Factor Xa in Mouse Fibroblasts May Induce Fibrosis More Than Thrombin

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Summary

Coagulation factors are known to play a role in wound healing by stimulating fibroblasts and might be associated with tissue fibrosis, however, only limited data exist. Protease-activated receptor 1 (PAR1), activated by thrombin or factor (F) Xa, and PAR2, activated by FXa, have recently been reported to play roles not only in the coagulation system, but also in cardiac fibrosis. Furthermore, a previous report found that FX deficiency in mice led to the development of cardiac fibrosis. Therefore, in the present study, we evaluated cellular biological function under conditions of overexpressed thrombin and FXa in fibroblasts.

Cell migration and proliferation with FXa (1U/mL) and thrombin (1U/mL) stimulation were evaluated. Cells incubated without FXa or thrombin were used as control. H2O2 and TGF-β1 production were measured using ELISA. Signal pathways were evaluated using a signal pathway reporter assay.

Cell migration and proliferation were increased in FXa-stimulated cells (1.3-fold increase for migration, 1.3-fold for proliferation compared with control, respectively) and thrombin (1.3-fold increase). H2O2 production was higher in FXa-stimulated cells compared to thrombin (1.3-fold increase) and control cells (1.4-fold increased). TGF-β1 production was up-regulated after FXa addition (12.6-fold increase compared with thrombin, 1.8-fold increase compared with control, respectively). In FXa-stimulated cells, AP-1 and NF-kB were increased compared to control (P < 0.05).

These data suggest that FXa and thrombin play important roles in the fibrotic process that could also lead to cardiac fibrosis, and that at least some of these signalings are more accelerated with FXa compared to thrombin. (Int Heart J 2014; 55: 357-361)

Key words: Cell signaling, Coagulation cascade, Cardiac fibrosis, Heart failure, Reactive oxygen species

The fibrotic response orchestrated by fibroblasts on injury is a critical component of wound healing, tissue remodelling, and repair in order to maintain the functional integrity of organs and systems. On the other hand, deregulation of normal healing that continues to chronic injury results in tissue fibrosis, massive deposition of extracellular matrix, scar formation, and organ failure. Concerning the heart, cardiac fibrosis is an important hallmark of maladaptive hypertrophy and heart failure, which is characterized by an increase in collagen and other extracellular matrix components in the interstitium and perivascular regions of the myocardium. Fibrogenesis also leads to events in the pathogenesis of systemic sclerosis that develops into vascular sclerosis, which contributes to the vascular thickening and production of pro-inflammatory cytokines that may be related to pericoronary arterial fibrosis. On the other hand, coagulation factors are known to play roles in wound healing by stimulating fibroblasts and might be associated with tissue fibrosis, however, only limited data exist. Protease-activated receptor 1 (PAR1) which is activated by thrombin or factor (F) Xa, and PAR2, which is activated by FXa, have recently been reported to play roles not only in the coagulation system but also in cardiac fibrosis. Furthermore, interestingly, there is also a report which says that mice with FX deficiency developed cardiac fibrosis. Therefore, we evaluated cellular biological function under conditions of overexpressed thrombin and FXa using mouse embryonic fibroblasts (NIH3T3 cells).

Methods

Cell culture: Growth-arrested fibroblasts NIH3T3 were kindly provided by Prof. Hidero Kitasato (Kitasato University, Kanagawa, Japan). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS). Unless stated otherwise, cells were starved with...
phosphate-buffered saline (PBS) for 2 hours, and subsequently stimulated as described.

**Reagents:** Human Factor Xa and thrombin were obtained from Kordia (Leiden, the Netherlands). Lipopolysaccharides from E. coli were purchased from Sigma-Aldrich (St. Louis, USA). TGF-β1 mouse ELISA kit (R&D, Minneapolis, USA), Cignal Finder 45-pathway Reporter Array and Attractene (QIAGEN, Düsseldorf, Germany), and Dual-Luciferase Reporter Assay System (Promega, Madison, USA) were also used.

**Proliferation assay (MTT assay):** Cells seeded at a density of 10^4/cm^2 in 96-well plates were stimulated for 24 hours with FXa (1 U/mL) and thrombin (1 U/mL). Cell survival was determined as the indicated intervals using MTT assay as described previously.\(^5\)

**Cell migration assay:** Wound-scratching assay was performed as described previously, with minor modification.\(^5\) NIH3T3 cells were cultured in 96-well plates. Confluent cell monolayers were scraped using sterilized 200-μL pipette tips and stimulated with FXa (1 U/mL) and thrombin (1 U/mL) for 24 hours. Cells were fixed and stained using Diff-Quick (SYSMEX, Hyogo, Japan). Images were captured immediately and at 24 hours after the wounding.

**H2O2 production:** Intracellular H2O2 levels in NIH3T3 were measured using the DCF-DA fluorescence method as described previously, with minor modification.\(^5\) NIH3T3 cells were seeded in 96-well plates to 60-70% confluence and starved with PBS. After supernatant aspiration, 20 μL DCF-DA was added to the cells followed by incubation for 2.5 hours at 37°C. After culture media aspiration, the cells were stimulated with FXa (1 U/mL) and thrombin (1 U/mL) for 30 minutes. Relative DCF-DA fluorescence was measured using a fluorescence microplate reader.

**TGF-β1 production:** TGF-β1 production was evaluated by ELISA using supernatants of NIH3T3 incubated with FXa (1 U/mL) and thrombin (1 U/mL) for 6, 18, 48, and 72 hours. Total TGF-β1 concentrations were measured by first activating (with 1N HCl) latent TGF-β1 to immunoreactive TGF-β1, and then free (active) TGF-β1 levels were measured without first acid activating the sample. ELISA immunoassay was performed according to the manufacturer’s protocol. Briefly, the wells were precoated with monoclonal antibody specific for TGF-β1. Standards, controls, and samples were added and incubated for 2 hours. After washing, an enzyme-linked polyclonal antibody specific for TGF-β1 was added. After another washing, the samples were incubated with a substrate solution for 30 minutes and the color reaction stopped by addition of a stop solution. The optical density of each well was measured immediately with a microplate reader at 450 nm.

**FXa pathway profiling:** NIH3T3 cells were seeded in 96-well cell culture plates and transected with a Cignal reporter array panel of transcription factor. The transcription factors used for 45 pathways were: Activating transcription factor (ATF) 2/3/4, androgen receptor, Nuclear factor (Nrf) 2/ Nrf1, ATF6, CCAAT-enhancer-binding proteins, CAMP response element binding protein, E2F, p53, early growth response protein 1, CBF/NF-YYY1, estrogen receptor, GATA, glucocorticoid receptor, heat shock transcription factor-1, metal regulatory transcription factor-1, Gli, hepatocyte nuclear factor-4, hypoxia inducible factor-1a, interferon regulatory factor 1, signal transducer and activator of transcription (STAT)1/STAT2, STAT1, Kruppel-like factor 4, liver X receptor, serum response factor/EIk-1, activator protein (AP)-1, monocyte enhancer factor-2, c-Myc, Nanog, recombining binding protein Suppressor of Hairless, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), oxtamer-binding transcription factor 4, Pax6, FOXO, nuclear factor of activated T-cells, peroxisome proliferator-activated receptor, progesterone receptor, retinoic acid receptor, retinoid X receptor, Sox2, SP1, STAT3, SMAD2/3/4, vitamin D receptor, TCF/LEF, and aryl hydrocarbon receptor. In each well, a pathway specific transcription factor was transfected along with renilla construct 20:1 using Attractene. Sixteen hours after transfection, the cells were stimulated with FXa (1 U/mL) and thrombin (1 U/mL) and then dual luciferase assays were performed as described previously.\(^5\)

**RESULTS**

**FXa-induced fibroblast proliferation and migration:** Because fibroblast proliferation is an important process in cardiac fibrosis, we assessed the functional consequences of FXa or thrombin-induced signalling on fibroblast proliferation. FXa and thrombin both enhanced the proliferation of NIH3T3 cells (Figure 1A) by 1.3-fold after 24 hours compared to control cells (P < 0.05). There was no significant difference between the effects of FXa and thrombin.

In addition to proliferation, we assessed FXa or thrombin-cell migration in NIH3T3 cells, which is another important process in fibrotic changes in the heart. Confluent NIH3T3 were wounded and compared after 24 hours of exposure to FXa or thrombin. Cell migration was significantly increased in FXa and thrombin-stimulated cells compared with control cells (4.1-fold increase, respectively, Figure 1B and 1C). There was no significant difference between FXa and thrombin-stimulated cells.

The above results indicate that FXa enhanced fibroblast proliferation and migration to the same extents as that of thrombin.

**FXa-induced ROS production in fibroblasts:** Next, we determined if ROS participated in FXa-induced and thrombin-induced cell proliferation or migration. As shown in Figure 1D, H2O2 production was significantly higher in FXa-stimulated cells than in thrombin-stimulated (1.3-fold increase) and control cells (1.4-fold increase) 30 minutes after stimulation with FXa or thrombin. Taken together, these results indicate that fibroblast activation is involved in ROS-dependent FXa signaling, and interestingly, that the elevation of ROS was more accelerated than that of thrombin signaling.

**FXa enhances TGF-β1 production:** TGF-β1 production is the key event in the pathogenesis of fibrosis and is known to be deeply associated with cardiovascular disease (CVD).\(^11\) TGF-β1 production exhibited a time-dependent increase and was significantly up-regulated after 18 hours of FXa stimulation (12.6-fold increase compared with thrombin, 1.8-fold increase compared with control, respectively) and then decreased. Although later than FXa, thrombin stimulation enhanced TGF-β1 production in a time-dependent manner and was dramatically increased after 48 hours (Figure 1E).

**Signal transduction of fibroblast activation induced by FXa:** We identified a set of transcription factors potentially involved
**Discussion**

Fibroblast proliferation and migration are central features of tissue repair and pathological fibrosis. Tissue fibrosis is the end stage of a heterogeneous group of disorders that can affect many organs, such as the lung, kidney, liver,\textsuperscript{12,14} and heart. Cardiac fibrosis is closely associated with cardiovascular diseases. For example, cardiac fibrosis and left ventricular (LV) hypertrophy induced by comorbidity such as pressure overload, ischemia, or diabetes lead to pathological cardiac remodeling, which is a cause of heart failure and diastolic LV dysfunction.\textsuperscript{5,6,10}

In this study, we demonstrated that FXa enhanced fibroblast proliferation and migration to the same extent as that of thrombin. These were accompanied by the elevation of ROS and TGF-β1 production, which were more accelerated compared with those of thrombin.

NF-κB and AP-1 have been reported to regulate the gene expression of TGF-β1.\textsuperscript{15} In FXa-stimulated cells, AP-1 and NF-κB were significantly increased compared to control. Downstream TGF-β receptors rely on activated nuclear Smad...
In FXa-stimulated cells, HIF-1 is the definitive difference with thrombin. Monomers. FXa is known to activate PAR1 or PAR2 and this ing PAR1 and by cleaving fibrinogen to generate fibrin.

Thrombin promotes coagulation by cleaving and activating growth factor; ROS, reactive oxygen species; ERK, extracellular signal-regulated kinase; AP1, activator protein 1; and JNK, c-jun N-terminal kinase.

On the other hand, ROS are known to activate HIF-1α and ER stress transcription. In FXa-stimulated cells, Elk-1 SRF and AP-1 were significantly higher than control. Compared to thrombin-stimulated cells, there were no significant differences in AP-1 and NF-κB, and Elk SRF signals were lower, which play a role not only in cell growth but also in the prevention of apoptosis or cell differentiation.

These data suggest that the activation of fibroblasts for the fibrotic process could also lead to cardiac fibrosis, and that at least part of those activations might be stimulated by FXa via AP-1 and the NF-κB pathway, accompanied by an elevation of ROS and fibrotic signal activation through the Erk and Jnk pathways (Figure 2). Furthermore, parts of these signalings were more accelerated than for thrombin.

When discussing these differences between thrombin and FXa, function must be taken into consideration. Coagulant tissue factor (TF) binds FVIIa and X to facilitate thrombin generation. Thrombin promotes coagulation by cleaving and activating PAR1 and by cleaving fibrinogen to generate fibrin monomers. FXa is known to activate PAR1 or PAR2 and this is the definitive difference with thrombin. PAR1 and PAR2 play roles not only in the coagulation system but also in cardiac fibrosis.

It has been reported that ROS could enhance the fibrogenic process or cardiovascular dysfunc-

Figure 2. Model of FXa signalings concerning cardiac fibrosis in fibroblast. FXa indicates factor Xa; PAR, protease-activated receptor; HIF1, hypoxia-inducible factor 1; ER, endoplasmic reticulum; TGF, transforming growth factor; ROS, reactive oxygen species; ERK, extracellular signal-regulated kinase; AP1, activator protein 1; and JNK, c-jun N-terminal kinase.

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