Thrombin Is a Potent Inducer of Connective Tissue Growth Factor Production via Proteolytic Activation of Protease-activated Receptor-1*

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The coagulation protease thrombin plays a critical role in hemostasis and exerts pro-inflammatory and pro-fibrotic effects via proteolytic activation of the major thrombin receptor, protease-activated receptor-1 (PAR-1). Connective tissue growth factor (CTGF) is a novel fibroblast mitogen and also promotes extracellular matrix protein production. It is selectively induced by transforming growth factor β (TGF-β) and is thought to be the autocrine agent responsible for mediating its pro-fibrotic effects. CTGF is up-regulated during tissue repair and in fibrotic conditions associated with activation of the coagulation cascade. We therefore hypothesized that coagulation proteases promote the production of CTGF by cells at sites of tissue injury. To begin to address this hypothesis, we assessed the effect of coagulation proteases on fibroblast CTGF expression in vitro, and we show that thrombin, at physiological concentrations, up-regulated CTGF mRNA levels 5-fold relative to baseline (p < 0.01) in fetal fibroblasts and 7-fold in primary adult fibroblasts (p < 0.01). These effects were cycloheximide-insensitive and were not blocked with a pan-specific TGF-β-neutralizing antibody. They were further paralleled by a concomitant increase in CTGF protein production and could be mimicked with selective PAR-1 agonists. In addition, fibroblasts derived from PAR-1 knockout mice were unresponsive to thrombin but responded normally to TGF-β1. Finally, factor Xa, which is responsible for activating prothrombin during blood coagulation, exerted similar stimulatory effects. We propose that coagulation proteases and PAR-1 may play a role in promoting connective tissue formation during normal tissue repair and the development of fibrosis by up-regulating fibroblast CTGF expression.

Thrombin is a pluripotent serine protease that plays a central role in hemostasis following tissue injury by converting soluble plasma fibrinogen into an insoluble fibrin clot and by promoting platelet aggregation. In addition to these procoagulant effects, thrombin also influences a number of cellular responses that play important roles in subsequent inflammatory and tissue repair processes. Thrombin influences the recruitment and trafficking of inflammatory cells and is a potent mitogen for a number of cell types, including endothelial cells, fibroblasts, and smooth muscle cells (reviewed in Ref. 1). Thrombin also promotes the production and secretion of extracellular matrix proteins (2, 3) and influences connective tissue remodeling processes (4). There is increasing in vivo evidence that the pro-inflammatory and pro-fibrotic effects of thrombin play an important role in both normal tissue and vascular repair (5), as well as in a number of pathological conditions associated with acute or persistent activation of the coagulation cascade, including restenosis and neo-intima formation following vascular injury (6, 7), atherosclerosis (8), pulmonary fibrosis (9), and glomerulonephritis (10).

Most of the cellular effects elicited by thrombin are mediated via a family of widely expressed G-protein-coupled receptors, termed protease-activated receptors (PARs)† that are activated by limited proteolytic cleavage of the N-terminal extracellular domain. The newly generated N terminus acts as a tethered ligand and interacts intramolecularly with the body of the receptor to initiate subsequent cell signaling events (11). To date, four PARs have been described, of which three (PAR-1, -3, and -4) are activated by thrombin. Synthetic peptides corresponding to the tethered ligand of PAR-1 and PAR-4 act as agonists for these receptors and have been useful tools for invoking the involvement of these receptors in mediating the cellular effects of thrombin. Studies with these agonists, as well as with PAR-1-deficient mice, have led to the conclusion that PAR-1 is the major receptor responsible for mediating most of the pro-inflammatory (10, 12) and pro-fibrotic effects (2, 13, 14) of thrombin. Once thrombin has interacted with its receptor, it exerts its cellular effects either directly or via the induction and release of secondary mediators, including classical growth factors, pro-inflammatory cytokines, and vasoactive peptides and amines (reviewed in Ref. 1).

Connective tissue growth factor (CTGF) is a novel potent cysteine-rich heparin-binding growth factor, originally isolated from human umbilical vein endothelial cells (15), that is also highly expressed by fibroblasts (16). It belongs to an emerging family of conserved and modular proteins (known as the CCN family) with diverse biological functions involved in the regulation of cell growth and differentiation. Six members have been described to date and include the gene products of the

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1 The abbreviations used are: PAR, protease-activated receptor; CTGF, connective tissue growth factor; DMEM, sterile Dulbecco's modified Eagle's medium; HFL1, human fetal lung fibroblasts; NCS, newborn calf serum; PDGF, platelet-derived growth factor; rTAP, recombinant tick anticoagulant protein; TGF-β, transforming growth factor β; kb, kilobase pair; PAI, plasminogen activator inhibitor; MLEC, mink lung epithelial cells.
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Recent studies have provided evidence that CTGF may play an important role in promoting connective tissue formation after tissue injury. As well as being a potent fibroblast mitogen and chemoattractant, CTGF stimulates fibroblast procollagen and fibronectin mRNA production and influences α1 integrin mRNA levels in vitro (22). CTGF mRNA levels are strongly up-regulated in skin wound healing models in vivo (16), and subcutaneous injection of CTGF into newborn mice results in increased connective tissue deposition (22). CTGF is also thought to be involved in the development of tissue fibrosis, based on the observation that CTGF expression is increased in skin and internal organ fibrosis (23–25) and the fibrotic areas of atherosclerotic lesions (26). There is also growing evidence that CTGF may be the downstream autocrine mediator responsible for mediating some of the cellular effects of TGF-β1, the most fibrogenic mediator characterized to date. CTGF expression by cultured fibroblasts is exclusively induced by TGF-β1, whereas other fibrotic mediators such as PDGF, epidermal growth factor, basic fibroblast growth factor, and insulin-like growth factor-1 have no effect (16, 27). This is consistent with the recent characterization of a novel TGF-β response element within the CTGF promoter (28). In addition, CTGF antisense constructs or neutralizing antibodies have been shown to block the effects of TGF-β on fibroblast proliferation and procollagen production (27, 29, 30), although this was not a universal finding (31).

In this study, we hypothesized that coagulation proteases promote the production of CTGF by cells at sites of tissue injury and repair. To address this hypothesis, we assessed the effect of thrombin on fibroblast CTGF expression in vitro and show for the first time that thrombin, at physiological concentrations, increases both CTGF mRNA levels and protein production via proteolytic activation of the major thrombin receptor, PAR-1. We further show that the coagulation protease factor X, responsible for the activation of prothrombin during blood coagulation, exerts similar stimulatory effects. These in vitro findings support a role for coagulation proteases and PAR-1 in promoting early wound healing responses and connective tissue formation by up-regulating the production of CTGF. Our results may further be relevant to a number of fibroproliferative and fibrotic disorders where both thrombin levels and CTGF expression are increased.

**EXPERIMENTAL PROCEDURES**

**Materials—**Human thrombin (catalog number T4393) and recombinant hirudin (catalog number H0392) and cycloheximide were from Sigma. Purified Russell's viper venom-activated human factor Xa was from Calbiochem. Recombinant tick anticoagulant peptide (rTAP) was a generous gift from Dr. Mike Scully (Thrombosis Research Institute, London, UK) was added at a 1:2000 dilution for 1 h followed by three washes with ice-cold phosphate-buffered saline, and the monolayer was washed twice with ice-cold phosphate-buffered saline, and cells were lysed by adding 100 μl of Laemmli loading buffer. The cell lysates were mixed several times to shear DNA, and 25 μl of each were heated for 5 min at 95 °C prior to electrophoresis on a 12% SDS-polyacrylamide gel with a 7% stacking gel. RNA loading and gel electrophoresis on a formaldehyde 1% (w/v) agarose gel. RNA loading and integrity was visualized and quantitated by a fluorescent scanning of the gel (Fujifilm FLA 3000) prior to transfer to nylon membranes (Hybond N, Amersham Pharmacia Biotech). Membranes were exposed to a phosphorImager storage screen (Fujifilm FLA 3000) and were visualized and quantitated using a fluorography phosphorImager analysis (Fujifilm FLA 3000).

**Western Analysis of CTGF—**Cells were seeded at 5 × 10^5 cells/ml in 2.4-cm diameter dishes in DMEM, 5% NCS. Upon reaching visual confluence, cells were quiesced in serum-free DMEM for 16 h and incubated in fresh serum-free DMEM containing thrombin, factor Xa, or the highly selective PAR-1 agonist TFLLR (32). For cycloheximide experiments, cells were preincubated with cycloheximide (25 μg/ml) for 2 h prior to addition to serum-free control media or thrombin. For thrombin and factor Xa proteolytic inhibition experiments, thrombin or factor Xa was incubated with either hirudin (in 2-fold molar excess) or rTAP (in 4-fold molar excess), respectively, and protease-inhibitor complex formation was allowed to proceed for 2 h at 37 °C with shaking, prior to addition to cell cultures. At the end of the incubation, the media were removed, and total RNA was isolated with Trizol reagent (Life Technologies, Inc.) according to the manufacturer's instructions. Five μg of total RNA were mixed with RNA loading buffer containing ethidium bromide (Sigma), heated to 65 °C for 10 min, and electrophoresed on a formaldehyde 1% (w/v) agarose gel. RNA loading and integrity was visualized and quantitated by a fluorescent scanning of the gel (Fujifilm FLA 3000) prior to transfer to nylon membranes (Hybond N, Amersham Pharmacia Biotech) by Northern transfer and fixation by UV cross-linking. Membranes were hybridized overnight at 65 °C in a rotating hybridization oven in standard Denhardt's containing hybridization solution in the presence of the [32P]dCTP-labeled cDNA probes for either CTGF or FISP12, generated by random priming using an oligolabeling kit (Amersham Pharmacia Biotech). At the end of the hybridization, filters were rinsed at low stringency (2× SSC, 0.1% SDS for 5 min at room temperature, followed by 15 min at 65 °C), once at high stringency (0.5× SSC, 0.1% SDS for 25 min at 65 °C), and once at high stringency (0.1× SSC, 0.1% SDS for 5 min at 65 °C). Membranes were exposed to a phosphorImager storage screen (Fujifilm) for 2–4 h, and CTGF/FISP12 mRNA levels were quantitated by phosphorImager analysis (Fujifilm FLA 3000).

**Northern Analysis of CTGF mRNA Levels—**Cells were seeded at 2 × 10^5 cells/ml in 6-cm diameter dishes in DMEM, 5% NCS. Upon reaching visual confluence, cells were quiesced in serum-free DMEM for 16 h and incubated in fresh serum-free DMEM containing thrombin, factor Xa, or the highly selective PAR-1 agonist TFLLR (32). For cycloheximide experiments, cells were preincubated with cycloheximide (25 μg/ml) for 2 h prior to addition to serum-free control media or thrombin. For thrombin and factor Xa proteolytic inhibition experiments, thrombin or factor Xa was incubated with either hirudin (in 2-fold molar excess) or rTAP (in 4-fold molar excess), respectively, and protease-inhibitor complex formation was allowed to proceed for 2 h at 37 °C with shaking, prior to addition to cell cultures. At the end of the incubation, the media were removed, and total RNA was isolated with Trizol reagent (Life Technologies, Inc.) according to the manufacturer's instructions. Five μg of total RNA were mixed with RNA loading buffer containing ethidium bromide (Sigma), heated to 65 °C for 10 min, and electrophoresed on a formaldehyde 1% (w/v) agarose gel. RNA loading and integrity was visualized and quantitated by a fluorescent scanning of the gel (Fujifilm FLA 3000) prior to transfer to nylon membranes (Hybond N, Amersham Pharmacia Biotech) by Northern transfer and fixation by UV cross-linking. Membranes were hybridized overnight at 65 °C in a rotating hybridization oven in standard Denhardt's containing hybridization solution in the presence of the [32P]dCTP-labeled cDNA probes for either CTGF or FISP12, generated by random priming using an oligolabeling kit (Amersham Pharmacia Biotech). At the end of the hybridization, filters were rinsed at low stringency (2× SSC, 0.1% SDS for 5 min at room temperature, followed by 15 min at 65 °C), once at high stringency (0.5× SSC, 0.1% SDS for 25 min at 65 °C), and once at high stringency (0.1× SSC, 0.1% SDS for 5 min at 65 °C). Membranes were exposed to a phosphorImager storage screen (Fujifilm) for 2–4 h, and CTGF/FISP12 mRNA levels were quantitated by phosphorImager analysis (Fujifilm FLA 3000).

**Cytotoxicity Assay—**In vitro transfection of primary human adult lung fibroblasts grown from explant cultures of responding wild type mice were a kind gift from Professor Shaun R. Coughlin (University of California, San Francisco, CA) and have been described previously (13). Cells were maintained in DME supplemented with penicillin (100 units/ml), streptomycin (100 units/ml), and 5% (v/v) NCS (DMEM, 5% NCS), in a humidified atmosphere containing 10% CO2. Cells were routinely passaged every 6–7 days and tested for mycoplasma infection. There were no noticeable effects on the parameters measured for cells used between passages 14 and 25.

**Materials—**Human thrombin (catalog number T4393) and recombinant hirudin (catalog number H0392) and cycloheximide were from Sigma. Purified Russell's viper venom-activated human factor Xa was from Calbiochem. Recombinant tick anticoagulant peptide (rTAP) was a generous gift from Dr. Mike Scully (Thrombosis Research Institute, London, UK) was added at a 1:2000 dilution for 1 h followed by three washes with ice-cold phosphate-buffered saline, and the monolayer was washed twice with ice-cold phosphate-buffered saline, and cells were lysed by adding 100 μl of Laemmli loading buffer. The cell lysates were mixed several times to shear DNA, and 25 μl of each were heated for 5 min at 95 °C prior to electrophoresis on a 12% SDS-polyacrylamide gel with a 7% stacking gel for 3 h at 125 V. Separated proteins were transferred onto Hybond-ECL nylon membranes (Amersham Pharmacia Biotech) for 1 h at 25 V. The membrane was blocked with TBST (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 0.1% Tween 20) containing 5% dry milk for 1 h, and the anti-CTGF antibody was added at a 1:1000 dilution overnight at 4 °C. A horseradish peroxidase-conjugated anti-rabbit IgG (Dako Ltd., Cambridge, UK) was added at a 1:2000 dilution for 1 h followed by three washes in TBST for 15 min. The CTGF band was visualized by enhanced chemiluminescence (ECL) according to the manufacturer’s protocol (Amersham Pharmacia Biotech). Membranes were also stripped and reblotted with a rabbit anti-human actin antibody (Sigma) at a 1:2000 dilution for 2 h at room temperature, followed by the same secondary antibody used above.

**TGFB Bioassay—**Active TGF-β conditioned medium from HFL1 cells exposed to control media, thrombin (25 nM), or TGF-β (0.25–1 ng/ml) in identical serum-free conditions for 90 min, as described above, was assessed using a highly quantitative bioassay, based on the ability of TGF-β to induce plasminogen activator inhibitor-1 (PAI-1) gene expression.
pression in mink lung epithelial cells (MLEC) stably transfected with an expression construct containing a truncated TGF-β-responsive PAI-1 promoter fused to a luciferase reporter gene. These cells were a kind gift from Dr. D. B. Rifkin (New York University Medical Center, New York), and the assay was performed as described previously (33). Briefly, cells were grown to 75% confluence and incubated with fibroblast conditioned media, naive media, or conditioned media spiked with thrombin and TGF-β1 for 16 h. At the end of the incubation, the media were removed, and the cell layer was washed with cold phosphate-buffered saline, and luciferase activity in cell lysates (passive buffer) was assayed using a luciferase assay kit (Promega, Southampton, UK) according to the manufacturer’s instructions, with a luminometer (Turner Designs-2020). The data are expressed in relative light units per well.

Statistical Analysis—All numerical data are presented as means ± S.E. from four replicate cultures, unless otherwise indicated. Statistical evaluation was performed using an unpaired Student’s t test or by one-way analysis of variance using the Neuman-Keuls procedure for multiple group comparisons. The mean values of various parameters were said 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

Thrombin Increases CTGF mRNA Levels and Protein Production—To determine the potential effect of thrombin on fibroblast CTGF expression, human fetal lung fibroblasts (HFL1) and primary human adult lung fibroblasts were exposed to a single concentration of thrombin (25 nM), and CTGF mRNA levels were assessed by Northern analysis of total cellular RNA at 1.5 h. In both cell lines, thrombin caused a dramatic increase in CTGF mRNA levels, with values increased 5-fold relative to base line for fetal lung fibroblasts and 7-fold for primary adult lung fibroblasts (Fig. 1A). For comparison, at the same time point, TGF-β1 (1 ng/ml) only induced a small non-significant increase in CTGF mRNA levels in both cell types examined.

We next performed detailed thrombin concentration-response experiments in human fetal lung fibroblasts at a range of physiological concentrations, from 10 pM to 500 nM. Fig. 1B shows the results obtained up to 10 nM thrombin after 1.5 h of exposure. Thrombin increased CTGF mRNA levels at concentrations as low as 10 pM with values increased 2.6-fold relative to base line. At 1 nM, the response was maximal with CTGF mRNA levels increased 4-fold. There was no further up-regulation with increasing concentrations of thrombin up to 500 nM, the highest concentration tested.

The effect of thrombin on CTGF protein levels was assessed by Western blotting of cell layer extracts after 6 h of exposure to control media, thrombin (25 nM), or TGF-β1 (1 ng/ml) (Fig. 1C). The anti-CTGF antibody recognized a faint immunoreactive 38-kDa protein in unstimulated fetal and adult fibroblasts as has been previously reported (22). The intensity of this band was dramatically increased in cells exposed to thrombin or TGF-β1 for both fetal and adult fibroblasts, with the greatest intensity observed for thrombin-stimulated cells.

The Stimulatory Effects of Thrombin on CTGF mRNA Levels Are Early, Occur Independently of de Novo Protein Synthesis, and Do Not Involve the Secretion or Release of TGF-β—In order to determine the time course by which thrombin stimulates CTGF mRNA levels, detailed time course experiments were performed with fetal fibroblasts exposed to a single dose of thrombin (25 nM) or TGF-β1 (1 ng/ml) up to 48 h. Combined data for four separate time course experiments are shown in Fig. 2A. Thrombin stimulated CTGF mRNA levels 2-fold relative to media control levels at the earliest time point examined (0.5 h). CTGF mRNA levels were maximally increased by at least 3-fold at 3 and 6 h and then gradually returned to baseline values by 30 h. For comparison, the stimulatory effects obtained with TGF-β1 were not apparent until after 1.5 h. At 3 and 6 h, CTGF mRNA levels were maximally increased and gradually returned to base-line values by 48 h.

The time course for the stimulatory effects of thrombin on CTGF mRNA levels suggested that CTGF may be responding to thrombin in a typical immediate-early gene response fashion. To test this, we examined the effect of cycloheximide on the ability of thrombin to stimulate CTGF mRNA levels by fetal fibroblasts. Fig. 2B shows a representative experiment where thrombin increased CTGF mRNA levels about 4-fold relative to media control levels. As expected, cycloheximide (250 μg/ml) did not block the stimulatory effects of thrombin on CTGF mRNA levels, indicating that thrombin exerts its stimulatory effects independently of de novo protein synthesis.

We also performed experiments to rule out more definitively the possibility that thrombin may be acting via the induction or the release of TGF-β, as this has been previously reported for thrombin in other cell types (34, 35). We first assessed whether the stimulatory effects of thrombin on CTGF mRNA levels could be blocked with pan-specific TGF-β neutralizing antibodies. Fig. 2C shows a representative Northern blot for fetal fibroblasts exposed to serum-free control media or thrombin in the presence of TGF-β blocking antibodies or isotype-matched control IgG for 1.5 h. The stimulatory effects of thrombin on CTGF mRNA levels were completely unaffected by the inclusion of TGF-β blocking antibodies. IgG control antibodies similarly had no effect on basal or thrombin-induced CTGF mRNA levels.

We also performed experiments to determine whether thrombin was capable of inducing the production or secretion of active TGF-β using a bioassay, based on the ability of TGF-β to induce plasminogen activator inhibitor-1 (PAI-1) gene expression in mink lung epithelial cells (MLEC), stably transfected with an expression construct containing a truncated TGF-β-responsive PAI-1 promoter fused to a luciferase reporter gene. In these experiments, active TGF-β in naive and conditioned media from fetal fibroblasts exposed to either thrombin (25 nM) or TGF-β1 (0.25 ng/ml) was measured by assessing luciferase activity in mink lung epithelial cell lysates (Fig. 2D). As expected, TGF-β1 added directly to these cells caused a dramatic increase in PAI-1 promoter activity with values increased 24-fold above naive media control-treated mink lung epithelial cells, whereas thrombin added to naive media caused a slight but significant increase in PAI-1 promoter activity, as has been previously reported (33). A similar slight increase in PAI-1 promoter activity was observed in conditioned media from fetal fibroblasts exposed to thrombin, whereas the conditioned media from fetal fibroblasts exposed to TGF-β1 increased mink lung epithelial cell PAI-1 promoter activity 25-fold. Finally, a similar increase in PAI-1 promoter activity was also observed for conditioned media from media control-treated fetal fibroblasts which was subsequently “spiked” with thrombin, whereas TGF-β1 added to the same conditioned media again caused a dramatic increase in PAI-1 promoter activity. Taken together these data show that the increase in PAI-1 promoter activity is entirely due to the direct effects of thrombin on the PAI-1 promoter rather than due to the release or secretion of active TGF-β by the fibroblasts used in this study.

The Stimulatory Effects of Thrombin on CTGF mRNA Levels Are Mediated via Proteolytic Cleavage of PAR-1—In order to begin to unravel the mechanism by which thrombin exerts its stimulatory effects on CTGF mRNA levels, we first assessed the role of thrombin proteolytic activity. In these experiments, thrombin was rendered proteolytically inactive by complexing with the highly specific thrombin inhibitor, hirudin, prior to addition to fetal fibroblasts. Fig. 3A shows a representative Northern blot where, as expected, proteolytically active throm-
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Confluent cultures of HFL1 fibroblasts and primary human adult lung fibroblasts were quiesced in serum-free conditions and exposed to serum-free control media (DMEM), thrombin (25 nM), or TGF-β1 (1 ng/ml) for 1.5 h. Total cellular RNA was extracted using Trizol, and 5 μg for each sample were electrophoresed in a 1% agarose-formaldehyde gel. The RNA was transferred to nylon membranes by Northern blotting, and membranes were probed with a radiolabeled CTGF cDNA probe for 16 h. After hybridization and stringency washing, the membranes were exposed to PhosphorImager storage screens (2–4 h) for densitometric quantitation. The figure shows images of the 2.4-kb CTGF transcripts and of the corresponding ethidium bromide-stained 28 S rRNA bands.

The figure shows images of the 2.4-kb CTGF transcripts and of the corresponding ethidium bromide-stained 28 S rRNA bands for a representative experiment (n = 3). C, thrombin stimulates fibroblast CTGF mRNA levels. Confluent cultures of HFL1 fibroblasts and primary adult lung fibroblasts were quiesced in serum-free conditions, exposed to serum-free control media (DMEM), thrombin (25 nM), or TGF-β1 (1 ng/ml) for 6 h, and CTGF protein levels associated with the cell layer were assessed by Western blotting using an anti-CTGF antibody (upper panel). Equal protein loading was verified by blotting with an anti-actin antibody (lower panel). The blot is representative of three separate experiments performed.

**DISCUSSION**

Thrombin Stimulates CTGF mRNA Levels in an Immediate-Early Gene Response Fashion—CTGF is a member of the CCN family of serum-induced immediate-early gene products, which, unlike other members of this family, is selectively induced by TGF-β and is responsible for mediating some of the cellular effects of TGF-β in connective tissue cells in an autocrine fashion (16, 28). In this paper, we show for the first time that thrombin, the final enzyme of the coagulation cascade, is a novel potent stimulator of fibroblast CTGF expression. We further show that the effects of thrombin are mediated via proteolytic activation of PAR-1 and that factor Xa exerts similar stimulatory effects. This is, to our knowledge, the first evidence that there may be additional physiological inducers of fibroblast CTGF expression in addition to TGF-β.

These findings further raise the possibility that coagulation proteases may influence connective tissue formation after tissue injury and during the development of tissue fibrosis by up-regulating fibroblast CTGF expression.
The stimulatory effects of thrombin on CTGF mRNA levels are rapid and occur independently of de novo protein synthesis and the secretion of active TGF-β. A time course for the stimulatory effects of thrombin on CTGF mRNA levels. Confuent cultures of HFL1 fibroblasts were quiesced in serum-free conditions and exposed to serum-free control media (DMEM), thrombin (25 nM), or TGF-β (1 ng/ml) for incubation times from 0.5 to 48 h. CTGF mRNA levels at each time point were assessed by Northern analysis as described under “Experimental Procedures” and in Fig. 1. The figure shows combined data as means and S.E. for four replicate cultures, expressed in arbitrary relative light units. Where no error bar is shown it is within the column representing the media control. The effect of thrombin on CTGF mRNA levels was very rapid (within 30 min), cycloheximide-insensitive, and maximal at 1.5 h, suggesting that the CTGF response to thrombin was typical of that of an immediate-early gene. For comparison, TGF-β used as a positive control at 40 pm (1 ng/ml) did not up-regulate CTGF mRNA levels until at least 3 h. The concentration of TGF-β used in these experiments was based on previous studies performed in our laboratory showing that this is a concentration at which TGF-β maximally stimulates procollagen synthesis by these fibroblasts (37). The CTGF mRNA response obtained with TGF-β is slightly delayed compared with the earliest time of induction reported by other investigators at high concentrations of TGF-β (10 ng/ml) in human foreskin fibroblasts (0.5 h (16)) but comparable to those reported at similar concentrations in other human lung fibroblasts (2 h (27)). For both thrombin and TGF-β, the stimulation in CTGF mRNA levels was prolonged up to at least 24 and 30 h, respectively. This is unusual for an immediate-early gene response and is again concordant with previous reports of the CTGF response to TGF-β (16). The concentrations at which thrombin exerts its stimulatory effects on CTGF mRNA levels and the magnitude of the maximum fold increase obtained was similar to that obtained with TGF-β, suggesting that thrombin is as efficient at stimulating CTGF mRNA levels but exerts its effects faster in the fibroblasts employed in this study. This is consistent with the CTGF response obtained at the protein level since the intensity of the CTGF band obtained by Western analysis was greater for thrombin in both adult and fetal fibroblasts at the single early time point examined (6 h).
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The stimulatory effects of thrombin on CTGF mRNA levels are mediated via proteolytic activation of PAR-1. A, the effects of thrombin on CTGF mRNA levels are dependent upon thrombin proteolytic activity. Confluent cultures of HFL1 fibroblasts were quiesced in serum-free conditions and exposed to serum-free control media (DMEM), thrombin (25 nM), hirudin (50 nM) alone, and thrombin complexed with hirudin for 2 h at 37 °C prior to addition to cell cultures. CTGF mRNA levels at 1.5 h were assessed by Northern analysis as described under “Experimental Procedures” and in Fig. 1. The figure shows the 2.4-kb CTGF transcripts and the corresponding ethidium bromide-stained 28 S rRNA bands for a representative experiment (n = 3). C = media control; Thr = thrombin; Hir = hirudin. B, the stimulatory effects of thrombin on CTGF mRNA levels can be mimicked with selective PAR-1 peptide agonist TFLLR (200 μM). CTGF mRNA levels at 1.5 h were assessed by Northern analysis as described under “Experimental Procedures” and Fig. 1. The bar graph shows data as means and S.E. for four replicates, expressed as fold relative to media control levels after normalization based on the intensity of the ethidium bromide-stained 28 S rRNA bands. A representative image of the 2.4-kb CTGF transcripts and the corresponding ethidium bromide-stained 28 S rRNA bands are also shown. C = media control; Thr = thrombin; FXa = factor Xa; p values are calculated against media control levels. C, PAR-1−/− fibroblasts do not respond to the stimulatory effects of thrombin on FISP12 mRNA levels. Confluent cultures of PAR-1−/− and wild type fibroblasts were grown to confluence, quiesced in serum-free conditions, and exposed to serum-free control media (DMEM), thrombin (25 nM), or TGF-β, (1 ng/ml). FISP12 mRNA levels at 1.5 h were assessed by Northern analysis with a radioabeled FISP12 cDNA probe as described under “Experimental Procedures.” The figure shows the FISP12 transcripts and the corresponding ethidium bromide-stained 28 S rRNA bands for a representative experiment (n = 3). C = media control; Thr = thrombin. D, the effects of factor Xa on CTGF mRNA levels are dependent upon its proteolytic activity. Confluent cultures of HFL-1 fibroblasts were quiesced in serum-free conditions and exposed to serum-free control media (DMEM), factor Xa (15 nM), factor Xa complexed with rTAP for 2 h at 37 °C prior to addition to cell cultures, and rTAP alone. CTGF mRNA levels at 1.5 h were assessed by Northern analysis as described under “Experimental Procedures” and Fig. 1. The figure shows the 2.4-kb CTGF transcripts and the corresponding ethidium bromide-stained 28 S rRNA bands for a representative experiment (n = 3). C = media control; FXa = factor Xa.

Thrombin elicits a number of its cellular responses, including its pro-inflammatory and pro-fibrotic effects, via the induction or release of secondary mediators. Of particular relevance to the present study, thrombin has been reported to promote TGF-β₁ secretion by human mesangial cells (34) and its release from the pericellular matrix of cultured fibroblasts (35), although it was produced in a latent form and over long incubation periods in both of these reports. In the present study, there was good reason to believe that thrombin was exerting its stimulatory effects on CTGF expression independently of TGF-β₁ production or release, based on the observations that CTGF mRNA levels were increased very early and independently of de novo protein synthesis in response to thrombin, whereas TGF-β₁, at concentrations that could not be generated by fibroblasts in our culture conditions, did not affect fibroblast CTGF mRNA levels until much later. However, we performed additional experiments to more definitively rule out the involvement of TGF-β₁. We first assessed the effect of a pan-specific TGF-β₁ blocking antibody on the CTGF mRNA response obtained with thrombin. In these experiments, the antibody, at concentrations that would be sufficient to neutralize the biological activities of TGF-β₁ present at 1 ng/ml, had no effect on the increase in CTGF mRNA levels obtained with thrombin. We also measured active TGF-β₁ in fibroblast conditioned media after exposure to thrombin for at least 1.5 h using a bioassay, based on mink lung epithelial cells stably transfected with a truncated TGF-β₁-responsive PAI-1 promoter fused to a luciferase reporter gene and capable of detecting concentrations of active TGF-β₁ as low as 0.2 pm (33). Although thrombin induced low levels of expression of this construct, we did not detect any activity that could be ascribed to active TGF-β₁ in the conditioned media from thrombin-exposed fibroblasts.

Thrombin Exerts Its Stimulatory Effects on CTGF Expression via Proteolytic Activation of PAR-1—Thrombin exerts most of its cellular effects via activation of at least three PARs (PAR-1, PAR-2, and PAR-4) by limited proteolytic cleavage of the N-terminal extracellular domain and the unmasking of a tethered ligand (11). In terms of the mitogenic and fibrogenic effects of thrombin, we and others (2, 13, 14) have shown that PAR-1 is the major receptor involved in mediating the effects of thrombin on fibroblast G-protein signaling, downstream mitogen-activated protein kinase activation, proliferation, and extracellular matrix protein production. Experiments performed in the present study with the potent direct thrombin inhibitor, hirudin, showed that the effects of thrombin were dependent on its proteolytic activity, but the critical involvement of PAR-1 in mediating these responses was demonstrated in experiments employing the highly selective PAR-1 peptide agonist, TFLLR. This agonist activates PAR-1 independent of receptor cleavage, and unlike the commonly used peptide agonists, based on the tethered ligand sequence of PAR-1 (SFLLRN), does not activate PAR-2 in human mesenchymal cells (32). This was important because we have previously shown that the fibroblasts employed in this study express PAR-2 and respond to PAR-2 agonists in proliferation studies (39). In the present study, there was no difference in the magnitude of the stimulation in CTGF mRNA levels obtained with an optimal concentration of thrombin, its cellular effects via activation of at least three PARs (PAR-1, PAR-2, and PAR-4) by limited proteolytic cleavage of the N-terminal extracellular domain and the unmasking of a tethered ligand (11). In terms of the mitogenic and fibrogenic effects of thrombin, we and others (2, 13, 14) have shown that PAR-1 is the major receptor involved in mediating the effects of thrombin on fibroblast G-protein signaling, downstream mitogen-activated protein kinase activation, proliferation, and extracellular matrix protein production. Experiments performed in the present study with the potent direct thrombin inhibitor, hirudin, showed that the effects of thrombin were dependent on its proteolytic activity, but the critical involvement of PAR-1 in mediating these responses was demonstrated in experiments employing the highly selective PAR-1 peptide agonist, TFLLR. This agonist activates PAR-1 independent of receptor cleavage, and unlike the commonly used peptide agonists, based on the tethered ligand sequence of PAR-1 (SFLLRN), does not activate PAR-2 in human mesenchymal cells (32). This was important because we have previously shown that the fibroblasts employed in this study express PAR-2 and respond to PAR-2 agonists in proliferation studies (39). In the present study, there was no difference in the magnitude of the stimulation in CTGF mRNA levels obtained with an optimal concentration of TFLLR and a maximal stimulatory concentration of thrombin, indicating that PAR-1 activation alone is sufficient to account for all of the stimulatory effects of thrombin on CTGF mRNA levels. The critical involvement of PAR-1 was further confirmed in experiments where FISP12 (mouse ortholog of CTGF) was not up-regulated by thrombin in fibroblasts derived from PAR-1 knockout mice (40), whereas these cells responded normally to TGF-β₁. In contrast, wild type mouse fibroblasts responded to both thrombin and TGF-β₁. Taken together, our data indicate that PAR-1 is both necessary and sufficient for mediating the stimulatory effects of thrombin on CTGF/FISP12 mRNA levels. They further show that these stimulatory effects are not restricted to human fibroblasts and confirm the critical
Role of CTGF in Mediating the Cellular Responses of Thrombin—The novel finding that thrombin induces the production of CTGF raises the possibility that CTGF may be involved in mediating some of the cellular effects of thrombin in an autocrine fashion. CTGF and thrombin elicit similar biological responses in a number of cell types. In fibroblasts, both mediators stimulate mitogenesis and chemotaxis and promote procollagen and fibronectin production (2, 13, 22, 41, 42). In our experiments, CTGF mRNA levels were already maximally increased at concentrations of thrombin below the EC50 we have previously reported for both proliferation and procollagen production responses by these fibroblasts (2). However, both DNA synthesis and up-regulation of procollagen α1(I) mRNA levels are delayed by about 16 h (2), consistent with the hypothesis that thrombin is acting via the induction of autocrine mediators. In this regard, there is good evidence that the mitogenic effects of thrombin for fibroblasts are mediated, at least in part, via the autocrine release of PDGF and up-regulation of PDGF receptors (43). Interestingly, PDGF has also been implicated in mediating some of the mitogenic effects of TGF-β in monolayer cultures of connective tissue cells (44), whereas a role for CTGF in mediating the effects of TGF-β on fibroblast anchorage-independent growth has been more firmly established (29). In addition, there is also evidence that the effects of TGF-β on procollagen α1(I) mRNA levels are mediated via both CTGF-dependent and CTGF-independent pathways (27, 30). The exact role of CTGF in mediating thrombin-mediated fibroblast mitogenic and fibrogenic responses is, at present, uncertain, but future studies employing CTGF blocking antibodies or antisense approaches should prove informative. We are presently considering the possibilities that CTGF may act, in concert with PDGF, to augment the mitogenic response to thrombin and that CTGF is involved in mediating the effects of thrombin on fibroblast connective tissue formation.

Factor Xa Also Up-regulates Fibroblast CTGF mRNA Levels—Factor Xa is generated at the point of convergence of the intrinsic and extrinsic coagulation pathways. It is an essential component of the prothrombinase complex, which is also composed of membrane phospholipids, factor Va, and Ca2+, and is responsible for the conversion of prothrombin to thrombin. Factor Xa has also been shown to exert cellular effects in a number of cell types (45). For fibroblasts, we have recently reported that this protease is a potent mitogen at similar concentrations as thrombin (36). The exact mechanisms by which factor Xa exerts its cellular effects are not clear, although we have preliminary data suggesting that its mitogenic effects for fibroblasts may be mediated via binding to the integral cell membrane receptor, effector cell-protease receptor-1 (EPR-1), and activation of PAR-12. However, there is also evidence that factor Xa may activate PAR-2, or a PAR-2 related receptor, in other cell types (45). In the present study, we show that factor Xa is also a potent promoter of CTGF mRNA levels and further that the magnitude of the response obtained was similar to that obtained with thrombin. We further demonstrate that, as for thrombin, the effects of factor Xa are critically dependent on its proteolytic activity. It is again possible that CTGF may also play a role in mediating the mitogenic and other cellular effects elicited by factor Xa.

Role of CTGF Induction in Tissue Repair and Fibrosis—Although the exact physiological role of CTGF is unclear, its fibrogenic effects have been proposed to play a central role in normal tissue repair. CTGF mRNA levels are up-regulated in skin wound healing models (16), and subcutaneous injection of CTGF into newborn mice produces large granulation tissue formation, fibroplasia, and increased extracellular matrix deposition (22). Overexpression of CTGF is also a feature of a number of fibrotic and fibroproliferative disorders, including localized scleroderma, keloid tissue (23), the fibrous stroma of mammary tumors (46), the fibrotic areas of atherosclerotic lesions (26), renal and pulmonary fibrosis (24, 25), and inflammatory bowel disease (47). The mechanism by which CTGF expression is up-regulated during tissue repair and the development of tissue fibrosis is still unknown, although there is good in vitro and in vivo evidence that TGF-β plays an important role. TGF-β and CTGF are coordinately overexpressed during wound healing and are also co-localized at sites of connective tissue formation in a variety of fibrotic disorders (16, 47).

Activation of the coagulation cascade is one of the earliest events following tissue injury and is also a common feature of a number of acute and chronic pathological conditions associated with proliferative responses and excess deposition of matrix proteins, including atherosclerosis (8), restenosis after vascular injury (6, 7), glomerulonephritis (10), and pulmonary fibrosis (9). These conditions are also associated with increased CTGF expression. Our novel finding that thrombin and factor Xa induce rapid increases in fibroblast CTGF expression in vitro raises the possibility that these proteases may contribute to increased CTGF expression during both normal tissue repair and the development of tissue fibrosis. In a related project, we have been addressing the potential role of thrombin and CTGF in tissue fibrosis by assessing the effect of a direct thrombin inhibitor in an animal model of bleomycin-induced lung injury and fibrosis.3 In this model, both thrombin and CTGF/FISP12 mRNA levels are elevated within the 1st week after bleomycin administration (48, 49). We have shown that continuous infusion of a direct thrombin inhibitor at an anticoagulant dose reduced the doubling in lung collagen accumulation observed in animals treated with bleomycin alone by up to 40% (50). Furthermore, at a time when thrombin levels and CTGF expression were maximally increased in bleomycin-treated animals, both CTGF and procollagen α1(I) mRNA levels were significantly reduced in animals receiving the direct thrombin inhibitor compared with animals receiving bleomycin alone. These data lend support to our hypothesis that thrombin influences CTGF mRNA levels in this in vivo model of lung injury and fibrosis. This is, to our knowledge, also the first report to show that a reduction in CTGF mRNA levels correlates with reduced procollagen α1(I) mRNA levels and ultimately collagen deposition. Finally, it is also tempting to speculate that our results may be pertinent to the recent finding that thrombin-activated platelets adhere to CTGF and its homolog Cyr61 (51). These proteins are associated with the extracellular matrix in arterial vessels, and platelet adhesion to the subendothelial matrix is a key mechanism by which platelets participate in hemostasis. The rapid up-regulation of CTGF by coagulation proteases may therefore serve to promote platelet adhesion to the subendothelial matrix of the damaged vessel. Our findings may therefore also have implications for platelet adhesion in atherosclerotic blood vessels, where a pathological role for CTGF (26, thrombin (6), and PAR-1 (8) has been proposed.

Summary and Implications—In this paper, we report that the coagulation cascade proteases, thrombin and factor Xa, are potent inducers of fibroblast CTGF expression and further for

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2 O. P. Blanc-Brude, R. C. Chambers, F. Archer, and G. J. Laurent, manuscript in preparation.

3 D. C. Howell, N. R. Goldsack, R. P. Marshall, and R. C. Chambers, manuscript in preparation.
Thrombin induces connective tissue growth factor production

Thrombin that these effects are mediated via activation of PAR-1. The stimulatory effects of thrombin on CTGF mRNA levels precede those induced in response to TGF-β, the only currently known physiological inducer of fibroblast CTGF expression. Signal transduction by TGF-β is initiated by ligand binding to specific transmembrane receptor serine kinases and activation of the Smad pathway (reviewed in 52). In contrast, PAR-1 is a seven transmembrane domain G-protein-coupled receptor which is activated by limited proteolysis leading to the activation of heterotrimeric G-proteins and the immediate mobilization of cytosolic free Ca2+ (11). Our study therefore has further implications for our understanding of the receptor systems and signal transduction pathways leading to CTGF expression in that it is the first to demonstrate a role for a G-protein coupled receptor in mediating these effects. Our results are further consistent with the hypothesis that coagulation proteases, beyond their critical role in blood coagulation, influence cellular responses that are central to subsequent tissue repair processes. These findings may also be relevant to fibrotic conditions where there is excessive and/or persistent tissue repair processes. These findings may also be relevant to fibrotic conditions where there is excessive and/or persistent tissue repair processes. These findings may also be relevant to fibrotic conditions where there is excessive and/or persistent tissue repair processes. These findings may also be relevant to fibrotic conditions where there is excessive and/or persistent tissue repair processes. These findings may also be relevant to fibrotic conditions where there is excessive and/or persistent tissue repair processes. These findings may also be relevant to fibrotic conditions where there is excessive and/or persistent tissue repair processes. These findings may also be relevant to fibrotic conditions where there is excessive and/or persistent tissue repair processes. These findings may also be relevant to fibrotic conditions where there is excessive and/or persistent tissue repair processes. These findings may also be relevant to fibrotic conditions where there is excessive and/or persistent tissue repair processes.