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Factor Xa stimulates fibroblast procollagen production, proliferation, and calcium signaling via PAR_1 activation

Olivier P. Blanc-Brude[a,*], Fabienne Archer[b], Patricia Leoni[a], Claudia Derian[c], Steven Bolsover[b], Geoffrey J. Laurent[a], Rachel C. Chambers[a]

[a]*Royal Free and University College London Medical School, Centre for Cardiopulmonary Biochemistry and Respiratory Medicine, 5 University Street, London WC1E 6JJ, UK*

[b]*University College London Department of Physiology, Bolsover Laboratory, Gower Street, London WC1E 6BT, UK*

[c]*Johnson and Johnson Pharmaceutical Research and Development, LLC, Welsch and McKean roads, Spring House, PA 19477-0776, USA*

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Abstract

Fibroblast proliferation and procollagen production are central features of tissue repair and fibrosis. In addition to its role in blood clotting, the coagulation cascade proteinase thrombin can contribute to tissue repair by stimulating fibroblasts via proteolytic activation of proteinase-activated receptor-1 (PAR_1). During hemostasis, the coagulation cascade proteinase factor X is converted into factor Xa. We have previously shown that factor Xa upregulates fibroblast proliferation via production of autocrine PDGF. In this study, we further examined the effects of factor Xa on fibroblast function and aimed to identify its signaling receptor. We showed that factor Xa stimulates procollagen promoter activity and protein production by human and mouse fibroblasts. This effect was independent of PDGF and thrombin production, but dependent on factor Xa proteolytic activity. We also showed that PAR_1-deficient mouse fibroblasts did not upregulate procollagen production, mobilize cytosolic calcium, or proliferate in response to factor Xa. Desensitization techniques and PAR_1-specific agonists and inhibitors were used to demonstrate that PAR_1 mediates factor Xa signaling in human fibroblasts. This is the first report that factor Xa stimulates extracellular matrix production. In contrast with endothelial cells and vascular smooth muscle cells, fibroblasts appear to be the only cell type in which the effects of factor Xa are mediated mainly via PAR_1 and not PAR_2. These findings are critical for our understanding of tissue repair and fibrotic mechanisms, and for the design of novel approaches to inhibit the profibrotic effects of the coagulation cascade without compromising blood hemostasis.

Keywords: Fibroblast; Coagulation; Factor Xa; PAR_1; Tissue repair; Fibrosis; Procollagen; Proliferation

Introduction

Fibroblast proliferation and extracellular matrix production are central features of normal tissue repair and pathological tissue fibrosis. Tissue fibrosis is the end stage of a heterogenous group of disorders that can affect many organs, including the lung [1], the kidney [2], and the liver [3]. Excessive fibroblast proliferation and collagen deposition are common to these diseases. Many normal responses to tissue injury are recapitulated in tissue fibrosis, but they are deregulated, exacerbated, and abnormally sustained. Eventually, the accumulation of connective tissue, mainly collagen types I and III, compromises the organ's function and often leads to premature death.

Abbreviations: $[Ca^{2+}]_i$, Cytosolic-free Ca^{2+} concentration; CTGF, Connective tissue growth factor; DEGR-ck, dansyl-Glu-Gly-Arg chloromethylketone dihydrochloride; EPR-1, Effector cell-protease receptor-1; HBS, HEPES-buffered saline; MAPK, Mitogen-activated protein kinase; PAR_1, Proteinase-activated receptor-1; PDGF, Platelet-derived growth factor; PTX, Pertussis toxin.

* Corresponding author. Present address: Olivier Blanc-Brude, INSERM Unit 541, Hôpital Lariboisière, 41 Boulevard de la Chapelle, 75010 Paris, France. Fax: +33 1 42 81 31 28.

E-mail address: olivier.blanc-brude@larib.inserm.fr (O.P. Blanc-Brude).

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In pulmonary fibrosis, increased levels of classical cytokines and growth factors, such as transforming growth factor-β_1, platelet-derived growth factor (PDGF), and insulin growth factor-1 promote fibroblast function [4]. However, blood coagulation and the dramatic activation of the coagulation cascade proteinase thrombin have also been extensively documented in association chronic lung injury [5], in pulmonary fibrosis associated with scleroderma, and in interstitial pulmonary fibrosis [6,7]. Activation of the coagulation cascade is particularly relevant to the lung and organs where the interstitial compartment is in close contact with an extensive microvascular bed. Persistent activation of the coagulation cascade is thought to result from an imbalance between pro- and anti-coagulant factors. For instance, bronchoalveolar lavage (BAL) fluid from patients with acute respiratory distress syndrome (ARDS) contains elevated levels of factor VIIa, a factor that initiates blood coagulation [8]. High levels of factor Xa are also generated following lung injury [8], during pulmonary fibrosis [9,10], and in a variety of fibrotic and infectious conditions [11,12]. On the other hand, abnormally low levels of anti-coagulant factors such as antithrombin III have been observed in ARDS, resulting in increased procoagulant activity [13].

There is increasing evidence that the coagulation cascade proteinase thrombin contributes to the pathogenesis of lung fibrosis. Thrombin stimulates fibroblast recruitment [14] and proliferation [15,16], increases procollagen production in vitro [17], and accelerates tissue fibrosis [18]. Thrombin can activate several seven transmembrane domain G protein-coupled cell surface receptors [19,20] termed proteinase-activated receptors (PAR_1, PAR_3 and PAR_4). PAR_1 is the principal receptor for thrombin in fibroblasts [17,20,21] and its activation leads to autocrine fibroblast simulation via the production of PDGF [16] and possibly CTGF [22]. We recently obtained the first evidence that direct thrombin inhibition attenuates connective tissue deposition in a model of experimental pulmonary fibrosis [23], supporting the idea that thrombin contributes to tissue fibrosis [24]. However, little is known about the potential role of other coagulation cascade proteinases.

Factor Xa plays a critical role at the point of convergence of the intrinsic and extrinsic coagulation pathways, by converting prothrombin into active thrombin during blood coagulation [11,12]. We have recently shown that factor Xa is also a potent stimulator of PDGF-A expression and a mitogen for human lung fibroblasts in vitro, and that factor Xa acts independently of thrombin generation [25]. In contrast, coagulation cascade proteinases activated upstream of factor Xa, such as factor IXa and VIIa, had no significant effect at physiological concentrations. We further showed that ligation of effector cell protease receptor-1 (EPR-1) by factor Xa enhances its mitogenic effect. However, the factor Xa receptor that transduces its mitogenic signal remains unknown. In pioneering studies, Riewald and Ruf [26] recently reported that factor Xa can activate PAR_1 and PAR_2 in heterologous transfection systems. PAR activation by factor Xa was greatly enhanced by cotransfection of tissue factor and the presence of factor VIIa, presumably associating at the cell surface. This group also showed that factor Xa can stimulate gene expression in HeLa cells that express PAR_1 and not PAR_2 [27].

In this study, we aimed to further investigate the profibrotic effects of factor Xa and begin to characterize the signaling receptors responsible for these effects in fibroblasts. Our experiments showed that factor Xa stimulates fibroblast procollagen production in human and mouse fibroblasts and put in light the essential role of PAR_1 in these events, even in the presence of other factor Xa receptors such as PAR_2 and EPR-1.

Materials and methods

Materials

Purified Russel's viper venom-activated human factor Xa, pertussis toxin (PTX) and dansyl-Glu-Gly-Arg chloromethylketone dihydrochloride (DEGR-ck) were from Calbiochem-Novabiochem UK Ltd. (Nottingham, UK). Catalytically inactivated factor Xa (DEGR-factor Xa) was prepared by Dr C. Goodwin (National Heart and Lung Institute, London, UK) by incubation of factor Xa with DEGR-ck until no proteolytic activity remained. Excess DEGR-ck was removed by extensive dialysis and the purity of DEGR-factor Xa was assessed by conventional SDS-PAGE. Human α-thrombin was purchased from Sigma Ltd. (Poole, UK), human airway mast cell tryptase was from Europa Bioproducts Ltd. (Wicken, UK) and the specific cell-permeant intracellular Ca^{2+} chelator BAPTA-AM was purchased from Molecular Probes Europe BV (Leiden, The Netherlands). Porcine TGF-β1, recombinant PDGF-AB were from R&D Systems (Minneapolis, MN). The specific PAR1 agonist TFLLR [28] was synthesized as an amidated peptide by Professor R. Mecham (University of Washington, St. Louis, MO, USA). The selective PAR_1 antagonist RWJ-58259 was previously described by Derian et al. [29]. Recombinant tick anticoagulant peptide (rTAP) [30] was a kind gift from Dr. M. Scully (National Heart and Lung Institute, London, UK) and originally prepared by Dr. G. Vlasuk (Corvas International, San Diego, CA). Antistasin core peptide $D–Arg^{32}–Pro^{38}$ (ASN peptide) [31] was purchased from Bachem (Saffron Walden, UK). Those well-characterized inhibitors are increasingly used to specifically inhibit factor Xa while leaving thrombin activity intact [25–27].

Human fetal, mouse PAR_1-deficient fibroblasts and tissue culture

Human fetal lung fibroblasts (HFL-1, ref. CCL-153) were obtained from ATCC (Rockville, MD, USA) and used

at passages below 20. Wild-type and PAR_1-deficient mouse lung fibroblasts [20] were kind gifts from Dr S. Coughlin (Cardiovascular Research Institute, University of California, San Francisco, CA, USA). Cells were routinely cultured at 37°C in DMEM supplemented with 10% neonatal calf serum and standard antibiotics. For proliferation and DNA synthesis experiments, fibroblasts were seeded in 96-well plates (5000 cells/100 μl/well), grown to 75% confluency for 24 h in 5% neonatal calf serum before treatment.

DNA synthesis and proliferation assays

Fibroblasts were grown to subconfluence, quiesced, and incubated in DMEM supplemented with test substances in the absence of serum. DNA synthesis was assayed by addition of [^{3}H]thymidine (2 μCi/ml) for the last 4 h of a 20-h incubation. DNA was collected, washed, and shaken in scintillation fluid overnight before radioactivity was evaluated. Fibroblast proliferation was assayed after 48 h of incubation using a colorimetric assay based on the uptake and elution of methylene blue dye [32] to reveal whether potential mitogens stimulate cell cycle progression through to cell division. Data were expressed as means ± standard error of the mean (SEM) in percent stimulation above control. Statistical evaluation was performed using the unpaired Student's *t* test. Significance was achieved when $P < 0.05$.

Immunoblotting

Subconfluent mouse wild-type and PAR1-deficient lung fibroblasts in the absence of serum were lysed in 2% SDS. Weight-normalized aliquots (80 μg) of whole cell lysates were boiled and separated in a 12% acrylamide gel under reducing conditions. Samples were electrophoresed at 150 V for 3 h. Proteins were then electroblotted onto Immobilon membranes (Millipore Corp., Bedford, MA, USA) for 1 h at 0.8 A. Membranes were blocked with 5% milk in Tris-buffered saline and incubated at 22°C for 2 h with 10 μg/ml of a sequence-specific anti-EPR-1 rabbit antibody (TAIL antibody) [33], followed by peroxidase-conjugated goat anti-rabbit IgG for 1 h (1:5000 dilution), and visualization with an enhanced chemiluminescence detection system.

Single cell cytosolic Ca^{2+} spectrofluorometry

Fibroblasts were plated in DMEM supplemented with 10% neonatal calf serum (5000 cells/ml) in hydrophilic Petriperm tissue culture plates (Heraeus Instruments GmbH, Hanau, Germany), grown to 50% confluence, and loaded with the calcium indicator Fura-2 by incubation with 2 μM Fura-2-AM plus 25 mg/L Pluronic F127 (Molecular Probes) for 30 min [34]. Cells were placed on the stage of an Axiovert 100TV fluorescence microscope (Carl Zeiss Ltd., Welwyn Garden City, UK) fitted with Fucal 5.12c imaging system (TILL Photonics GmbH, Martinsreid, Germany). Cells were rinsed twice with HBS and test solutions were delivered by dish perfusion. 340/380 nm ratio pairs were acquired for 10 to 20 cells every 2 s for up to 4 min. Cytosolic Ca^{2+} values were calculated using the standard calibration equation $[Ca^{2+}] = K_d^{Ca2+} \times S \times (R - R_{min}) / (R_{max} - R)$ [35]. Individual Ca^{2+} transients obtained in all cells in each treatment group were averaged to obtain a single representative $[Ca^{2+}]_i$ curve used for comparison purposes. Variations in $[Ca^{2+}]_i$ after stimulation were influenced by the number of responsive cells, the peak increase in $[Ca^{2+}]_i$, and the average increase over the complete stimulation period. We analyzed all three parameters since any variation in $[Ca^{2+}]_i$ may significantly affect cell function, and because individual proteinases stimulated either delayed non-synchronous responses or immediate synchronous responses. In such conditions, we and others have shown that factor Xa signaling via cytosolic Ca^{2+} transients and cGMP production occurs independently of thrombin generation [25,34,36].

To investigate the effects of receptor desensitization on calcium responses, fibroblasts were washed twice with PBS to remove serum, pretreated with factor Xa (25 nM), thrombin (25 nM) or agonist vehicle (HEPES-buffered saline, HBS) in serum-free conditions for 90 min. Fura-2 was loaded during the last 30 min. Our desensitization protocol (25 nM proteinase for 90 min) was selected after determination of the minimal thrombin concentration (25 nM) and incubation time (60 min) necessary for total PAR_1 desensitization in human fibroblasts, as previously described in endothelial cells [37,38].

To inhibit PAR_1 specifically, fibroblasts were plated on glass cover slips, pretreated with HBS or the selective PAR_1 antagonist RWJ-58259 for 5 min before stimulation with factor Xa or thrombin (25 nM). For dosage, we selected a concentration of RWJ-58259 (1 μM) sufficient to entirely inhibit thrombin-induced proliferation of smooth muscle cells [29]. For comparative purposes, cytosolic Ca^{2+} mobilization was expressed in % relative calcium rise above basal levels in HBS-treated controls.

Determination of procollagen production

Procollagen production was determined as described elsewhere [39,40]. Briefly, fibroblasts were grown to full confluence for 5 days. Media were replaced with serum-free DMEM supplemented with 50 μg/ml ascorbic acid and 0.2 mM proline for 24 h. These conditions of confluency and serum starvation that do not allow further significant proliferation. The cells were then treated with or without factor Xa, thrombin or TGF-β_1. After 48 h, the cell layer and medium were harvested together (human fibroblasts) or separately (mouse fibroblasts) and proteins were ethanol-precipitated. Ethanol-insoluble proteins were separated from free amino acids by filtration, washed twice

with ethanol, and hydrolyzed overnight in 6 M HCl at 110°C. Hydrolysates were cleared with activated charcoal and filtered and derivatized with 4-chloro-7-nitrobenzofurazan (NBD-Cl) (Sigma). Hydroxyproline in ethanol-insoluble fractions was quantified by reverse-phase HPLC using a Beckman System Gold (Beckman, High Wycombe, Bucks., UK), and taken as an index of procollagen production. Data are expressed as nmol of hydroxyproline per well per 48 h. Hydroxyproline associated with the cell layer at the onset of the experiment was subtracted from all values.

Determination of procollagen-α_1(I) promoter activity

For the analysis of procollagen-α1(I) transcriptional activity, a human procollagen-α_1(I) promoter–reporter construct was transiently transfected into approximately 90% confluent human fetal lung fibroblasts, mouse wild type, and PAR_1-deficient lung fibroblasts. The construct was a kind gift from Dr Raghow (University of Tennessee, USA) and contained 804 bp of the procollagen-α_1(I) promoter region upstream from the initiation site, including 117 bp of the first intron coupled to the luciferase coding sequence [41,42]. This promoter was selected as it gave maximal responses to TGF-β_1 in our assay conditions. The sequence identity for human and mouse type I collagen genes (COL1A1) is very high in these regions with several highly conserved cis-acting elements. This construct has previously been successfully employed to examine the transcriptional regulation of COL1A1 gene expression using mouse NIH3T3 fibroblasts [42]. The transfection efficiency, estimated with the number of positively stained cells after transfection with a control construct encoding the LacZ reporter gene, was estimated to be about 30%. A novel peptide comprising an RGD moiety for integrin binding and a polylysine moiety for DNA binding (peptide-6), was used to enhance transfection efficiency [43]. Forty-microliter peptide-6 was mixed with 0.75 μl lipofectin (Gibco BRL Life Technologies, Paisley, Scotland, UK), in 100 μl OptiMEM (Gibco), and 0.5 μg promoter–reporter construct DNA was mixed with 100 μl OptiMEM. The two solutions were mixed for 45 min at RT, diluted to make a final volume of 500 μl with OptiMEM before addition to the cells in 12 well plates for 6 h. Cells were washed with PBS and placed in fresh DMEM with 10% serum overnight. Transfected cells were placed in fresh DMEM without serum, with or without additions for 24 h. For catalytic site inhibition, factor Xa was incubated with rTAP or ASN for 2 h at 37°C, or in combination with a polyclonal pan-specific neutralizing antibody directed against PDGF (10 μg/ml) to block the effects of the growth factor as described elsewhere [25]. Cells were lysed and aliquots (10 μl) of the soluble fraction were added to 100 μl luciferase substrate at RT. Chemiluminescence was measured immediately in triplicates with a TD-20/20 luminometer (Turner Designs, Promega UK, Southampton, UK) using a Luciferase Assay System (Promega). Each experiment was repeated twice and the data expressed in relative light units (RLU).

Results

Factor Xa stimulates fibroblast procollagen production

We determined the effects of factor Xa on procollagen-α_1(I) promoter activity and procollagen protein production in human lung fibroblasts. Fig. 1A shows that factor Xa stimulated procollagen protein production by up to 125% in fully confluent and quiesced human fibroblasts in vitro over 48 h. The effect of factor Xa was dose-dependent and peaked at 25 nM ($P < 0.01$). Thrombin (25 nM) and TGF-β_1 (1 ng/ml) stimulated rates of procollagen production by about 200% and 250%, respectively. In contrast, coagulation factor IXa had no significant effect.

Factor Xa stimulates fibroblast procollagen-α_1(I) promoter activity

The effect of factor Xa on procollagen-α_1(I) promoter activity was evaluated using a luciferase-linked procollagen-α_1(I)-promoter construct transiently transfected into human fetal lung fibroblasts. Fig. 1B shows that factor Xa (25 nM) upregulated procollagen-α_1(I) promoter activity by almost 5-fold in serum-starved confluent human fibroblasts. In contrast, PDGF-AB (10 ng/ml) was a poorer stimulus for procollagen-α_1(I) gene transcription, stimulating less than a doubling in promoter activity. Since we have previously reported that factor Xa can stimulate PDGF-A expression in fibroblasts, we determined the effect of a polyclonal pan-specific neutralizing antibody directed against PDGF at a concentration (10 μg/ml) necessary to completely block the mitogenic effect of factor Xa in our cells [25]. The neutralizing anti-PDGF antibody completely blocked the effect of PDGF-AB, but only reduced the effect of factor Xa by about 25% ($P < 0.01$), (Fig. 1B). In order to determine the role of factor Xa catalytic activity, we used the highly specific inhibitors, rTAP and ASN peptide [30,31] which we have previously shown to block the mitogenic effect of factor Xa on human lung fibroblasts [25]. Fig. 1C shows that rTAP and ASN blocked factor Xa-stimulated procollagen-α_1(I) promoter activity by 60 % and 40 %, respectively ($P < 0.01$).

Procollagen-α_1(I) promoter activity and procollagen production in PAR_1-deficient mouse lung fibroblasts

We next assessed the role of PAR_1 in mediating the stimulation by factor Xa of procollagen-α_1(I) promoter activity in wild-type and PAR_1-deficient mouse lung fibroblasts transfected with a procollagen-α_1(I)-promoter reporter construct. Fig. 2A shows that TGF-β_1 (1 ng/ml),

factor Xa (25 nM) and thrombin (25 nM) stimulated procollagen-α_1(I) promoter activity by about 3- to 4-fold over control levels in wild-type fibroblasts ($P < 0.01$ for all). In PAR_1-deficient fibroblasts, TGF-β_1 (positive control) [40] stimulated a similar increase in procollagen promoter activity ($P < 0.01$), but factor Xa and thrombin had no significant effect. In other experiments, wild-type and PAR_1-deficient mouse lung fibroblasts were treated with factor Xa. Fig. 2B shows that procollagen production was increased by 45.5 % over 48 h ($P < 0.01$) by factor Xa in wild-type fibroblasts, whereas PAR_1-deficient fibroblasts were unresponsive. Note that a significant difference in basal procollagen production rates was observed between wild-type (0.33 nmol/well/48 h) and PAR_1-deficient fibroblasts (7.11 nmol/well/48 h). A difference in procollagen promoter activity was also observed (see legend to Fig. 2A). Since fibroblasts are known to be heterogeneous in their capacity to proliferate and produce collagen and other extracellular matrix proteins, this difference may arise from clonal selection during primary fibroblast isolation.

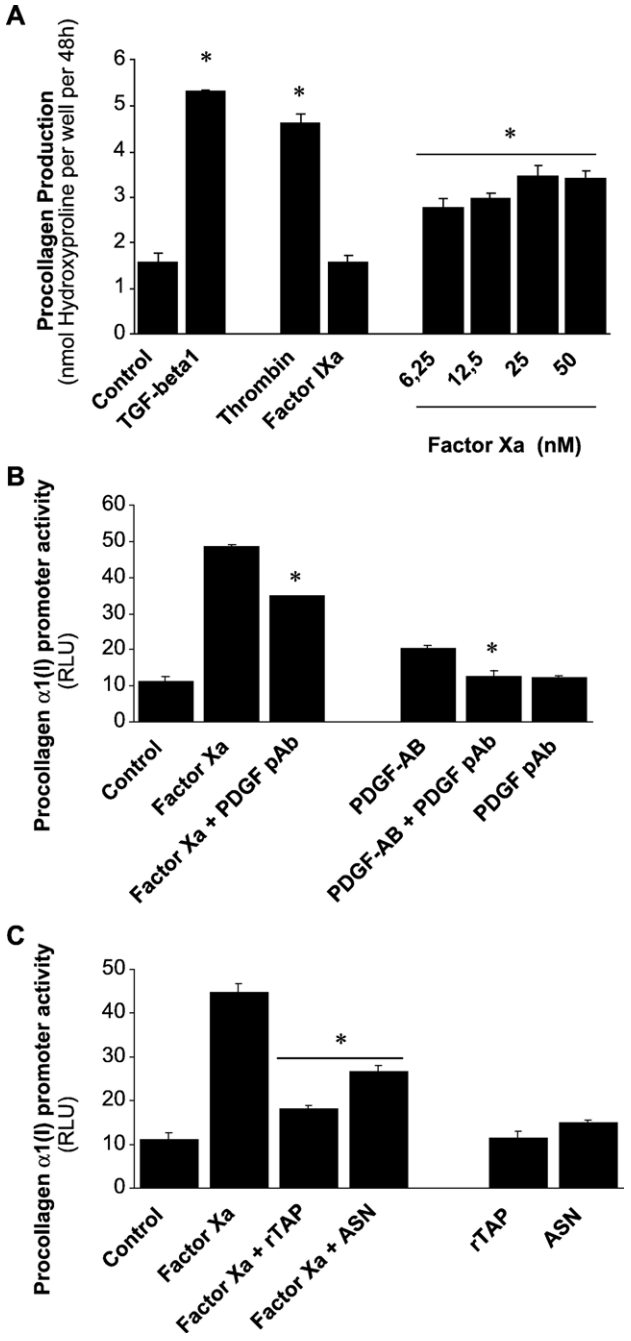

Fig. 1. Factor Xa stimulates fibroblast procollagen production. (Panel A) Procollagen production by human fibroblasts. Confluent human fetal lung fibroblasts were washed and incubated with either serum-free control medium, factor Xa (6.25, 12.5, 25 and 50 nM), thrombin (25 nM), factor IXa (25 nM), or TGF-β_1 (40 pM), for 48 h and harvested. Total hydroxyproline content was quantified by reverse-phase HPLC. Data are expressed as the mean of six replicates from a representative experiment that was repeated three times and expressed as nmol of hydroxyproline per well per 48 h ± SEM * $P < 0.01$; P values are calculated against control treatment. (Panel B) Procollagen-α_1(I) promoter activity in human fibroblasts. Serum-deprived confluent human fetal lung fibroblasts were transiently transfected with a human procollagen-α_1(I) promoter-luciferase reporter construct. The transfected cells were washed and incubated with factor Xa (25 nM), PDGF-AB (10 ng/ml), or with a polyclonal pan-specific anti-PDGF neutralizing antibody (15 μg/ml) for 24 h. Luciferase activity was determined as described in Materials and methods. Data are expressed as mean of three replicates in relative light units (RLU) ± SEM from a representative experiment that was repeated twice. *P values <0.01 against treatment without anti-PDGF blocking antibody. (Panel C) Procollagen-α_1(I) promoter activity in human fibroblasts. The experimental protocol was as described for panel B, but fibroblasts were transfected with the human procollagen-α_1(I) promoter-reporter construct were incubated with medium only, factor Xa (25 nM), rTAP (125 nM), or ASN (1 mM) for 24 h. Data are expressed as mean of three replicates in relative light units (RLU) ± SEM from a representative experiment that was repeated twice. *P values <0.01 against treatment with factor Xa alone.

Proliferation of PAR_1-deficient mouse lung fibroblasts

We also assessed proliferation and immediate transient cytosolic Ca^{2+} signals provoked by factor Xa in PAR_1-deficient mouse lung fibroblasts in serum-free conditions. Factor Xa and thrombin stimulated a doubling in wild-type mouse fibroblast proliferation ($P < 0.01$), but PAR_1-deficient fibroblasts completely failed to respond (Fig. 3A). In contrast, mast cell tryptase, known to activate PAR_2, stimulated proliferation equally in wild-type and PAR_1-deficient fibroblasts (45% increase; $P < 0.01$ versus controls).

Cytosolic Ca^{2+} signals in PAR_1-deficient mouse lung fibroblasts

In wild-type mouse lung fibroblasts, factor Xa elicited a rise in cytosolic Ca^{2+} (60 cells evaluated per condition) 70 s after stimulation, reaching up to 310% above basal levels, (Fig. 3B). Thrombin elicited a Ca^{2+} rise 30 s after stimulation, reaching up to 800% above basal levels. In contrast, PAR_1-deficient fibroblasts showed greatly reduced responses. Cytosolic Ca^{2+} signals provoked by factor Xa and thrombin exhibited certain differences: the average Ca^{2+} signal

triggered by factor Xa was modest in amplitude, and its onset was delayed compared with thrombin. However, factor Xa stimulated cytosolic Ca^{2+} signals in over 90% mouse wild-type fibroblasts, compared to less than 10% for PAR_1-deficient fibroblasts. Thrombin stimulated Ca^{2+} transients in 100% wild-type fibroblasts but none in PAR_1-deficient fibroblasts, in agreement with previous observations [20].

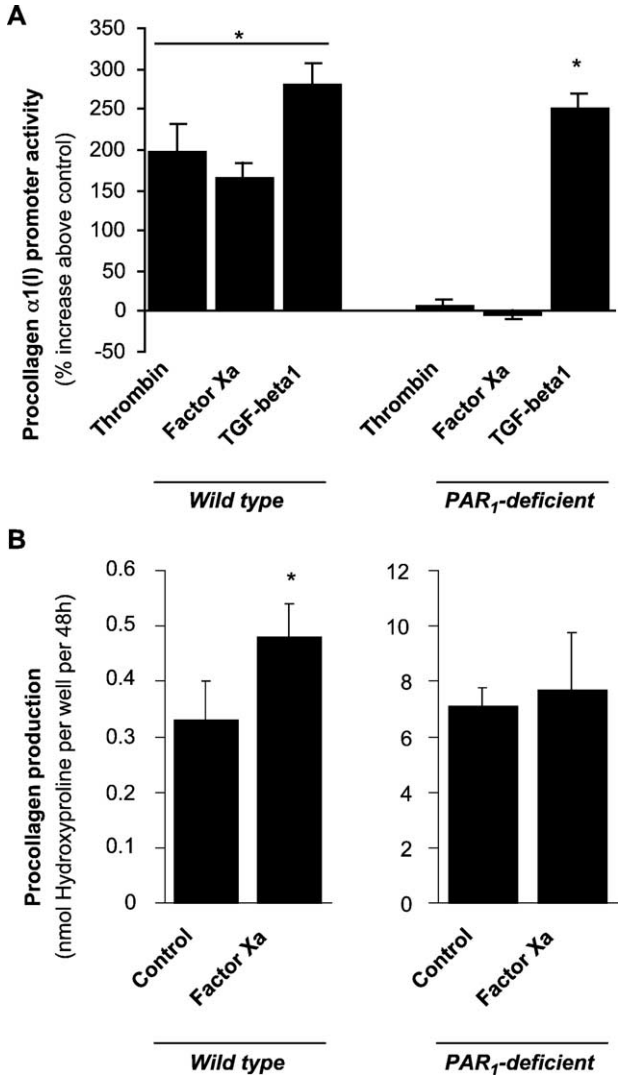

Fig. 2. PAR_1 mediates factor Xa procollagen gene expression in mouse fibroblasts. (Panel A) Procollagen-α_1(I) promoter activity in PAR_1-deficient mouse fibroblasts. The experimental protocol was as described in Fig. 1B, but experiments were performed with wild-type and PAR_1-deficient fibroblasts transfected with the procollagen-α_1(I) promoter-reporter construct and incubated with medium only, TGF-β_1 (1 ng/ml), factor Xa (25 nM), or thrombin (25 nM) for 24 h. Data are expressed as mean of four replicates in % increase above controls ± SEM from a representative experiment that was repeated twice. Data are presented in % stimulation above control levels to facilitate comparisons. *P values <0.01 against control treatment. (Data in Relative light units: Control, 22.0 ± 1.6, factor Xa 51.9 ± 3.0, thrombin 57.8 ± 6.2, TGF-β_1 80.1 ± 5.6, in control fibroblasts. Control, 66.2 ± 6.1, factor Xa 62.9 ± 5.2, thrombin 69.0 ± 3.2, TGF-β_1 173.6 ± 9.7, in PAR_1-deficient fibroblasts). (Panel B) Procollagen production in PAR_1-deficient mouse fibroblasts. Experiments were performed with wild-type and PAR_1-deficient fibroblasts incubated with medium only, TGF-β_1 (1 ng/ml), factor Xa (25 nM), or thrombin (25 nM) for 48 h. The data represent the amount of hydroxyproline associated with the cell layer and is expressed as the mean of six replicates in % increase above controls ± SEM from a representative experiment that was repeated twice. *P values <0.01 against control treatment.

EPR-1 expression in PAR_1-deficient mouse lung fibroblasts

Since we have previously implicated EPR-1 in the mitogenic effects of factor Xa on human fibroblasts [25], we also investigated EPR-1 expression in mouse fibroblasts by Western blot analysis with a sequence-specific antibody directed against the cytoplasmic domain of the receptor. A predominant immunoreactive band of 65 kDa corresponded to the molecular weight of human EPR-1 (Fig. 3C). The intensity of the EPR-1 band was comparable for wild-type and PAR_1-deficient lung fibroblasts.

Cytosolic Ca^{2+} signals in human lung fibroblasts

To determine the role of PAR_1 in factor Xa signaling in human fibroblasts, we studied the effects of factor Xa, catalytically inhibited factor Xa, thrombin and the highly specific PAR_1 agonist peptide, TFLLR [28] on $[Ca^{2+}]_i$ in naive human fibroblasts. Fig. 4A shows that factor Xa provoked an average increase in $[Ca^{2+}]_i$ of 195% above basal levels over the monitoring period ($P < 0.05$). The peak increase in $[Ca^{2+}]_i$ reached 225% above basal levels about 50 s after stimulation. However, each Ca^{2+} transient in individual cells was only about 100 s long. Since the signals were non-synchronous and delayed by up to 80 s, the combined transient signal (average of about 60 cells) thus appeared to be of lower intensity but of more sustained duration. In addition, 15% of fibroblasts did not respond to factor Xa, reflecting a heterogeneous response. Catalytic site-inhibited DEGR-factor Xa did not elicit Ca^{2+} signals (Fig. 4A, left). Thrombin provoked an average increase in $[Ca^{2+}]_i$ of 285% above basal levels over the monitoring period ($P < 0.05$), which peaked at 410% above basal levels 40 s after stimulation (Fig. 4A, right). At the individual cell level, the thrombin response was similar in time and intensity to that of factor Xa, however, all fibroblasts responded in a synchronous fashion within 10 s of stimulation. Responses to the specific PAR_1 agonist peptide TFLLR [28] were equivalent to those of thrombin, as previously observed in other cells [20,44].

Cytosolic Ca^{2+} signals in human lung fibroblasts desensitized with thrombin

Following PAR_1 activation, thrombin is known to cause a rapid and transient desensitization of PAR_1 signaling responses [20]. We measured factor Xa-induced Ca^{2+} transients in thrombin-desensitized human fibroblasts. Fig. 4B shows that pretreatment with thrombin (25 nM) for 90 min totally desensitized cells to subsequent thrombin stimulation (complete inhibition, $P < 0.01$). Fibroblasts

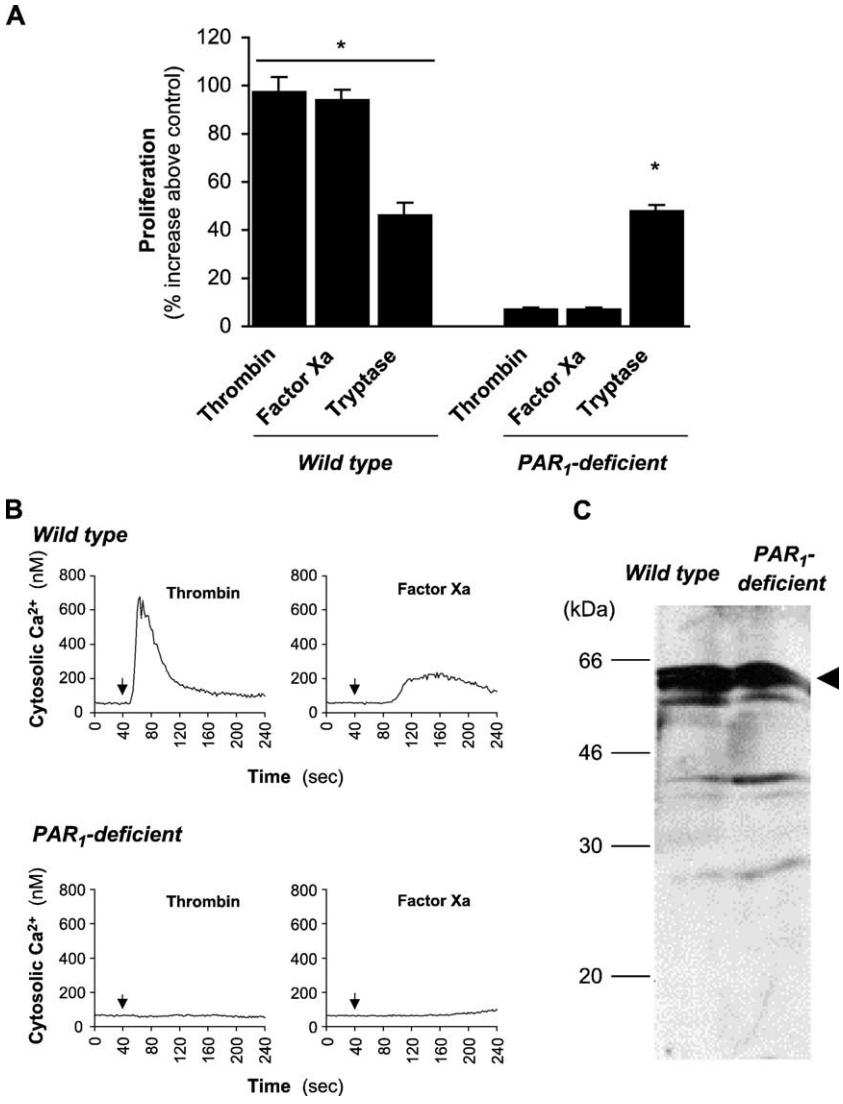

Fig. 3. PAR_1 mediates factor Xa signaling and proliferation in mouse fibroblasts. (Panel A) PAR_1-deficient mouse fibroblasts. Lung fibroblasts derived from wild-type and PAR_1-deficient mice were grown to subconfluence, quiesced for 24 h and incubated for 48 h in serum-free control medium, thrombin (25 nM), factor Xa (25 nM) or tryptase (25 nM). The fibroblast monolayer was fixed and stained with methylene blue which was quantified by spectrophotometry following elution. Data are expressed as mean of 12 replicates in % increase above control ± SEM from a representative experiment that was repeated four times. *P values <0.01 against control treatment. (Panel B) Calcium signaling. Subconfluent mouse lung fibroblasts were washed with HBS and incubated for 30 min with 2 μM of the AM ester of the Ca^{2+} fluorescent indicator FURA-2. The fibroblast monolayers were washed twice with HBS and mounted on the stage of an inverted microscope fitted with an imaging system. Cytosolic-free Ca^{2+} was quantified by recording differential fluorescence after excitation of the cells at 340 and 380 nm. $[Ca^{2+}]_i$ was monitored for 40 s before cells were exposed to factor Xa (100 nM) or thrombin (100 nM) and for 200 s thereafter. Arrowheads show time of proteinase delivery. Data are expressed as mean of three independent experiments, each with 10 to 12 cells monitored at each time point (n = 30 to 36). (Panel C) EPR-1 expression by mouse PAR_1-deficient and wild-type fibroblasts. Wild-type and PAR_1-deficient mouse lung fibroblasts were grown to subconfluence, quiesced by serum deprivation and homogenized in SDS buffer and boiled. Aliquots of whole cell lysate normalized for total protein content (80 μg) were electrophoresed on a 12% acrylamide gel under reducing conditions, transferred onto Immobilon membranes and immunoreacted with a rabbit polyclonal anti-EPR-1 antibody (10 μg/ml). EPR-1 appeared as a predominant band of about 65 kDa.

pretreated with thrombin also failed to respond to factor Xa (complete inhibition, $P < 0.01$), consistent with the desensitization of factor Xa cytosolic Ca^{2+} signals.

Cytosolic Ca^{2+} signals in human lung fibroblasts desensitized with factor Xa

We next determined whether the signaling receptor for factor Xa is subject to homologous desensitization. Fig. 4C shows that fibroblasts pretreated with factor Xa did not mobilize cytosolic Ca^{2+} in response to subsequent stimulation with factor Xa (complete inhibition, $P < 0.01$). Moreover, fibroblasts pretreated with factor Xa showed greatly diminished responses to the PAR_1 agonist TFLLR (84% inhibition, $P < 0.01$), both in intensity and duration, suggesting that PAR_1 is desensitized by factor Xa in fibroblasts in a manner reminiscent of thrombin [45].

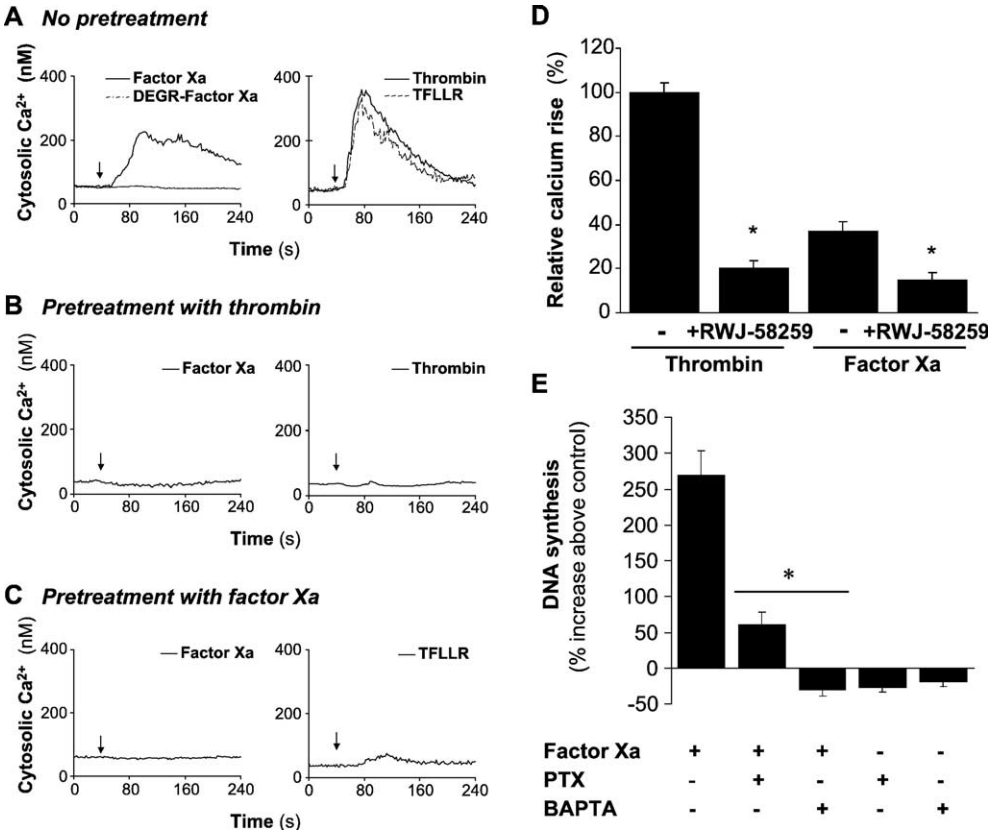

Fig. 4. PAR_1 activation mediates factor Xa signaling in human and mouse fibroblasts. Subconfluent human fetal lung fibroblasts were exposed to serum-free control medium (Panel A), 25 nM thrombin (Panel B) or 25 nM factor Xa (Panel C) for 90 min. Treated fibroblasts were washed twice with HBS, Fura-2 loaded, and mounted on the stage of an inverted fluorescence microscope fitted with imaging system for cytosolic-free Ca^{2+} quantification. $[Ca^{2+}]_i$ was then monitored for 40 s before cells were exposed to factor Xa (100 nM), thrombin (100 nM), or 100 μM PAR_1 agonist peptide TFLLR, and for 200 s thereafter. Arrowheads show agonist delivery. Data are expressed as mean of three independent experiments, each with 10 to 12 cells monitored at each time point ($n = 30$ to 36). (Panel D) Relative inhibition of cytosolic-free Ca^{2+} mobilization in human fetal lung fibroblast treated with thrombin, or factor Xa in the absence or presence of 1 μM of the PAR_1 antagonist RWJ-58259. Data are mean cytosolic-free Ca^{2+} rise in three independent experiments and expressed as % of thrombin signal ± SEM. *P values <0.01 against treatment without RWJ-58259. (Panel E) Human fibroblast proliferation. Human fetal lung fibroblasts were grown to subconfluence, quiesced by serum deprivation, washed, and incubated with 100 μM BAPTA-AM or 100 ng/ml PTX for 2 h. At this point, 25 nM factor Xa or control medium was added and the fibroblasts were further incubated for 16 h. Fibroblasts were then incubated with [^{3}H]thymidine (0.5 μCi/well) for 4 h, harvested, and incorporated radioactivity was quantified. Data are expressed as mean of six replicates in % increase above control ± SEM from a representative experiment that was repeated three times. *P values <0.01 against treatment with factor Xa alone.

Factor Xa cytosolic Ca^{2+} signals in the presence of the PAR_1 antagonist RWJ-58259

To further invoke the role of PAR_1 in mediating fibroblast responses to factor Xa, we examined the effect of a potent and selective PAR1 antagonist, RWJ-58259. Fig. 4D shows that thrombin and factor Xa elicited strong cytosolic Ca^{2+} rises in human lung fibroblasts pretreated with vehicle (HBS). However, pretreatment with RWJ-58259 for 5 min inhibited thrombin and factor Xa signals by 80.0% ($P < 0.05$) and 59.2% ($P < 0.05$), respectively.

Factor Xa mitogenesis is Pertussis toxin and BAPTA-sensitive

To obtain additional supporting evidence that factor Xa modulates fibroblast function via PAR_1, we further examined fibroblast proliferation in response to factor Xa. First, we found that the mitogenic effect of factor Xa at 50 nM was exactly equivalent to that of thrombin at 50 nM ($P > 0.8$), or to that of 25 nM factor Xa in the presence of 25 nM thrombin ($P > 0.9$), (methylene blue dye binding assay; data not shown). We next measured fibroblast proliferation in response to factor Xa in the presence of the Gαi protein inhibitor, PTX, and the intracellular Ca^{2+} chelator, BAPTA [46], which are known to block PAR_1 signaling in response to thrombin [47,48]. Fig. 4E shows that PTX and BAPTA completely inhibit the mitogenic effect of factor Xa on human fetal lung fibroblasts.

Discussion

Our previous report that factor Xa is mitogenic for human lung fibroblasts suggested that other coagulation cascade factors, and not just thrombin, may exert pro-

fibrotic effects. In this study, we investigated further the effects of factor Xa on fibroblast procollagen production and proliferation, and focused on identifying its signaling receptor. We show for the first time that factor Xa stimulates procollagen production by both human and mouse fibroblasts. We also demonstrate that the effects of factor Xa on fibroblast calcium signaling, procollagen production, and proliferation are mediated predominantly by the proteinase receptor, PAR_1.

Factor Xa stimulates procollagen production by human and mouse fibroblasts

We used both a luciferase-reporter gene assay and a biochemical method for the quantification of hydroxyproline and showed that factor Xa stimulates procollagen promoter activity and protein production. The effect of factor Xa on procollagen protein production was dose-dependent and of similar magnitude to that observed with thrombin [17]. In contrast, other coagulation cascade proteinases, such as factor IXa, failed to increase procollagen production at concentrations thought to be generated in vivo [46]. This suggests that factor Xa and thrombin, which are activated during the final steps of the coagulation cascade, play a specific role in the regulation of fibroblast procollagen production that is not shared by upstream coagulation cascade proteinases.

We have previously shown that factor Xa induces human fibroblast proliferation via the upregulation of PDGF-AB production, release, and autocrine stimulation [25]. To determine the potential role of PDGF proliferation in factor Xa-stimulated fibroblast procollagen-α_1(I) promoter activity, we used a pan-specific anti-PDGF neutralizing antibody. This antibody completely blocked the effect of purified PDGF-AB in our procollagen promoter assay, but only blocked the effect of factor Xa by about 25%. In addition, purified recombinant PDGF-AB at optimal mitogenic concentrations only had a modest effect on procollagen-α_1(I) gene transcription compared with factor Xa. Moreover, increased cell proliferation would have little or no effect on procollagen-α_1(I) promoter activity in our conditions as it is unlikely that the construct would be replicated during cell division. We and others have further shown that factor Xa acts independently of prothrombin activation in conditions of serum depletion and with careful washes of the cell layer at the onset of the experiments [25,34,36]. Together, these data imply that autocrine stimulation by PDGF and proliferation would only contribute marginally to procollagen gene expression induced by factor Xa in fibroblasts.

PAR_1 mediates factor Xa stimulation of fibroblast procollagen promoter activity and procollagen production

We used two highly specific factor Xa inhibitors, rTAP and ASN peptide, to show that the induction of procollagen-α_1(I) promoter activity is chiefly dependent on factor Xa catalytic activity in fibroblasts. We further hypothesized that the proteolytic activation of the signaling receptor, PAR_1, may mediate this effect. We obtained PAR_1-deficient lung fibroblasts and showed that factor Xa had no significant effect on procollagen promoter activity and procollagen production in these cells, nor did the control PAR_1 agonist thrombin. In contrast, factor Xa and thrombin stimulated a 3-fold increase in procollagen-α_1(I) promoter activity ($n = 3$, $P < 0.05$), and a 45.5% increase in procollagen production ($n = 3$, $P < 0.05$) in wild-type control fibroblasts. In contrast, TGF-β_1 (positive control) had similar effects on procollagen promoter activity (4-fold increase) in both wild-type and PAR_1-deficient fibroblasts. This suggests that the stimulatory effects of factor Xa on fibroblast procollagen promoter activity, gene transcription and protein production are dependent on PAR_1 expression and proteolytic activation.

PAR_1 mediates factor Xa signaling and proliferation in mouse fibroblasts

We have previously shown that the mitogenic effect of factor Xa for fibroblasts is dependent on its proteolytic activity, but the signaling receptor remained unknown [25]. Here, we provide compelling genetic evidence that PAR_1 plays an essential role in the activation of fibroblasts by factor Xa. Indeed, PAR_1-deficient mouse lung fibroblasts fail to either proliferate or mobilize cytosolic Ca^{2+} in response to factor Xa, whereas these cells proliferate in response to mast cell tryptase [49,50]. Although it has previously been shown that factor Xa can activate PAR_1 in transfected cell lines [26,27], this is to our knowledge the first report that PAR_1 is necessary for factor Xa-stimulated Ca^{2+} signaling and function in fibroblasts.

The only previous observations in normal differentiated cells were obtained using vascular smooth muscle cells [51] and endothelial cells [52,53]. These studies show that PAR_2, rather than PAR_1, mediates factor Xa signaling in the vasculature and are supported by reports that factor Xa evokes aorta relaxation via PAR_2 activation [54]. Our results therefore show for the first time that PAR_1 is the main transducer of factor Xa signaling in fibroblasts, in contrast with the vasculature where PAR_2 plays the major role.

PAR_1 mediates factor Xa signaling and proliferation in human fibroblasts

We aimed to further characterize the specific role of PAR_1 in the activation of human fibroblasts by factor Xa, and examined cytosolic Ca^{2+} signals in human fetal lung fibroblasts. Factor Xa, like thrombin and the highly specific PAR_1 agonist peptide, TFLLR, was able to stimulate strong transient cytosolic Ca^{2+} signals. However, catalytically inhibited factor Xa, DEGR-factor Xa, failed to elicit Ca^{2+} signals in human fibroblasts, suggesting a critical role for factor Xa proteolytic activity. One noticeable difference

between factor Xa signals and those of thrombin and TFLLR is the characteristic delay in its onset seen in human and mouse fibroblasts. The delayed kinetics of PAR_1 cleavage by Xa, previously observed in HeLa cells [27], are difficult to explain fully at this time, but could stem from a rate limitation arising from the requirement for protease binding to the cell surface before proteolytic activation of PAR_1. Thrombin, which directly interacts with specific, high-affinity hirudin-like regions of PAR_1 [19], could show faster binding kinetics than factor Xa, which lacks known specific PAR_1 interaction mechanisms, as discussed by Riewald et al. [27].

Following PAR_1 activation, a period of receptor desensitization follows until new receptors appear at the cell surface [20]. This enabled us to specifically abrogate PAR_1 responses and evaluate potential agonists in our human fibroblasts. Thrombin elicited immediate Ca^{2+} signals that were completely desensitized by thrombin pretreatment, as previously reported for human kidney cells [55], platelets [56], and megakaryocytic cells [38]. PAR_1 desensitization with thrombin abrogated subsequent factor Xa signals (heterologous desensitization). Similarly, desensitization with factor Xa blocked subsequent factor Xa signals (homologous desensitization). Moreover, desensitization with factor Xa prevented subsequent signaling with the highly specific PAR_1 agonist peptide TFLLR [28]. These data suggest that factor Xa signaling is sensitive to specific PAR_1 desensitization, and that factor Xa desensitizes subsequent PAR_1-mediated responses. To further demonstrate the critical role of PAR_1 in factor Xa signaling, we used the specific PAR_1 antagonist RWJ-58259 which prevents PAR_1 signaling without altering other proteinase receptor responses [29]. These studies would allow us to eliminate the possibility that PAR_1 may in fact be desensitized by indirect mechanisms [57] rather than direct factor Xa activation. Fibroblast pretreatment with 1 μM RWJ-58259 prevented thrombin signaling by 80%, and factor Xa signaling by about 60%. When taken altogether, these experiments point toward a central role for PAR_1 in factor Xa signaling in human lung fibroblasts, and specific PAR_1 inhibition suggests that over two thirds of factor Xa signaling in fibroblasts are dependent upon PAR_1 activation.

Additional supportive evidence that factor Xa signaling is mediated by PAR_1 in human fibroblasts was obtained in experiments showing that factor Xa-induced proliferation is dependent on Gαi protein activation and cytosolic Ca^{2+} mobilization, two features of PAR signaling [58]. In addition, the mitogenic effects of factor Xa and thrombin were equivalent at equal concentrations, and their effects were strictly additive, suggesting that the proteolytic activity of thrombin and factor Xa can be substituted to activate a common signaling pathway and directly stimulate fibroblast function. Taken together, our data in human and mouse lung fibroblasts provide compelling evidence that factor Xa activates PAR_1 to stimulate fibroblast intracellular Ca^{2+} signals, procollagen production and proliferation. This is the first report that PAR_1 is the main endogenous signaling receptor for factor Xa in human fetal fibroblasts where PAR_2, PAR_3, and PAR_4 are known to be expressed [49] (and data not shown).

Role of other receptors in factor Xa signaling responses in fibroblasts

In the absence of PAR_1 expression, factor Xa stimulated no significant increase in fibroblast proliferation, procollagen promoter activity, or protein production, suggesting that PAR_1 is necessary to mediate the effects of factor Xa on fibroblast function. However, factor Xa was able to stimulate transient Ca^{2+} signals in about 10% of PAR_1-deficient mouse fibroblasts, and in about 30% of human fetal fibroblasts after specific PAR_1 inhibition. It is likely that some of the remaining detectable signals are mediated by an independent receptor. Since factor Xa binds to EPR-1 or activates PAR_2 in cell types such as smooth muscle and endothelial cells, it is possible that factor Xa activates these receptors in fibroblasts. It is known that factor Xa does not cleave EPR-1 [59] but that catalytically inactive DEGR-factor Xa binds EPR-1 in a similar way to factor Xa [60]. We have previously shown that EPR-1 ligation by factor Xa enhances, but is not sufficient to stimulate fibroblast proliferation [25]. We also showed that the effects of factor Xa on procollagen production (this work) and proliferation [25] depend on factor Xa catalytic activity, even in the presence of EPR-1. Together, these observations suggest that EPR-1 ligation may play an accessory role in the regulation of fibroblast function by factor Xa in vitro. Since PAR_2 is activated by factor Xa in other cell types and expressed by human fetal lung fibroblasts [49], PAR_2 may mediate a proportion of factor Xa-dependent signaling. Finally, it is tempting to speculate that the expression of additional coagulation cascade proteinase receptors such as tissue factor with the simultaneous presence of small amounts of factor VIIa may increase the effects of factor Xa on fibroblasts, as they do for factor Xa in HeLa cells [27]. However, PAR_1-deficient fibroblast studies suggest that the signals mediated by putative additional receptors are not sufficient to engage human or mouse fibroblasts proliferation or procollagen production.

Summary and conclusion

This is, to our knowledge, the first report that factor Xa or any coagulation cascade proteinase other than thrombin, stimulates fibroblast procollagen production. This is also the first study demonstrating that PAR_1 is the major signaling receptor mediating the effects of factor Xa on fibroblast procollagen production and proliferation, implying that factor Xa activates and exerts its cellular effects via distinct PAR receptors in the vasculature and the lung parenchyma. This information is critical to our understanding of tissue

repair mechanisms and lends support to the emergence of PAR_1 as a dominant sensor of multiple signals elicited by coagulation cascade proteinases in fibroblasts. Our observations may take particular significance in the design of antifibrotic therapies. PAR_1 and the signaling pathways triggered by both factor Xa and thrombin may represent attractive targets for interfering with tissue fibrosis while preserving their role in blood coagulation.

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