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Independent Anti-Angiogenic Capacities of Coagulation Factors X and Xa

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Knockout models have shown that the coagulation system has a role in vascular development and angiogenesis. Herein, we report for the first time that zymogen FX and its active form (FXa) possess anti-angiogenic properties. Both the recombinant FX and FXa inhibit angiogenesis in vitro using endothelial EA.hy926 and human umbilical cord vascular endothelial cells (HUVEC). This effect is dependent on the Gla domain of FX. We demonstrate that FX and FXa use different mechanisms: the use of Rivaroxaban (RX) a specific inhibitor of FXa attenuated its anti-angiogenic properties but did not modify the anti-angiogenic effect of FX. Furthermore, only the anti-angiogenic activity of FXa is PAR-1 dependent. Using in vivo models, we show that FX and FXa are anti-angiogenic in the zebrafish intersegmental vasculature (ISV) formation and in the chick embryo chorioallantoic membrane (CAM) assays. Our results provide further evidence for the non-hemostatic functions of FX and FXa and demonstrate for the first time a biological role for the zymogen FX.

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Upon vascular injury, plasma coagulation factor VII (FVII) forms a complex with its cofactor, the cell surface receptor tissue factor (TF). After autocatalytic activation, activated FVII (FVIIa) converts coagulation zymogen factor X (FX) into its active form, FXa (via the extrinsic pathway). This cleavage can also occur through the protease action of FVIIIa–FIXa complex (intrinsic pathway). FXa with the assistance of cofactor Va (FVa) then converts prothrombin to thrombin, which

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generates fibrin from circulating fibrinogen (Furie and Furie, 1988). Coagulation factors can also mediate intracellular signaling on a wide variety of cell types in both physiological and pathological conditions (Borensztajn and Spek, 2008). In FX knockout mice, approximately one-third of mice show embryonic lethality, while the neonates die within 5 days due to intra-abdominal bleeding (Dewerchin et al., 2000). The non-hemostatic functions coagulation factors are mediated by their ability to proteolytically cleave and activate protease activated receptors (PARs), a family of G-protein-coupled receptors (Ossovskaya and Bunnett, 2004). The family of PARs includes PAR-1, -2, -3, and -4. Current dogma states that PAR-1, -3, and -4 are cleaved by thrombin, PAR-2 is a target for TF/FVIIa, whereas FXa can activate both PAR-1 and PAR-2 (Camerer et al., 1999; Riewald et al., 2001). Extracellular cleavage of PARs triggers an intracellular activation that can initiate signaling cascades, regulating cellular functions such as migration, survival, and cytoskeleton reorganization (Coughlin, 2000; Macfarlane et al., 2001; O'Brien et al., 2001; Ossovskaya and Bunnett, 2004). Changes in vasculature in PAR-1 knockdown models confirm the role of this receptor in this process (Connolly et al., 1996).

Angiogenesis is the process of formation of new capillaries from pre-existing vessels. It is essential for a variety of physiological processes such as embryonic development, growth, formation of the corpus luteum and endometrium, regeneration, and wound healing. Abnormal angiogenesis is characteristic in many malignancies, increasing tumor growth, and metastasis. The angiogenic process is complex and tightly regulated by a balance between pro- and anti-angiogenic factors. Pro-angiogenic factors include: vascular endothelial growth factor (VEGF) (Veikkola et al., 2000), basic fibroblast growth factor (bFGF) (Dow and deVere White, 2000) and angiopoietin (ANG) (Tsigkos et al., 2003), Many factors involved in coagulation are also known to influence angiogenesis; these include TF, thrombin, fibrin, heparin, tissue plasminogen activator (Browder et al., 2000). In vitro angiogenesis, also known as formation of capillary-like structures in matrigel or tubule formation assay has been widely characterized and used to test potential angiogenic activities or compounds. In particular, the primary culture of Human Umbilical cord Vascular Endothelial Cells (HUVEC) and the permanent human endothelial hybrid cell line EA.hy926 offer widely utilized and quantifiable evaluations of angiogenesis (Edgell et al., 1983; Aranda and Owen, 2009).

FXa occupies a central position within the coagulation cascade as a convergence point between the intrinsic and extrinsic pathways and along with thrombin mediates signal transduction through cleavage and activation of PARs (Rao and Pendurthi, 2005). In contrast, zymogen FX is not known to activate any specific signaling pathway. However, the existence of an unidentified FX receptor has been speculated and it has been suggested that this receptor may trigger activation of PAR-1 (Bae et al., 2010). To date, the accepted role of FX is only as an inactive zymogen, which may be converted to FXa by the TF complex. FXa has been previously reported to regulate both physiological and pathological processes as a mitogenic agent in vascular smooth muscle cells and by stimulating acute inflammatory response (Herbert et al., 1998). FXa also induces cytokine production and adhesion molecules in HUVEC (Senden et al., 1998). Conditioned medium of murine fibroblasts stimulated with FXa enhances murine endothelial tubule formation in vitro suggesting a pro-angiogenic function of FXa. This effect is observed only on fibroblasts and not on endothelial cells (Borensztajn et al., 2009a), this is in line with previous studies suggesting that the presence and activity of the TF complex is associated with angiogenic progression (Versteeg et al., 2003). However, the direct effect of FX and FXa on angiogenesis in human endothelial cells or in in vivo models has not been determined. Herein, we investigated if the zymogen FX or its active form FXa could modulate angiogenesis in vitro and in vivo and explored potential mechanisms of action. Our data demonstrate that FX and FXa inhibit angiogenesis in vitro and in vivo. Furthermore, we postulate that the zymogen FX is a biologically active factor in its own right.

Materials and Methods

Cell culture

Endothelial EA.hy926 and breast cancer ZR-75-1 cell lines were maintained as described (Kato et al., 2005; Aranda and Owen, 2009). HUVEC were isolated from umbilical cords obtained with patient consents and approved by the ethical committee at the Hospital Clínico Universidad Católica de Chile. HUVEC were obtained by collagenase treatment and used in the first passage, as described (Jaffe et al., 1973).

Reagents

FVIIa, FX, and FXa were purchased from American Diagnostica, Inc. (Stamford, CT). FX lacking the Gla domain was purchased from USBiological (Swampscott, MA). VEGF was from Invitrogen (Grand Island, NY). Rivaroxaban (RX; Bayer, Leverkusen, Germany) was reconstituted in DMSO and used at a final concentration of 0.5 μM. Hirudin (H) was from Abcam (Cambridge, UK) (ab73660) reconstituted in sterile water and used at 100 nM. RX and H were applied 30 min prior to treatment with coagulation factors. Matrigel was purchased from Becton-Dickinson (San Diego, CA). Activating and blocking peptides for PAR-1, PAR-2 were obtained from Genscript (Piscataway, NJ). Sequences were: activator of PAR-1 TFLLRN (H-Thr-Phe-Leu-Leu-Arg-NH2), activator of PAR-2 SLIGKV (H-Ser-Leu-Ile-Gly-Lys-Val-NH2), blocking peptide PAR-1 FLLRN (H-Phe-Leu-Leu-Arg-Asn-OH), and blocking peptide for PAR-2 FSLLRY (H-Thr-Phe-Leu-Leu-Arg-NH2). Activating peptides were used at 100 μM. Blocking peptides were added at 200 μM either alone or along with 130 nM of FXa. TF primers have been published previously [23]. Antibody against TF was from Calbiochem (San Diego, CA) and β-actin from Sigma–Aldrich Co. (St. Louis, MO).

Tubule formation assay

The tubule formation assay was performed using EA.hy926 cells as described (Aranda and Owen, 2009). Briefly, EA.hy926 cells (4×10^4/ml in DMEM/F12 0% FBS and VEGF 10 ng/ml) were plated on top of Matrigel-coated plates and incubated at 37°C for 6–8 h. Cultures were photographed (10× magnification) and results were quantified as reported (Aranda and Owen, 2009).

Migration assay

Migration of EA.hy926 cells was evaluated using Transwell inserts (Nunc, Rochester, NY) 8-μm pore size as described (Diaz et al., 2012). Briefly, cell suspension was loaded into upper wells along with FX, FXa (130 nM) or vehicle (water) for 5 h at 37°C. Inserts were then fixed and analyzed by immunocytochemistry against β-actin (Sigma–Aldrich Co.), for quantification a minimum of twenty fields were counted for every condition.

Coagulation Assay

The pro-coagulant activity assay indirectly measures TF levels evaluating the ability of cell line lysates to accumulate colorimetric factor Xa substrate (Chromozym X) in the presence of FVII. This assay was used to measure TF activity in EA.hy926 cells (Kato et al., 2005). Briefly, 50,000 cells of EA.hy926 and ZR-75 (TF positive control) were harvested and incubated with a reagent

mixture containing FVII (1 U/ml), FX (1.2 U/ml), and $CaCl_2$ (25 mM) and Chromozym X (Roche Molecular Biochemicals, Indianapolis, IN, 1 mm). Cells were incubated for 40 min at 37°C and read at 405 nm on a microplate reader (Molecular Devices, Sunnyvale, CA). FXa generation was measured by colorimetric change and subsequently converted to TF procoagulant activity (units per milliliter) for graphical display.

Cell viability and flow cytometry cycle analysis

Cell viability was assessed using the CellTiter 96® AQueous Cell Proliferation MTS assay (Promega, WI) (Kato et al., 2007). Cell cycle distribution was obtained in a FACScan flow cytometer using the Cell Quest software (Becton Dickinson, CA) as described (Kato et al., 2007).

Chicken embryo chorioallantoic membrane (CAM) assay

Angiogenesis was assessed by the chicken embryo chorioallantoic membrane (CAM) assay in 8-day-old embryos (Don Pollo, Santiago, Chile), with institutional bioethics committee approval. Briefly, a 2-cm^2 window was cleared on the egg to allow the placement of a coverslide upon the CAM. Slides were pre-treated with 50 ng of VEGF or ringer buffer, FX (750 ng/egg), FXa (750 ng/egg), PAR1 activating peptide (67 mg/egg). At the time of the stimulus and each day for 4 days, the area below the coverslip was photographed. Quantification was performed by comparing the number of new vessels formed, with that of the previous day. Results were normalized to the corresponding control.

Zebrafish *fli1::GFP* model

Transgenic zebrafish embryos of the *fli1::GFP* strain that express GFP in endothelial cells allowing visualization of the vasculature (Lawson and Weinstein, 2002) were reared at the zebrafish facility at the University of Chile, Santiago, Chile. Protocol was approved by the institutional bioethics committee. Immediately after fertilization (prior to 1 h post-fertilization), one cell stage embryos were injected with FX, FXa, or vehicle (13 or 130 nM). Five *fli1::GFP* transgenic embryos were raised in individual wells in a 12-well multiplate for 33 h, before fixation in paraformaldehyde. Angiogenesis was quantified by the formation of the intersegmental vessels (ISVs) in the trunk/tail region of *fli1::GFP* larvae using a fluorescence dissecting microscope. Correct formation of an ISV, generated by angiogenesis (Quezada et al., 2013), was evaluated depending on whether it spanned the distance between the ventral and dorsal longitudinal vessels. The five ISVs immediately anterior to the end of the yolk extension were scored in this manner and the average number of correctly formed vessels was calculated. A minimum of 50 individuals were evaluated per experimental condition.

Results

To determine the effects of FX and FXa in an in vitro angiogenesis assay we utilized the characterized human endothelial cell line EA.hy926 (Edgell et al., 1983), these cells form capillary-like structures in Matrigel in the presence of VEGF. Figure 1A shows that VEGF applied for 7 h causes the formation of capillary-like structures. Incubation with FX or FXa, but not FVIIa inhibits their formation. Inhibition was not time-dependent as 48 h later cells still did not form such structures. This experiment was repeated replacing VEGF with FGF with identical results (not shown). A recombinant FX lacking the GLA-domain also failed to inhibit capillary-like structure formation. Quantification revealed that FXa at 130 nM produced a significantly higher inhibition compared to FX (Fig. 1B). Concentration response curves confirmed that at lower concentrations, FXa was over an order of magnitude more potent than FX (Fig. 1C). In order to define a mechanism for this inhibitory effect of FX and FXa first we investigated if FXa was affecting cell death or altering cell cycle. Figure 2A shows that neither FX nor FXa alter cell cycle distribution or cell death. To understand better how FX and FXa were mediating their anti-angiogenic effects, we performed time-lapse photography of the endothelial cells in matrigel. These cells in culture show some (limited) migration along with tube formation, however we observed that the effect of FX seems to be more related to the inhibition of cytoskeletal expansion/remodeling. The cell nucleus remained relatively fixed while the cytoplasm expanded and joined with neighboring cells to

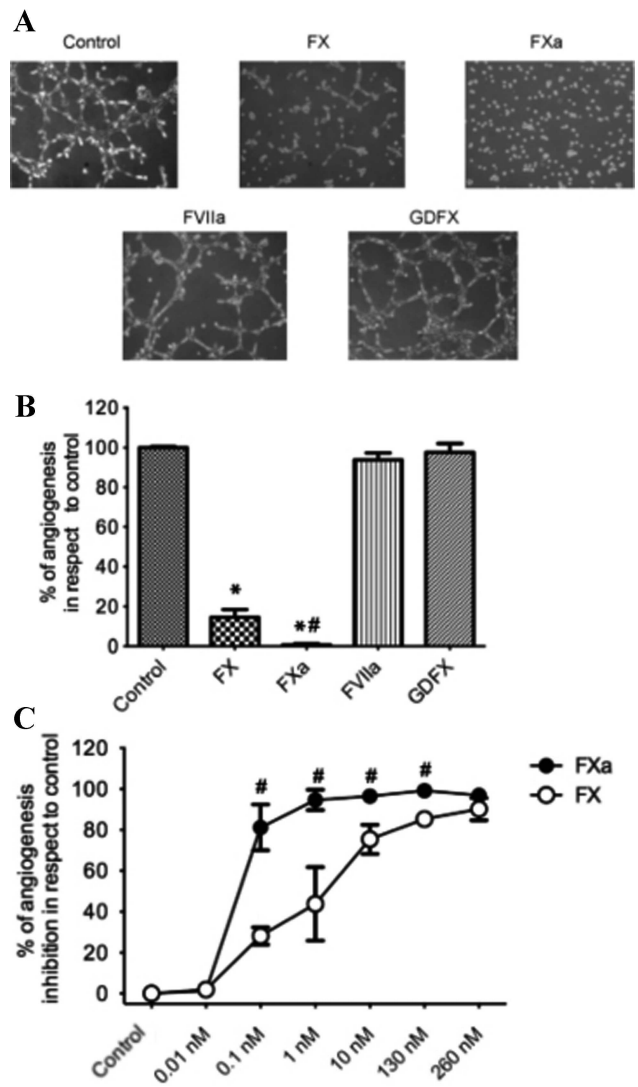

Fig. 1. FX and FXa inhibit endothelial tube formation in a concentration dependent manner. A: EA.hy926 cells were seeded onto Matrigel supplemented with VEGF (10 ng/ml) and incubated in the presence of control (water), FX, FXa, FVIIa, or the FX lacking the Gla-domain (GDFX) for 7 h. B: Quantified angiogenic index (% of angiogenesis) relative to control. n = 3, *$P < 0.05$ vs. control, #$P < 0.05$ vs. FX, ANOVA followed by Bonferroni post-test. C: Concentration response curves, EA.hy926 cells were treated with FX or FXa in the presence of VEGF 10 ng/ml. The graph shows the percentage of angiogenesis inhibition in comparison to control. n = 3, *$P < 0.05$ vs. control, #$P < 0.05$ vs. FX at the same concentration, statistical test ANOVA followed by Bonferroni post-test.

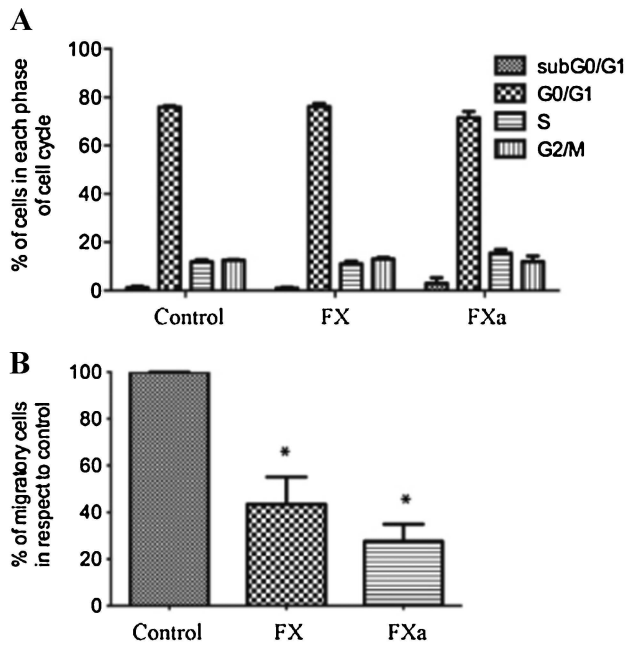

Fig. 2. FX and FXa inhibit endothelial migration, but do not alter cell cycle. A: Cell cycle analysis was performed by flow cytometry in EA.hy926 treated for 24 h with control (water), FX, or FXa (both at 130 nM). Cell cycle was divided into four categories: cell death (sub G0/G1 peak), non-proliferative (G0/G1), proliferative (S), and mitotic (G2/M). n = 3, *$P < 0.05$ vs. control by ANOVA followed by Bonferroni post-test. B: Cell migration in control (water), FX, or FXa (both at 130 nM) VEGF (10 ng/ml) was used as a chemoattractant. n = 3, *$P < 0.05$ vs. control, by ANOVA followed by Bonferroni post-test.

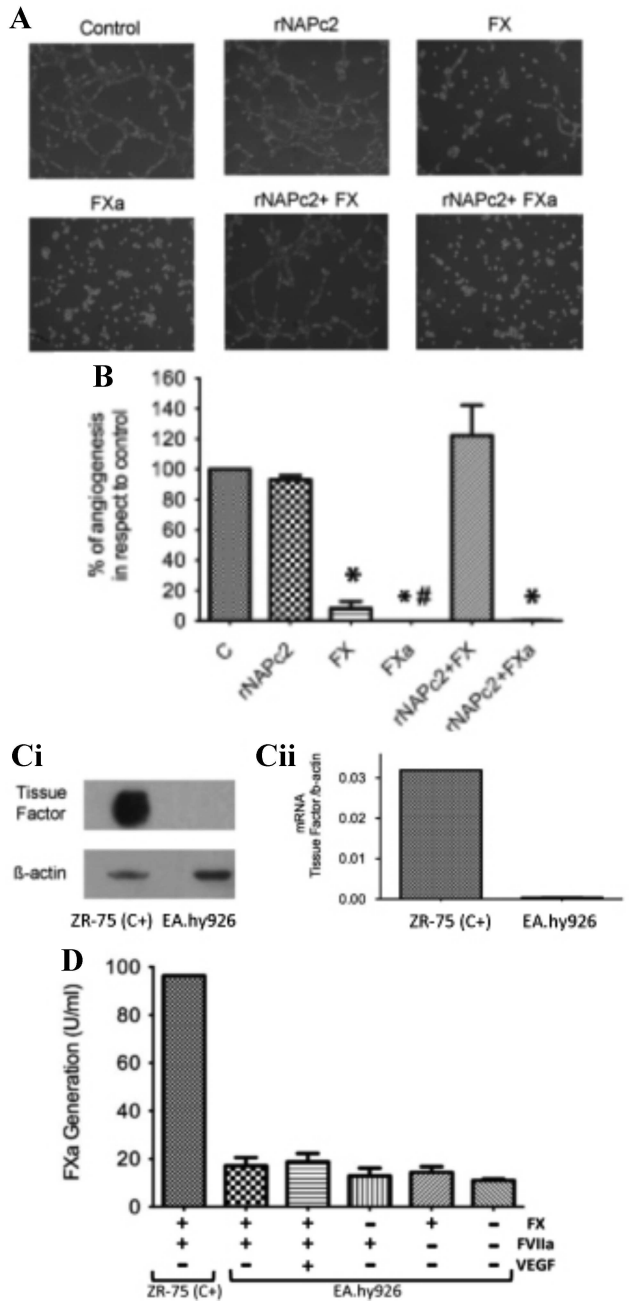

Fig. 3. rNAPc2 prevents FX but not FXa anti-angiogenic activity. A: EA.hy926 cells in Matrigel supplemented with VEGF (10 ng/ml) were treated with control (water), rNAPc2, FX, FXa (both at 130 nM), or the combinations rNAPc2 + FX and rNAPc2 + FXa. B: Angiogenic index (% of angiogenesis) relative to control. n = 3, *$P < 0.05$ vs. control, by ANOVA followed by Bonferroni post-test. C: TF expression: (Ci) Western blot showing absence of TF in EA. hy926. Progesterone-treated ZR-75 cells were used as a positive control (labeled C+) β-actin was used as a loading control. (Cii) TF mRNA levels by Real-time PCR mean values ± SEM. D: Pro-coagulant activity in ZR-75 and EA.hy926 cells. ZR-75 were used as a positive control (C+, first bar left). n = 3 per condition.

form tubular structures. Angiogenesis in vivo has been reported to be dependent on cell migration therefore we utilized the well-characterized transwell assay to investigate if FX could inhibit endothelial cell migration. Figure 2B shows that both FX or FXa significantly reduce migration of EA.hy926.

Our results suggested that the effect of FX (zymogen) was due to its conversion FXa (active form). The inhibitor, rNAPc2, has been shown to bind FX in a TF-independent manner (Lee and Vlasuk, 2003). In Figure 3, the use of rNAPc2 blocked the anti-angiogenic activity of FX but not FXa (Fig. 3A,B). The postulated mechanism of rNAPc2 is by sequestration of FX within the TF complex, however endothelial cells do not express TF. Indeed, Figure 3C shows that EA.hy926 cells do not express TF by Western blot or real time PCR. Furthermore, in our pro-coagulant activity assay, no increase in pro-coagulant activity is observed when compared to control (Fig. 3D, far right), suggesting that the EA.hy926 cell line is not producing factors that cleave FX to FXa. In order to rule out the possibility that VEGF was increasing TF or creating an environment favorable for FX cleavage, we repeated this assay using EA.hy926 cells pretreated with VEGF for 12 h, obtaining identical results (Fig. 3D, third bar from the left).

Our results suggest that FX signaling is independent of TF or its conversion to FXa. To test this, we turned to the anti-coagulant drug RX which specifically binds and blocks the activity of FXa (Gulseth et al., 2008; Rupprecht and Blank, 2010). Figure 4 shows that RX reverts only FXa but not FX anti-angiogenic activity, confirming that the anti-angiogenic activity of FX is independent of its conversion to the active FXa. Hirudin a specific thrombin inhibitor did not inhibit FX or FXa anti-angiogenic activity. This demonstrates that FXa function is not through proteolytic conversion of prothrombin to thrombin. Prothrombin was not detected in the EAhy926 cell line (not shown).

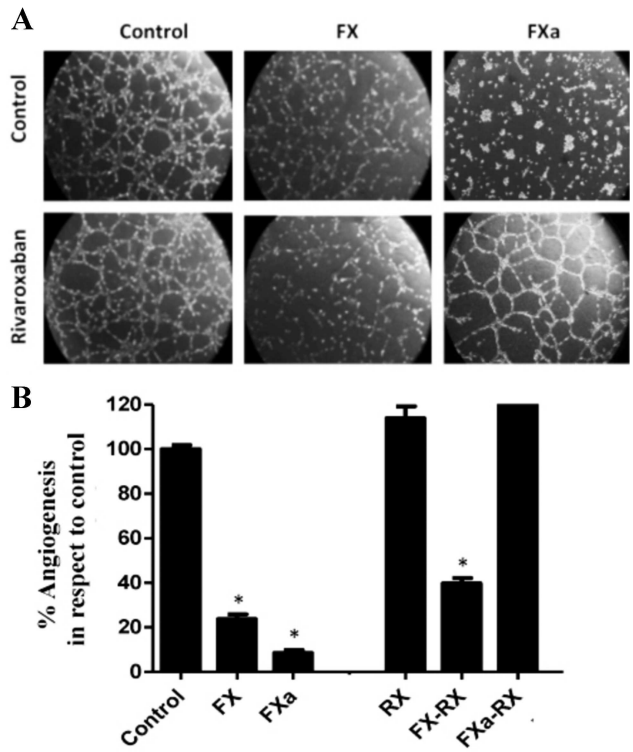

Fig. 4. The anti-angiogenic effect of FX is not dependent on the conversion to FXa. A: Tubule formation assay. EA.hy926 cells in Matrigel supplemented with VEGF (10 ng/ml) were treated with control (water), FX, or FXa in the presence of specific FXa inhibitor Rivaroxaban (RX, 0.5 μm) or the thrombin inhibitor Hirudin (H, 100 nM) or control (vehicle: DMSO). B: Quantification of the angiogenic index (% of angiogenesis) relative to control. n = 3, *$P < 0.05$ vs. control, ANOVA followed by Bonferroni post-test.

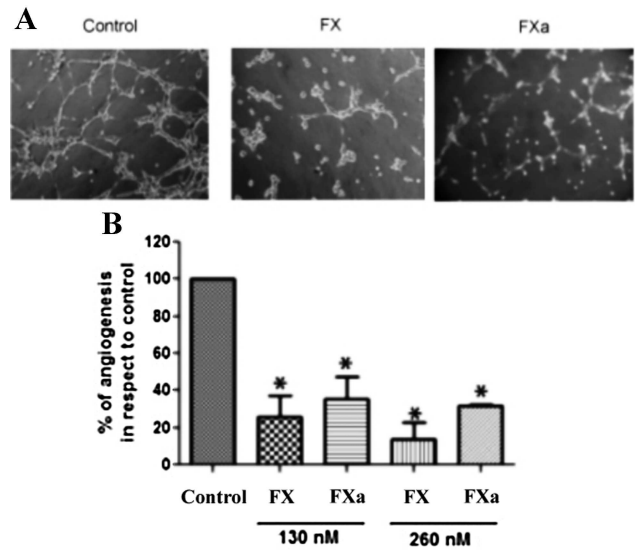

Fig. 5. The anti-angiogenic effect of FXa, but not FX, is dependent on PAR-1. A: Tubule formation assay. EA.hy926 cells supplemented with VEGF (10 ng/ml) were seeded in Matrigel and treated with control (water), FX, FXa, activating peptides for PAR-1 and PAR-2 (aPAR1, aPAR2), inhibitory peptide for PAR-1 (iPAR1) or the combinations iPAR1 + FX and iPAR1 + FXa. Concentrations stated in methods section. B: Quantification of angiogenic index (% of angiogenesis) relative to control. n = 3, *$P < 0.05$ vs. control, ANOVA followed by Bonferroni post-test.

As mentioned previously extracellular coagulation factors can generate intracellular signaling by PARs, therefore, we examined the role of PAR-1 and -2 using activating and inhibitory PAR binding peptides (Hollenberg et al., 1992; Vassallo et al., 1992; Bohm et al., 1996; Al-Ani et al., 2002). Figure 5 shows that an activating peptide of PAR-1 blocks the formation of capillary-like structures in EA.hy926. In contrast, neither an activator of PAR-2, nor an inhibitor peptide, altered tubule formation. The inhibitory PAR-1 peptide blocked the anti-angiogenic effect of FXa, but surprisingly not that of FX (Fig. 5). To confirm our observations were not restricted to EA.hy926 cells, we cultured HUVEC. Figure 6 shows that both FX and FXa inhibit the formation of capillary like structures in HUVEC.

Next, we evaluated angiogenesis using two widely recognized in vivo models. First, we used the CAM assay. Here, FX and FXa were evaporated onto glass slides and placed directly upon the open chicken egg at day 8 of development. As shown in Figure 7A, in the subsequent four days the presence of FXa significantly reduced angiogenesis measured as the normalized number of blood vessels. The addition of VEGF increased the number of new vessels and FXa also inhibited this process. Interestingly, analysis of the vessel formation suggested that both FXa and the PAR-1 activating peptide were preventing sprouting and formation of small blood vessels. Second, we evaluated FXa in a developmental model of angiogenesis, using larvae of the transgenic zebrafish line *fli1::GFP*. The vascular system development is well conserved between mammals and zebrafish, as is the homology of FX. The zebrafish larva is transparent allowing the quantification of fluorescent vasculature, a measure of angiogenesis (Serbedzija et al., 1999). Zebrafish embryos were injected with FX or FXa at either 13 or 130 nM and observed at 33 h, when ISVs have just formed. Angiogenesis was assessed by quantification of the presence or lack of ISVs (Robinson et al., 2009). Figure 7B shows that both FX and FXa possess anti-angiogenic activity and FXa effect is significantly stronger when compared to FX. In both cases the anti-angiogenic effect is concentration dependent.

Discussion

FXa plays a central role in coagulation as a convergence point for the intrinsic and extrinsic pathways. Recent studies have also demonstrated that FXa have significant non-hemostatic functions, many mediated by PARs. FX deficiency (albeit frequently combined with FVII deficiency) is associated with mental retardation, microcephaly, cleft palate carotid body tumors, atrial septal defect, and ventricular septal defect among many other pathologies (Girolami et al., 2008). FX knockout mice have high embryonic lethality and most animals die at embryonic day E11.5-12.5 with evidence of massive bleeding. However, embryos of these animals exhibit a normal vasculature with no histological defects in the yolk sac (Dewerchin et al., 2000; Rosen, 2002). Our results may be consistent with this observation. The early $FX^{-/-}$ lethality may be masking a physiological role of FX in the inhibition of angiogenesis. Alternatively, this anti-angiogenic function of FX may not affect embryogenesis. Our in vivo data support this idea; often we observe that larger vessels (maybe vasculogenesis) are mostly intact. In contrast, smaller spouting vessels

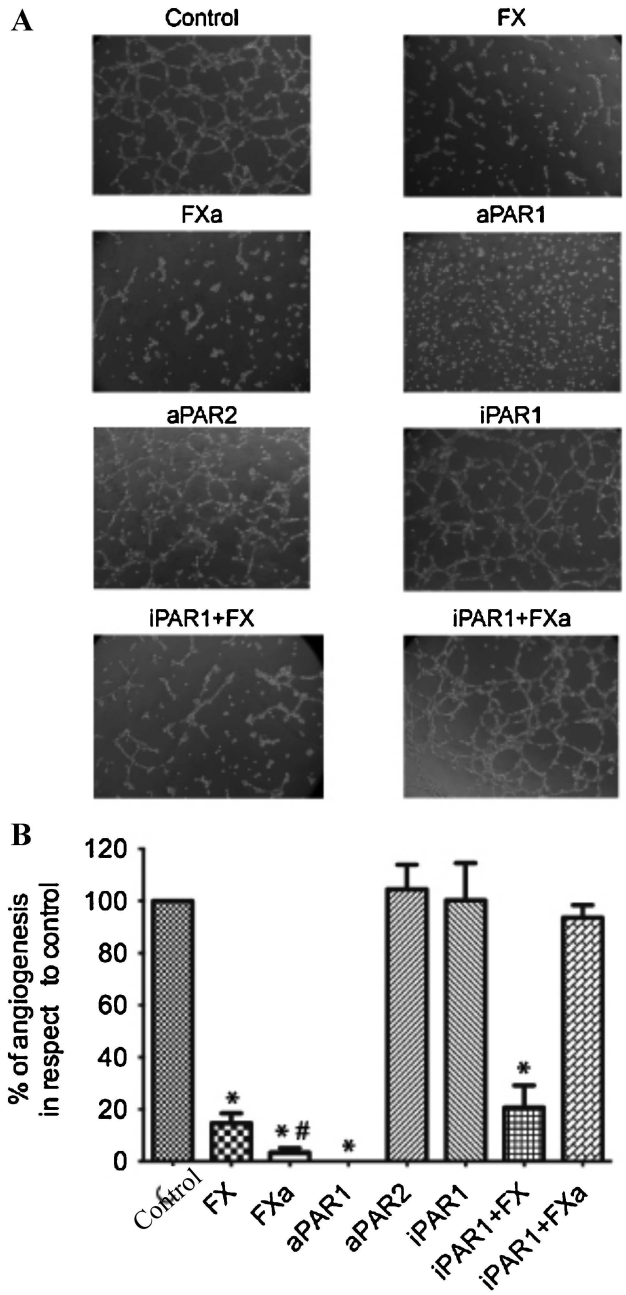

Fig. 6. FX and FXa inhibit angiogenesis in HUVEC. A: Tubule formation assay. HUVEC supplemented with VEGF (10 ng/ml) were seeded in Matrigel and treated with control (water), FX, or FXa (130 nM) for 7 h. B: Quantification of angiogenic index (% of angiogenesis) relative to control. n = 3 for both FX and FXa concentrations tested, *$P < 0.05$ vs. control, ANOVA followed by Bonferroni post-test.

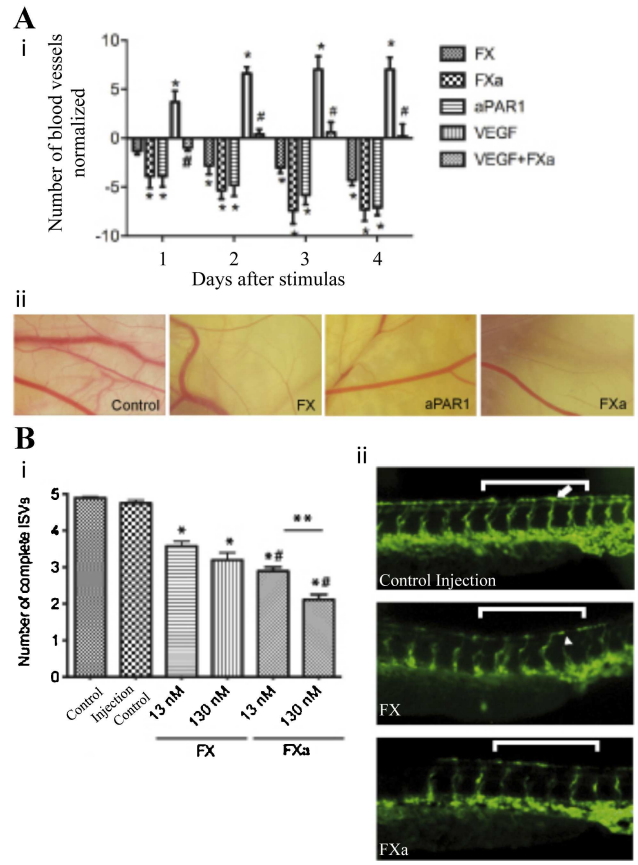

Fig. 7. FX and FXa display anti-angiogenic activity in vivo. A.i: Chick embryo chorioallantoic membrane (CAM). Quantification of vessel formation (number of blood vessels normalized) after four days relative to vehicle (ringer buffer). Embryos were treated with FX, FXa, VEGF, activating PAR-1 peptide (aPAR1), or the combination VEGF + FX. A.ii: Representative images showing a reduction in the number of newly formed blood vessels with FX, FXa, or aPAR1 treatment. n = 9 per condition *$P < 0.05$ vs. control, #$P < 0.05$ vs. VEGF Student's *t*-test. B.i: Intersegmental vessels (ISVs) assay in zebrafish at 33 h post-injections. Zebrafish embryos were left intact (control) or injected with vehicle (injection control), FX, or FXa (at 13 and 130 nM). B.ii: Representative images of ISV formation in control injection, FX, or FXa treated embryos. n = 30 embryos/condition. *$P < 0.05$ vs. control, #$P < 0.05$ vs. FX at the same concentration, **$P < 0.05$ vs. at the lower concentration, ANOVA statistical test.

(maybe angiogenesis) are absent. There is also the possibility of redundancy of this anti-angiogenic function by other members of the coagulation pathway this would explain why this phenotype is not manifested before E 11.5. However, we acknowledge, that despite the use of physiological concentrations, an anti-angiogenic function for FX/FXa may not be a physiological or a pathological process, but merely a result of forcing the system with recombinant protein. A possible option is that the presence of the Gla-domain of FX is sequestrating extracellular calcium and thus deregulating plasma membrane, for example intregin, mediated angiogenesis. It has been demonstrated that mutations in the Gla-domain result in a FX protein with decreased ability to bind calcium (Girolami et al., 2008).

However, this is most likely not the case as we observed that GLA-domain containing FVIIa did not manifest anti-angiogenic activity. The FVIIa Gla-domain has been demonstrated to bind calcium, resulting in conformational change. Moreover, the failure of FVIIa to inhibit angiogenesis rules out the possibility that this observation is a result of non-specific protease activity at the cell surface. Our results suggest that this is a physiological process, as the anti-angiogenic pathway is triggered by PAR-1 activation (thus an intracellular downstream anti-angiogenic pathway exists).

Thrombin, although absent in our model system (our experiments are conducted in serum free conditions and PCR failed to detect prothrombin), has been reported to inhibit tubule formation in in vitro angiogenesis assays. We confirmed this observation in our assay using recombinant thrombin and noted that Hirudin (a specific thrombin inhibitor) blocked thrombin action but not FX or FXa (Fig. 4). Interestingly, suggesting that other coagulation factors can also have an anti-angiogenic function, thrombin inhibits the growth of vascular tubules and branching in vitro but stimulates vascular smooth muscle cells proliferation and induces VEGF (Wang et al., 2010). We hypothesize that at the site of vascular injury, this mechanism operates as an advantage for organisms inhibiting local angiogenesis until the endothelium is fully repaired, promoting the secretion of pro-angiogenic factors in the surrounding tissues. Of course, the anti-angiogenic function of FX as a physiologically relevant mechanism remains to be demonstrated, however our data opens the possibility for important biomedical applications in diseases that affect the vascular system especially in the case of zymogen FX which would have virtually no side effects due to the lack of protease activity.

FXa has a variety of functions in cellular signaling (Rosen, 2002; Schuepbach and Riewald, 2010). Both FX and FXa can signal through PARs, specifically PAR-1 and PAR-2 (Feistritzer et al., 2005). Herein, we demonstrate that the anti-angiogenic effect of FXa is mediated through PAR-1 and not PAR-2. PAR-1 has been reported to be both pro- and anti-angiogenic. PAR-1 activation accompanied by low levels of thrombin enhances angiogenesis in HUVEC (Haralabopoulos et al., 1997), while high concentrations of thrombin inhibit angiogenesis (Chan et al., 2003). Interestingly, in our studies FXa displayed anti-angiogenic activity through the four orders of magnitude of concentrations tested in both EA.hy926 and HUVEC. Similarly, previous studies have shown that thrombin can increase endothelial cell permeability following PAR-1 activation while in the same model PAR-1 activating peptides decrease permeability (McLaughlin et al., 2005). This effect might be explained through the simultaneous activation of multiple PARs by thrombin.

Interestingly, a study shows that fibroblasts secrete VEGF in response to FXa stimulating angiogenesis in a murine model (Kato et al., 2007). The same study shows no anti-angiogenic effect of FXa in a rat endothelial cell line (2H11). We confirmed this observation in a similar cell line (3B11). We speculate that this discrepancy could be explained by a loss of this function following immortalization of these cell lines. A change of function in the rat, although not impossible, is unlikely as we have observed the same anti-angiogenic effect of FX in fish (zebrafish), chicken (CAM), and human (EA.hy926 and HUVEC).

Results from time-lapse photography suggest that at least in vitro tube formation, the mechanism of FX and FXa action is through inhibition of cytoskeletal expansion. Cytoskeletal remodeling is the initial step in the migratory process and thus this inhibition would account for the observed reduction in in vitro migration assays and the lack of vessel formation in the in vivo models. The relationship between FXa and migration is controversial. In breast cancer MDA-MB-231 and MCF-7 cells, FXa has been shown to decrease migration via PAR-1 (Borensztajn et al., 2009b), In contrast, breast cancer ZR-75-1 and T47D cells increase their migration and invasive potential following PAR-1 activation (Diaz et al., 2012).

As previously mentioned, our results obtained with the zymogen FX constitute a novel observation. Initially we assumed this observation was explained by conversion of FX to FXa, however we conclusively demonstrated using in vitro and in vivo models that this is not the case: firstly, we showed that TF is not expressed by EA.hy926 cells, this was confirmed by a coagulation assay. Our migration assays in the absence of Matrigel and serum as possible contaminants of other factors showed that FX was capable of inhibiting this process. We further demonstrated this is a specific effect of FX by using RX, a specific inhibitor of FXa. Our most conclusive result is that FX, unlike FXa, does not require either PAR-1 activation to inhibit angiogenesis in our models and thus both FX and FXa possess anti-angiogenic properties but mediated by different mechanisms. A study by Bae et al. (2010) suggests FX as a signaling factor. The latter authors demonstrate that FX interacts with endothelial cells leading to PAR-1 activation through the dissociation of the membrane receptor EPCR from caveolin-1. They also speculate on the existence of an as yet unknown FX receptor. Currently we have several clues as to the nature of FX zymogen signaling pathway. Our results obtained with the TF complex inhibitor rNAPc2 show inhibition of FX activity, although not upon the already formed FXa. Other studies suggest that rNAPc2 can bind to FX in a TF-independent manner (Bergum et al., 2001) and thus in our model may be blocking an interface for protein-protein interaction or a folding necessity for FX. Our results also show that deletion the plasma membrane binding domain of FX (Gla-domain mutant) abolishes its anti-angiogenic activity suggesting cell surface binding may be required for this effect. In the above mentioned article (Bae et al., 2010), the interaction between ECPR and PAR-1 also required the Gla domain of FX and thus the mechanism proposed is unlikely to be the mechanism reported herein. Studies with FX constructs deleted in the active site and EGF-like domains will clarify this and are currently ongoing.

Intriguingly, our in vivo data show that both FX and FXa presented anti-angiogenic properties. In the CAM model, we observed that it was the small capillary structures or the initial branches that were reduced in the presence of FXa. This result confirms our in vitro results demonstrating an absence of sprouting from the endothelial cell. In the zebrafish, we noted a reduction in the intersegmental vasculature along the length of the embryo at 33 h after injection. A total reduction in ISVs was not observed, further suggesting that FXa is preventing a specific aspect of the angiogenic process that may be compensated for by other processes before the pharingula stage. As FX concentrations in the zebrafish are unknown, although FX homology is high, the amounts of FX used in this experiment corresponded to the human physiological (130 nM) concentrations and a concentration one order of magnitude less (13 nM). We demonstrate that both FX and FXa give a highly significant reduction in angiogenesis at a concentration of 13 nM. In these experiments we are examining angiogenesis at 33 h post-fertilization. In the zebrafish embryo the blood circulation does not form until hour 26 (Isogai et al., 2001), thus this system has to be regarded as a model and not a reflection of a role for FX in zebrafish development.

It is important to note that our study concentrates only on the effect of FX on isolated endothelial cells (in vitro) and immature vessels (in vivo CAM and zebrafish larva models). No mural cells surround the endothelial cells before day 3 in the zebrafish embryo (Santoro et al., 2009) and pericytes are not present in the developing chick egg until day 12 (Nico et al., 2004; Kurz et al., 2008). Thus, the role of this coagulation factor on mature vessels remains to be elucidated. The presence of pericytes or associated cellular structures surrounding the endothelial cells (absent in our models) may modify the response to FXa. As mentioned previously FXa can increase VEGF secretion by stromal fibroblasts (Borensztajn et al., 2009a). Thus, FXa may play an anti-angiogenic function on the endothelium, yet favor the secretion of factors that promote vascular stability and size from fibroblasts/pericytes /smooth muscle cells of the vessel; the balance between FX and

other pro- and anti-angiogenic factors will determine the eventual cell fate. Experiments to fully address this question are currently ongoing.

Studies with elevated FX levels in circulation are scarce. Few studies show that when the coagulation pathway is elevated FX does not modify its levels. As speculated above, FXa accumulates primarily at a wound site and thus may represent an advantage for the organism if FXa prevents angiogenesis until the endothelium is fully repaired.

In summary, we report for the first time that recombinant FX and FXa display anti-angiogenic properties. We demonstrate that FXa and FX use different mechanisms to mediate this activity, with only FXa being dependent on PAR-1 activation. Using in vivo models we show that FX and FXa are anti-angiogenic. Our results shed further light on the non-hemostatic function of FXa and show for the first time a biological role for the zymogen FX.

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