Action and Function of coagulation FXa on cellular signaling

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Chapter 6

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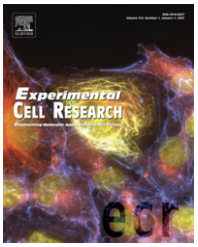

Research Article

Coagulation factor Xa drives tumor cells into apoptosis through BH3-only protein Bim up-regulation

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ABSTRACT

Coagulation Factor (F)Xa is a serine protease that plays a crucial role during blood coagulation by converting prothrombin into active thrombin. Recently, however, it emerged that besides this role in coagulation, FXa induces intracellular signaling leading to different cellular effects. Here, we show that coagulation factor (F)Xa drives tumor cells of epithelial origin, but not endothelial cells or monocytes, into apoptosis, whereas it even enhances fibroblast survival. FXa signals through the protease activated receptor (PAR)-1 to activate extracellular-signal regulated kinase (ERK) 1/2 and p38. This activation is associated with phosphorylation of the transcription factor CREB, and in tumor cells with up-regulation of the BH3-only pro-apoptotic protein Bim, leading to caspase-3 cleavage, the main hallmark of apoptosis. Transfection of tumor cells with dominant negative forms of CREB or siRNA for either PAR-1, Bim, ERK1 and/or p38 inhibited the pro-apoptotic effect of FXa. In fibroblasts, FXa-induced PAR-1 activation leads to down-regulation of Bim and pre-treatment with PAR-1 or Bim siRNA abolishes proliferation. We thus provide evidence that beyond its role in blood coagulation, FXa plays a key role in cellular processes in which Bim is the central player in determining cell survival.

Introduction

Coagulation factor (F)X is a vitamin-K-dependant plasma protein that plays a key role in blood coagulation. Upon vascular injury, FVII forms a 1:1 stoichiometric complex with its cofactor, the cell surface receptor tissue factor (TF). After autocatalytic activation, activated FVII (FVIIa) converts FX into its active form, FXa. In the coagulation cascade, FXa occupies a central position as a convergence point between the intrinsic and extrinsic coagulation pathways, and converts prothrombin into thrombin [1]. Beside its well known role as a coagulation factor, FXa, like the other serine-proteases FVIIa and thrombin, induces intracellular signal transduction [2].

A family of G-protein-coupled receptors, the proteases-activated receptors (PARs), that are activated by proteolytic cleavage rather than ligand binding, has been identified in recent years [3]. PARs are widely distributed in a variety of cell types, including circulating blood cells, endothelial and vascular smooth muscle cells, as well as in cells constituting the nervous system, the gastrointestinal tract and the respiratory system. Among the 4 currently known PARs, PAR-1,-3 and-4 are cleaved by thrombin. PAR-2 is a target for cleavage

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by the TF/FVIIa complex, whereas FXa can activate both PAR-1 and PAR-2 [4,5]. Proteolytic cleavage of either receptor exposes a new N-terminus that binds to the body of the receptor to induce transmembrane signaling to G proteins. The activated G proteins in turn impact on a substantial network of signaling pathways, which trigger a cascade of downstream events, thereby regulating many cellular functions like cytoskeleton reorganization and cell survival [3]. For instance, in neuronal and epithelial cells, as well as tumor cells, activation of PAR-1 induces apoptosis [6]. G-protein-dependent signaling pathways regulated through activation of PARs turn on the migratory and invasive program in tumor cells [3,7], and PAR-1 over-expression is correlated with the invasive properties of various human cancers [8]. Overall, these data provide compelling evidence for a link between PAR-induced signaling and cancer progression.

As FXa induces signal transduction through PARs, it is commonly accepted that FXa might modulate tumor cell growth, invasion and metastasis [9]. Indeed, FXa elicits numerous cellular events, especially through activation of PAR-1. FXa is mitogenic and induces proliferation of fibroblasts, smooth muscle cells and mesenchymal cells [10–12]. It provokes expression of adhesion molecules on monocytes [13], it induces NK-κB and Cyr61 gene expression in HeLa cells and up-regulates the early growth response gene-1 in HaCaT cells [4,5]. The contribution of these events to pathophysiological processes has only just begun to unfold. For instance, mitogenic effects exerted by FXa in aortic smooth muscle cells may contribute to intimal hyperplasia in arterial restenosis [14].

A recent study using Baby Hamster Kidney (BHK) cells transfected with TF showed that FXa induces prolonged phosphorylation of p42/p44 MAPK and Phosphatidylinositide-3-(OH) kinase pathways thereby enhancing cell survival suggesting that FXa facilitates metastasis [15]. However, as BHK cells are fibroblasts which are not tumorigenic by nature, extrapolation of these data to human tumor biology in order to understand the mechanism of metastasis remains speculative. Confirmation of the anti-apoptotic effect of FXa in tumor cells and/or stromal cells surrounding tumors seems essential for establishing the actual role of FXa in tumor biology. Therefore, we determined the effect of FXa on cell growth and survival using different human cancer cell lines.

Material and methods

Cell culture and treatment

MDA-MB-231, MFC-7, SW-480, DLD-1, HT-29, A549, B16F10, HL-60 and HEK293 cells were purchased from ATCC and maintained in DMEM supplemented with 10% FCS. The normal small intestine epithelial cell line FHs Int 74 was purchased from ATCC and maintained in Hybri-Care medium supplemented with 10% FCS and 30 ng/mL EGF according to the manufacturer's recommendations. The origin of the cancer cell lines used in this study, as well as the genetic lesions, is indicated in Table 1. Human monocytes and leukocytes were isolated from whole blood using Ficoll reagent according to the manufacturer's recommendations. Human Umbilical Vein Endothelial Cells (HUVECs) were purchased from ATCC and maintained in EBM-2 medium supplemented with 10% FCS and containing VEGF, human fibroblast growth factor-B, human epidermal growth factor, R3-insulin like growth factor-1, heparin, hydrocortisone and ascorbate (EGM-2 SingleQuots, BioWhittaker Takara Shuzo).

Cell survival assay

Cells (10^4) plated onto 96-well plates in DMEM supplemented with 1% FCS were pretreated as indicated in the figure legends. Cell survival was determined using MTT assays as described previously [15].

Anoikis

To induce anoikis, cells were plated in DMEM supplemented with 1% FCS on culture plates coated with polyHEMA (10 mg/mL). At the indicated time points, cells were harvested and viability was determined by MTT assay.

Immunoblotting

Cells were pre-treated for 60 min with either 20 μM PD98059, 20 μM SB203580 (both Promega, Madison), Hirudin (25 U/mL; Calbiochem, Germany) or 200 nM TAP (kindly provided by Dr. Vlasuk, Corvas International, San Diego, CA). If indicated, cells

Table 1 – Cell lines used in this study and their origin

Designation	Organ	Disease	Genetic lesions according to ATCC[a]	References
MCF-7	Mammary gland, breast	Adenocarcinoma	p53–	[51]
MDA-MB-231	Mammary gland, breast	Adenocarcinoma	p53+	[52]
B16F10	Skin	Melanoma	p53+	[53]
HEK-293	Kidney	N/A	p53–	[54]
SW-480	Colon	Colo-rectal adenocarcinoma	myc+; myb+; ras+; fos+; sis+; p53+; abl–; ros–; src–	[55]
DLD-1	Colon	Colo-rectal adenocarcinoma	myc+; myb+; ras+; fos+; sis+; p53+; abl–; ros–; src–	[56]
HT-29	Colon	Colo-rectal adenocarcinoma	myc+; ras+; myb+; fos+; sis+; p53+; abl–; ros–; src–	[55]
A-549	Lung	Carcinoma	p53–	[57]
HL-60	Peripheral blood	Acute promyelocytic leukemia	myc+	[58]
FHs 74 Int	Small intestine	N/A	N/A	[59]

[a] American Type Culture Collection.

were pre-treated with 2 μg PAR-1 H-111 blocking antibody (Santa Cruz Biotechnologies, CA) for 1 h. Next (unless stated otherwise), cells were stimulated with 0.75 U/mL FXa (Kordia, the Netherlands) or PBS. To determine the effect of receptor desensitization on ERK1/2 phosphorylation, cells were washed twice with PBS, pretreated with FXa (0.75 U/mL) or Thrombin (1U/mL; Kordia, the Netherlands) in serum-free conditions for 90 min. Phospho-ERK 1/2, phospho-p38, phospho-JNK, phospho-CREB, Bim and cleaved-caspase 3 antibodies were from Cell Signaling Technology (Beverly, MA); whereas β-actin and PAR-1 antibodies were from Santa Cruz Biotechnologies. Cell extracts were prepared and subjected to Western blot analysis as described previously [15].

DAPI staining

Cells at 20–30% of confluence, grown on coverslips and treated with 0.75U FXa or PBS for 24 h, were washed with PBS, and fixed with 3.7% formaldehyde for 20 min at room temperature. After fixation, cells were permeabilized in PBS/0.1% Triton X-100 (PBS-T) supplemented with 10% FCS for 1 h. Cell nuclei were stained with DAPI (200 ng/mL; Roche) in PBS-T for 30 min. Next, cells were washed and mounted in Mowiol/DABCO aqueous mounting medium (Vector Laboratories, Burlingame, CA). Stained nuclei were visualized and photographed using an epifluorescence microscope Leica DMRA (Wetzlar, Germany). Images were captured on a cooled charge coupled camera (KX Series, Apogee, Auburn, CA) operated by imagePro Plus software (Media Cybernetics, Silver Spring, MD).

CREB vectors and siRNA

pCMV-CREB, pCMV-KCREB and pCMV-S133A expression constructs were purchased from Clontech (BD Biosciences, USA). Pre-designed human control (scrambled sequence not leading to the specific degradation of any of our targets) and p38-siRNA (signal silence pool siRNA kit) was purchased from Cell Signaling. Pre-designed human ERK1, PAR-1 (reference ID #4377 and #158457) and PAR-2-siRNAs (reference #1960) were obtained from Ambion Inc (Austin, TX). Pre-designed Bim siRNA was purchased from Santa Cruz Biotechnologies.

Transfections

DNA transfections were performed using Effectene (Qiagen, Hilden, Germany) according to the manufacturers' protocol. For transfections in 6-wells plates, 2 μg DNA was used at a 1:6 ratio of DNA/Effectene. Cells were incubated with transfection complexes for 16 h, after which fresh medium was added for 6 h preceding further experimentation. Transfections of pre-designed siRNAs were performed using RNAiFect (Qiagen, Hilden, Germany). For transfections in 6-wells plates, 0.6 μg siRNA was used at a 1:3 ratio of RNA/RNAiFect.

RNA isolation and MLPA procedure

After 0–8 h of the indicated treatment, total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendation. Next, 100 ng total RNA was used for the MLPA reaction as described previously [16,17].

Statistical analysis

The experiments were repeated independently as indicated in the corresponding section. Statistical analysis was performed with Graph Prism using two-tailed Student's *t* tests.

Results

MCF-7 and MDA-MB-231 human breast carcinoma cell lines were incubated with different concentrations of FXa for 24 h. Surprisingly, FXa inhibited cell survival (Fig. 1A), with optimal inhibition observed at 0.75 U/mL (inhibition of survival by 38.3±4% in MCF-7 ($P<0.0001$) and 45.6±3.8% in MDA-MB-231 cells ($P=0.0002$). FXa-mediated inhibition of survival was specific since Tick Anticoagulant Peptide (TAP; FXa inhibitor) but not Hirudin (thrombin inhibitor) abolished the FXa effect (Fig. 1B). To establish whether FXa specifically inhibits survival of breast cancer cells or whether FXa exerts its effect also in other tumor cell lines, we selected colon tumor cell lines, HT-29, SW480 and DLD-1, one lung tumor cell line A549, a murine melanoma cell line B16F10 and the epithelial cell line HEK293. Indeed, FXa impaired cell survival of all these cell lines to a similar extent as it impaired survival of the breast cancer cell lines (Fig. 1C). Inhibition of survival was dose-dependent and FXa-specific for all cell lines (data not shown). Primary tumor cells are normally outside of the vasculature unless they enter the bloodstream at the early stage of metastasis to travel to distant organs. To confirm that FXa inhibits the growth and survival of circulating tumor cells without affecting cells constitutively in contact with blood (and thus FXa) under normal conditions or upon vascular injury, the effect of FXa on monocytes, endothelial cells and fibroblasts was assessed. As shown in Fig. 1C, FXa did not impair monocyte and/or endothelial cell survival and even enhanced fibroblast survival suggesting that FXa specifically inhibits tumor cell survival. Alternatively, based on the epithelial origin of the selected tumor cells, one could argue that FXa exerts a specific inhibitory effect on epithelial cells. To prove or refute this hypothesis, non tumorigenic epithelial cells from normal small intestine (FHs 74 Int) were treated with FXa. Reciprocally, the same experiment was conducted using HL-60 cells, which are non-epithelial promyelocytic cells derived from acute promyelocytic leukemia (see Table 1). As shown in Fig. 1C, FXa impaired the survival of the FHs 74 Int cells to a similar extent as that observed for the tumor cells. Furthermore, FXa had no effect on the survival of the non-epithelial HL-60 cell line, suggesting that FXa indeed specifically targets epithelial (or epithelial-derived) cells.

Tumor cells lose matrix attachment during metastasis and as a consequence undergo anoikis [18]. We therefore analyzed the effect of FXa on the proliferation of cells grown in suspension. As shown in Fig. 1D, FXa impaired cell survival by approximately 35% (at $t=24$ h).

The morphological characteristics accompanying FXa-mediated inhibition of survival, i.e. gradually rounding, detachment and shrinkage of the cells (Fig. 1E), were suggestive of apoptosis. These observations prompted us to

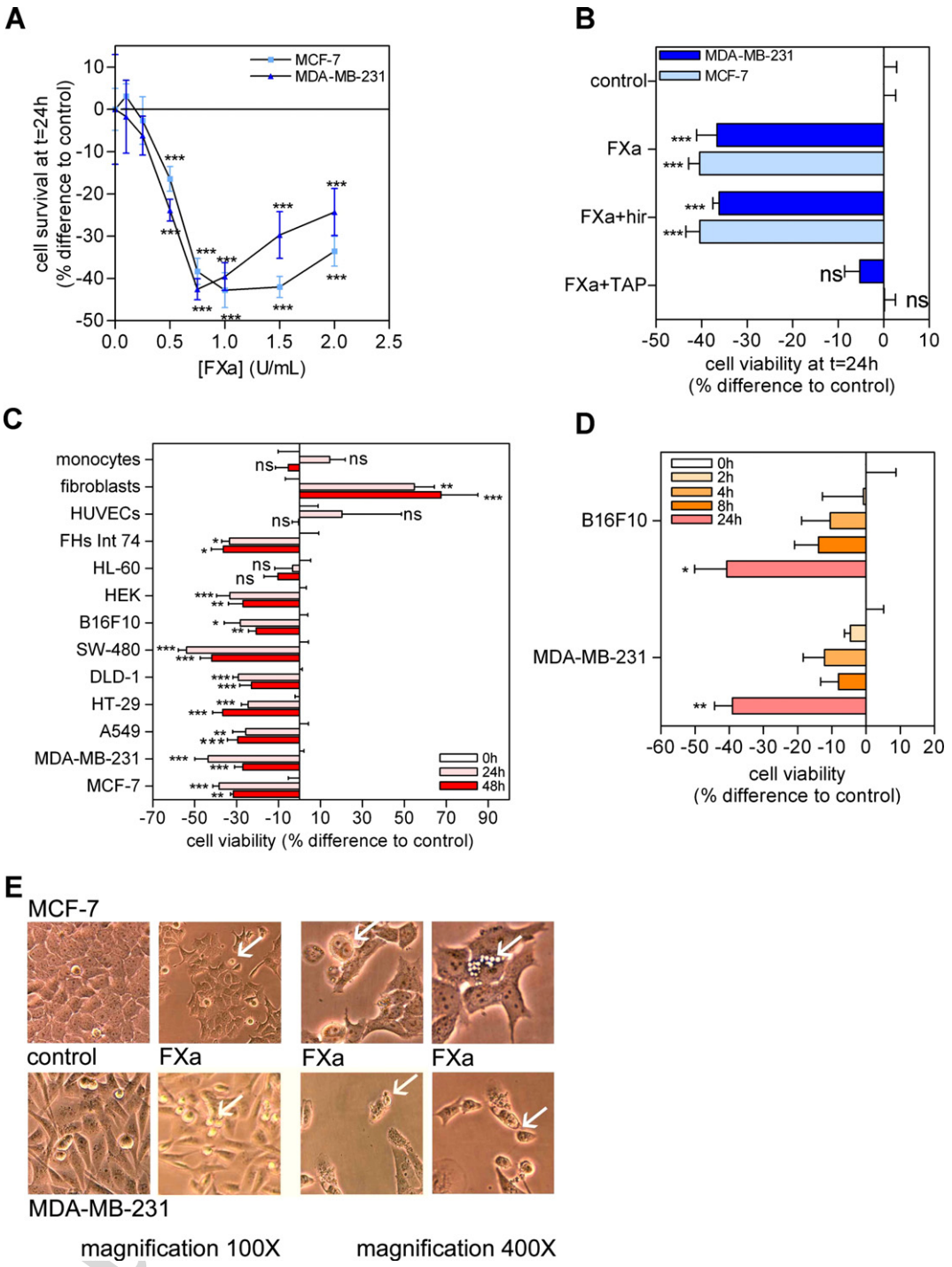

Fig. 1 – FXa specifically impairs the growth of epithelial cells. (A) MCF-7 and MDA-MB-231 cells were incubated for 24 h with the indicated concentration of FXa, or with PBS (control). (B) FXa effects on MCF-7 cell survival were specific and did not rely upon downstream thrombin formation, as the effect was almost completely abolished by TAP but not by Hirudin. (C) FXa (0.75 U/mL) specifically inhibited the growth of normal (FHs 74 Int) and tumor cells with epithelial phenotype, but did not affect the growth of monocytes and endothelial cells. In contrast, FXa enhanced the growth of fibroblasts. (D) FXa enhanced anoikis in MDA-MB-231 and B16F10 cells. (E) Addition of FXa caused cells to become rounded, detached, shrunk in size and vesiculated, which are hallmarks of apoptosis. Shown are untreated (control) and FXa-treated cells 24 h after the addition of FXa or PBS. Note the difference in confluence between treated versus untreated cells, confirming impaired cell survival by FXa. The four panels on the right show details (magnification 400×) of individual cells which allow a better appreciation of morphological changes induced by FXa. Results of panels A to D represent the mean ± SEM of experiments performed twice in octuplicate. $^{***}P<0.0001$, $^{**}P<0.001$.

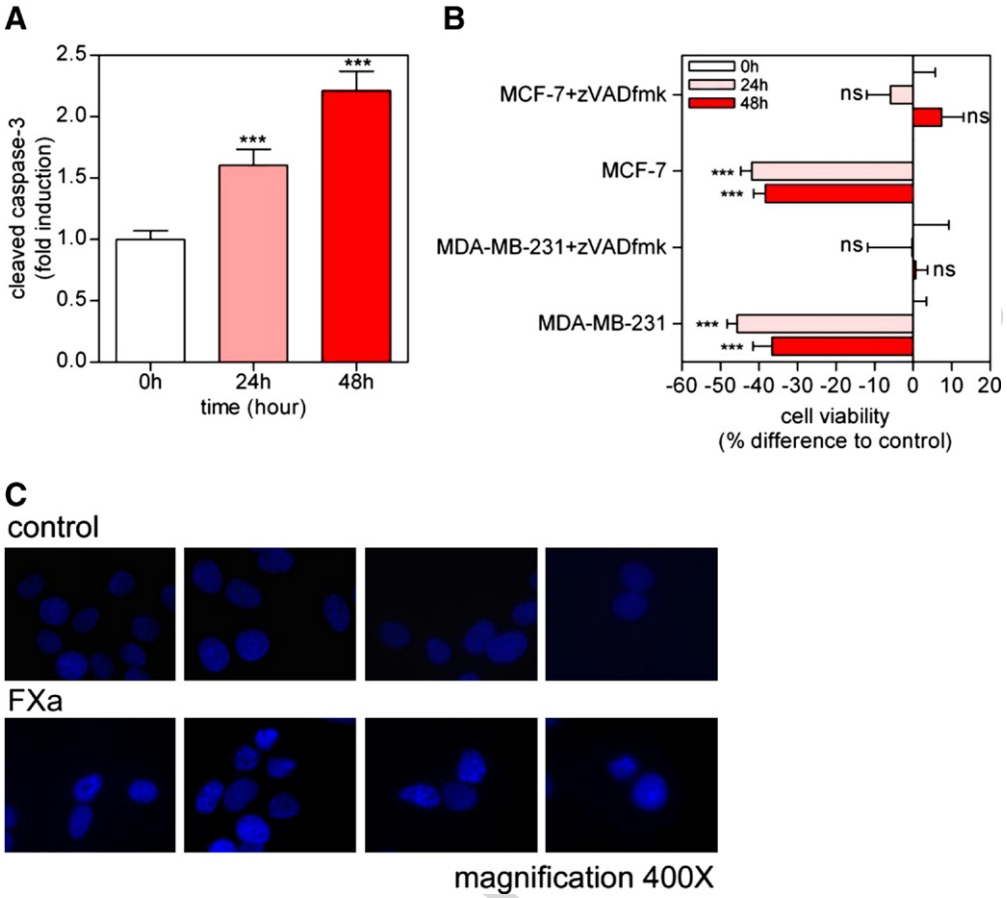

Fig. 2 – FXa induces apoptosis in tumor cells. (A) FXa induced caspase-3 cleavage, as determined on Western Blot. Equal loading was verified using an β-actin antibody. Data are the mean ± SEM ($n=3$). (B) FXa effects were abolished by pre-treatment with zVADfmk. Depicted is the mean ± SEM of experiments performed twice in octuplicate. (C) FXa induced chromatin condensation as evident from enhanced fixation of DAPI in the nucleus of FXa-treated cells. The pictures were taken 24 h after the addition of 0.75U FXa or PBS. ***$P<0.0001$.

investigate the involvement of apoptosis in FXa-induced inhibition of cell survival. To this end, MCF-7 and MDA-MB-231 cells were treated with FXa to assess caspase-3 cleavage as this is the central executioner caspase in apoptosis [19]. In both cell lines, FXa treatment enhanced caspase-3 activation, which was most prominent after 48 h treatment (Fig. 2A for MCF-7 cells, data for MDA-MB-231 not shown). To confirm the actual involvement of apoptosis in FXa-induced inhibition of cell survival, both cell lines were pre-treated with zVAD, a broad-range peptide inhibitor of caspases, but a poor inhibitor of non-caspase proteases, which completely abrogated the FXa effect (Fig. 2B). Nuclear staining using the DNA-binding dye DAPI showed that FXa induced chromatin condensation and nuclear fragmentation which are hallmarks of apoptosis [20] (Fig. 2C for MCF-7, data not shown for MDA-MB-231). Similar experiments performed in fibroblasts did not show differences between control and FXa-treated cells (data not shown).

The cellular effects described for FXa, so far, are mediated through the activation of PAR-1 or -2 [2,14]. To establish the involvement of these PARs in FXa-induced apoptosis, MCF-7 and MDA-MB-231 cell were transfected with PAR-1 or PAR-2 siRNA. As shown in Fig. 3A, siRNA transfection was efficient and specific. Knocking-down PAR-1 expression almost completely abolished FXa-induced apoptosis, whereas PAR-2 siRNA treatment had no effect (Fig. 3B for MCF-7, data not shown for MDA-MB-231). These data indicate that FXa induces apoptosis via PAR-1 cleavage and rule out the involvement of PAR-2. To identify the intracellular pathway downstream of PAR-1 underlying FXa-induced apoptosis, we analyzed phosphorylation of the major MAPKinase proteins and showed that FXa specifically leads to transient ERK1/2 and p38, but not JNK1/2, phosphorylation (Fig. 3D for MCF-7, data not shown for MDA-MB-231). FXa-induced phosphorylation of both kinases was dose-dependent (data not shown) and specific (Fig. 3D) as phosphorylation was blocked by TAP but not hirudin. Pre-treatment with a PAR-1 blocking antibody reduced ERK1/2 and p38 phosphorylation by nearly 70% (Fig. 3E). Similarly, transfection with PAR-1 siRNA abolished phosphorylation of these kinases (data not shown). Transfecting cells with ERK1 or p38 siRNA both partially inhibited FXa-induced apoptosis (Fig. 3B for MCF-7, data not shown for MDA-MB-231), implying the involvement of both these pathways in FXa-induced apoptosis.

Following PAR-1 activation, thrombin is known to cause a rapid and transient desensitization of PAR-1 signaling [21]. We therefore evaluated FXa-induced ERK1/2 phosphorylation in

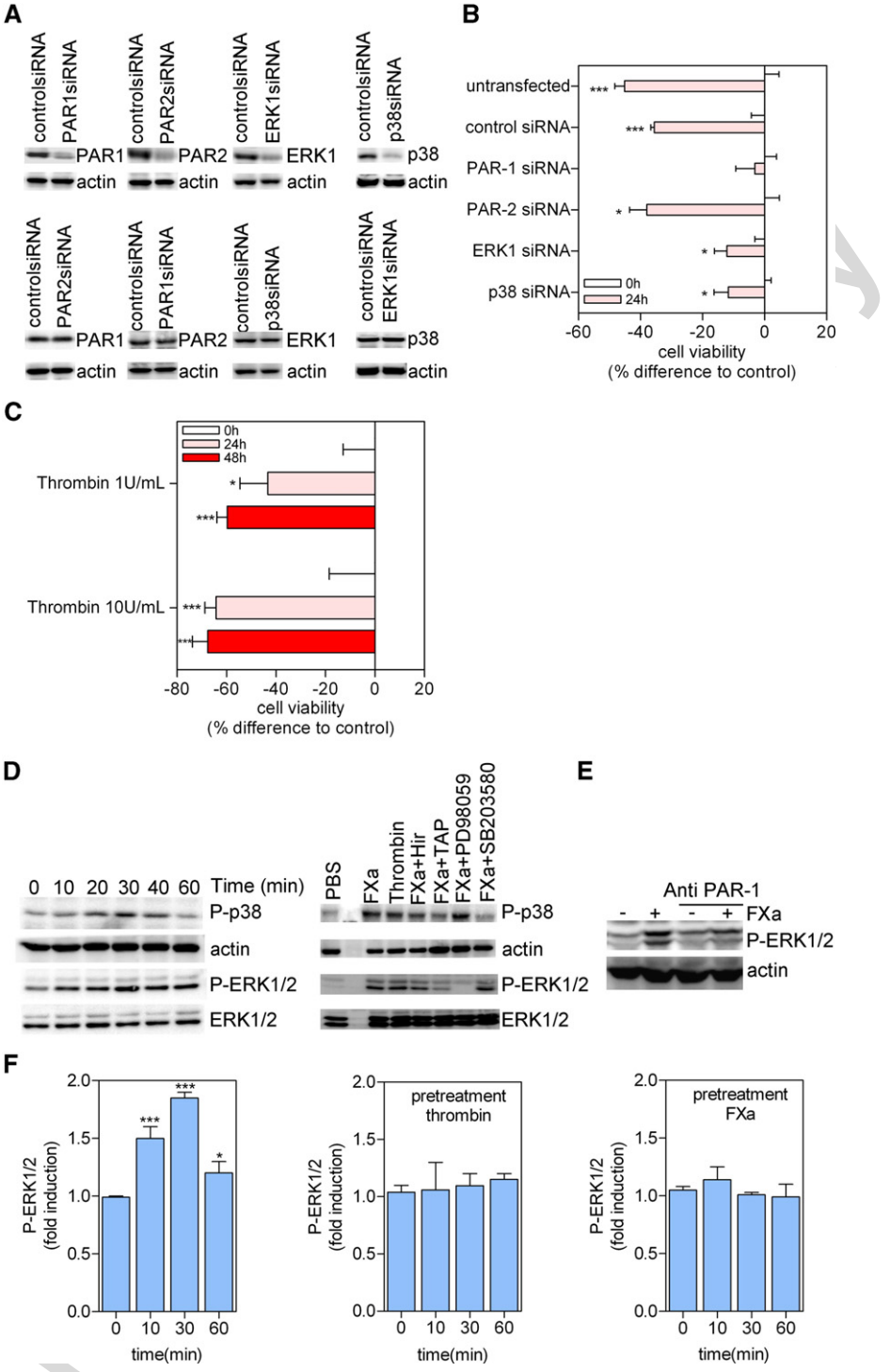

Fig. 3 – FXa-induced apoptosis involves PAR-1-mediated ERK- and p38-activation. (A) siRNA transfections efficiently knock-down the targeted protein (top panels), whereas it did not affect the expression of non-targeted proteins (bottom panel). (B) Involvement of PAR-1, ERK and p38 pathways in FXa-mediated apoptosis. FXa treatment of cells transfected with control or PAR-2 siRNA induced apoptosis after 24 h, whereas in cells transfected with PAR-1-, p38- or ERK1-siRNA, the effects of FXa were abolished. Depicted is the mean ± SEM of experiments performed twice in octuplicate. (C) MCF-7 cells were incubated for 0–48 h with the indicated concentration of thrombin, or with PBS (control). (D) FXa induced p38 and ERK1/2 phosphorylation as shown by Western blot analysis using actin and total ERK1/2 as loading control (left panel). FXa-induced phosphorylation is specific as it was reverted by TAP but not hirudin (right panel). The activity of the p38 (SB203580) and ERK1/2 (PD098059) inhibitors is indicated on the right part of this panel. (E) Inhibition of PAR-1 activation using a blocking antibody impaired FXa-induced phosphorylation of ERK1/2. (F) In cells pre-treated with Thrombin (1 U/mL) or FXa (0.75 U/mL), the transient ERK1/2 phosphorylation induced by subsequent FXa treatment was abolished. Data shown are phosphorylated ERK1/2 raw volumes divided by β-actin raw volumes (mean ± SEM; $n=3$).

thrombin-desensitized tumor cells. As shown in Fig. 3F for MCF-7 cells, tumor cells pretreated with thrombin failed to respond to FXa with respect to ERK1/2 phosphorylation. Furthermore, FXa desensitized PAR-1 in a manner reminiscent of thrombin. The fact that thrombin and FXa desensitization of PAR-1 blocks FXa-induced phosphorylation of ERK1/2 confirms the involvement of PAR-1 in FXa-mediated signal transduction.

To confirm that PAR-1 cleavage can drive cells into apoptosis, we incubated MCF-7 and MDA-MB-231 cells with 1 and 10 U/mL (10 and 100 nM) thrombin, a specific PAR-1 agonist that cannot activate PAR-2 [22,23]). As shown in Fig. 3C for MCF-7 cells, thrombin indeed impaired cell survival thereby confirming previous data [24–26] showing the involvement of PAR-1 in apoptosis.

CREB regulates the transcription of a range of pro-and/or anti-apoptotic targeted genes [27] making it a likely candidate player in FXa-induced apoptosis. Indeed, FXa induced CREB phosphorylation, and the effect was specific since pretreatment with TAP but not hirudin prevented CREB phosphorylation (Fig. 4A). Phosphorylation was partially inhibited by pretreatment with U0126, an ERK-1 and ERK-2 inhibitor, PD098059, an inhibitor specific for MEK1/2 and SB203580, which inhibits p38 phosphorylation, suggesting that both pathways are involved in CREB phosphorylation (the activity of these inhibitors was assessed using Western blot analysis as indicted in Fig. 3D). To assess the relevance of CREB phosphorylation for FXa induced-apoptosis, cells were transfected with vectors either expressing wild-type CREB, or expressing dominant negative mutants (KCREB or CREB S133A). KCREB forms an inactive dimer with endogenous CREB and blocks its ability to bind to its DNA binding sequence, whereas CREB S133A contains a serine to alanine mutation at position 133 that prevents its phosphorylation and thus its ability to initiate target gene transcription. As shown in Fig. 4B, cells over-expressing wild-type CREB are driven into apoptosis by FXa. In contrast, cells transfected with the dominant negative forms of CREB are (almost) unresponsive to FXa. Thus, CREB plays an essential role in FXa-induced cell apoptosis.

The phosphorylation of CREB by FXa indicates a direct effect on gene transcription. Through expression profiling of 37 genes involved in apoptosis using multiplex ligation-dependent probe amplification (MLPA) [17], we observed that only two pro-apoptotic genes are induced by FXa (Table 2) of which the induction of the BH3-only protein Bim could be confirmed on the protein level (Fig. 5A for MCF-7, data not shown for MDA-MB-231). Maximum FXa-induced Bim levels were observed after 8 h (mRNA) or 24 h (protein). Based on these expression profiling data, we hypothesized that Bim, which is known to act as a death ligand that neutralizes members of the pro-survival Bcl-2 subfamily [28], is a central player in FXa-induced apoptosis. In keeping with this hypothesis, Bim expression was up-regulated by FXa in non-

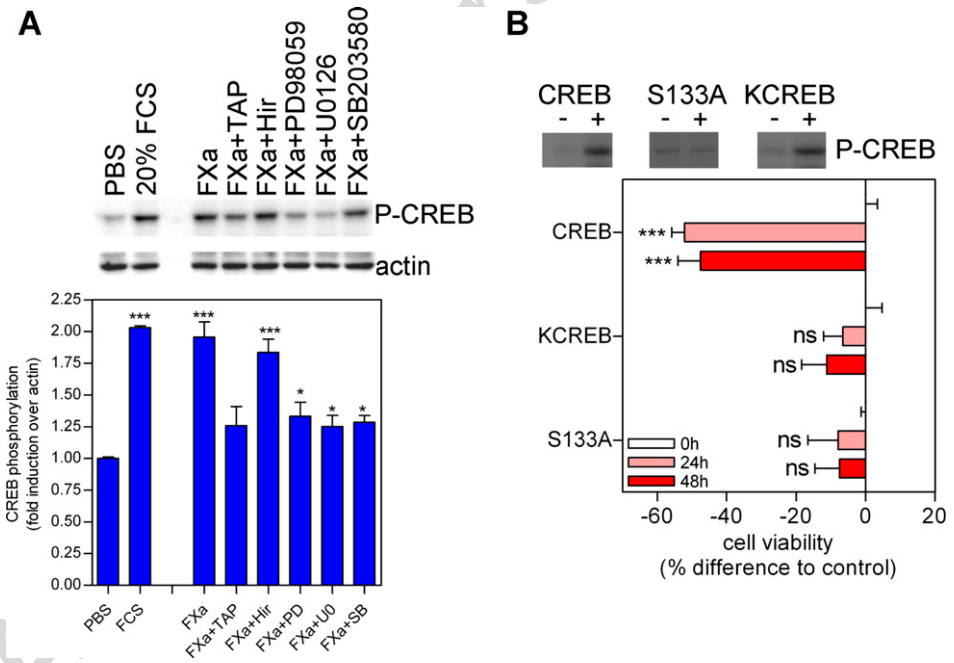

Fig. 4 – FXa-mediated apoptosis involves the phosphorylation of the transcription factor CREB via p38 and ERK1/2 MAP kinases. (A) FXa induced phosphorylation of CREB. The Western Blot (top panel) is representative of three independent experiments. Shown in the bottom panel are density measurements of three independent Western blots (mean ± SEM). FXa specifically induced CREB phosphorylation, since its effects are reverted by TAP but not by Hirudin. Molecular mechanisms underlying CREB activation involve ERK1/2 and p38 MAP Kinase, since CREB activation was diminished by the use of U0126, PD098059 and SB203580. (B) CREB activation is required for FXa-induced apoptosis as cells expressing the native form of CREB or the KCREB mutant but not cells expressing the S133A mutant showed phosphorylated CREB after FXa treatment. Cells over-expressing wild-type CREB are driven into apoptosis, whereas cells expressing the mutant CREB forms do not respond to FXa treatment. Depicted is the mean ± SEM of experiments performed in octuplicate. ***$P<0.0001$, *$P<0.01$.

Table 2 – Transcriptional changes induced by FXa compared with untreated cells as determined by MLPA expression profiling

Gene ID	HUGO name	Genbank	Alias	0 h	2 h	4 h	8 h
APAF	APAF1	AF149794	Apoptotic protease activating factor 1	1	1.01±0.01	0.98±0.03	1.03±0.01
APAF-XL	APAF1	NM_013229	Apoptotic protease activating factor 1 (splice variant)	1	1.14±0.06	1.04±0.01	1.01±0.01
B2M	B2M	AB021288	Beta-2-microglobulin	1	1	1	1
BAD	BAD	AF021792	BCL2 antagonist of cell death; BCL2-binding protein; BCL2L8	1	0.99±0.02	0.92±0.04	0.95±0.02
BAK	BAK	U16811	BCL2L7; BCL2 antagonist killer 1	1	1.04±0.04	0.97±0.03	1.06±0.02
BAX1	BAX	L22473	BCL2-associated X protein	1	1.00±0.13	0.99±0.06	0.98±0.12
BAX2	BAX2	L22473	BCL2-associated X protein	1	1.06±0.01	0.97±0.02	0.98±0.01
PUMA	**BBC3**	AF354654	**Bcl-2 binding component 3; Bcl-2 binding component 3**	**1**	**1.18±0.03**	**1.31±0.01**	**1.44±0.01**
BCL2	BCL2	M14745	BCL2	1	1.19±0.02	1.03±0.01	1.09±0.02
A1	BCL2A1	U29680	BCL2-related protein A1; BFL1	ND	ND	ND	ND
BCL-XL	BCL2L1	Z231115	BCL2-like 1	1	1.06±0.03	1.16±0.1	1.10±0.02
BIM	**BCL2L11**	AF032457.1	**BCL2-like 11; BCL2-interacting protein Bim**	**1**	**1.20±0.03**	**1.48±0.08**	**1.59±0.05**
BCL-W	BCL2L2	D87461	BCL2-like 2	1	1.11±0.02	1.19±0.03	1.05±0.01
BCL-G	BCL-G	AF281254	Apoptosis regulator Bcl-G	ND	ND	ND	ND
BCL-RMB	BCL-RAMBO	AF325209	BCL-Rambo	1	1.05±0.04	0.98±0.02	0.99±0.02
BID	BID	AF042083	BH3-interacting domain death agonist	1	1.02±0.05	1.08±0.01	1.09±0.01
BIK	BIK	NM_001197	BCL2-interacting killer (apoptosis-inducing NBK	1	1.06±0.03	1.05±0.01	1.03±0.02
NIAP	BIRC1	NM_004536	Baculoviral IAP repeat-containing protein 1	1	1.07±0.01	1.10±0.002	1.00±0.02
IAP2	BIRC2	U37547	Baculoviral IAP repeat-containing protein 2; API1, HIAP2, CIAP1	ND	ND	ND	ND
IAP1	BIRC3	NM_001165	Baculoviral IAP repeat-containing protein 3; HIAP1; CIAP2	1	1.02±0.03	1.07±0.08	1.03±0.07
XIAP	BIRC4	NM_001167	Baculoviral IAP repeat-containing protein 4; API3; XIAP; ILP	1	1.05±0.04	0.99±0.04	1.05±0.01
SURVIVIN	BIRC5	NM_001168	Baculoviral IAP repeat-containing 5, API4	1	1.01±0.05	0.98±0.002	0.98±0.01
APOLLON	BIRC6	AF265555	Baculoviral IAP repeat-containing protein 6; KIAA1289	1	1.14±0.06	1.07±0.03	1.12±0.04
LIVIN	BIRC7	AF311388	Baculoviral IAP repeat-containing 7; MLIAP, KIAP	ND	ND	ND	ND
BMF	BMF	NM_033503	Bcl-2 modifying factor	1	1.16±0.03	0.98±0.01	1.10±0.01
NIP3	BNIP3	AF002697	BCL2/adenovirus E1B 19-kDa protein-interacting protein 3	1	0.97±0.03	0.99±0.02	1.00±0.01
NIX	BNIP3L	AF067396	BCL2/adenovirus E1B 19-kDa protein-interacting protein 3-Like	1	1.01±0.03	1.08±0.02	1.13±0.06
Flip	CFLAR	U97074	CASP8 and FADD-like apoptosis regulator, FLICE, CASP8	1	1.02±0.01	1.10±0.01	1.18±0.03
FLT	FLT	M11147	Ferritin light chain	1	0.98±0.04	0.98±0.04	1.07±0.06
HRK	HRK	U76376	HARAKIRI;BCL2-interacting protein; DP5	1	0.97±0.03	1.01±0.02	1.01±0.01
MAP1	MAP1	AK024029	Modulator of apoptosis 1	1	1.06±0.05	0.96±0.04	1.10±0.01
MCL1-S	MCL1	AF118124	Myeloid cell leukemia sequence 1 (BCL2-related), short form	1	0.97±0.01	1.06±0.006	1.07±0.01
MCL1-L	MCL1	AF118124	Myeloid cell leukemia sequence 1 (BCL2-related), long form	1	1.02±0.03	1.10±0.05	1.00±0.03
PARN	PARN	AJ005698	POLY(A)-specific ribonuclease; deadenylating nuclease; DAN	1	1.10±0.04	1.04±0.005	1.07±0.03
AIF	PDCD8	AF100928	Programmed cell death 8; apoptosis-inducing factor; AIF	1	1.04±0.04	0.98±0.03	0.99±0.04
Noxa	PMAIP1	D90070	Phorbol-12-myristate-13-acetate-induced protein 1; APR	1	1.05±0.02	1.03±0.01	0.99±0.01
GUS	SADS	AK000602	Hypothetical protein FLJ20595	1	1.00±0.04	1.04±0.01	0.99±0.02
PI-9	SERPINB9	U71364	Serine (or cysteine) proteinase inhibitor, clade B, member 9	1	1.11±0.06	1.10±0.03	1.03±0.04
DIABLO	SMAC	AK024768	Second mitochondria-derived activator of caspase	1	1.02±0.06	1.03±0.02	1.02±0.03

Expression values are normalized to B2M expression levels and fold induction compared to *t*=0 h is indicated (mean±SEM). Significant up-regulation is highlighted by bold characters. ND: not detectable.

tumorigenic epithelial cells. To obtain support for this hypothesis, Bim expression was transiently knocked down in MCF-7 and MDA-MB-231 cells using Bim siRNA. Indeed, FXa-induced apoptosis was completely abolished in both cell lines (Fig. 5B for MCF-7, data not shown for MDA-MB-231) establishing that FXa-induced apoptosis is dependent on Bim.

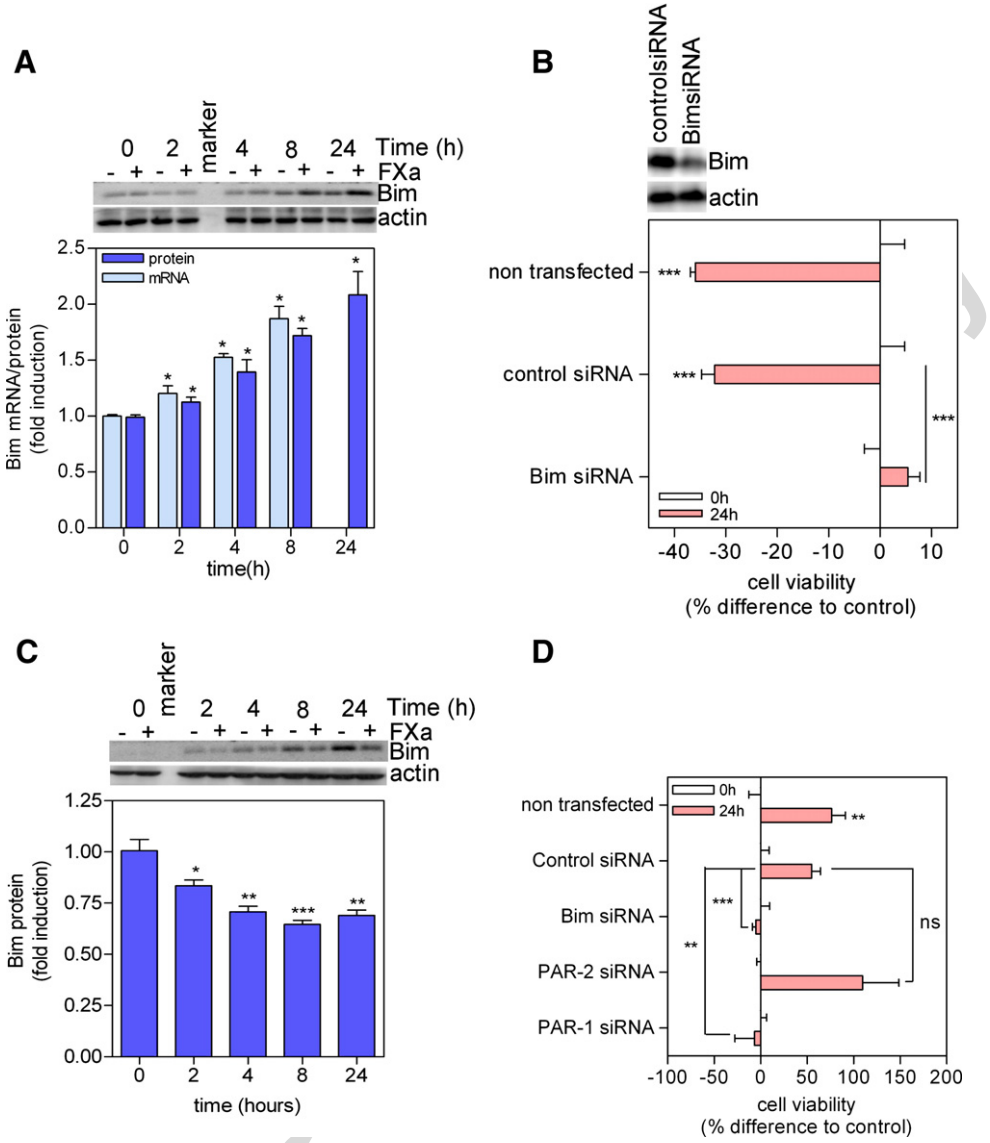

Fig. 5 – FXa-induced apoptosis is mediated through BH3-only Bim protein. (A) FXa treatment of MCF-7 cells induces Bim expression at the mRNA level as determined using MLPA, and at the protein level as determined by Western blotting. Shown on top is a representative Western blot of Bim expression, whereas the bottom panel represents the MLPA data (mRNA) and density measurements of Western blots (mean ± SEM (n=3)). (B) Efficacy of Bim silencing is indicated in the anti-Bim Western blot. The anti-actin Western blot is indicated as a loading control. In non-transfected cells and cells transfected with control siRNA, FXa induced apoptosis as shown previously. In contrast, in cells transfected with Bim siRNA, the effect of FXa was abolished. (C) In fibroblasts, FXa treatment down-regulates Bim protein expression. Shown on top is a representative Western blot of Bim expression. (D) In non-transfected fibroblasts and fibroblasts transfected with control siRNA, FXa enhances cell survival. In contrast, in cells transfected with Bim siRNA, the effect of FXa was abolished. Depicted is the mean ± SEM of experiments repeated twice performed in octuplicate; $^{***}P<0.0001$, $^{**}P<0.001$, $^{*}P<0.01$.

The cell type-specific response to FXa made us speculate about the underlying mechanism and the potential involvement of Bim. To this end, we determined Bim expression in fibroblasts treated with FXa. Unlike in epithelial cells (either tumorigenic or not), FXa down-regulated Bim expression in fibroblasts (Fig. 5C). PAR-1 but not PAR-2 siRNA pretreatment abolished FXa-induced Bim down-regulation and reversed the anti-apoptotic effect of FXa, suggesting that FXa acts via PAR-1 to prevent fibroblast apoptosis (Fig. 5D). Importantly, transfection of fibroblasts with Bim siRNA blocked FXa-induced cell survival, further demonstrating that the switch between pro- and anti-apoptotic effects of FXa is controlled by Bim regulation.

Discussion

Our results uncover several unexpected facets of coagulation FXa in cell biology. First we show that FXa exerts a cell-type-specific effect on cell survival. Indeed, FXa-induced intracellular

signaling drives cells of epithelial origin (either tumorigenic or not) into apoptosis, and enhances tumor cell anoikis, but has no effect on endothelial cell or monocyte survival, whereas it enhances fibroblast survival. This latter result supports previous studies on an anti-apoptotic effect of FXa in fibroblasts [12,15], smooth muscle cells [10,29,30] and mesangial cells [11]. However, the finding of a pro-apoptotic effect of FXa on tumor cells has not been reported previously, and indicates that, aside from its well known effect on blood coagulation and cell proliferation, FXa may also directly inhibit the growth of cells.

Using a specific PAR-1 blocking antibody, we demonstrated that FXa-induced signaling was mediated by PAR-1 activation. In accordance, desensitization of cells with thrombin, which specifically cleaves PAR-1, but not by trypsin (unpublished observations), which specifically activates PAR-2 [3], totally inhibited FXa signaling. Although thrombin can also activate PAR-3 and-4, it has been shown that these receptors are not present on MCF-7 and MDA-MB-231 cells [25], further confirming that thrombin stimulation specifically desensitized PAR-1 for further FXa activation. In addition, knocking-down PAR-1, but not PAR-2 expression, abolished the effect of FXa on cell survival. Taken together, our results establish that PAR-1 activation by FXa mediates apoptosis. At first, these results might be surprising as several papers describe a crucial role for PAR-1 in tumor cell growth and metastasis [7,31–34]. In addition, thrombin is shown to act as a growth factor via PAR-1 activation in a variety of cancer cell lines [34]. More recently, however, PAR-1 activation has been shown to induce apoptosis in different cell types [6,35,36]. For instance, thrombin levels above 0.3 U/mL (3 nM) induced apoptosis via PAR-1 in several tumor cell lines including MCF-7 and MDA-MB-231 cells [24–26]. In this study, we confirmed the pro-apoptotic effect of thrombin on the latter cell lines (Fig. 3C). In addition, thrombin-induced PAR-1 activation also drives primary cells (neurons [6,37] and epithelial cells [36,38]) into apoptosis. Importantly, PAR-1 agonist peptides mimicked thrombin effects on apoptosis though at much higher concentrations (25–100 μM) due to different affinities. Thus, PAR-1 activation might mediate tumor growth but might also induce apoptosis indicating a pleiotropic role of this receptor in tumor biology.

The effect of FXa on tumor cell apoptosis differs in at least two ways from FXa-induced apoptosis. First, the effect of thrombin on cell survival is shown to be biphasic as it induces cell proliferation at low concentrations [23] but (as indicated above) induces apoptosis at higher concentrations [24–26]. In the present study, we show the absence of such a biphasic effect for FXa as the induction of apoptosis was directly correlated with increasing FXa levels. Second, thrombin induces apoptosis of HL-60 cells (this study, data not shown and [26]) and its effect is thus not restricted to epithelial cells, whereas FXa has no effect on HL-60 cell survival (Fig. 1C). The underlying mechanism for these differential effects of thrombin and FXa is not yet resolved. However, it is well-established that by signaling through the same receptor different PAR-1 agonists can exert distinct biological effects. For instance, it has already been shown that PAR-1 agonists were not able to mimic thrombin-induced PAR-1 cleavage in fibroblasts, or endothelial cells [39–41]. Also activated protein C-dependent PAR-1 cleavage triggers signaling pathways which differ from that induced by thrombin-mediated PAR-1 cleavage [42].

Interestingly, FXa induced apoptosis of all epithelial cells to a similar extent despite the fact that PAR-1 expression levels differ between the different cells. For instance, MDA-MB-231 cells are known to express high levels of PAR-1, whereas MCF-7 cells express low levels of this receptor [24,25,43]. All other tumor cell-lines express intermediate levels of PAR-1 (data not shown). This suggests that PAR-1 levels in these cell lines are not rate-limiting in FXa-induced apoptosis.

A recent study reported that the TF–FVIIa–FXa complex prevented apoptosis in MCF-7Adr cells, which are MCF-7 cells transfected with TF to allow FVII activation and ternary complex formation [44]. Unfortunately, however, the effect of FXa alone on apoptosis was not determined in this study. As the TF-FVIIa-FXa also activates PAR-2 it is attractive to hypothesize that direct cleavage of PAR-1 by FXa induces apoptosis whereas incorporation of FXa in the ternary complex switches the affinity towards PAR-2 thereby inducing cell survival. However, the receptor involved in the prevention of apoptosis in the abovementioned study [44] has not been investigated.

Strikingly, we established that the pro- (epithelial cells) or anti-apoptotic (fibroblasts) effect exerted by FXa seems to be regulated by Bim, a well-known key factor in the regulation of apoptosis [45]. In the absence of death signals, Bim is sequestered away from its site of action at the mitochondrial outer membrane through interactions with the micro tubular dynein motor [46]. Death signals trigger its release and rapid translocation to the mitochondrial surface, where it neutralizes the pro-survival activity of Bcl-2 family members, inducing cell death [47]. In accordance with our results thrombin down-regulates Bim expression in fibroblasts, thereby preventing apoptosis [39]. The underlying mechanism by which FXa-induced PAR-1 activation leads to Bim up-regulation in epithelial cells but to Bim down-regulation in fibroblasts is currently not known. The rationale behind this important dilemma of how PAR-cleavage induces different cellular responses dependent on either ligand or cell type is currently subject to an intense research effort.

The present study is the first report showing FXa-induced apoptosis of tumor cells together with fibroblast survival via the modulation of Bim. These data strongly suggest that, beyond its role in blood coagulation, FXa might play a key role in maintaining organ homeostasis or in pathological processes. Especially a role in metastasis could be hypothesized as many carcinomas shed epithelial cells into the circulation. An increase of up to 100-fold of circulating neoplasic epithelial cells is observed in patients with organ-confined carcinoma, nodal involvement and/or distant metastases [48,49]. Therefore, it is tempting to speculate that direct FXa-induced signaling might limit the survival of circulating epithelial cells. Further studies to investigate whether FXa can mediate these anti-tumor effects *in vivo* would be highly desirable, as pharmacological interference with FXa-dependent thrombin generation without affecting FXa-induced signal transduction, as it has been done for activated protein C [50], might have interesting therapeutic implications not only in cancer biology but also in other diseases where activated coagulation factors play a key role.

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