Effect of FXIII on Monocyte and Fibroblast Function

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Key Words

FXIII • Proliferation • Migration • Apoptosis • Monocytes • Fibroblasts

giogenic activity of FXIIIa, further substantiate a significant role of FXIII in tissue repair.

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Abstract

Factor XIII is a plasma transglutaminase that participates in the final stage of the coagulation cascade. Thrombin-activated FXIII (FXIIIa) catalyzes the formation of covalent crosslinks between γ -glutamyl and ϵ -lysyl residues on fibrin molecules to yield the mature clot. In addition to its role in hemostasis, FXIIIa was previously shown by us to stimulate endothelial cells to exhibit pro-angiogenic activity. In this work, we studied the effect of FXIIIa on other cells that participate in angiogenesis and tissue repair, such as monocytes and fibroblasts. FXIIIa significantly enhanced migration and proliferation, and inhibited apoptosis of monocytes and fibroblasts. Similar to our previous observations with endothelial cells, the stimulating effect of FXIIIa on monocytes and fibroblasts was elicited via its binding to $\alpha_v\beta_3$ integrin leading to cJun upregulation and TSP-1 downregulation. Since monocytes and fibroblasts are essential components of the tissue repair process, the results of this study, together with the proan-

Introduction

Angiogenesis involves both proangiogenic and antiangiogenic factors, and consists of several steps, which include stimulation of endothelial cells by growth factors, degradation of the surrounding extracellular matrix by proteolytic enzymes, migration and proliferation of endothelial cells, and, ultimately, capillary tube formation [1]. Hemostasis and angiogenesis are closely interrelated. Following injury, proteins generated by the hemostatic system coordinate the spatial localization and stabilization of endothelial cells, followed by growth and repair of the damaged blood vessel. Once clot stabilization is achieved, angiogenesis is modulated by proteins and cryptic peptide fragments generated from the coagulation and the

fibrinolytic systems [2]. The well-characterized proangiogenic coagulation proteins are thrombin [3], factor VII, and tissue factor [4]. The antiangiogenic proteins include cleaved anti-thrombin III [5], as well as cryptic fragments of coagulation proteins, e.g., high-molecular-weight kininogen domain 5 [6], prothrombin fragments 1 and 2 [7], and angiostatin derived from plasmin [8]. Our work has been focused on an additional proangiogenic coagulation protein – factor XIII (FXIII).

FXIII is a plasma transglutaminase that participates at the final stage of the coagulation cascade. Following activation by thrombin, activated FXIII (FXIIIa) then catalyzes the formation of covalent crosslinks between γ -glutamyl and ϵ -lysyl residues on adjacent fibrin chains in polymerized fibrin to yield the mature clot [9].

We have previously shown that FXIIIa regulates the key steps of angiogenesis by increasing endothelial cell migration, proliferation, and survival [10]. All of these proangiogenic effect of FXIIIa were found to be dependent on its transglutaminase activity, since the proangiogenic actions of FXIIIa was completely abrogated by blockade of its active site. We also observed that the proangiogenic effect of FXIIIa on endothelial cells is accompanied by downregulation of thrombospondin 1 (TSP-1), one of the best characterized antiangiogenic factors [10]. In addition, FXIIIa was also shown to induce new vessel formation in different animal models, such as rabbit cornea [10] and murine heart implant [11]. Finally, angiogenesis was significantly impaired in FXIII-knock out mice [11]. In line with our observations, two recent reports demonstrated proangiogenic activity of FXIII in animal models [12, 13].

The important role of FXIII in wound healing has been recently substantiated by some of us using FXIII-knock out mice [14]. We have showed that cutaneous wound healing was delayed in these mice and almost normalized following treatment with FXIII concentrate [14]. Thus, it seems that, similar to tissue transglutaminase [15-17], plasma transglutaminase - FXIII - is essential for wound healing. This conclusion is based on several lines of evidence: 1) FXIII can modulate the composition and stability of the fibrin clot via crosslinking several extracellular matrix proteins [9]; 2) some FXIII-deficient patients [9], as well as in FXIII-deficient mice [14], manifest delayed wound healing, and FXIII concentrate facilitates wound repair [18-21]; and 3) FXIII can stimulate angiogenesis [10, 11, 22], thus, providing new blood vessels to supply nutrients for the newly formed tissue.

At present, the information on the effect of FXIII

on the behavior of other cells that participate in wound healing, such as monocytes and fibroblasts, is insufficient, although some stimulating effect of FXIII on these cells was demonstrated in preliminary reports [23-27]. In the present work, we systematically studied the effect of FXIII on the proliferation, migration, and survival of monocytes and fibroblasts originating from different tissues, as well as the mechanism underlying the observed effect.

Materials and Methods

FXIII activation

The source of FXIII was FXIII concentrate, Fibrogammin-P (ZLB- Behring, Marburg, Germany). After reconstitution, 2 mL of 100 U/mL (approximately 1000 μ g/mL) FXIII was incubated with thrombin immobilized on Affi-gel 10 beads. One milliliter of packed bead volume contained 200 U of bovine thrombin. Ten millimolar CaCl_2 was added, and the mixture was incubated at 37°C for 2 hours. FXIII activation was monitored by measuring FXIII activity using a chromogenic assay (Berichrome, Dade Behring). FXIIIa was inactivated by treatment with 3 mmol/L iodoacetamide (Sigma, Rehovot) for 30 minutes at 22°C to block transglutaminase activity, and free iodoacetamide was then removed by dialysis. For all the experiments, a concentration of 50 μ g/ml was used, because a plateau of the studied effects was achieved at this concentration in our previous study [10].

Cell culture

Monocytes were isolated from the peripheral blood of healthy donors by Ficoll-Hypaque gradient centrifugation. Subsequently, monocytes were cultured in six-well plates (0.5 to 1.5 $\times 10^6$ cells/ml) in fresh, complete Iscove's medium supplemented with 1 ng/ml GM-CSF (PeproTech, London, UK). Cells were cultured for 5 days and subsequently incubated with either 50 μ g/mL FXIIIa, 10 ng/ml GM-CSF, or iodoacetamide-inactivated FXIII in serum-free media for 24 hours.

Skin and lung fibroblasts were purchased from Clonetics (Baltimore, MD) and grown in media supplied by the manufacturer according to the manufacturer's instructions.

Migration Assays

Migration of monocytes was measured using a modified Boyden chamber (Neuro Probe) migration assay as described previously [10]. Monocytes grown overnight in culture media were treated with trypsin, resuspended in culture media at 200 cells/ μ L, and added to the upper chamber (500 μ L). Fifty μ g/mL FXIIIa or 10 ng/ml GM-CSF in serum-containing medium was added to the lower chamber. A polycarbonate filter (8- μ m pores) was placed between the upper and lower chambers, and the chambers were incubated for 24 hours at 37°C. After incubation, the filters were removed and the cells migrating through the filter and adhering to the lower chamber were counted by light microscopy.

Fig. 1. Effect of FXIIIa on monocyte proliferation, migration, and apoptosis. Proliferation, migration, and apoptosis of monocytes were induced by 50 μ g/mL FXIIIa, 50 μ g/mL I-FXIIIa, and 10 ng/mL GM-CSF. Data are presented as percent of control (mean $\pm$ SD of 3 separate experiments each performed in triplicate). * p <0.05.

Migration of fibroblasts was measured using the wound-migration assay [10] with slight modification. Briefly, monolayers of fibroblasts were grown in Petri dishes. A small incision was made adjacent to the confluent cell-dense region using a small razor blade. Cells were removed from approximately half of the plate by gentle scraping. After wounding, the cells were allowed to migrate for 24 h. Migration of the cells across the sharp wound edge to the cell-free region was assessed by phase microscopy and counting the cells grown in serum-containing cell culture medium in the absence or presence of 50 μ g/mL FXIIIa or 10 ng/mL PDGF-B (Peprotech).

[³H]-Thymidine Incorporation

Monocytes and fibroblasts were seeded in 24-well tissue culture plates at a density of 1×10^4 cells/well. After 24 hours, the cell culture medium was replaced with serum-free medium, and 48 hours after seeding, 50 μ g/mL FXIIIa, 50 μ g/mL Iodo-FXIIIa, 10 ng/mL GM-CSF (for monocytes), or 10 ng/mL PDGF-B (for fibroblasts) was added. Seventy-two hours after seeding, 2% FBS was added to the media along with 1 μ Ci/well [³H]-thymidine, and the cells were incubated for an additional 16 h. After this time period, the cells were washed twice with PBS and incubated in 10% TCA overnight at 4°C. The cells were then washed twice with 100% ethanol and dried. Precipitated DNA and protein were solubilized with 1 N NaOH, and the radioactivity was counted in a liquid scintillation counter.

Apoptosis Assay

Apoptosis was measured using the cell death detection ELISA kit (Roche, Indianapolis, IN). The test was performed according to the manufacturer's instructions.

Flow cytometry

Monocytes were incubated with either FXIII, FXIIIa or I-FXIIIa (50 μ g/mL) for 1 h at 37°C. Some samples were preincubated with LM609 (Chemicon, Temecula, CA), a mouse monoclonal anti- $\alpha_v\beta_3$ integrin antibody, prior to the incubation with FXIIIa. The cells were then washed in TBS and incubated with sheep anti-FXIIIa antibody (The Binding Site, 1:200 dilution) for 30 min at 37°C, followed by FITC-conjugated

donkey anti-sheep IgG (Jackson ImmunoResearch Laboratories). The samples were analyzed by flow cytometry (Beckman-Coulter, High Wycombe, UK).

Real-Time PCR

Total RNA extracted from one 75-cm² flask of either untreated or FXIIIa-treated monocytes and fibroblasts (50 μ g/mL for 24 hours at 37°C) was isolated using the Qiagen RNA isolation kit (Qiagen, Valencia, CA). RNA was then reverse transcribed into cDNA by MMLV reverse transcriptase (Promega, Madison, WI). cDNA samples were then amplified using specific primers for TSP-1 (Forward primer: CTC AGG AAC AAA GGC TGC TC; Reverse primer: CTG GAC AGC TCA TCA CAG GA), for c-Jun, Egr-1 and GAPDH as previously described [22] and the Quanti 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, Foster City, CA).

Coimmunoprecipitation Experiments

Triton-X-100 extracts of untreated cells (monocytes or fibroblasts), or cells treated with either FXIIIa or I-FXIIIa for 16 hours at 37°C were incubated with anti- $\alpha_{v\beta_3}$ antibody LM609 (Chemicon, Temecula, California), followed by addition of goat-anti-mouse IgG-Agarose beads (Sigma, Rehovot, Israel). The beads were washed 3x with 1% Triton X-100 lysis buffer and boiled in SDS-PAGE sample buffer for 3 minutes. The precipitated proteins were resolved by 4% to 12% SDS-PAGE and electrophoretically transferred to a nylon membrane. Blots containing the proteins immunoprecipitated with LM609 antibody were probed with anti-gamma-glutamyl-epsilonyl-lysine isopeptide bond antibody (clone 814-MAM, Covalab, France), or anti- β_3 rabbit polyclonal antibody (Chemicon). The signals were detected using the appropriate horseradish peroxidase-conjugated antibodies followed by enhanced chemiluminescence.

Statistical analysis

Data were analyzed using Graphpad® Prism (ver. 4) for Windows. ANOVA test was performed to determine statistical

Fig. 2. Effect of FXIIIa on skin (A) and lung (B) fibroblast proliferation, migration, and apoptosis. Proliferation, migration, and apoptosis of fibroblasts were induced by 50 $\mu\text{g/mL}$ FXIIIa, 50 $\mu\text{g/mL}$ I-FXIIIa, and 10 ng/mL PDGF-B. Data are presented as percent of control (mean $\pm$ SD of 3 separate experiments each performed in triplicate). * $p < 0.05$.

significance. Results are presented as mean $\pm$ standard deviation. A p value < 0.05 was considered significant.

Results

Effect of FXIIIa on proliferation, migration and apoptosis of monocytes

The effect of FXIIIa on proliferation, migration, and survival of monocytes was compared to the effect of GM-CSF (positive control). As shown in Fig. 1, FXIIIa enhanced monocyte proliferation 1.4-fold compared to control ($P=0.027$). Similarly, a significant increase in monocyte migration (1.9-fold) was observed with FXIIIa treatment ($p=0.01$). FXIIIa inhibited monocyte apoptosis by 57% ($p=0.001$). No effect was observed when FXIIIa transglutaminase activity was blocked with iodoacetamide (I-FXIIIa).

Effect of FXIIIa on proliferation, migration and apoptosis of fibroblasts

The effect of FXIIIa on proliferation, migration, and survival of skin and lung fibroblasts as compared to PDGF-B (positive control) is shown in Fig. 2. FXIIIa

enhanced migration 5.5-fold ($p = 0.019$), and inhibited apoptosis by 25% ($p = 0.022$) in skin fibroblasts (Fig. 2 A). Similarly, a significant increase in migration and survival of lung fibroblasts was observed (Fig. 2 B). There was no effect of FXIIIa on proliferation of skin fibroblast, whereas there was a 1.75-fold increase in proliferation of lung fibroblasts following FXIIIa treatment (Fig. 2 B). No effect was observed when FXIIIa transglutaminase activity was blocked with iodoacetamide (I-FXIIIa).

Effect of FXIIIa on TSP-1, c-Jun and Egr-1 gene expression.

We have previously showed that the FXIIIa-stimulating effect on endothelial cells was associated with upregulation of cJun and Egr-1, and consequent downregulation of TSP-1 [22]. To test if FXIIIa has similar effect on monocytes and fibroblasts, we studied expression of cJun, Egr-1 and TSP-1 in these cells in response to FXIIIa treatment. FXIIIa upregulated the expression of cJun by 42% and 45% in monocytes and fibroblasts, respectively ($p < 0.05$). The effect of FXIIIa on Egr-1 expression in monocytes and fibroblasts was even more pronounced, leading to 170% and 230% enhancement, respectively ($p < 0.05$). Furthermore, FXIIIa

Fig. 3. Effect of FXIIIa on TSP-1, c-Jun and Egr-1 mRNA levels in monocytes and fibroblasts. mRNA levels were measured by real-time RT-PCR. The results represent ratios between TSP-1 (hatched bars), c-Jun (white bars), Egr-1 (black bars) and GAPDH levels obtained in control, FXIIIa-treated, and I-FXIIIa-treated cells. * $p < 0.05$.

Fig. 4. Binding of FXIIIa to monocytes and fibroblasts. Analysis of FXIIIa binding to the monocyte and fibroblast surface by flow cytometry. Histograms show binding of I-FXIIIa and FXIIIa with or without preincubation with LM609, as indicated.

treatment downregulated the expression of TSP-1 in monocytes and fibroblasts by 42% and 45%, respectively ($p < 0.05$). (Fig 3).

FXIIIa binding to monocytes and fibroblasts

FXIIIa binding to the cells was analyzed by flow cytometry. As shown in Fig. 4, both monocytes and fibroblasts bind FXIIIa. In contrast, no binding was observed with non-activated FXIII (data not shown) or iodoacetamide-inactivated FXIIIa (I-FXIIIa). Since we and others have previously shown that FXIIIa binds to $\alpha_v\beta_3$ of endothelial cells [28, 29], we studied whether its binding to monocytes and fibroblasts is also mediated by the $\alpha_v\beta_3$ integrin. As shown in Fig. 4, the anti $\alpha_v\beta_3$ antibody LM609 significantly reduced FXIIIa binding to monocytes and fibroblasts. Figure 5 demonstrates that FXIIIa binding to the $\alpha_v\beta_3$ integrin leads to crosslinking of the integrin into a high molecular weight complex.

Discussion

In our previous work, we showed that activated coagulation factor XIII - FXIIIa- has a proangiogenic effect on endothelial cells by enhancing their migration, proliferation and survival [10]. In this study, we studied the effect of FXIIIa on other cells that participate in tissue repair and angiogenesis, monocytes and fibroblasts. We have shown that, similar to its effect on endothelial cells, FXIIIa significantly enhanced proliferation and migration, and inhibited apoptosis of monocytes and fibroblasts. Since FXIIIa's effect on fibroblasts varied among sources, we cannot exclude the possibility that some tissue fibroblasts are more sensitive to its action than others. Similarly to FXIIIa-mediated effect on endothelial cells [10], the transglutaminase activity was essential for the stimulating the effect on monocytes and fibroblasts.

In accordance with our results, previous publications

Fig. 5. Coimmunoprecipitation of monocyte and fibroblast membrane proteins with LM609 and anti-γ-glutamyl-ε-lysine isopeptide bond antibodies. The representative picture of 2 separate experiments is shown. A and B, Triton X-100 soluble proteins of either untreated or cells treated with FXIIIa or I-FXIIIa. The samples were subjected to immunoprecipitation (IP) with LM609 antibody and analyzed by WB using anti-γ-glutamyl-ε-lysine isopeptide bond, or anti-β₃ (as IP control) antibodies.

showed that FXIIIa has some stimulatory effect on monocytes and fibroblasts. One report demonstrated FXIII-mediated proliferation of fibroblasts [30], and another group reported that activated FXIII can bind to a putative fibroblast surface receptor that mediates its internalization and degradation [26]. FXIIIa was also shown to be a modulator of the amount and type of collagen synthesized by fibroblasts [31]. Finally, it was previously demonstrated that FXIII-induced crosslinking of fibrin or fibrin-fibronectin gel matrices to promote the migration of fibroblasts [24] or macrophages [23], respectively, and mediates phagocytosis of monocytes/macrophages [27]. However, none of these reports characterized the molecular mechanisms underlying the effects of FXIIIa, nor identified the cellular receptors mediating these effects.

The early phase of tissue repair involves wound contraction, granulation tissue invasion, and fibroblast proliferation and migration [32-34]. Monocytes are known to play an important role in wound healing by producing a variety of growth factors and cytokines (eg, TGF-α, TGF-β, IGF-1). Monocytes are also responsible for wound debridement by phagocytosis. In fact, inhibition of proliferation of monocytes significantly impairs the healing process [1, 20]. Wound healing is also characterized by increased nutritional needs of rapidly vascularized, hyperpermeable granulation tissue supporting the

proliferating and migrating fibroblasts [35]. Thus, in view of the important role of macrophages and fibroblasts at various stages of tissue repair, the ability of FXIII to stimulate these cells further supports its role in the repair process.

In our previous report, FXIIIa-mediated enhancement of endothelial cell migration, proliferation, and survival was associated with downregulation of TSP-1 [10]. TSP-1 downregulation occurred in response to FXIIIa binding to α_vβ₃ integrin, thereby eliciting an intracellular signaling cascade leading to cJun upregulation, which in turn downregulated TSP-1 [22]. In line with these observations in endothelial cells, a significant increase in cJun mRNA and decrease in TSP-1 mRNA were observed following FXIIIa treatment of monocytes and fibroblasts in the present study. We have also shown that FXIIIa binds to α_vβ₃ integrin on monocytes and fibroblasts, and crosslinks it to other membrane proteins. α_vβ₃ is one of the most important integrins involved in angiogenesis and vasculogenesis. This integrin is highly expressed by endothelial cells in tumors and wounds where it functions as a regulator of the balance between the proliferative and apoptotic signals [36]. Thus, it seems very likely that FXIIIa binding to and crosslinking of α_vβ₃ of monocytes and fibroblasts, followed by upregulation of c-Jun and Egr-1, and downregulation of TSP-1, is responsible for modulation of migration, proliferation, and apoptosis of

these cells, similar, to that observed with endothelial cells.

FXIIIa-mediated TSP-1 downregulation in fibroblasts can contribute to the repair process, as overexpression of TSP-1 has been reported to inhibit fibroblast migration [37]. Skin fibroblast proliferation was not, however, affected by TSP-1 [37]. Similarly, in the present work, no proliferative effect of FXIIIa on skin fibroblasts was observed, in contrast to significant enhancement of proliferation of lung fibroblasts, indicating that FXIIIa's effects may vary among fibroblasts of different origins. Additional evidence for the negative effect of TSP-1 on the tissue repair process comes from the observation showing that chronic overexpression of TSP-1 in the skin of transgenic mice inhibits cutaneous tissue repair, granulation tissue formation, and wound angiogenesis [37].

That TSP-1 has inhibitory effects on monocytes can be inferred from publications showing that monocyte differentiation to macrophages is associated with an increase in CD36 and a decrease in TSP-1 expression [38], and that CD36-dependent phagocytosis is inhibited by TSP-1 [39]. Thus, an FXIIIa-mediated decrease in TSP-1 of monocytes can further contribute to their phagocytic activity required for elimination of cellular debris in the wound.

FXIII belongs to the transglutaminase family of proteins apparently participating in the stabilization of the provisional matrix and cell-matrix interactions fundamental for the repair process. Following injury, tissue transglutaminase crosslinks fibrin, collagen, fibronectin, and other ECM proteins that take part in a concerted tissue repair process to ensure cell adhesion, survival, and proliferation [16, 40]. A definitive role for tissue transglutaminase as well as for plasma transglutaminase - FXIII in tissue repair has been recently confirmed in deficient mice, which displayed impaired skin wound healing [14, 41].

Taken together, the literature, our present observations, and our previous observations suggest that the role of FXIIIa in tissue repair can be attributed to: 1 - stabilization of the fibrin clot and/or other proteins of the extracellular matrix to provide a provisional matrix for attachment of different cells involved in the wound healing process; 2 - stimulation of monocytes/macrophages functions, such as migration, proliferation, and phagocytosis of cellular debris; 3 - migration and proliferation of fibroblasts to synthesize essential ECM proteins; 4 - promotion of new vessel formation to provide nutrients for the newly formed tissue.

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