Factor Xa is a fibroblast mitogen via binding to effector-cell protease receptor-1 and autocrine release of PDGF

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Factor Xa is a fibroblast mitogen via binding to effector-cell protease receptor-1 and autocrine release of PDGF. Am J Physiol Cell Physiol 281: C681–C689, 2001.—The coagulation cascade protease thrombin is a fibroblast mitogen, but the proliferative potential of other coagulation proteases is not known. In this study we show that factor Xa stimulated human fetal lung fibroblast DNA synthesis in a concentration-dependent manner from 1 nM onward with a fourfold increase at 200 nM. The mitogenic effect of factor Xa was confirmed using a colorimetric proliferation assay and direct cell counting. Factor Xa and thrombin had equivalent potencies, and their stimulatory effects followed a similar time course. Comparable results were also obtained with primary human adult fibroblasts derived from lung, kidney, heart, skin, and liver. Factor VIIa also stimulated fibroblast proliferation, but only at concentrations >10 nM, whereas factor IXa had no effect. To begin to address the mechanism by which factor Xa is acting, we show that human fibroblasts express effector-cell protease receptor-1 and that blocking antibodies to this receptor and the catalytic site of factor Xa inhibited its mitogenic effect. Furthermore, factor Xa upregulated platelet-derived growth factor-A (PDGF-A) mRNA expression, whereas PDGF-B could not be detected, and a blocking antibody to PDGF inhibited the mitogenic effect of factor Xa. We conclude that factor Xa acts as a fibroblast mitogen via binding to effector-cell protease receptor-1 and the autocrine release of PDGF.

FIBROBLAST PROLIFERATION and synthesis of extracellular matrix proteins are key features of connective tissue deposition during normal tissue repair and fibrosis. These fibroblast functions are regulated in part by cytokines and growth factors such as platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β). However, there is increasing evidence that thrombin, a key protease of the coagulation cascade, also promotes tissue repair responses in vivo (10) and promotes fibroblast proliferation (35) and procollagen production in vitro (11). These effects are independent of thrombin’s procoagulant function. In contrast to thrombin, the effects of other coagulation cascade proteases on fibroblasts have been little studied apart from one report suggesting that coagulation factors Xa and VIIa have no effects on fibroblast proliferation (13).

After tissue injury, several proteases structurally related to thrombin are activated in the blood coagulation cascade, including factors VIIa, IXa, and Xa. The binding of factor VIIa to tissue factor on cell surfaces is the primary trigger of the coagulation cascade extrinsic pathway. This binding enhances the proteolytic activity of factor VIIa (14) and leads to the generation of small amounts of factor Xa (12). Feedback mechanisms trigger the intrinsic pathway and the formation of factor IXa, which results in greater amounts of factor Xa (12, 24). Subsequently, factor Xa activates prothrombin into thrombin, which cleaves blood-borne fibrinogen into an insoluble fibrin clot.

During blood coagulation, factors Xa and IXa assemble into macromolecular complexes on cell surfaces involving membrane phospholipids, calcium ions, and cofactors, such as factor VIIIa for factor IXa, and factor Va for factor Xa. These cofactors greatly enhance the proteolytic activity of these proteases (12). In addition, factor Xa can bind to a novel protease receptor termed effector-cell protease receptor-1 (EPR-1) on the surface of platelets (8) and vascular (16) and inflammatory cells (4). EPR-1 is immunologically related to factor Va (3) and thought to help localize factor Xa to cell surfaces. EPR-1 may also enhance factor Xa-prothrombin interactions during blood coagulation (8) and promote the proteolytic activity of factor Xa (1, 8). In addition to its procoagulant effects, factor Xa acts as a mitogen for smooth muscle (13) and endothelial cells (24) by upregulating the production of PDGF and autocrine stimulation (16, 20).

The recurrent and excessive activation of the coagulation cascade extrinsic pathway is a feature common
to diseases that are characterized by excessive fibroblast proliferation and connective tissue deposition. These include fibrotic disorders of the lung (17, 18, 21), kidney (34), and liver (32). Despite this, the effects of the coagulation factors on fibroblast proliferation, as well as the expression and role of EPR-1 in these cells, have never been investigated.

In this study, we hypothesized that factors VIIa, IXa, and Xa, which share high structure homology with thrombin, have similar effects on fibroblasts and found that factor VIIa and Xa are strong mitogens for human fibroblasts. We also investigated the expression of EPR-1 and began to examine the molecular basis of the effect of factor Xa in fibroblasts.

METHODS

Materials. Preparations of purified Russel viper venom-activated human factor Xa, factor IXa, and factor VIIa, as well as dansyl-Glu-Gly-Arg chloromethylketone dihydrochloride (DEGR-CK), were purchased from Calbiochem-Novabiochem (Nottingham, UK). Preparations of purified human thrombin, factor X, trypsin, and the specific thrombin inhibitor recombinant leech hirudin (rHir) were purchased from Sigma Chemical (Poole, UK). Recombinant human PDGF-AB was obtained from R&D Systems Europe (Abingdon, UK). Recombinant tick anticoagulant peptide (rTAP) 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). Antistain coreptide d-Arg32-Pro38 (ASN) was purchased from Bachem (Safforn Walden, UK).

Antibodies. The polyclonal antibody JC15 is a mouse antibody raised against a synthetic peptide mimicking the inter-EGF repeats residues Leu36-Leu48 of human factor Xa (6). The mouse monoclonal antibody B6 blocks factor Xa binding to endothelial cells by 50% (25), and it is directed against residues Pro29-Ala34 of human EPR-1 (5). Both antibodies were generous gifts from Dr. D. Altieri (Yale University, New Haven, CT). A polyclonal, pan-specific anti-human PDGF antibody due to the shorter incubation time (20 h vs. 48 h). Key results were confirmed by direct cell counting. Quiescent human fibroblasts were incubated in DMEM supplemented with test substances in the absence of serum. After 48 h, cells were washed in PBS, fixed in 10% formol saline for 24 h, and stained with 0.1% methylene blue in borate saline for 1 h. Methylen blue binding to negative charges in proteins correlates with cell number (30). The cells were then washed extensively with borate saline, and the remaining dye was eluted with 50% acidified ethanol and quantified by spectrophotometry at 650 nm. DNA synthesis was monitored by addition of [3H]thymidine (2 µCi/ml) for the last 4 h of a 20-h incubation with the test substances. DNA was harvested on filter paper with a cell harvester. Filters were washed with excess H2O and shaken in scintillation fluid. Radiolabel incorporation was measured in disintegrations per min in a scintillation counter.

Data were expressed as means ± SE in percent stimulation above control. Single comparison statistical evaluation was performed using the unpaired Student t-test. ANOVA tests were used for single-parameter multiple measurement comparisons such as concentration-response curves. Significance was achieved when P < 0.05.

Protease inhibition protocols. Factor Xa was incubated with rTAP or a peptide mimicking the core inhibitory region of ASN for 2 h at 37°C. Catalytically inactivated factor Xa (DEGR-factor Xa) was prepared by Dr. C. Goodwin (Thrombosis Research Institute, London, UK) by incubating factor Xa with DEGR-CK until no remaining proteolytic activity was detectable in chromogenic assays. Excess DEGR-CK was removed by extensive dialysis, and the purity of the DEGR-factor Xa was assessed by conventional SDS-PAGE and silver staining.

Assessment of PDGF-A mRNA levels by RT-PCR. Cells were seeded in 6-cm-diameter dishes (2 × 10^5 cells/ml well^-1^) and grown for 24 h in DMEM supplemented with 5% serum. By this time, the fibroblasts had reached ~75% visual confluency. Cells were quiesced in serum-free medium for 24 h and incubated in fresh serum-free DMEM for 6 h in the presence or absence of thrombin (25 nM) or factor Xa (25 nM). Total RNA was isolated with TRIzol reagent (GIBCO BRL, Paisley, UK).

For cDNA synthesis, 1 µg of total RNA in 5 µl of diethyl pyrocarbonate-treated water was incubated at 65°C for 10
A reverse transcriptase (RT) reaction mixture was added, yielding a total reaction volume of 20 µl containing 1× avian myeloblastosis virus (AMV) RT buffer (Promega, Southampton, UK), 1 mM of GTP, ATP, TTP, and CTP (Perkin-Elmer; PE Biosystems, Warrington, UK), 0.05 U/µl RNase inhibitor (Perkin-Elmer N808-0119), 0.125 µM random hexamer (Perkin-Elmer N808-0127), and 0.5 U/µl AMV RTase (Promega M5101). This mixture was incubated for 55 min at 41°C and stored at −20°C before use.

PCR reactions were performed by incubating 2 µl of cDNA with 1× KCl buffer, 0.2 mM dNTPs, 2.5 mM MgCl2, 1 pM sense and antisense primers, and 0.25 U/µl Taq polymerase in a total volume of 20 µl. Hypoxanthine phosphatidyl ribosyltransferase (HPRT) was used as an internal control for total cellular mRNA levels. Primers (5’ to 3’) used were PDGF-A sense (CTG GAG ATA GAC TCC GT) and PDGF-A antisense (CCT GAC GTA TTC CAC CT) (36). Primers were synthesized in the Department of Immunology, Erasmus University (Rotterdam, The Netherlands). Amplified cDNA products were 336 and 454 bp, respectively. PDGF PCR was performed for 45 cycles (denaturation 94°C, 1 min; annealing 60°C, 1 min; extension 72°C, 1 min), followed by an extension period (72°C, 10 min). HPRT PCR was performed for 30 cycles (denaturation 94°C, 30 s; annealing 55°C, 30 s; extension 72°C, 1 min), followed by an extension period (72°C, 10 min). Ten-microliter aliquots of PCR reaction mixture were electrophoresed on 2% agarose gel with ethidium bromide. PCR products were visualized using a combined phosphoimager-fluorescent laser scanner (Fuji FLA 3000).

**Northern blot analysis.** Quiescent fibroblasts were homogenized into TRIzol reagent (Life Technologies, Paisley, UK). Total RNA was isolated according to the manufacturer’s instructions. Twenty micrograms of total RNA were mixed with loading buffer and loaded into 1% agarose gels. After electrophoresis, RNA was transferred to nylon filters and UV cross-linked. A 1.6-kb EPR-1 cDNA probe was prepared from the full-length EPR-1 cDNA construct kindly provided by Dr. D. Altieri (Yale University, New Haven, CT) according to published protocols (25). The full-length EPR-1-coding cDNA sequence was grown in a bacterial expression vector, cut out, and used as a template for cDNA probe synthesis in the presence of [32P]dCTP, using a random priming DNA labeling system. Hybridization with the radiolabeled cDNA probe for EPR-1 was allowed to occur on the filter overnight at 65°C. Filters were washed once in 2× standard saline citrate (SSC), 1% SDS for 10 min at room temperature, once in 2× SSC, 0.5% SDS for 10 min at room temperature, and once in 1× SSC, 0.1% SDS for 10 min at 65°C in shaker incubator. After washing, filters were exposed to autoradiographic film at −70°C with intensifying screens for 5 days.

**RESULTS**

**Effect of factors VIIa, IXa, and Xa and thrombin on human fibroblast proliferation.** Figure 1 shows the effect of factor Xa (A), thrombin (B), factor VIIa (C), and factor IXa (D) on human fetal lung fibroblast mitogenesis. Factor Xa stimulated fibroblast DNA synthesis in a concentration-dependent fashion (F<sub>max</sub>) between 100 and 200 nM. The half-maximal response (EC<sub>50</sub>) was ~25 nM. All results were confirmed by direct cell counting, as well as a dye-binding assay (Table 1). There was a good correlation between the trends obtained with the three assays employed, but the DNA synthesis assay generally gave greater stimulations. In addition, factor Xa at 100 nM produced the same mitogenic response as 0.4 nM PDGF-AB. This represented about one-half of the effect of

**Fig. 1.** Effects of factor Xa (A), thrombin (B), factor VIIa (C), and factor IXa (D) on human fetal lung fibroblast DNA synthesis. Quiescent human fetal lung fibroblasts were incubated with the proteases in serum-free medium for 20 h. As an indication of physiological levels, protease concentrations above that of their corresponding zymogens in normal plasma are shown in gray fields. Factor Xa and thrombin were assessed at concentrations up to 200 nM, above which they displayed trypsin-like activity and detached cells from their substratum. Factors VIIa and IXa were assessed at concentrations up to ~10-fold that of their corresponding zymogens in normal human plasma. Data shown were obtained with the [3H]thymidine uptake assay and are expressed as means ± SE of 6 replicates in % increase above control from a representative experiment of at least 3 repeats. P values are calculated against medium-treated controls.
To determine dependent on proteolytic activity. Figure 3 shows that ASN peptide D-Arg32-Pro38 blocked this effect with specific direct catalytic site inhibitors. Figure 4 shows that a pan-specific PDGF neutralizing antibody almost completely blocked fibroblast DNA synthesis in response to factor Xa (P < 0.05). In contrast, factor IXa had little effect on fibroblast proliferation at concentrations up to 1,000 nM (P > 0.1).

Figure 2 shows the time course of fibroblast proliferation in response to factor Xa and thrombin (25 nM) over 72 h. The amplitude of the mitogenic effect of the proteases was similar at all time points.

Table 2 shows the mitogenic potential of factor Xa in primary human fibroblasts derived from adult normal lung, heart, skin, and kidney tissue. Factor Xa stimulated the proliferation of all adult human fibroblast types tested. However, the magnitude of the response was between one-half and two-thirds of that seen with human fetal fibroblasts.

The mitogenic effect of factor Xa for fibroblasts is dependent on proteolytic activity. To determine whether the mitogenic effect of factor Xa on fibroblasts is dependent on its proteolytic activity, we tried to block this effect with specific direct catalytic site inhibitors. Figure 3 shows that ASN peptide D-Arg32-Pro38 and rTAP, used at concentrations that did not affect basal proliferation, completely blocked the mitogenic effect of factor Xa. DEGR-factor Xa, an inactivated form of factor Xa, also failed to stimulate fibroblast proliferation.

To investigate the possibility that the mitogenic effect of factor Xa is mediated by local production of thrombin or contamination of the factor Xa preparation, the mitogenic effect of factor Xa was determined in the presence of rHir, a specific thrombin inhibitor. rHir did not block the mitogenic effect of factor Xa but completely inhibited that of thrombin at concentrations that did not affect basal proliferation. Similar results were obtained with the thrombin inhibitor phenylalanyl-prolyl-arginine chloromethyl ketone (PPACK; results not shown). Finally, the inactive zymogen factor X only had a modest mitogenic effect on fibroblast proliferation compared with factor Xa and thrombin used at similar concentrations.

The mitogenic effect of factor Xa in fibroblasts is mediated by autocrine PDGF stimulation. We investigated the possibility that autocrine PDGF may mediate the mitogenic effect of factor Xa in fibroblasts. Figure 4 shows that a pan-specific PDGF neutralizing antibody almost completely blocked fibroblast DNA synthesis in response to factor Xa (>75% inhibition), as well as thrombin (85% inhibition). The antibody alone had no effect on basal proliferation.

We then determined whether factor Xa stimulates PDGF gene expression and measured PDGF-A mRNA levels by RT-PCR in fibroblasts stimulated with factor Xa, with thrombin as positive control. Figure 5 shows that PDGF-A mRNA was undetectable in unstimulated fibroblasts. Table 2 shows the mitogenic effect of factor Xa on human fibroblast proliferation.

Table 2. Effect of factor Xa on human fibroblast proliferation

<table>
<thead>
<tr>
<th>Fibroblast Tissue of Origin</th>
<th>Proliferation, % increase above control</th>
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<tbody>
<tr>
<td>Fetal lung</td>
<td>92.3 ± 4.6</td>
</tr>
<tr>
<td>Adult lung</td>
<td>45.8 ± 3.0</td>
</tr>
<tr>
<td>Adult skin</td>
<td>35.7 ± 1.3</td>
</tr>
<tr>
<td>Adult heart</td>
<td>39.9 ± 1.2</td>
</tr>
<tr>
<td>Adult kidney (SV40 transformed)</td>
<td>55.7 ± 6.5</td>
</tr>
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</table>

Data shown are expressed as means ± SE of 6 replicates in % increase above control from a representative experiment of 2 repeats for each cell type. Factor Xa stimulated the proliferation of human fetal fibroblasts as well as fibroblasts derived from human adult lung, skin, heart, and kidney tissue. Quiescent fibroblasts were incubated in the presence of factor Xa (25 nM) or medium alone for 48 h. P values were calculated against medium-treated controls (P < 0.001 for all experiments).
lated fibroblasts, but PDGF-A gene expression was induced in fibroblasts stimulated by factor Xa or thrombin. As control for equal mRNA loading, we show that HPRT mRNA levels were comparable in all samples.

The mitogenic effect of factor Xa in fibroblasts is dependent on binding to EPR-1. To determine whether human fetal lung fibroblasts express EPR-1, we examined EPR-1 mRNA and protein expression in our cells by Northern and Western blot analysis. Figure 6 shows that two EPR-1 mRNA transcripts of about 1.8 kb and 3.0 kb were detected with an EPR-1-specific radiolabeled cDNA probe (Fig. 6A). The 1.8-kb band appeared to predominate based on signal intensity. The anti-EPR-1 monoclonal antibody B6 detected three proteins with approximate molecular mass values of 52, 58, and 65 kDa (Fig. 6B). No proteins were detected when a nonspecific mouse antiserum was used.

We employed three different blocking antibodies to identify the regions of factor Xa and EPR-1 that mediate the mitogenic effect of factor Xa in fibroblasts (Table 3). A polyclonal antibody directed against the EPR-1-binding region of factor Xa (JC15) inhibited factor Xa-stimulated DNA synthesis by up to 72%. In addition, monoclonal antibodies directed against the catalytic site of factor Xa (5224) or against the factor Xa-binding site of EPR-1 (B6) inhibited the mitogenic effect of factor Xa by up to 69% and 38%, respectively ($P < 0.01$ for all experiments). In contrast, these three antibodies had no effect on thrombin-stimulated proliferation, nor on basal fibroblast proliferation when used alone. Finally, purified mouse IgG1 antibodies at concentrations up to 50 μg/ml did not inhibit the effects of factor Xa and thrombin (data not shown).

DISCUSSION

We examined the mitogenic potential of factors VIIa, IXa, and Xa on fibroblasts on the basis of the hypothesis that coagulation cascade proteases structurally related to thrombin may have similar effects. We found that factor Xa is mitogenic to human fibroblasts.

Factor Xa is a potent mitogen for human fibroblasts. We show for the first time that factor Xa stimulates human fetal lung fibroblast proliferation in a concentration-dependent manner from 5 nM upward. Three methods, i.e., [3H]thymidine uptake, methylene blue

Fig. 4. A pan-specific anti-platelet-derived growth factor (anti-PDGF) polyclonal antibody abrogates the mitogenic effect of factor Xa in fibroblasts. Quiescent human fetal lung fibroblasts were incubated with factor Xa (25 nM), factor Xa + a polyclonal pan-specific anti-PDGF antibody (15 μg/ml), thrombin (25 nM), thrombin + anti-PDGF antibody, PDGF-AB (0.4 nM), PDGF-AB + anti-PDGF antibody, anti-PDGF antibody alone or serum-free medium alone for 20 h. Data shown were obtained with the [3H]thymidine uptake assay and are expressed as means ± SE of 6 replicates in %increase above control from a representative experiment of 3 repeats. P values are calculated between treatments.

Fig. 5. PDGF mRNA expression in human fetal lung fibroblasts. mRNA analysis: nonconfluent quiesced fibroblasts were treated with factor Xa (25 nM), factor Xa + recombinant factor Xa (25 nM), factor Xa + recombinant leech hirudin (rHir; 200 nM), DEGR-factor Xa (25 nM), factor X (25 nM), thrombin (25 nM), or thrombin + rHir for 48 h. The protease inhibitors rTAP, ASN, and rHir had no effect alone. The protease-inhibitor complexes were allowed to form for 2 h at 37°C before addition to the cells. Data shown were obtained with the methylene blue dye assay and are expressed as means ± SE of 6 replicates in %increase above control from a representative experiment of 3 repeats. P values are calculated between treatments.
dye binding, and direct cell counting, confirmed this effect. Together, these assays suggested that factor Xa stimulates entry and progression through the complete cell cycle. Preparations of factor Xa from alternative leading manufacturers (Calbiochem and American Diagnostica, data not shown) produced similar results, confirming that the mitogenic effect of factor Xa is not the eccentric characteristic of a single preparation.

Table 3. Effect of neutralizing antibodies on human fibroblast proliferation

<table>
<thead>
<tr>
<th>Protease</th>
<th>Antibody</th>
<th>DNA Synthesis, %increase over medium alone</th>
<th>Specific %Inhibition vs. Protease Alone</th>
<th>P values</th>
</tr>
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<tbody>
<tr>
<td>Factor Xa</td>
<td></td>
<td>254 ± 13%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Factor Xa</td>
<td>mAb 5224</td>
<td>76 ± 20%</td>
<td>69%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Factor Xa</td>
<td>pAb JC15</td>
<td>86 ± 20%</td>
<td>72%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Factor Xa</td>
<td>mAb B6</td>
<td>173 ± 13%</td>
<td>38%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Thrombin</td>
<td></td>
<td>240 ± 38%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombin</td>
<td>mAb 5224</td>
<td>215 ± 23%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombin</td>
<td>pAb JC15</td>
<td>248 ± 33%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombin</td>
<td>mAb B6</td>
<td>264 ± 27%</td>
<td></td>
<td></td>
</tr>
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</table>

Results shown are expressed as means ± SE of 6 replicates in %increase above control from a representative experiment of 3 repeats. The effects of neutralizing antibodies on factor Xa- and thrombin-stimulated fibroblast DNA synthesis were assessed with the [3H]thymidine uptake assay. Quiescent fibroblasts were incubated with factor Xa (25 nM), thrombin (25 nM), or medium alone and in the presence or absence of antibodies 5224 (5 μg/ml), JC15 (10 μg/ml), and B6 (50 μg/ml) for 48 h. The monoclonal antibody (mAb) 5224 neutralizes the catalytic site of factor Xa, the polyclonal antibody (pAb) JC15 is directed against the effector-cell protease receptor-1 (EPR-1) binding site of factor Xa (residues Leu53-Leu68 of factor Xa), and the mAb B6 is directed against the factor Xa binding site of EPR-1 (see Materials). These antibodies were all raised in mice and used at the highest concentration that did not alter basal fibroblast DNA synthesis. Purified mouse IgG1 antibodies did not inhibit factor Xa- or thrombin-induced DNA synthesis at concentrations up to 50 μg/ml (data not shown). P values were calculated between protease alone and protease + antibody. NS, not significant.

Factor Xa stimulated proliferation in primary cultures of fibroblasts derived from human adult lung, skin, heart, and kidney tissue. Fibroblasts exhibited a greater response to factor Xa than primary adult cells. They also proliferated more in response to serum, implying that fetal fibroblasts have a higher proliferative potential than adult cells. The proliferative responses to factor Xa were determined at the concentration of 25 nM for comparison. However, all fibroblasts responded to factor Xa following a concentration-response curve similar to that of fetal lung fibroblasts, reaching a maximum between 100 and 200 nM. The proliferative response to factor Xa at near-physiological concentrations (25 nM) was similar to optimal concentrations of PDGF-AB (0.4 nM) and equivalent to about one-half the effect of 10% serum in fetal and adult cells.

In normal human plasma, the concentration of factor X (zymogen) is ~140 nM (24). Thus concentrations of factor Xa could theoretically reach 100 nM or more at sites of blood coagulation and cause fibroblast proliferation in vivo.

The observation that factor Xa is mitogenic for fibroblasts and comparable to thrombin contrasts with a previous report that showed the mitogenic effects of factor Xa in smooth muscle cells, but not in mouse Swiss 3T3 and calf dermal fibroblasts (13). The reasons for this discordance are uncertain, but species differences between some fibroblasts (mouse) and proteases (bovine) used are a possible explanation. Our study also shows that human dermal fibroblasts are the least responsive cells tested. Thus the lack of responsiveness of their calf skin fibroblasts (13) may be characteristic of skin tissue.

Our data show that the time course of the mitogenic effects of factor Xa and thrombin on fibroblasts is
The increased proliferation (13 previously been demonstrated (9, 22), and it is possible that the protease activity of fibroblasts has previously been shown to have no effect on fibroblast proliferation (35). Incidentally, the fact that factor VII has no effect on fibroblast proliferation during normal tissue repair, and the principal mitogenic coagulation cascade proteases may be thrombin and factors Xa of the extrinsic pathway. In addition, factor VIIa may increase fibroblast mitogenesis during excessive or recurrent coagulation cascade activation.

Effects of factor VIIa and factor IXa on human fibroblast proliferation. Factor VIIa stimulated fibroblast DNA synthesis from 10 nM upward, whereas the concentration of factor VII in normal human plasma approximates 7 nM (24). Factor IXa had no significant effect on fibroblast proliferation at concentrations up to 10-fold higher than its zymogen in normal human plasma, 89 nM (24). Thus factor IXa stimulates fibroblast proliferation at concentrations that may not occur during normal tissue repair, and the principal mitogenic coagulation cascade proteases may be thrombin and factors Xa of the extrinsic pathway. In addition, factor VIIa may increase fibroblast mitogenesis during excessive or recurrent coagulation cascade activation.

Factor Xa upregulates fibroblast proliferation via its proteolytic activity and PDGF autocrine stimulation. rTAP and ASN, which are known to inhibit the catalytic site of factor Xa via highly specific but reversible binding (19, 29), completely abrogated the mitogenic effect of factor Xa on fibroblasts. In addition, targeting the catalytic site of factor Xa with a monoclonal antibody (5224) (Ref. 37) inhibited 69% of its mitogenic effect (Table 3), whereas antibody 5224 had no effect on thrombin-stimulated proliferation. Furthermore, the specific thrombin inhibitors rhir and PPACK had no effect on factor Xa-induced proliferation.

First, these data show that the mitogenic effect of factor Xa is dependent on its proteolytic activity, and factor Xa must gain its mitogenic potential on activation during blood coagulation. This is confirmed by the fact that factor X had few effects on fibroblast proliferation. Moreover, a similar paradigm of activation/gain-of-function applies to thrombin, because prothrombin has no effect on fibroblast proliferation (35). Incidentally, the protease activity of fibroblasts has previously been demonstrated (9, 22), and it is possible that the increased proliferation (13 ± 4%) stimulated by factor X over 48 h is due to its conversion into factor Xa on the fibroblast surface. Second, the possibility that mitogens such as PDGF or bacterial lipopolysaccharide may contaminate the factor Xa preparation is excluded by our results since rTAP, ASN, and the specific antibodies have no effect on these possible contaminants. Third, we can conclude that the mitogenic effect of factor Xa is not mediated by the local generation of thrombin on the fibroblast surface. Taken together, these observations suggest that the production of factor Xa and thrombin may link blood coagulation to fibroblast proliferation.

When we examined the potential involvement of secondary mediators in the mitogenic effect of factor Xa, we found that a pan-specific PDGF neutralizing antibody inhibited nearly completely the effect of factor Xa. This suggests that factor Xa does not act directly, but via PDGF production and autocrine stimulation. Furthermore, we measured PDGF mRNA levels in our fibroblasts and found that factor Xa induced PDGF-A expression, whereas PDGF-B mRNA could not be detected in either factor Xa-, thrombin-, or control medium-treated cells (data not shown). This is in agreement with previous studies where PDGF-A was found to be expressed by proliferating fibroblasts, whereas PDGF-B expression was undetectable (23). Similarly, thrombin is known to stimulate fibroblast proliferation by upregulating PDGF-AA production and PDGF receptor expression in fibroblasts (27, 28). It is possible that this PDGF-A-mediated mitogenic pathway is shared by several proteases. Finally, factor Xa is known to elicit smooth muscle cell proliferation via autocrine stimulation involving a yet unidentified isoform of PDGF (20). Fibroblasts and smooth muscle cells appear to respond to coagulation cascade proteases via a common signaling mechanism involving PDGF-A.

Human fibroblasts express the factor Xa receptor EPR-1. EPR-1 transcripts of about 1.8 kb and 3.0 kb were detected in our human fetal lung fibroblasts. Two EPR-1 mRNA splice variants were previously described in mononuclear cells (2), smooth muscle cells (16), and endothelial cells (25). In addition, smooth muscle cells express a third EPR-1 transcript of 1.3 kb (25) that was not found in our fibroblasts.

It was previously shown that two forms of mRNA can arise from the EPR-1 gene. The low-molecular-weight product, termed EPR-1a (1.5 kb), gives rise to a 337-amino acid protein with a transmembrane domain, the receptor for factor Xa (1). The second splice variant, EPR-1b (1.9 kb), retains an intronic sequence containing several stop-of-translation signals (2). It encodes a truncated protein of 110 amino acids only that is not found on the cell surface. In fibroblasts, EPR-1a appeared to be the most highly expressed, suggesting that fibroblasts express the cell surface form of EPR-1.

We detected three EPR-1-related protein bands in human fetal lung fibroblasts (52-, 58-, and 65-kDa molecular mass). EPR-1-related proteins of different molecular mass have previously been identified in smooth muscle cells (65 kDa), endothelial cells (2 bands, 54 and 58 kDa (24)), platelets, and megakaryocytic cells (65 kDa (8)) by using monoclonal antibody B6. EPR-1 was also detected in mononuclear cells [58 and 65 kDa (3)] and lymphocytes [63 kb (1)]. We found that human fetal lung fibroblasts express all three EPR-1-related proteins reported to date. These different molecular masses may reflect cell-specific post-translational modifications as previously suggested (1), but their functional relevance has yet to be explained.

Binding to EPR-1 mediates the mitogenic effect of factor Xa. Upon activation, factor X is subjected to a translational modification as previously suggested (1), but their functional relevance has yet to be explained.
that factor Xa inhibitors such as ASN and rTAP abrogate levels of thrombin and fibrinogen-derived peptides. This suggests that the proliferative activity of factor Xa, like its prothrombinase activity, is greatly enhanced by the interaction between factor Xa and EPR-1. This was previously observed in endothelial and smooth muscle cells (16, 25).

The interaction between factor Xa and cell surface EPR-1 is dependent on the conformation of factor Xa (6, 7). Catalytically inactive factor Xa, DEGR-factor Xa, or factor Xa inhibited with rTAP retains a conformation similar to the active protease and binds to EPR-1 (6, 7). However, we found that DEGR-factor Xa did not stimulate fibroblast proliferation. In addition, thezymogen factor X had little effects on our cells. This implies that only active factor Xa is mitogenic for fibroblasts and that binding to EPR-1 is necessary but not sufficient to stimulate proliferation.

**The role of EPR-1 in the mitogenic effect of factor Xa in fibroblasts.** EPR-1 may mediate the mitogenic effects of factor Xa in fibroblasts, but this receptor has not been linked to a specific intracellular signaling pathway (1). Our data and that of others (6, 16) suggest that binding to EPR-1 alone is not sufficient to stimulate proliferation. We speculate that the function of EPR-1 in fibroblast proliferation is mainly to promote the proteolytic activity of factor Xa at the cell surface, as it does during blood coagulation (8). It is likely that a second protease receptor is involved in transducing the mitogenic signal of factor Xa. In addition, factor Xa does not cleave EPR-1 (5), whereas its proteolytic activity is necessary for its mitogenic effects. The substrate required for factor Xa to stimulate fibroblast proliferation may be a proteolytically activated receptor. Because protease-activated receptor-1 mediates the effects of thrombin on fibroblast proliferation (11), it constitutes a good candidate. The identical potency and time course of action observed for factor Xa and thrombin also point in that direction.

**Significance to physiology and pathology.** The extrinsic coagulation pathway and the generation of factors VIIa and Xa is the primary trigger of blood coagulation in response to tissue injury. High levels of activation of this pathway are also observed during lung fibrosis (17, 18, 21) and several fibrotic conditions (32, 34). The finding that coagulation factor Xa is mitogenic for human fibroblasts suggests that the extrinsic pathway may contribute to the development of tissue fibrosis. Manipulating the coagulation cascade pathway may be beneficial for the treatment of fibrotic diseases. In particular, early mediators such as factors VIIa and Xa may be targeted to prevent the generation of excessive levels of thrombin and fibrinogen-derived peptides.

Studies in experimental models of arterial injury (33) and balloon angioplasty (15, 26, 31) have shown that factor Xa inhibitors such as ASN and rTAP abrogate smooth muscle cell proliferation, neointima formation, and extracellular matrix deposition in vivo. These factor Xa inhibitors elicited fewer bleeding complications than direct thrombin inhibitors. Our data in fibroblasts combined with these studies invite an urgent investigation of the antifibrotic potential of factor Xa and factor VIIa inhibitors in vivo.

In summary, we found that factor Xa, like thrombin, is a fibroblast mitogen at physiological concentrations, but not factor IXa or VIIa. In addition, EPR-1 is constitutively expressed in human fibroblasts and mediates the mitogenic effects of factor Xa in vitro. Thus proteases of the extrinsic coagulation pathway may play a role in the regulation of fibroblast function and connective tissue deposition during normal tissue repair and fibrotic diseases associated with excessive activation of the coagulation cascade.

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