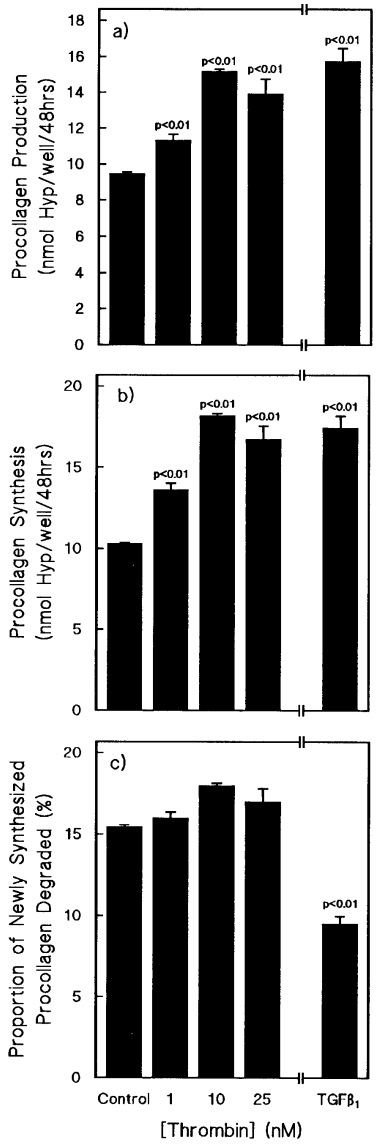

Figure 1 Effect of thrombin on fibroblast procollagen metabolism

The figure shows the effect of increasing concentrations of thrombin on procollagen production (**a**), procollagen synthesis (**b**) and the proportion of newly synthesized procollagen degraded (**c**) over a 48 h incubation period. Procollagen production rates represent hydroxyproline in intact procollagen, whereas procollagen synthesis rates represent hydroxyproline in both intact procollagen and procollagen synthesized and subsequently degraded during the incubation period. The proportion of newly synthesized procollagen degraded is derived by dividing the amount of hydroxyproline in newly synthesized procollagen degraded by the total amount of hydroxyproline in procollagen synthesized. All values have been corrected for hydroxyproline present in the cell layer at the onset of the 48 h incubation. These values were 0.75 ± 0.02 nmol of hydroxyproline in procollegen per well for ethanol-insoluble proteins and 0.65 ± 0.10 nmol hydroxyproline per well for ethanol-soluble proteins. Each value is the mean ± S.E.M. for six replicate cultures.

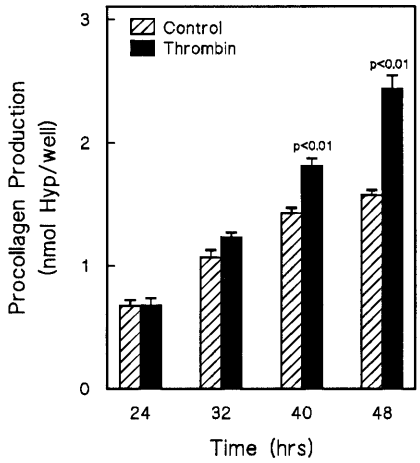

Figure 2 Effect of thrombin on fibroblast procollagen production over time

The figure compares procollagen production measured at various time points by cells exposed to an optimal concentration of thrombin (10 nM) with that of cells exposed to culture medium alone. Hydroxyproline values are corrected for hydroxyproline in the cell layer at the onset of the exposure period (0.70 ± 0.01 nmol of hydroxyproline per well) and are means $\pm$ S.E.M. for six replicate cultures.

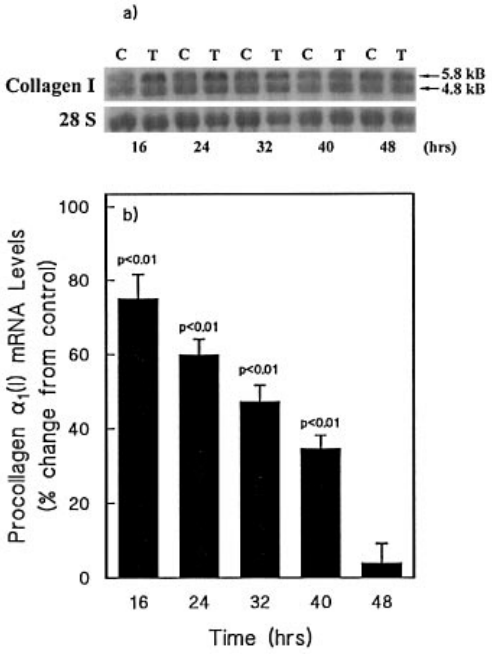

Figure 3 Effect of thrombin on fibroblast α1(I) procollagen mRNA levels over time

(**a**) Representative Northern blot from three separate experiments performed. Total RNA was extracted from confluent cultures exposed to control medium or to 10 nM thrombin for various durations up to 48 h. The RNA was fractionated by agarose gel electrophoresis and hybridized with a cDNA probe for α_1(I) procollagen. Also shown is the Northern blot obtained on hybridization with a gene-specific oligonucleotide probe for human 28 S rRNA. C and T denote media control and thrombin-treated cells respectively. (**b**) Data expressed as percentage changes (means $\pm$ S.E.M.) in procollagen α_1(I) mRNA levels for thrombin-treated cells compared with the corresponding media controls for three replicate cultures at each time point.

Figure 2 shows the effect of an optimal stimulatory concentration of thrombin (10 nM) on fibroblast procollagen production at several time points up to 48 h. For control cells there was a highly significant linear correlation between procollagen production and time ($r = 0.992$, $P < 0.001$), indicating that procollagen was being produced at a constant rate over the incubation period. At 24 and 32 h there was no significant difference in procollagen production between media control and thrombin-treated cells, whereas at 40 and 48 h the production of procollagen by thrombin-treated cells was increased significantly by $27 \pm 4\%$ and $55 \pm 6\%$ (both $P < 0.01$) respectively.

Effect of thrombin on fibroblast procollagen mRNA levels

Figure 3 shows the effect of 10 nM thrombin on procollagen α_1(I) mRNA levels for a representative experiment. At 4 and 8 h, thrombin had no effect on procollagen α_1(I) mRNA levels, but these levels were consistently elevated from 16 h onwards. In the experiment shown, densitometric quantification of the bands obtained by Northern analysis, normalized to 28 S rRNA, showed that procollagen α_1(I) mRNA levels were increased above the corresponding media controls by approx. 78 % at 16 h, remained significantly elevated until least 40 h, and returned to basal levels by 48 h.

Effect of thrombin inhibitors on fibroblast procollagen production

Figure 4 examines whether thrombin proteolytic activity was required in order to mediate the stimulatory effects of thrombin on procollagen production. In these experiments thrombin was incubated with two highly specific and irreversible inhibitors, hirudin (Figure 4a) and PPACK (Figure 4b), before addition to cell cultures. In these experiments, an optimal concentration of thrombin stimulated the production of procollagen by between $63 \pm 2\%$ (Figure 4b) and $117 \pm 6\%$ (Figure 4a) (both $P < 0.01$) above cells grown in medium alone. The stimulatory effects of thrombin were completely blocked when hirudin and PPACK were added at concentrations that had no effect on basal procollagen production.

Effect of TRAP on fibroblast procollagen production

The results shown in Figure 4 raised the possibility that thrombin was exerting its stimulatory effects on procollagen production by proteolytically activating PAR-1. Figure 5 shows results obtained with cells exposed to TRAPs for this receptor. In these experiments, 10 nM thrombin stimulated the production of procollagen by $84 \pm 12\%$ ($P < 0.01$), whereas TRAP stimulated the production of procollagen by $43 \pm 5\%$ and $44 \pm 8\%$ (both $P < 0.01$) at 25 and 100 μM respectively.

DISCUSSION

Effect of thrombin on procollagen production, synthesis and degradation of newly synthesized procollagen

In this paper we report the novel observation that thrombin is a potent promoter of fibroblast procollagen production, with maximal stimulatory effects ranging from 60 % to 117 % with an optimal concentration of thrombin of 10 nM over a 48 h incubation period. We also show that these stimulatory effects are both dose-dependent and time-dependent and occur independently of changes in fibroblast cell number.

In these experiments procollagen production measurements represent the amount of hydroxyproline in intact procollagen secreted into the medium or deposited into the cell layer during the incubation period. We also assessed the effect of thrombin on procollagen synthesis, as well as on the degradation of newly synthesized procollagen, because some stimulatory mediators

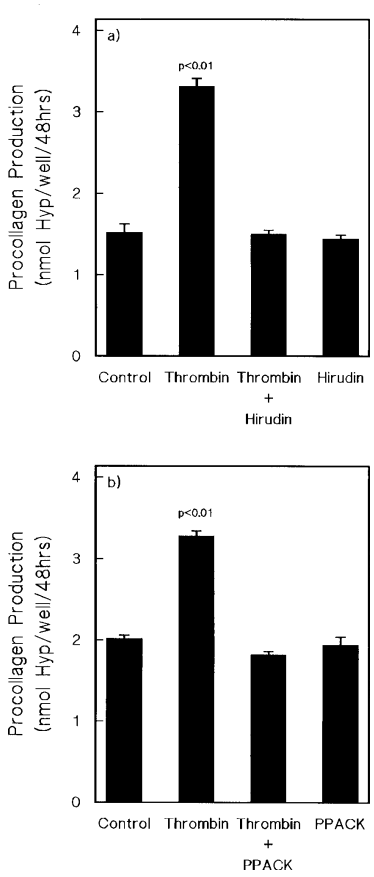

Figure 4 Effect of inhibiting thrombin proteolytic activity on thrombin-induced stimulation of fibroblast procollagen production

The figure shows the effects obtained with hirudin (**a**) and PPACK (**b**). In these experiments an optimal concentration of thrombin (10 nM) was preincubated with hirudin (50 nM) or PPACK (10 nM) at 37 °C for 1 h before addition to cell cultures. Hydroxyproline values are corrected for hydroxyproline present in the cell layer at the onset of the exposure period (0.95 ± 0.01 nmol of hydroxyproline per well) and are means ± S.E.M. for six replicate cultures. The results are representative of three experiments performed.

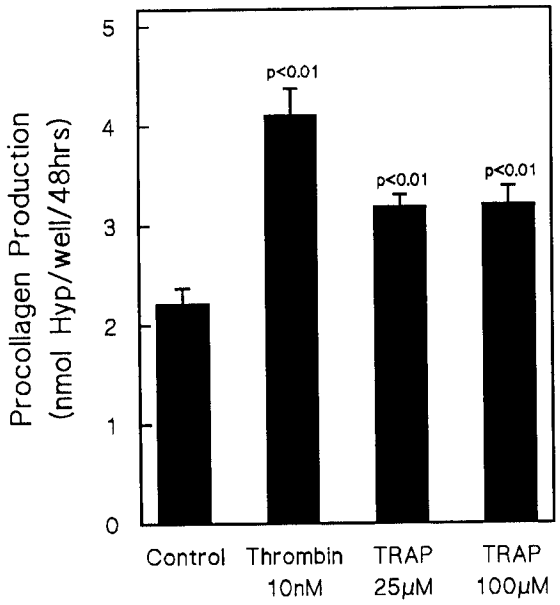

Figure 5 Effect of TRAP on fibroblast procollagen production

Hydroxyproline values are corrected for hydroxyproline present in the cell layer at the onset of the exposure period (0.85 ± 0.01 nmol of hydroxyproline per well) and are means ± S.E.M. for six replicate cultures. The results are representative of three experiments performed.

such as TGFβ_1 have been shown to increase the amount of procollagen produced by decreasing the proportion of newly synthesized procollagen degraded [29]. Degradation of newly synthesized procollagen was assessed by measuring hydroxyproline in the ethanol-soluble protein fraction (after correction for hydroxyproline at the onset of the experiment) because this pool can be taken to represent procollagen that has been synthesized and subsequently degraded intracellularly during the incubation period [27]. Procollagen synthesis rates were calculated by combining the values for hydroxyproline in the ethanol-insoluble and ethanol-soluble protein fractions. The trend obtained with thrombin for procollagen synthesis was similar to that observed for procollagen production. Similarly, degradation of newly synthesized procollagen, expressed as an amount of hydroxyproline, was increased in thrombin-treated cells. As these values were increased in proportion to the values obtained for procollagen synthesis, when expressed as a percentage of proccollagen synthesis, these values were unchanged in thrombin-treated cells, so that, unlike TGFβ_1, thrombin was exerting its stimulatory effects on procollagen production by increasing procollagen synthesis only. However, although thrombin had no effect on the proportion of newly synthesized procollagen degraded, the magnitude of the stimulatory effect obtained for procollagen production with an optimal concentration of thrombin was very similar to that obtained with a maximal concentration of TGFβ_1 with these cells.

In all experiments performed, although thrombin consistently stimulated procollagen production, basal rates of procollagen production obtained with this cell line and their responsiveness to thrombin was variable from one experiment to the next. This did not seem to be related to variations in cell number but might, in part, relate to changes in procollagen production rates at different stages of aging of the cell line *in vitro*, as has been described by others [30]. Furthermore, although attempts were made to keep cell culture conditions and experimental protocol constant, small variations in these parameters might also have contributed to variable basal rates and responsiveness to thrombin.

Experiments performed to examine the mechanism by which thrombin was exerting its stimulatory effects on procollagen production showed that these effects were critically dependent on thrombin proteolytic activity, because the stimulatory effects were completely abrogated when thrombin was complexed with two highly specific and irreversible inhibitors of thrombin proteolytic activity, PPACK and hirudin. In contrast, synthetic TRAPs designed to activate PAR-1 were capable of mimicking the stimulatory effects of thrombin. Taken together, these observations suggest that thrombin was acting, at least in part, via proteolytic activation of PAR-1. The concentrations of TRAP required to elicit a response were at least three orders of magnitude higher than for thrombin; the maximal stimulation obtained was almost 50% lower. Similar differences in potency and efficacy between thrombin and TRAP have been reported for several other thrombin-mediated cellular effects, including its ability to stimulate human platelets [31], mesenchymal cell mitogenesis and signal transduction pathways [32,33]. Several explanations have been proposed for these differences, including

the possibility that the tethered ligand might adopt a more appropriate orientation after thrombin receptor cleavage than free-floating TRAP or that receptor cleavage itself might be necessary to obtain a full response. It is also possible that signals that might be generated by the interaction of thrombin with the receptor in addition to those produced by proteolysis might be required. Finally, these differences have also been interpreted as circumstantial evidence for the existence of another thrombin receptor in addition to PAR-1. The existence of such a protein has now been confirmed by the recent molecular cloning of a second thrombin receptor, termed PAR-3 [34]. This receptor is also activated by proteolytic cleavage but the TRAP peptides used in our experiments are not capable of activating PAR-3 [34]. Our results therefore allow us to conclude that PAR-1 is involved in mediating thrombin's effects on procollagen production, but whether PAR-3 also has a role remains uncertain.

In this study we also began to examine the molecular mechanism by which thrombin is acting on procollagen production after receptor activation and showed that procollagen α_1(I) mRNA levels were increased in cells exposed to thrombin from 16 h onwards. However, we do not yet know whether thrombin is acting in a direct or indirect manner, although the delay required for thrombin to act at the mRNA level would be consistent with an indirect mechanism of action, possibly involving the production of an autocrine mediator. Thrombin-induced cell proliferation has been shown to be mediated via the induction of platelet-derived growth factor in fibroblasts [23] and smooth-muscle cells [35], and basic fibroblast growth factor in endothelial cells [36]. A similar indirect mechanism might also be involved in mediating thrombin's effect on procollagen production.

Relevance to wound healing and human disease

The novel results reported here are consistent with a wider role for thrombin in normal tissue repair and suggest that thrombin, in addition to increasing fibroblast migration and proliferation, might also contribute to the deposition of extracellular matrix proteins at these sites by directly stimulating fibroblast procollagen production. This might be relevant to both acute and chronic situations associated with activation of the coagulation cascade. Thrombin can remain functional when bound to the extracellular matrix or when sequestered within fibrin clots [16], and can therefore remain available for cellular interactions even after haemostasis has occurred. Our results might therefore be particularly pertinent to fibrotic conditions associated with the endothelial leakage of blood proteins, including pulmonary fibrosis associated with adult respiratory distress syndrome and systemic sclerosis, a disease in which we have recently shown that thrombin has an important role as a fibroblast mitogen [22]. Our observations might also be applicable to vascular conditions associated with excess deposition of matrix proteins, including adventitial thickening in pulmonary hypertension, atherosclerosis and restenosis after vascular surgery. Finally, they might also provide a biochemical explanation for the reported observation that the administration of thrombin and TRAPs increases the production of mature collagen without producing abnormally high numbers of fibroblasts during incisional wound healing in experimental animals [37]. In terms of therapeutic interventions for fibrotic conditions, the recent findings from PAR-1 knockout mouse studies by Coughlin and colleagues showing that PAR-1 is critical for fibroblast responses to thrombin, but not for platelets [38], raise the possibility that the pro-fibrotic effects of thrombin might be blocked selectively by targeting PAR-1 without affecting haemostatic responses.

Conclusion

We have shown that thrombin is a potent promoter of fibroblast procollagen production and that these effects are mediated, at least in part, via the proteolytic activation of PAR-1 and increased procollagen mRNA levels. This is, to our knowledge, the first report of a direct receptor-mediated effect of thrombin on procollagen production by cultured fibroblasts and represents a significant advance in our understanding of the modulation of fibroblast function by thrombin. We propose that thrombin might influence the deposition of connective tissue proteins at sites of tissue injury via the activation of PAR-1, and further that PAR-1 might merit validation as a novel target for anti-fibrotic therapy in conditions associated with the extravasation of coagulation proteins.

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