RAPID COMMUNICATION

Tissue Factor-Dependent Vascular Endothelial Growth Factor Production by Human Fibroblasts in Response to Activated Factor VII

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The transmembrane protein tissue factor (TF) is the cell surface receptor for coagulation factor VII (FVII) and activated factor VII (FVIIa). Recently, TF has been identified as a regulator of angiogenesis, tumor growth, and metastasis. This study was designed to link the binding of FVII(a) to its receptor, TF, with the subsequent triggering of angiogenesis through vascular endothelial growth factor (VEGF) production by human lung fibroblasts. We report that incubation of fibroblasts, which express constitutive surface TF, with FVII(a) induces VEGF synthesis. FVII(a)-induced VEGF secretion, assessed by a specific enzyme-linked immunosorbent assay, was time- and concentration-dependent. VEGF secretion was maximal after 24 hours of incubation of the cells with 100 nmol/L FVII(a) and represented a threefold induction of the basal VEGF level. Reverse transcriptase-polymerase chain reaction analysis of VEGF detected three mRNA species of 180, 312, and 384 bp corresponding, respectively, to $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$. A 2.5- to 3.5-fold increase was observed for the 180- and 312-bp transcripts at 12 and 24 hours, respectively. FVII(a)-dependent VEGF production was inhibited by a pool of antibodies against TF, pointing to the involvement of this receptor. On specific active-site inhibition with dansyl-glutamyl-glycinyl-arginyl chloromethyl ketone, FVIIa lost 70% of its capacity to elicit VEGF production. Consistent with this, the native form (zymogen) of FVII only had a 1.8-fold stimulating effect. Protein tyrosine kinase and protein kinase C are involved in signal transduction leading to VEGF production, as shown by the inhibitory effects of genistein and GF 109203X. The results of this study indicate that TF is essential for VIIa-induced VEGF production by human fibroblasts and that its role is mainly linked to the proteolytic activity of the TF-VIIa complex.

TISSUE FACTOR (TF) is an integral membrane glycoprotein that, assembled with activated factor VII (FVIIa), initiates coagulation. The TF-VIIa complex is the main initiator of the coagulation cascade via activation of factors IX and X,[1] thereby resulting in thrombin production. The molecular structure of TF indicates that it may be classified in the class 2 cytokine receptor family that includes the receptors for interferon-α/β and interferon-γ,[2] with a hydrophilic extracellular domain, a membrane-spanning region, and a short cytoplasmic tail consisting of 21 amino acids. The functional ligand for TF is FVIIa[3] and the crystal structure of the TF-VIIa complex has recently been described.[4] Supporting the hypothesis of intracellular signal transduction activated by the binding of FVIIa to its receptor TF, Rottingen et al[5] have demonstrated that binding of FVIIa to TF induces Ca^{2+} oscillations in several cell types. More recently, Masuda et al[6] reported that binding of FVIIa to cell-surface TF increases protein tyrosine phosphorylation in human monocytes and that TF is associated with the γ chain homodimer identified as a component of FcεRI.

Vascular endothelial growth factor (VEGF), or vascular permeability factor, is a 45-kD tumor-derived cytokine that acts specifically on vascular endothelial cells to promote vascular permeability, endothelial cell growth, and angiogenesis.[7,8] Five VEGF isoforms varying in length from 121 to 209 aminoacids ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{209}$) are encoded by a single gene and generated by alternative splicing.[9,10] Several cell types, including monocytes, macrophages, T lymphocytes, keratinocytes, vascular smooth muscle cells, and fibroblasts, can produce VEGF.[11-14] Because TF has been reported to play an important role in the angiogenic-antiangiogenic balance,[15] we evaluated whether the binding of FVII(a) to tissue factor-expressing human lung fibroblasts induced the production of VEGF.

MATERIALS AND METHODS

Materials. A pool of neutralizing anti-TF antibodies (TF8-5G9, TF8-6B4, and TF9-9C3) was generously provided by T.S. Edgington and N. Mackman (The Scripps Research Institute, La Jolla, CA). The inhibition of TF-cofactor function by the majority of anti-TF antibodies is mediated by competition with FVIIa for association with TF.[16] In addition, one of these antibodies (TF8-5G9) has been shown to inhibit the catalytic activity of the preformed TF-VIIa complex.[17] A mouse irrelevant IgG1 antibody directed against *Aspergillus niger* glucose oxidase was purchased from DAKO (Glostrup, Denmark). Genistein and GF 109203X were purchased respectively from RBI (Natick, MA) and France Biochem (Meudon, France).

ACSET. ACSET (plasma-derived human FVIIa concentrate; LFB, Les Ulis, France), hereafter designated FVII(a), was prepared as previously described.[18] FVII antigen was determined by an enzyme-linked immunosorbent assay (ELISA) method using the Asserachrom FVII kit from Diagnostica-Stago (Asnières, France). FVII coagulant activity was measured in a one-step assay using reference standards calibrated against international standards (WHO 84/665 and 89/668 for native FVII and FVIIa, respectively). The ACSET preparation contains 30% FVIIa and 70% FVII and some contaminating proteins, including protein S, prothrombin, and factor X.

Preparation of FVII(a)-immunodepleted ACSET [$A_{(ID)}$]. To obtain a negative control, ACSET was submitted to affinity chromatography with an insolubilized anti-factor VII monoclonal antibody (anti-VII-MoAb). Four milligrams of anti-VII-MoAb (Sigma, St Louis, MO) was coupled to 10 mL of CNBr-activated sepharose 4B (Pharmacia, Uppsala, Sweden). The unbound fraction was recovered and stored at −40°C until use. This treatment removed 90% of the FVII(a) antigen, whereas other contaminating proteins were unaffected.

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Isolation of native human plasma FVII. Human native FVII was prepared to discriminate between the effects of FVIIa and FVII. Human plasma cryosupernatant was adsorbed onto DEAE-Sephadex. Elution was performed with 0.2 mol/L NaCl, pH 7, in the presence of 20 mmol/L benzamidine. The eluate, after diafiltration against a buffer containing 0.02 mol/L Tris, 0.15 mol/L NaCl, and 0.02 mol/L benzamidine, pH 7, was run through the anti-FVII MoAb-Sepharose column as described for the immunodepletion of ACSET. The highly purified FVII was eluted with 3 mol/L Na thiocyanate in the same buffer. This fraction was then dialysed against a buffer containing 20 mmol/L Tris, 0.075 mol/L NaCl, and 20 mmol/L benzamidine, pH 7.5. The FVII solution was concentrated by adsorption on Q-Sepharose equilibrated with the same buffer. Elution was performed with buffer containing 20 mmol/L Tris and 0.5 mol/L NaCl, pH 7.5. The preparation was submitted to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in reducing conditions and transfered to ECL nitrocellulose membranes (Amersham, Arlington Heights, IL). After washing, the membranes were incubated with an anti-FVII polyclonal antibody (Stago) showing only one band of 53 kD, corresponding to native FVII.

Inactivation of the active site of FVIIa. The active site of FVIIa was blocked by dansyl-glutamyl-glycinyl-arginyl chloromethylketone (DEGR-ck; purchased from Calbiochem, La Jolla, CA) by incubating VII(a) (containing 27 μg FVII ag/mL) with a 200-fold molar excess of peptidyl chloromethyl ketone. The inhibitor was added to the mixture four times every hour at room temperature. After 4 hours, the excess of inhibitor was removed by extensive dialysis at 4°C overnight against buffer containing 50 mmol/L Tris, 0.15 mol/L NaCl, pH 7.4. As a control, untreated VII(a) was dialyzed in the same conditions. More than 98% inactivation was obtained, as showed by the determination of amidolytic activity on the chromogenic substrate S 2288, even in the presence of soluble TF (sTF; Diagnostica Stago). The clotting activity of inactivated FVII(a) was 11 IU/mL, as compared with 660 IU/mL for the control. This residual clotting activity is due to the native form of FVII, which is not impaired by DEGR-ck.

Cells and culture. Human lung fibroblasts (CCD-11Lu) were obtained from American Type Culture Collection (ATCC; Rockville, MD) and grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 1% nonessential amino acids, 2 mmol/L glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin (all from Life Technologies, Cergy Pontoise, France), and 10% heat-inactivated fetal calf serum (DAP, Neuf Brisach, France). Cells were used between passages 8 and 12. Cells (4×10^4/mL) were seeded in 6- or 96-well plates in complete medium. Subconfluent fibroblasts were starved for 24 hours in DMEM without heat-inactivated fetal calf serum and subsequently incubated with human FVII(a) in DMEM supplemented with 6 mmol/L $CaCl_2$. Cell viability, monitored by lactate dehydrogenase release, was not altered by any of the experimental conditions (data not shown).

Estimation of cellularity. To avoid modifications of VEGF production due to changes in cell proliferation in different experimental conditions, a colorimetric method was used to evaluate the amount of cells present in the well at the time of the assay.[19] Briefly, after fixation of fibroblasts in paraformaldehyde and washes, staining was performed with 100 μL of 0.1% crystal violet in 2% ethanol for 20 minutes. The cells were then washed vigorously with water and, after drying, the dye was extracted from the cells with 100 μL of 20% acetic acid. The extracted dye was quantitated by reading absorbance on an ELISA reader (Molecular Devices, Menlo Park, CA) at 550 nm. Absorbance is directly proportional to the amount of viable cells present in the well.

TF assay. TF activity was assessed in intact fibroblasts by a two-step amidolytic assay based on the activation of factor X by the TF-VIIa-Ca^{2+} complex. Fresh cells were incubated for 5 minutes at 37°C with 20 μL of human factor VIIa (10 nmol/L, initial concentration) and 50 μL of $CaCl_2$ (25 mmol/L). Then, 20 μL of human factor X (52 μg/mL) was added and factor Xa was assayed by adding 60 μL of the chromogenic substrate S2765 (2.33 mmol/L). Kinetic changes in absorbance at 405 nm were read on a ELISA reader (Molecular Devices) at 37°C. A calibration curve was established by using dilutions of a standard TF reagent (Thromborel; Behring, Rueil Malmaisan, France). The 1:60 dilution of this reagent was arbitrarily taken to represent 1,000 mU/mL.

Human VEGF immunoassay. Human VEGF concentrations in fibroblast culture supernatants was determined by using the Quantikine human VEGF kit (R&D Systems Europe, Abingdon, UK), a quantitative sandwich enzyme immunoassay technique. The kit was used according to manufacter's recommendations and as previously reported.[20,21] The detecting limit of the VEGF assay was 5 pg/mL, and there is no cross-reaction with other cytokines.

VEGF reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was extracted from 6-well plate fibroblast cultures (2 wells per experimental point) using the TRIzol Reagent (Life Technologies, Cergy Pontoise, France). Total RNA (5 μg) was incubated for 5 minutes at 65°C with 3 μmol/L of random hexamers (Perkin Helmer, Roissy CDG, France). After 10 minutes at room temperature, reverse transcription was performed for 1 hour at 37°C in a 50 μL reaction volume containing 50 U of murine leukemia virus (MuLV) reverse transcriptase (Perkin Helmer), 40 U of RNAse inhibitor (Perkin Helmer), and all four deoxyribonucleoside triphosphates (dNTPs; 1 mmol/L each) in reaction buffer (50 mmol/L KCl, 10 mmol/L Tris HCl, pH 8.3, 5 mmol/L $MgCl_2$). PCR reactions were performed in a Trio-Thermoblock device (Biometra, Göttingen, Germany) using 2 μL of the first-strand reaction in a total volume of 100 μL with 0.2 mmol/L of each dNTP, 0.4 μmol/L of each primer, and 1 U of Taq DNA polymerase (ATGC, Noisy Le Grand, France). The 28 reaction cycles were at 94°C for 1 minute, 55°C for 2 minutes, and 72°C for 3 minutes. The sense primer for VEGF was 5′-GAGTGTGTGCCCACTGAGGAGTCCAAC-3′ and the antisense primer was 5′-CTCCTGCCCGGCTCACCGCCTCGGCTT-3′.[22] A semiquantitative competitive RT-PCR method was used to correct for variations in amplification efficiency in each reaction and to calculate relative changes in mRNA levels. This was performed using the PCR MIMIC construction kit (Clontech Laboratories, Palo Alto, CA), which enables the generation of a PCR mimic used as competitive internal standard in PCR amplification. The PCR mimic consists of a nonhomologous DNA fragment that possesses, at each extremity, the recognition sequences for VEGF primers. The PCR mimic and the target template thus compete for the same primers in the same reaction. Equal amounts of the mimic were included in each PCR reaction. Amplification products were subjected to electrophoresis in 2% agarose gels containing ethidium bromide and photographed with Polaroid 665 negative film (Polaroid Corp, Cambridge, MA). RNA was quantified by scanning the bands with a Macintosh One-Scanner densitometer (Apple Computer, Inc, Cupertino, CA); the density of each band was normalized to the density of the mimic band and plotted in arbitrary units.

Statistical analysis. Results are given as means ± SEM. Data were compared using analysis of variance (ANOVA). Where significant differences were inferred, the two sample means were compared using the Student's paired *t*-test.

RESULTS

Cell surface expression of TF in human fibroblasts. Cell surface expression of TF in resting human fibroblasts was measured after 24 hours of starvation of the cells in DMEM without fetal calf serum. This expression was 14.4 ± 4.9 mU/10^3 cells, indicating that TF is constitutively produced in fibroblasts with high surface availability, because the total TF expression in lysed cells was 54.8 ± 8.5 mU/10^3 cells.

Effect of FVII(a) binding to TF in human fibroblasts on VEGF production. VEGF production by fibroblasts was evaluated by using a specific ELISA and normalized to the amount of cells present in the well at the time of the assay (crystal violet

results). VEGF production induced by 100 nmol/L FVII(a) was studied after 24 and 48 hours of incubation of fibroblasts at 37°C. As shown in Fig 1A, VEGF baseline levels increased at 24 hours with a significant ($P < .01$) 2.95-fold induction as compared with baseline in the absence of FVII(a) (156.2 ± 32.9 pg/mL). At 48 hours, basal VEGF secretion increased, gradually reaching 385.3 ± 66.8 pg/mL, and FVII(a) stimulation resulted in a 2.5-fold induction of VEGF secretion. Incubation of fibroblasts with increasing concentrations of FVII(a) (10 to 100 nmol/L) stimulated VEGF production in a concentration-dependent manner (Fig 1B). The increase in VEGF production was significant ($P < .05$) from 25 nmol/L. The use of higher concentrations of factor VIIa (150 and 200 nmol/L) did not further increase VEGF production, which was maximal at 100 nmol/L FVII(a) (data not shown). When FVII(a) was coincubated with the cells for 10 minutes, 30 minutes, 1 hour, or 2 hours and subsequently washed, VEGF secretion at 24 hours remained at the basal level (data not shown), indicating that lengthy stimulation was necessary for the increase in VEGF . The role of contaminating LPS in FVII(a) preparation was ruled out, because incubation of fibroblasts with increasing concentrations of LPS (0.01 to 10 μg/mL) did not induce VEGF production (data not shown). The role of contaminating protein S was also ruled out by incubation of fibroblasts with 24 μg/mL of purified human protein S (Stago), which led to no significant increase in VEGF production. The addition of cycloheximide (10 μg/mL) completely blocked VEGF production by unstimulated and FVII(a)-stimulated fibroblasts, indicating the role of new protein synthesis in the observed effect. The specificity of the FVII(a)-TF interaction for VEGF production by human fibroblasts was verified by using several approaches. First, preincubation of fibroblasts with specific antibodies against TF inhibited the FVII(a)-induced VEGF production by 86% ± 2%, whereas an irrelevant antibody had no effect (Fig 2). These antibodies had no effect on the basal VEGF level. Second, a preparation of FVII(a)-immunodepleted ACSET [$A_{(ID)}$] that contained 10 nmol/L (final concentration) residual FVII(a) (as compared with 100 nmol/L in native ACSET) but used at the same other protein concentration than the native ACSET had a mean stimulating effect of 1.2-fold on VEGF production by human fibroblasts (Fig 3) as compared with 2.7-fold with FVII(a) ($P < .01$). Native nonactivated FVII, containing no contaminating proteins and used at a concentration of 100 nmol/L, induced VEGF production by 1.8- ± 0.34-fold (data not shown).

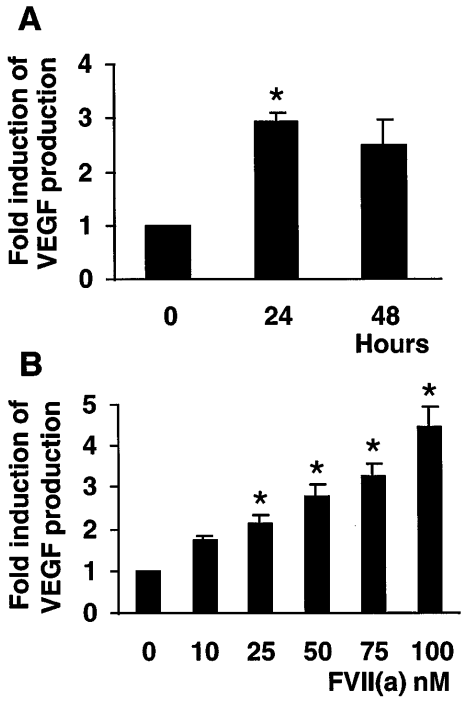

Fig 1. (A) Kinetics of FVII(a)-induced VEGF secretion by human fibroblasts. Confluent fibroblasts were incubated with 100 nmol/L FVII(a) for 24 and 48 hours at 37°C. Secreted VEGF was assessed in a specific ELISA. Results are expressed as the fold induction of VEGF in FVII(a)-treated fibroblasts as compared with unstimulated fibroblasts at the same time. Each point represents the mean ± SEM of four different determinations each performed in triplicate. (B) Concentration effect of FVII(a) on VEGF production. Confluent fibroblasts were incubated for 24 hours with or without 10, 25, 50, 75, or 100 nmol/L of FVII(a) at 37°C. Secreted VEGF was assessed by a specific ELISA. Results are expressed as the fold induction of VEGF secretion as compared with unstimulated fibroblasts (basal VEGF level, 82.29 ± 11.8 pg/mL). Each point represents the mean ± SEM of three different determinations each performed in triplicate. *$P < .05$ versus unstimulated fibroblasts.

Effect of DEGRck-inactivated FVII(a). Because FVIIa has little proteolytic activity without TF, we used DEGRck-inactivated FVII(a) to determine whether VEGF upregulation was dependent on the proteolytic activity of FVIIa. The cells were incubated for 24 hours with 100 nmol/L of either VII(a) or inactivated VII(a). Inactivation of FVII(a) was associated with a 70% reduction in VII(a)-induced VEGF secretion ($P < .001$), from 446 ± 59 to 138 ± 35 pg/mL (baseline VEGF production was deduced from these values).

Binding of FVII(a) to TF modulates VEGF production at the mRNA level. To assess the level of action of FVII(a), VEGF mRNA was studied after reverse transcription and amplification by PCR. Three VEGF transcripts of 180, 312, and 384 bp coding for $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$, respectively, were detected. After 12 hours of treatment with FVIIa, the 180-bp transcript showed a 2.67-fold increase and, at 24 hours, a significant ($P < .01$) 3.51-fold increase when compared with the constitutive 180-bp transcript observed at the same times (Fig 4). The 312-bp transcript increased in a similar manner to the 180-bp transcript. Induction of the 312-bp transcript was 2.63-fold after 12 hours with FVII(a) and 3.1-fold at 24 hours when compared with constitutive levels of the 312-bp transcript at the same times. The 384-bp transcript was less expressed increased 2.29-fold and 2.11-fold when compared with its basal level at 12 and 24 hours, respectively.

Effect of protein tyrosine kinase and protein kinase C inhibitors on FVII(a)-induced VEGF production. Genistein, a tyrosine kinase inhibitor, and GF 109203X, a protein kinase C inhibitor, were preincubated with fibroblasts for 30 minutes before 24 hours of stimulation of the cells with 100 nmol/L FVII(a). Inhibition of VEGF production by 100 μmol/L genistein was 62% ± 13% ($P < .05$) and, by 1 μmol/L GF 109203X, 57% ± 6% ($P < .01$).

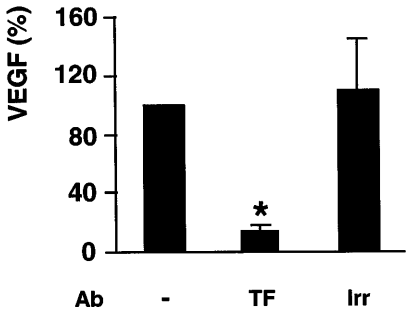

Fig 2. Effect of anti-TF antibodies on FVII(a)-induced VEGF production. Confluent fibroblasts were incubated with 10 μg/mL of anti-TF or irrelevant (Irr) antibodies for 30 minutes at 4°C before 24 hours of incubation at 37°C with 100 nmol/L FVII(a). Secreted VEGF was assessed by a specific ELISA. Results are expressed as the percentage of VEGF production in response to 100 nmol/L FVII(a), with 100% corresponding to 279.9 ± 35.5 pg/mL. Each point represents the mean ± SEM of three different determinations each performed in triplicate. *$P < .01$ versus stimulated fibroblasts in the absence of antibodies.

DISCUSSION

The results of this study indicate that TF is essential for the VIIa-induced signaling events leading to VEGF synthesis by human fibroblasts. Normal human lung fibroblasts incubated for 24 hours in culture medium synthesize and release low levels (156.2 ± 32.9 pg/mL) of VEGF, as assessed by ELISA. Incubation of these cells, which express surface TF constitutively (14.4 ± 4.9 mU/10^3 cells), with increasing amounts (10 to 200 nmol/L) of FVII(a) led to a concentration-dependent increase in VEGF, maximal at 100 nmol/L FVII(a), which was threefold the constitutive level at 24 hours and fell slightly at 48 hours. RT-PCR analysis of VEGF mRNA with primers flanking the VEGF variable portion[22] showed that unstimulated cells expressed low levels of three of the four VEGF mRNA species (384, 312, and 180 bp) corresponding to $VEGF_{189}$, $VEGF_{165}$, and $VEGF_{121}$, respectively. In untreated cells, the mRNA level

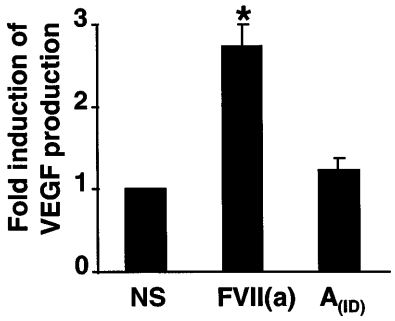

Fig 3. Effect of FVII(a)-immunodepleted ACSET [$A_{(ID)}$] on VEGF production. Confluent fibroblasts were incubated for 24 hours in the presence of 100 nmol/L FVII(a) or immunodepleted ACSET [$A_{(ID)}$] used at the same protein concentration as FVII(a). Secreted VEGF was assessed by a specific ELISA. Results are expressed as the fold induction of VEGF production, with VEGF secretion by unstimulated fibroblasts (NS) being considered as onefold induction and corresponding to 109.4 ± 14.6 pg/mL of secreted VEGF. Each point represents the mean ± SEM of at least eight different determinations each performed in triplicate. *$P < .01$ versus unstimulated and $A_{(ID)}$-stimulated fibroblasts.

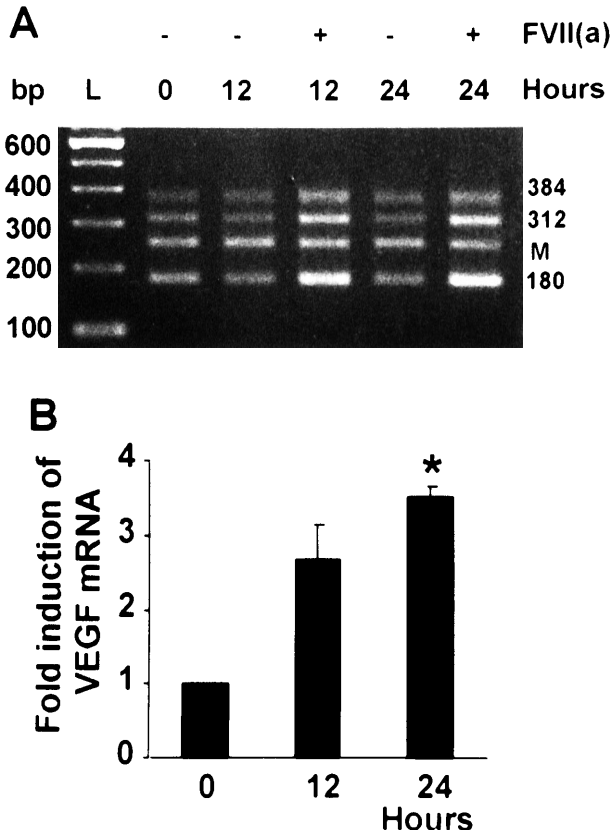

Fig 4. VEGF mRNA induction by FVII(a). Confluent fibroblasts were incubated with or without 100 nmol/L FVII(a) for the times indicated. Five micrograms of total RNA was analyzed by RT-PCR. (A) Photograph from a representative experiment. Three VEGF mRNA transcripts are seen (180, 312, and 384 bp). The internal standard, mimic (M), is also visible. (B) RNA was quantified was achieved by scanning the Polaroid negative by laser densitometry. The density of the 180-bp band was normalized to the density of the mimic, and the fold induction of VEGF mRNA induced by FVII(a) compared with unstimulated cells at the same time was plotted. Each point represents the mean ± SEM of three experiments. *$P < .01$ versus unstimulated fibroblasts.

of each species remained stable at 12 and 24 hours. By contrast, treatment of the cells with FVII(a) induced a 2.5- to 3.5-fold increase in the 312- and 180-bp mRNA species at 12 and 24 hours and a twofold increase in the 384-bp transcript, which remained stable between 12 and 24 hours. FVII(a)-induced VEGF synthesis was inhibited by specific anti-TF antibodies but not by an irrelevant antibody, thus demonstrating that FVII(a) stimulates VEGF production by a TF-mediated pathway. VEGF synthesis appears to be FVII(a)-specific, because it was not induced by the FVII(a)-immunodepleted ACSET preparation [$A_{(ID)}$]. In fact, we observed only a slight increase in VEGF induction [1.2-fold, compared with 2.7-fold in the presence of 100 nmol/L FVII(a)], which might be due to the 10 nmol/L residual FVII(a) that remained in this preparation. However, these results did not discriminate between FVIIa- and FVII-dependent induction of VEGF, because the FVII(a) preparation (ACSET) used in most experiments contained approximately 30% FVIIa and 70% FVII. Native nonactivated FVII

induced VEGF production 1.8-fold, suggesting a minor role of FVII in VEGF induction. To further examine this question, we used DEGRck-inactivated FVII(a). Our results clearly show that the proteolytic activity of FVIIa is required for VEGF induction, because inactivation abolished 70% of the effect. The role in this effect of the secondary production of other molecules such as activated clotting factors or cytokines is currently under investigation. The partial stimulation by purified factor VII can be explained by residual FVIIa activity, which can result in autoactivation of FVII in the presence of TF on cells.

VEGF can be produced by several cell types.[11-14] In most studies, including ours, simultaneous expression of $VEGF_{121}$, $VEGF_{165}$, and, in some cases, $VEGF_{189}$ is observed. Most studies on the induction of VEGF synthesis are based on RT-PCR analysis, Northern blot, and in situ hybridization, and few data on the levels of secreted VEGF are available. Recently, Volpert et al[23] observed that relative VEGF mRNA levels measured by semiquantitative RT-PCR were very low in normal fibroblasts, but increased 3.6-fold upon immortalization, whereas secreted protein levels increased fivefold (from 5 to 25 pg/μg total protein in the culture medium). They also found that doubling the amount of secreted protein was sufficient to account for the doubling of net angiogenic activity as assessed by capillary endothelial cell migration. This finding and the extremely tight concentration dependence of vessel formation on VEGF suggest that the threefold induction of VEGF in response to FVII(a) might be relevant for such an angiogenic activity. Indeed, small changes in the local concentration of VEGF in target tissues may have profound effects on angiogenesis in vivo.[24]

The FVII(a) concentration used (100 nmol/L) is higher than the plasma concentration of FVII (10 nmol/L). However, the concentration of 100 nmol/L is in keeping with the results obtained by Rottingen et al.[5] In this last work, elevated FVIIa concentrations (200 nmol/L) had to be used to observe Ca^{2+} oscillations in 100% of the cells. The investigators suggested that binding of FVII to TF-phospholipid membranes may increase the local concentration of FVII in vivo.

TF is a member of the class-2 cytokine receptor family that includes the receptors for interferon-α/β and interferon-γ.[3] Several receptors of this family contain no tyrosine kinase domains in their cytoplasmic regions but require a receptor-associated molecule for transmission of a cytoplasmic signal.[25] TF is associated with the γ chain homodimer identified as a component of FcεRI.[6] This γ chain of the IgE receptor type I is classified as a signal transducing component, like the ξ chain of the TcR complex.[26] Our observation that genistein and GF 109203X inhibed FVII(a)-induced VEGF synthesis suggests that VEGF production involves the activation of protein tyrosine kinase and that of protein kinase C. These results are in keeping with those obtained by Masuda et al,[6] who showed that binding of FVIIa to TF induced tyrosine phosphorylation in human monocytes/macrophages.

Our results are in accordance with those obtained by Rottingen et al,[5] who showed that binding of FVIIa to the extracellular domain of TF on the cell membranes induced Ca^{2+} oscillations in a variety of TF-expressing cells and that this effect was lost when FVIIa was inactivated by DEGR-ck.[27] Surprisingly, however, human fibroblasts did not respond to ligand binding with Ca^{2+} oscillations.[27] Moreover, and at variance with our results, FVII(a)-induced Ca^{2+} signals did not involve tyrosine kinase activation.[27] These discrepancies again suggest that several activation pathways may be induced by FVIIa binding.

The physiological consequences of FVII(a)-induced VEGF synthesis mediated by TF are potentially very important. The concept that TF functions as a haemostatic envelope should be enlarged to a role in endothelial recovery after vascular damage. The involvement of TF in vasculogenesis is emphasized by the very recent report on the effect of deleting TF gene expression in mice.[28] In vascular disorders, VEGF production by cells of the atherosclerotic plaque might be beneficial in promoting collateral vessel formation[29]; by contrast, as recently suggested,[30] VEGF may contribute to atheromatous lesion progression by enhancing neovascularisation and neointimal formation within the lesion. TF expression by cancer cells has also been shown to be an independent marker of bad outcome in melanoma and breast cancer.[31] In this work, we show that VEGF expression by normal fibroblasts is controlled by the binding of the natural TF ligand, FVII(a). Whether this is also true for malignant cells is currently under investigation.

TF clearly has properties beyond its role as the primary regulator of blood coagulation in mammals. We found that VEGF synthesis can occur after binding of FVII(a) to its receptor TF. Whether this is a direct effect of TF involving transduction by the intracellular domain in response to VIIa binding or is mediated by other molecules produced secondarily is an intringuing question.

REFERENCES

1. Bach RR: Initiation of coagulation by tissue factor. CRC Crit Rev Biochem 23:339, 1988

2. Bazan JF: Structural design and molecular evolution of a cytokine receptor superfamily. Proc Natl Acad Sci USA 87:6934, 1990

3. Edgington TS, Mackman N, Brand K, Ruf W: The structural biology of expression and function of tissue factor. Thromb Haemost 66:67, 1991

4. Banner DW, Darcy A, Chene C, Winkler FK, Guha A, Konigsberg WH, Nemerson Y, Kirchhofer D: The crystal structure of the complex of blood coagulation factor VIIa with soluble tissue factor. Nature 380:41, 1996

5. Rottingen JA, Enden T, Camerer E, Iversen JG, Prydz H: Binding of human factor VIIa to tissue factor induces cytosolic Ca2+ signals in J82 cells, transfected COS-1 cells, Madin-Darby canine kidney cells and in human endothelial cells induced to synthesize tissue factor. J Biol Chem 270:4650, 1995

6. Masuda M, Nakamura S, Murakami T, Komiyama Y, Takahashi H: Association of tissue factor with a gamma chain homodimer of the IgE receptor type I in cultured human monocytes. Eur J Immunol 26:2529, 1996

7. Connolly DT, Heurelman DM, Nelson R, Olander JV, Eppley BL, Delfino JJ, Siegel NR, Leimgruber RM, Feder J: Tumor vascular permeability factor stimulates endothelial growth and angiogenesis. J Clin Invest 84:1470, 1989

8. Ferrara N, Davis-Smyth T: The biology of vascular endothelial growth factor. Endocrine Rev 18:4, 1997

9. Tischer E, Mitchell R, Hartman T, Silva M, Gospodarowicz D, Fiddes JC, Abraham JA: The human gene for vascular endothelial growth factor: Multiple proteins are encoded through alternative exon splicing. J Biol Chem 266:11947, 1991

10. Poltorak Z, Cohen T, Sivan R, Kandelis Y, Spira G, Vlodavsky I, Keshet E, Neufeld G: VEGF145, a secreted vascular endothelial growth

factor isoform that binds to extracellular matrix. J Biol Chem 272:7151, 1997

11. Freeman MR, Schneck FX, Gagnon ML, Corless C, Soker S, Niknejad K, Peoples GE, Klagsburn M: Peripheral blood T lymphocytes and lymphocytes infiltrating human cancers express vascular endothelial growth factor: A potential role for T cells in angiogenesis. Cancer Res 55:4140, 1995

12. Namiki A, Brogi E, Kearney M, Kim EA, Wu TG, Couffinhal T, Varticovski L, Isner JM: Hypoxia induces vascular endothelial growth factor in cultured human endothelial cells. J Biol Chem 270:31189, 1995

13. Pertovaara L, Kaipainen A, Mustonen T, Orpana A, Ferrara N, Saksela O, Alitalo K: Vascular endothelial growth factor is induced in response to transforming growth factor-β in fibroblastic and epithelial cells. J Biol Chem 269:6271, 1994

14. Sunderkotter C, Steinbrink K, Goebeler M, Bhardwaj R, Sorg C: Macrophages and angiogenesis. J Leukoc Biol 55:410, 1994

15. Zhang YM, Deng YH, Luther T, Muller M, Ziegler R, Waldherr R, Stern DM, Nawroth PP: Tissue factor controls the balance of angiogenic and antiangiogenic properties of tumor cells in mice. J Clin Invest 94:1320, 1994

16. Morrissey JH, Fair DS, Edgington TS: Monoclonal antibody analysis of purified and cell-associated tissue factor. Thromb Res 52:247, 1988

17. Ruf W, Edgington TS: An anti-tissue factor monoclonal antibody which inhibits TF-VIIa complex is a potent anticoagulant in plasma. Thromb Haemost 66:529, 1991

18. Chabbat J, Hampikian-Lenin S, Toully V, Gaillandre A, Pejaudier L, Steinbuch M: A human factor VIIa concentrate and its effects in the hemophilic A dog. Thromb Res 54:603, 1989

19. Kueng W, Silber E, Eppenberg U: Quantification of cells cultured on 96-wells plates. Anal Biochem 182:16, 1989

20. Gabrilovich DI, Chen HL, Girgis KR, Cunningham HT, Meny GM, Nadaf S, Kavanaugh D, Carbone DP: Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. Nat Med 2:1096, 1996

21. Gaudry M, Brégerie O, Andrieu V, El Benna J, Pocidalo MA, Hakim J: Intracellular pool of vascular endothelial growth factor in human neutrophils. Blood 90:4153, 1997

22. Ben-Av P, Crofford LJ, Wilder RL, Hla T: Induction of vascular endothelial growth factor expression in synovial fibroblasts by prostaglandin E and interleukin-1: A potential mechanism for inflammatory angiogenesis. FEBS Lett 372:83, 1995

23. Volpert OV, Dameron KM, Bouck N: Sequential development of an angiogenic phenotype by human fibroblasts progressing to tumorigenicity. Oncogene 14:1495, 1997

24. Majesky MW: A little VEGF goes a long way. Circulation 94:3062, 1996

25. Taga T, Kishimoto T: Cytokine receptors and signal transduction. FASEB J 6:3387, 1992

26. Paolini R, Jouvin MH, Kinet JP: Phosphorylation and dephosphorylation of the high-affinity receptor for immunoglobulin E immediately after receptor engagement and disengagement. Nature 353:855, 1991

27. Camerer E, Rottingen JA, Iversen JG, Prydz H: Coagulation factors VII and X induce Ca^{++} oscillations in Madin-Darby canine kidney cells only when proteolytically active. J Biol Chem 271:29034, 1996

28. Carmeliet P, Mackman N, Moons L, Luther T, Gressens P, Vanvlaenderen I, Demunck H, Kasper M, Breier G, Evrard P, Muller M, Risau W, Edgington T, Collen D: Role of tissue factor in embryonic blood vessel development. Nature 383:73, 1996

29. Tsurumi Y, Takeshita S, Chen D, Kearney M, Rossow ST, Passeri J, Horowitz JR, Symes JF, Isner JM: Direct intramuscular gene transfer of naked DNA encoding vascular endothelial growth factor augments collateral development and tissue perfusion. Circulation 94:3281, 1996

30. Ruef J, Hu ZY, Yin LY, Wu Y, Hanson SR, Kelly AB, Harker LA, Rao GN, Runge MS, Patterson C: Induction of vascular endothelial growth factor in balloon-injured baboon arteries. Circ Res 81:24, 1997

31. Contrino J, Hair G, Kreutzer DL, Rickles FR: In situ detection of tissue factor in vascular endothelial cells: Correlation with the malignant phanotype of human breast disease. Nat Med 2:209, 1996