Molecular Mechanisms Underlying the Proangiogenic Effect of Factor XIII

Rima Dardik, Joseph Loscalzo, Regina Eskaraev, Aida Inbal

Objective—Coagulation Factor XIII (FXIII) was previously shown by us to induce angiogenesis. The aim of this study was to elucidate the molecular events underlying the proangiogenic effects of activated FXIII (FXIIIa) on human umbilical vein endothelial cells (HUVECs).

Methods and Results—As shown by coimmunoprecipitation studies, FXIIIa crosslinked $\alpha_\beta$ with vascular endothelial growth factor receptor 2 (VEGFR-2) and enhanced the noncovalent interaction between the 2 receptors. In addition, FXIIIa induced tyrosine phosphorylation of VEGFR-2 in both the crosslinked high-molecular-weight and the noncovalent VEGFR-2/$\alpha_\beta$ complexes. These effects as well as FXIIIa-induced proliferation and migration of HUVECs were abolished by iodoacetamide treatment of FXIIIa (I-FXIII) or by PTKI, an inhibitor of VEGFR-2. FXIIIa induced upregulation of c-Jun and Egr-1 as revealed by quantitative RT-PCR. Electrophoretic mobility-shift assay experiments showed that FXIIIa treatment of HUVECs enhanced binding of Wilms’s tumor-1 (WT-1) but not of early growth response (Egr)-1 to the thrombospondin-1 (TSP-1) promoter sequence, suggesting that WT-1 but not Egr-1 is involved in downregulation of TSP-1 expression.

Conclusions—The proangiogenic effect of FXIIIa is mediated by (1) enhancement of crosslinked and noncovalent $\alpha_\beta$/VEGFR-2 complex formation; (2) tyrosine phosphorylation and activation of VEGFR-2; (3) upregulation of c-Jun and Egr-1; and (4) downregulation of TSP-1 induced indirectly by c-Jun through WT-1. These processes may clarify FXIII role in vascular remodeling and tissue repair. (Arterioscler Thromb Vasc Biol. 2005;25:526-532.)

Key Words: Factor XIII • angiogenesis • vascular endothelial growth factor receptor 2 • c-Jun • early growth response-1

Angiogenesis is a complex process involving both proangiogenic and antiangiogenic factors. Vascular endothelial growth factor A (VEGF-A) is a major angiogenic growth factor regulating the key steps of angiogenesis, including endothelial cell (EC) proliferation, migration, and survival. VEGF-A exerts its proangiogenic effects by binding to the EC-specific tyrosine-kinase receptor VEGFR-2. After binding of VEGF-A to VEGFR-2, the receptor is autophosphorylated on tyrosine residues, followed by activation of the phosphatidylinositol kinase/protein kinase B (PI3K/PKB) and phospholipase C 3/3 complexes. These effects as well as FXIIIa-induced proliferation and migration of HUVECs were abolished by iodoacetamide treatment of FXIIIa (I-FXIII) or by PTKI, an inhibitor of VEGFR-2. FXIIIa induced upregulation of c-Jun and Egr-1 as revealed by quantitative RT-PCR. Electrophoretic mobility-shift assay experiments showed that FXIIIa treatment of HUVECs enhanced binding of Wilms’s tumor-1 (WT-1) but not of early growth response (Egr)-1 to the thrombospondin-1 (TSP-1) promoter sequence, suggesting that WT-1 but not Egr-1 is involved in downregulation of TSP-1 expression.

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$\alpha_\beta$ is one of the most important integrins involved in angiogenesis and vasculogenesis. This integrin is highly expressed by angiogenic ECs in tumors and wounds where it functions as a regulator of the balance between the proliferative and apoptotic signals. In addition to a broad spectrum of adhesive glycoproteins, $\alpha_\beta$ is capable of interacting with several growth factor receptors, such as the insulin receptor, the platelet-derived growth factor $\beta$ (PDGF-$\beta$) receptor, and VEGFR-2. The association of $\alpha_\beta$ with VEGFR-2 was found to be essential for VEGFR-2 activation and phosphorylation in response to VEGF-A. Interestingly, VEGFR-2 levels are significantly elevated in mice lacking $\alpha_\beta$, suggesting that the $\alpha_\beta$/VEGFR-2 interaction might also regulate VEGFR-2 expression in ECs.

Factor FXIII (FXIII) is a plasma transglutaminase that participates at the final step of the coagulation cascade. Thrombin-activated FXIII (FXIIIa) catalyzes formation of covalent crosslinks between $\gamma$-glutamyl and $\epsilon$-lysyl residues of adjacent fibrin chains in polymerized fibrin to yield the mature clot. Besides its role in hemostasis, FXIII participates in tissue remodeling, wound healing, and embryo implantation as can be inferred from defects in these processes in patients with

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inherited FXIII deficiency. Wound healing and embryo implantation are complex processes that involve cell proliferation and angiogenesis. Until recently, the precise role of FXIII in these processes has not been sufficiently studied. In our previous work we showed for the first time that FXIIIa supports angiogenesis by enhancing EC migration, proliferation, and survival. In addition, FXIIIa induced new vessel formation in a rabbit cornea model, and this proangiogenic effect was associated with downregulation of thrombospondin-1 (TSP-1). In this study, we explore the molecular mechanism(s) underlying the proangiogenic activity of FXIII.

Materials and Methods

Cells

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords and maintained in EGM-2 medium (Clonetics) as described previously. Dermal microvascular ECs were purchased from Clonetics and grown according to the manufacturer’s instructions. Subconfluent cells from passages 1 to 6 plated on fibronectin (unless stated otherwise) were used in all the experiments.

FXIII Activation

FXIII was activated by immobilized thrombin as described previously. FXIIIa was inactivated by treatment with 2 mmol/L iodoacetamide (i-FXIII), an inhibitor of transglutamination (Sigma) as described previously. (Detailed procedure appears in online Methods, available at http://atvb.ahajournals.org.)

Western Blot Analysis of HUVEC Proteins

Detailed procedure appears in online Methods.

Coimmunoprecipitation Experiments

Triton-X-100 extracts of untreated HUVECs or HUVECs treated with either FXIIIa or i-FXIII for 16 hours at 37°C were incubated with the appropriate primary antibody (goat anti-human VEGFR-2 [R&D Systems, Minneapolis, Minn] or sheep anti-human β3 [Affinity Biological Inc]), or anti-αβ3 complex LM609 (Chemicon, Temecula, Calif), followed by addition of protein A-Sepharose beads (Pharmacia). The beads were washed 3× with 1% Triton X-100 buffer and boiled in SDS-PAGE sample buffer for 3 minutes. The precipitated proteins were resolved by 4% to 12% SDS-PAGE and transferred to a nylon membrane as described above. In some experiments, HUVECs were preincubated with 500 nM VEGFR-2 kinase inhibitor (Z)-5-bromo-3-(4,5,7-tetrahydro-1H-indol-2-ylmethylene)1,3-dihydroindol-2-one (Calbiochem; Cat. No. 676485) or sheep anti-human WT-1, Santa Cruz Biotechnology Inc, Santa Cruz, Calif), followed by addition of protein A-Sepharose beads (Pharmacia). The beads were washed 3× with 1% Triton X-100 buffer and boiled in SDS-PAGE sample buffer for 3 minutes. The precipitated proteins were resolved by 4% to 12% SDS-PAGE and transferred to a nylon membrane as described above. In some experiments, HUVECs were preincubated with 500 nM VEGFR-2 kinase inhibitor (Z)-5-bromo-3-(4,5,7-tetrahydro-1H-indol-2-ylmethylene)1,3-dihydroindol-2-one (Calbiochem; Cat. No. 676485) for 30 minutes at 37°C before the addition of FXIIIa. This compound is a membrane-permeable tyrosine kinase inhibitor with IC50 = 70 nM for VEGFR-2. Blots containing the proteins immunoprecipitated with anti-VEGFR-2 antibody were probed with mouse anti-β3 (AP3, GTI) or mouse anti-PY (4G10; Upstate Biotechnology, Lake Placid, NY) antibodies. Blots containing the proteins immunoprecipitated with the anti-β3 antibody were probed with either anti-VEGFR-2, anti-PY, anti-γ-glutamyl-ε-lysine isopeptide bond (clone 814-MAM, CovalAb, France), or anti-β3 (either sheep polyclonal or mouse monoclonal) antibodies. The signals were detected using the appropriate horseradish peroxidase–conjugated antibodies followed by enhanced chemiluminescence. The expression of VEGFR-2 on HUVECs was tested and found to be the same throughout the 1 to 6 passages used in these experiments.

HUVEC Migration and Proliferation

HUVEC migration was analyzed by a modified Boyden chamber assay; proliferation was assessed by measuring 3H- Thymidine incorporation. Detailed migration and proliferation procedures are provided in online Methods.

Real-Time PCR

Total RNA extracted from one 75-cm2 flask of either untreated HUVECs or HUVECs treated with 50 μg/mL FXIIIa for 16 hours at 37°C was isolated using the Qiagen RNA isolation kit (Qiagen). RNA was then reverse transcribed into cDNA by MMLV reverse transcriptase (Promega). cDNA samples were amplified using specific primers for either c-Jun (forward: 5′-gtgccctggcggccacctact; reverse: 5′-ctctgtctcatgctgcctt; accession number: J04111.1), Egr-1 (forward: 5′-gtctctgagaccctgctcg; reverse: 5′-gtctctgagaccctgctcg; accession number: NM_001964), or GAPDH (forward: cactctggtcagcgggggt; reverse: gttctctgggtgcgag; accession number: M33197) and the Quant i Test Sybr Green RT-PCR kit (Qiagen). Amplification was monitored by real-time PCR analysis using the ABI Prism 7700 Sequence Detector System (Applied Biosystems).

Electrophoretic Mobility-Shift Assay Experiments

Nuclear proteins were prepared as described.16 Protein concentrations were measured using Bradford analysis. The oligonucleotides used for electrophoretic mobility-shift assay (EMSA) experiments were: probe 1 forward: 5′-gtgccctggcggccacctact-3′; probe reverse: 5′-agctgtgctccggccctc-3′; probe 2 forward: 5′-ccgaccggccctcctc-3′; probe 2 reverse: 5′-gaagggggggcccgtgg-3′. Each oligonucleotide (10 pmol) was end-labeled with γ32P-ATP using polynucleotide kinase (New England Biolabs Inc). The radiolabeled oligonucleotides were purified from unincorporated γ32P-ATP using G-50 Spin columns (New England Biolabs Inc), mixed, and incubated for 15 minutes at room temperature to allow annealing. Ten μg of HUVEC nuclear proteins prepared as described above were mixed with 1 μg of poly-(df-dc) and 1 μL (~50 000 cpm) of the double-stranded oligonucleotides in binding buffer (20 mmol/L HEPES, pH 7.5, 1 mmol/L EDTA, 5 mmol/L MgCl2, 50 mmol/L KCl, 1 mmol/L DTT, and 10% glycerol). For supershift experiments, the nuclear extracts were preincubated with either anti–Egr-1 (rabbit anti-human Egr-1, Santa Cruz Biotechnology Inc, Santa Cruz, Calif), or anti–WT-1 antibodies (mouse anti-human WT-1, Santa Cruz Biotechnology Inc, Santa Cruz, Calif) at a ratio of 10 μg IgG/10 μg of total nuclear protein for 30 minutes at room temperature before the addition of the radiolabeled probe. To test the specificity of the formed complexes, competition assays using a 100-fold excess of cold double-stranded oligonucleotides were performed. The DNA-protein complexes were resolved on 4% nondenaturating polyacrylamide gels and detected by autoradiography.

Analysis of αβ3 Activation

αβ3 activation was analyzed by flow cytometry using WOW-1 antibody (kindly provided by Dr S. Shattil, Scripps Research Institute), which recognizes the αβ3 integrin in its active conformation. Detailed procedure appears in online Methods.

Statistical Analysis

Statistical analysis was performed by unpaired Student t test. Results are expressed as mean±SD. P<0.05 was considered significant.

Results

Tyrosine Phosphorylation of HUVEC Proteins Induced by FXIIIa

Triton-X-100 soluble lysates of control HUVECs or HUVECs incubated in the presence of either FXIIIa or I-FXIII were tested for tyrosine phosphorylation by Western blot (WB) analysis. As shown in Figure 1A, FXIIIa, but not I-FXIII, stimulated tyrosine phosphorylation of 2 proteins with <Mr> of 250 and 230 kDa. Another phosphorylated protein with <Mr> of 120 kDa was present in untreated cells and was not affected by FXIIIa or I-FXIII treatment.

To identify the candidate protein undergoing tyrosine-phosphorylation in response to FXIIIa, the blot shown in Figure 1A was stripped and incubated with VEGFR-2 anti-
body, which bound to the 230-kDa tyrosine-phosphorylated band (not shown).

Tyrosine phosphorylation of VEGFR-2 was further verified by immunoprecipitation of VEGFR-2 followed by WB analysis with anti-PY antibody (Figure 1B). VEGFR-2 was tyrosine phosphorylated in FXIIIa-treated and in VEGF-A-treated samples. VEGF-A– or FXIIIa-induced VEGFR-2 phosphorylation was abolished by a specific VEGFR-2 tyrosine kinase inhibitor PTKI (an inhibitor of VEGFR-2) or depletion of the FXIIIa preparation by passage over an immobilized anti-FXIII antibody (D), respectively (Figure 1B).

The Role of FXIIIa Crosslinking in αβ3–VEGFR-2 Interaction

We tested whether FXIIIa affects the αβ3 and VEGFR-2 interaction, because this interaction was previously shown to play an important role in VEGFR-2–induced signaling.7,10 Triton X-100 lysates of control and FXIIIa or I-FXIII–treated HUVECs were immunoprecipitated with anti-β3 antibody followed by WB analysis using anti–VEGFR-2 antibody (Figure 2A). A high-molecular-weight complex recognized by the 2 antibodies was observed at the interface between stacking and running gels in the lanes containing FXIIIa-treated HUVECs. This complex was absent in the cells treated with I-FXIII3. In addition, a 230-kDa band corresponding to the mature form of VEGFR-2 was observed in all the lanes, with an increase in intensity in a FXIIIa dose-dependent manner indicating VEGFR-2/β3 complex formation without crosslinking. To determine whether the VEGFR-2/β3 complex undergoes phosphorylation, this blot was stripped and reprobed with anti-PY antibody. WB with anti-PY revealed that VEGFR-2 in both the high-molecular-weight and the noncovalent VEGFR-2/β3 complexes underwent phosphorylation (Figure 2A). To prove that VEGFR-2 and β3 within the high-molecular-weight complex are crosslinked by FXIIIa, the blot containing immunoprecipitated VEGFR-2 and β3 proteins was subjected to WB analysis using an antibody recognizing the γ-glutamyl-e-lysine isopeptide bond antibodies, or anti-β3 (A), anti-β3, or anti-VEGFR-2 (B through D).
as demonstrated by lack of WOW-1 antibody binding to FXIIIa-treated cells (Figure I, available online at http://atvb.ahajournals.org).

To evaluate the role of the tyrosine kinase activity of VEGFR-2 in the VEGFR-2/β3 interaction, we performed a coimmunoprecipitation experiment using lysates of control and FXIIIa-treated HUVECs preincubated with a specific VEGFR-2 tyrosine kinase inhibitor (PTKI; Figure 2D). This inhibitor blocks VEGF-A–induced VEGFR-2 phosphorylation as shown by Sun et al15 and by us in Figure 1B. As demonstrated in Figure 2D, this inhibitor markedly decreased VEGFR-2/β3 complex formation in nontreated or FXIIIa-treated cells and completely abolished crosslinking of the VEGFR-2/β3 complex induced by FXIIIa. FXIIIa mediated VEGFR-2/α,β3 crosslinking, and VEGFR-2 phosphorylation was also observed in dermal microvascular ECs (data not shown).

To find out whether VEGFR-2/β3 crosslinking follows kinetics similar to that of downstream signaling, time-course experiments were performed and are presented in Figure 3. Weak bands corresponding to phosphorylated VEGFR-2 (Figure 3A) and VEGFR-2/β3 crosslinked complex (Figure 3B) were observed only after 1 hour of incubation with FXIIIa. Both bands increased in intensity in a time-dependent manner, reaching a plateau after 8 hours of exposure to FXIIIa. Similar kinetics was observed for downstream activation of Akt (Figure 3C). In contrast, maximal activation of ERK was observed already after 2 hours of incubation (Figure 3C). It is possible that partial activation of VEGFR-2 is sufficient to trigger a full activation of the MEK/ERK system. Treatment of HUVECs with VEGF resulted in more rapid maximal activation of both ERK and Akt (after only 10 minutes of incubation; Figure 3C). Densitometric assessment of the time course experiments is shown in Figure II (available online at http://atvb.ahajournals.org).

To provide functional evidence that the pathways studied are responsible for the angiogenic action of F-XIII, we performed migration and proliferation assays in the presence of FXIIIa, VEGF, or both. As shown in Figure 4, FXIIIa significantly increased EC migration and proliferation, and both processes were inhibited by VEGFR-2 tyrosine kinase inhibitor PTKI. Similar results were observed with VEGF. In addition, these experiments showed that FXIIIa had no additional effect on VEGF-A–induced migration and proliferation.

**Effect of FXIIIa on Gene Expression of Transcription Factors and Signaling Molecules**

After treatment of HUVECs with FXIIIa, suspected upregulation of cyclin D1 and Egr-1 was observed by microarray analysis (data not shown). However, validation of the upregu-
Effect of FXIIIa on Binding of Nuclear Proteins to the TSP-1 Promoter

c-Jun was previously shown to downregulate TSP-1 by inducing WT-1 binding to the TSP-1 promoter. We examined whether WT-1 binding to TSP-1 promoter is affected by FXIIIa by an EMSA experiment using radiolabeled oligonucleotide probes containing a GC-rich TSP-1 promoter sequence (probe 1) as outlined in Methods. As shown in Figure 5A, the treatment of HUVECs with FXIIIa increased complex formation between the probe and one of the nuclear proteins, which was significantly reduced by addition of an anti–WT-1 antibody. Because the amount of the WT-1 protein in the nuclear extracts of HUVECs was not affected by FXIIIa (Figure 5B), the downregulation of TSP-1 associated with FXIIIa may be caused by modulation of WT-1 binding to TSP-1 rather than WT-1 protein synthesis. Similarly, an EMSA experiment using radiolabeled oligonucleotide probes 1 and 2 (Methods) was undertaken to analyze binding of Egr-1 to TSP-1 promoter after FXIIIa treatment. Figure 5A shows that anti–Egr-1 antibody had no effect on FXIIIa-induced complex formation between probe 1 and nuclear proteins. Binding of probe 2 to nuclear proteins was not affected by FXIIIa although it was inhibited by anti–Egr-1 antibody. WB analysis of FXIIIa-treated HUVECs with anti–Egr-1 antibody revealed that Egr-1 was markedly upregulated (Figure 5B). Taken together, these data suggest that although Egr-1 protein is upregulated after FXIIIa treatment, its binding to the TSP-1 gene is not visibly regulated by FXIIIa.

Discussion

In this study, we have explored the molecular mechanisms that underlie the proangiogenic activity of FXIII reported previously by us. Our results show that FXIIIa crosslinks β1 integrin to VEGFR-2 as well as enhances the noncovalent complex formation between the β1 integrin and VEGFR-2. Moreover, FXIIIa induces tyrosine phosphorylation of VEGFR-2 in both the crosslinked and noncovalent β1-VEGFR-2 complexes. The formation of the complexes appears to be dependent on the intact catalytic site of VEGFR-2, because it is inhibited by a specific VEGFR-2 tyrosine kinase inhibitor (PTKI). It is, however, unclear why VEGFR-2/β1 noncovalent and crosslinked complexes depend on the intact catalytic site of VEGFR-2. One of the possibilities is that the inhibitor binding induces a conformational change in the receptor, thereby interfering with VEGFR-2/αβ3 interaction.

The effects of FXIIIa on both the αβ3 integrin–VEGFR-2 association and tyrosine phosphorylation of VEGFR-2 were found to be absolutely dependent on its transglutaminase activity, as indicated by the lack of effect of iodoacetamide-inhibited FXIIIa. Taken together, these data suggest that the proangiogenic effect of FXIIIa, in part, involves crosslinking of the 2 receptors αβ3 integrin and VEGFR-2 followed by activation of VEGFR-2 similarly to that elicited by VEGF-A. The faster kinetics of VEGF-induced as compared with FXIIIa-induced phosphorylation of signaling proteins may suggest that VEGF is the primary proangiogenic factor, whose effect is dependent on the transglutaminase activity and therefore acts at a much slower pace. This is further supported by the incapability of FXIIIa to enhance maximal VEGF-A–induced migration and proliferation of HUVECs.

The tyrosine-phosphorylated VEGF-2 was shown to mediate activation of MAP kinase, PI-3 kinase, c-GMP-dependent kinase, Jun-N-terminal kinase, and focal adhesion kinase, all of which are involved in regulation of the angiogenic response of ECs to VEGF-A. Our data indicate that, similar to VEGF-A, FXIIIa induces tyrosine-phosphorylation of VEGFR-2 in HUVECs; however, it does not increase the amounts of VEGF-A or VEGFR-2 as shown by us previously. It seems that VEGF-2 phosphorylation is the major and

Figure 5. EMSA of FXIIIa-induced binding of TSP-1 promoter to WT-1. The representative picture of 4 separate experiments is shown. A, Ten μg of nuclear proteins extracted from untreated HUVECs (−) or HUVECs treated with 50 μg/mL of FXIIIa (+) were subjected to EMSA analysis using radiolabeled probe 1 or 2 (50 000 cpm) from the TSP-1 promoter as described in Materials and Methods. Anti–WT-1 or anti–Egr-1 antibodies were used to identify the DNA-protein complexes (denoted by arrows). NSB indicates nonspecific binding. B, Fifty μg of nuclear proteins extracted from untreated HUVECs (−) or HUVECs treated with 50 μg/mL of FXIIIa (+) were subjected to WB analysis using either anti–WT-1 or anti–Egr-1 antibody.
vital step in the initiation of FXIIIa-induced angiogenesis. Inhibition of FXIII-mediated proliferation and migration by PTKI further confirm that VEGFR-2 phosphorylation is the event responsible for the angiogenic action of FXIII.

Activated tyrosine-phosphorylated VEGFR-2 binds and activates PLCγ, which in turn activates PKC. Activated PKC induces activation of MAPK, which in accordance with our results showing that FXIIIa-mediated VEGFR-2/αvβ3 crosslinking with subsequent VEGFR-2 phosphorylation is associated with MAPK activation. Activation of VEGFR-2 by VEGF-A induces upregulation of Egr-1 in a PKC- and MAPK-dependent manner, which is also in agreement with our data showing that the FXIIIa-induced proangiogenic effect is associated with upregulation of Egr-1.

Egr-1 is a zinc-finger transcription factor involved in cell proliferation and differentiation and regulation of multiple genes implicated in vasculogenesis and angiogenesis. Up-regulation of Egr-1 demonstrated in this study indicates that Egr-1 may serve as a direct mediator of FXIIIa-induced EC proliferation rather than mediating TSP-1 suppression, because its binding to the TSP-1 promoter is not affected by FXIIIa. That Egr-1 participates in angiogenesis and in wound healing supports its involvement in FXIII-induced wound healing owing to the ability of FXIIIa to upregulate Egr-1.

We have recently shown that the proangiogenic activity of FXIIIa is associated with downregulation of TSP-1 in vitro and in vivo models. Several lines of evidence indicate that downregulation of TSP-1 is linked to overexpression of the transcription factor c-Jun, a positive regulator of cell proliferation. In rat fibroblasts, the negative regulation of TSP-1 transcription by c-Jun was shown to be mediated by transcription factor WT-1 rather than by direct repression by c-Jun. In this study, c-Jun was found to be upregulated by FXIIIa at both mRNA and protein levels. In addition, FXIIIa induced an increase in binding of WT-1 but not Egr-1 to the TSP-1 promoter. Based on these results and the work of Dejong and colleagues, it seems most likely that the downregulation of TSP-1 by FXIIIa stems from indirect suppression of TSP-1 transcription by c-Jun through WT-1.

We and others have previously reported that FXIIIa binds to the αvβ3 integrin expressed on the surface of ECs. In this study, FXIIIa-αvβ3 interaction on HUVECs was not associated with αvβ3 activation. Because FXIIIa does not contain the conventional integrin recognition and activation sequence RGD, its binding to αvβ3 may be mediated by the LDV sequence without activating the integrin. LDV was previously shown to mediate the interaction of fibronectin with αvβ3.

Hemostasis and angiogenesis are interrelated. Proteins generated by the hemostatic system coordinate the spatial localization and stabilization of ECs followed by growth and repair of a damaged blood vessel. After clot stabilization, angiogenesis is regulated through proteins secreted by platelets and cryptic fragments generated from coagulation and fibrinolytic system. The well-characterized proangiogenic coagulation proteins are thrombin, FVII, and tissue factor. The antiangiogenic proteins are antithrombin, domain 5 of the high-molecular-weight kinogen, prothrombin fragments 1 and 2, u-PA, plasminogen activator inhibitor-1, and angiostatin. In this study, we focused on the regulation of angiogenesis mediated by clot stabilization protein-FXIII. Based on our results from this and previous studies, we propose the following model for the proangiogenic effect of FXIIIa (Figure IV, available online at http://atvb.ahajournals.org): FXIIIa binding to the EC αvβ3 integrin is followed by enhancement of the αvβ3–VEGFR-2 interaction resulting in tyrosine phosphorylation and activation of VEGFR-2. Activated VEGFR-2 then initiates a signaling cascade leading to upregulation of Egr-1 and c-Jun, which may then be responsible for the enhanced cell proliferation and survival induced by FXIIIa. In addition, downregulation of TSP-1 by c-Jun through WT-1 may further contribute to the proangiogenic activities exhibited by FXIIIa. This FXIIIa-mediated proangiogenic effect may finally clarify the role of FXIII in vascular remodeling and tissue repair.

References


A. 

![Graph showing c-Jun/GAPDH mRNA levels withFXIIIa and PTKI treatments.](image)

B. 

![Graph showing Egr-1/GAPDH mRNA levels withFXIIIa and PTKI treatments.](image)

C. 

![Western blot image showing c-Jun (39 kDa) withFXIIIa and PTKI treatments.](image)

D. 

![Western blot image showing Egr-1 (82 kDa) withFXIIIa and PTKI treatments.](image)
Endothelial cell

FXIIIa → ♂♀ β₃

VEGFR-2

Tyr-p-VEGFR-2

c-Jun ↑

WT-1 binding to TSP-1 promoter

Egr-1 ↑

TSP-1↓

ANGIOGENESIS

Proliferation ↑
Migration ↑
Survival ↑
Materials and Methods

FXIII activation

The source of FXIII was FXIII concentrate, Fibrogammin-P (Aventis Behring, Mamburg, Germany). After reconstitution, 2 ml of 100 units /ml (approximately 1000 µg/ml) FXIII were activated by thrombin immobilized on Affi-gel 10 beads as described previously (1). 1 U/ml of activated FXIII corresponds to approximately 10 µg/ml of FXIIIA (2). Contamination by traces of thrombin in the activated FXIII solution was excluded by chromogenic assay (S2238, Chromogenix, Sweden; sensitivity <0.01 u/ml thrombin). FXIIIa was inactivated by treatment with 2 mM iodoacetamide (I-FXIII) – an inhibitor of transglutamination (Sigma, Israel) as described previously (1).

Depletion of FXIII from the Fibrogammin preparation was achieved by passing it over agarose-anti- goat IgG beads (Sigma) coated with a polyclonal goat anti-FXIII antibody (Affinity Biologicals).

Western Blot analysis of HUVEC proteins

HUVEC were synchronized in EBM-2 serum-free medium for 24 hours. The serum-free medium was replaced with EBM-2 (Clonetics) containing 1/10 (vol) of EGM-2 (Clonetics; 0.2% FCS final concentration) and either 10 or 50 µg/ml FXIIIa or 50 µg/ml iodoacetamide-inhibited FXIIIa (I-FXIII) for 16 h at 37°C. In time course experiments, HUVEC were incubated with 50 µg/ml FXIIIa for time periods ranging from 5 min to 16 hr. Control (untreated) cells were incubated in EBM-2 containing 0.2% FCS with no other additives under the above conditions. Triton-soluble (membrane and cytoplasmic) proteins were extracted in ice-cold 1%-Triton X-100
lysis buffer (TBS = 0.15 M NaCl, 20 mM TrisHCl, pH 7.5) containing protease inhibitors (Boeringer-Manheim, Germany), 1mM NaVO₄, and 2 mM PMSF. Protein concentrations were measured using the Bradford assay (Bio-Rad, Germany). Proteins (100 µg/lane) were resolved on 4-12% SDS-PAGE under non-reducing conditions and electrophoretically transferred to a nylon membrane (PVDF; Millipore, USA). The blot was then blocked with blocking buffer (5% skim milk in TBS) and incubated with either mouse anti-PY antibody (clone number - 4G10; UBI, USA; 1:1000 in blocking buffer), mouse anti-phospho p-44/42 MAPK (Cell Signaling, USA; 1:1000 in blocking buffer), or rabbit anti- p-44/42 MAPK (Cell Signaling, USA; 1:1000 in blocking buffer) for 1 hr at room temperature. After incubation, the blot was washed 3 times in TBS containing 0.01 % Tween-20 (TBS-T) and incubated with the appropriate HRP-conjugated secondary antibody (Jackson, USA; 1:10000 in TBS-T). The signals were detected using ECL (Amersham, UK) according to the manufacturer's instructions.

[^H]-Thymidine incorporation

[^H]-Thymidine incorporation experiments were conducted as described by us previously (1). Briefly, HUVECs were seeded on 24-well tissue culture plates, and 48 hr after seeding 50 µg/ml FXIIIa, 10 ng/ml VEGF-A (R&D Systems) or a combination of 50 µg/ml FXIIIa with 10 ng/ml VEGF-A were added, followed by incubation with 1 µCi/well[^H]-thymidine. The samples were processed and the radioactivity counted as described previously (1).

Cell Migration Assay

Cell migration was studied by a modified Boyden chamber assay as described (1). Briefly, serum–starved cells were trypsinized, resuspended in EBM-2 containing 0.5% FCS at 1x10⁶ cells/ml, and 0.25 ml of this cell suspension (2.5x10⁴ cells/well)
were added to Transwell fibronectin-coated plates (6.5 mm diameter, 8.0 μm pore size). Added to the lower chamber were: EBM-2 with 0.5% FCS supplemented with either 50 μg/ml FXIIIa, 50 μg/ml I-FXIII, 10 ng/ml VEGF-A, or 10 ng/ml VEGF-A in combination with 50 μg/ml FXIIIa. The cells were incubated at 37°C for either 4h or 16 h. Cells adhering to the lower surface of the Transwell membrane were stained with May-Grunwald and migration was quantified by counting 10 random microscopic fields/well.

**Flow cytometry**

HUVEC were harvested by incubation in PBS containing 5 mM EDTA and resuspended in EBM-2 containing 0.5% FCS at 1x10^7 cells/ml. FXIIIa (50 μg/ml) or 1 mM MnCl₂ was added for 1 h at 37°C. Cells were then washed with PBS and incubated with WOW-1 for 30 min (3), followed by FITC-conjugated goat-anti-mouse IgG (Jackson). For analysis of FXIIIa binding, cells incubated with FXIIIa were washed and incubated with mouse anti-FXIII antibody (Acris Antibodies, Germany), followed by FITC-conjugated goat-anti-mouse IgG (Jackson). Flow cytometry was performed using the Coulter FACS analyzer.

**Densitometric analysis**

Densitometric analysis was performed using the ScanImage software (NIH).
Results

Quantitative assessment of the fraction of total VEGFR2 crosslinked to $\alpha_v\beta_3$ and fraction of total $\alpha_v\beta_3$ crosslinked to VEGFR2.

Quantitative assessment of VEGFR2/$\alpha_v\beta_3$ was performed by densitometric analysis of the gels shown in Fig. 2 A third lane and Fig. 2 B second lane. The ratio of the intensity of the band representing VEGFR2 crosslinked to $\alpha_v\beta_3$ to that representing VEGFR2 non-covalently bound to beta3 was 1:3 (Fig.2A). This indicates that one of total 4 parts (25%) of VEGFR2 interacting with $\alpha_v\beta_3$ was crosslinked. In order to estimate what fraction of VEGFR2 out of total VEGFR2 amount obtained from the cells interacts with $\beta_3$, we first immunoprecipitated the cells with anti $\beta_3$, then the VEGFR2 in the supernatant (non bound) was immunoprecipitated with anti-VEGFR2, followed by WB analysis with anti-VEGFR2. This experiment revealed that only 50% of VEGFR2 was bound to $\beta_3$, out of which 25% (namely, 12.5% of total VEGFR2) was crosslinked.

Similarly, $\alpha_v\beta_3$ interacting with VEGFR2 (Fig.2B, second lane) showed a ratio of 1:3 between crosslinked and non-covalently bound $\beta_3$. Similar experiments with 2 steps of immunoprecipitation, first with anti-VEGFR2 and then with anti- $\beta_3$, followed by WB analysis with anti –$\beta_3$ revealed that only 30 % of total $\alpha_v\beta_3$ extracted from the cells was bound to VEGFR2, 25% of which became crosslinked to VEGFR2 - about 8% of total $\alpha_v\beta_3$.

References


Figure Legends

**Figure I online. Effect of FXIIIa on WOW-1 binding.**

A. FACS analysis of WOW-1 binding to untreated cells and to cells treated with either FXIIIa or 1 mM MnCl$_2$. The results shown represent mean ± SD of three separate experiments. B. FACS analysis of FXIIIa binding to HUVEC. The experimental procedure is detailed in Methods online. Cells incubated with anti-FXIII antibody without preincubation with FXIIIa were used as a negative control.

**Figure II online. Densitometric analysis of the time course of FXIIIa-induced events shown in Fig. 3**

Densitometric analysis of:

A. FXIIIa-induced VEGFR2 phosphorylation;

B. FXIIIa-induced VEGFR2/α$_v$β$_3$ crosslinking;

C. VEGF-A and FXIIIa-induced phosphorylation of ERK1, ERK2 and Akt.

The numbers beneath the bars indicate incubation periods in minutes and hours for VEGF-A and FXIIIa, respectively.

Data are presented in number of pixels as mean ± SD of three separate experiments.

**Figure III online. Analysis of c-Jun mRNA (A) and protein (B) levels in untreated and FXIIIa – treated HUVEC.**

The representative picture of three separate experiments is shown.

A. cDNA of untreated HUVEC (-) or HUVEC treated with 50 µg/ml of FXIIIa (+) was subjected to real-time PCR analysis using either c-Jun, Egr-1 or GAPDH specific primers. The figure shows ratios c-Jun / GAPDH and Egr-1/GAPDH mRNA levels (mean ± SD of two separate experiments performed in triplicates). * - denotes p< 0.05
B. Fifty µg of nuclear proteins extracted from untreated HUVEC (-) or HUVEC treated with 50 µg/ml of FXIIIa (+) were subjected to WB analysis with either anti-c-Jun or anti Egr-1.

Figure IV online. Schematic presentation of FXIII-induced angiogenesis by signals downstream of VEGFR-2, αvβ3, and TSP-1 suppression.