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FXa-induced intracellular signaling links coagulation to neoangiogenesis: Potential implications for fibrosis

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ABSTRACT

Fibrosis represents the end-stage of a broad range of disorders affecting organ function. These disorders are often associated with aberrant angiogenesis, but whether vascular abnormalities during fibrosis are characterized by excessive or diminished neo-vascularization remains questionable. Strikingly, activation of the coagulation cascade is frequently observed in association with the progression of fibroproliferative disorders. As we recently showed that coagulation factor (F)Xa induced fibrotic responses in fibroblasts, we hypothesized that FXa might indirectly induce angiogenesis by triggering fibroblasts to secrete proangiogenic factors. In the present study, we show that although FXa induces p42/44 MAP Kinase phosphorylation in endothelial cells, it has no direct effect on endothelial cell proliferation, protein synthesis and tube formation. In contrast, conditioned medium of fibroblasts stimulated with FXa enhanced endothelial cell proliferation, extra cellular matrix synthesis, wound healing and endothelial tube formation. FXa induced VEGF production by fibroblasts and a VEGF neutralizing antibody blocked the indirect effect of FXa on proliferation and realignment of endothelial cells identifying VEGF as a crucial player in angiogenesis during coagulation factor-induced fibrosis. Overall, our results establish a link between the coagulation cascade and angiogenesis during fibrosis.

1. Introduction

Fibrotic disease constitutes the end-stage of a broad range of pathologies affecting organ function [1]. Fibrosis might occur in many different organs, but is most commonly observed in the skin [2], liver [3], lung [4], kidney [5] and heart [6]. At the cellular level, fibrosis involves aberrant fibroblast proliferation and accumulation of extracellular matrix (ECM) impairing normal organ function. Strikingly, activation of the coagulation cascade is frequently observed in association with the progression of fibroproliferative disorders. For instance, post-ischaemic renal damage is associated with fibrin deposition [7]. Moreover, alveolar fibrin deposition after acute lung injury leads to accelerated pulmonary fibrosis [8]. Physiologically, activation of the coagulation cascade is initiated when vascular injury exposes tissue factor (TF) to the circulating blood. Upon such vascular injury, TF is exposed to the blood allowing complex formation with circulating (activated) factor (F) VII (FVIIa). The TF/FVIIa complex in turn activates FX into FXa. FXa converts prothrombin into thrombin which in turn cleaves fibrinogen into fibrin, leading to the formation of a clot [9].

A mechanistic link between activation of the coagulation cascade and the progression of fibrosis is provided by the ability of activated coagulation factors to proteolytically cleave and activate protease-activated receptors (PARs), a family of G-protein-coupled receptors. Four PARs (PAR-1 to -4), of which PAR-1, PAR-3 and PAR-4 can be activated by thrombin, have been identified so far. The TF/FVIIa complex can activate PAR-2 only, and FXa is able to activate PAR-1 and PAR-2 [10]. The role of thrombin in fibrotic processes has been extensively studied *in vitro* [11–14], and thrombin signaling via PAR-1 plays indeed a crucial role in the progression of fibrosis [15,16]. Recently, we and others showed that FXa also triggers fibro-proliferative responses in fibroblasts and smooth muscle (SM) cells. FXa enhanced cell proliferation and migration, it induced the secretion of pro-fibrotic and pro-inflammatory cytokines and stimulated ECM deposition [17–22].

Increasing evidence suggests that vascular endothelial growth factor (VEGF) is a major regulator of fibrosis and tissue remodeling (like for instance asthma) [23,24]. Generally, fibroblasts of the connective tissue produce very low levels of VEGF but during fibrosis activated fibroblasts secrete increased VEGF levels [25]. VEGF is a potent angiogenic protein [26] involved in the formation of new capillary blood vessels by a sequence of complex and precisely programmed events that include proliferation and migration of endothelial cells (ECs) into the surrounding tissue, the reconstruction of a matrix and the assembly of ECs into capillary tubes [27]. Angiogenesis is instrumental during embryogenesis, tissue repair after injury and the female reproductive cycle: indeed, even if during adulthood most blood vessels remain quiescent, ECs retain their remarkable ability to rapidly divide in response to physiological

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stimuli. However, angiogenesis requires very tight regulation and a disbalance between pro- and anti-angiogenic factors is intrinsically linked to disease progression. For instance, regression of angiogenesis contributes to the progression from adaptive cardiac hypertrophy to heart failure [28]. Also, in women with preeclampsia, increased levels of anti-angiogenic proteins are identified in the placenta [29,30] and serum [30–33]. In contrast, aberrant neo-angiogenesis is well known to be responsible for tumor growth and metastasis [34,35].

The associations between modulation of angiogenesis and the progression of the heterogeneous group of fibrotic diseases are numerous and very complex. Numerous studies report changes in VEGF production and consequently in the density of the vascular network during fibrosis but contradictory both regression and/or expansion of the vasculature has been observed. For instance, during idiopathic pulmonary fibrosis (IPF) both increased [36–40] and decreased VEGF levels have been observed [41,42]. Also with respect to vascular density, both increases [43–45] and decreases [45,46] within fibrotic lesions of IPF patients have been observed. Overall, these studies pinpoint VEGF as a crucial player in angiogenesis during fibrosis. However, whether regression or proliferation of the vascular network is a cause or consequence of fibrosis remains elusive. Consequently, it also remains questionable whether angiogenesis accompanying fibrosis is beneficial of deleterious. Further elucidation of the molecular mechanisms modulating VEGF production is therefore of critical importance for understanding the relationship between angiogenesis and fibrosis.

Next to its role in fibrosis, the coagulation cascade is well-known to be a crucial regulator of angiogenesis [47], and the role of individual coagulation factors in angiogenesis has been especially studied during the progression of cancer. For instance, TF/FVIIa and thrombin both induce angiogenesis during cancer progression [35,48,49]. In contrast, FXa does not seem to affect angiogenesis as, for instance, it does not promote tube formation *in vitro* [50]. Interestingly, we recently showed that FXa induced fibro-proliferative responses in fibroblasts, whereas it has also been shown that FXa, but not thrombin or the TF/FVIIa complex, induced VEGF release by human lung fibroblasts [20]. Consequently, we hypothesized that FXa might indirectly induce angiogenesis by triggering fibroblasts to secrete pro-angiogenic factors thereby establishing a link between activation of the coagulation cascade, angiogenesis and fibrosis. To prove or refute this hypothesis, we transferred medium of FXa-stimulated fibroblasts to endothelial cells, and examined the consequences on cell viability, protein synthesis and tube formation.

2. Materials and methods

2.1. Cell lines, cell culture and reagents

The murine endothelial cells 2H11 and the murine fibroblast cell lines C2C12 and C3H10T1/2 were purchased from American Type Culture Collection (ATCC, Manassas, VA), and maintained in Dulbecco's minimal essential media (DMEM) supplemented with 10% FCS according to routine procedures. Unless specified otherwise, cells were washed twice with phosphate-buffered saline (PBS), then serum starved overnight or for 4 h for 2H11 and C2C12 or C3H10T1/2 respectively prior to stimulation. Human FXa was obtained from Kordia (Leiden, the Netherlands). VEGF-A blocking antibody (aVEGF) was obtained from R&D Systems (Minneapolis, MN).

2.2. Preparation of conditioned media (CMs)

Murine fibroblasts were grown in DMEM in the presence of 10% FCS until 70–80% confluence. The growth medium was then removed, cell layers were washed twice with PBS and cells were cultured for 48 h in either serum-free medium supplemented with PBS (CM) or 174 nM FXa (CM FXa). Fibroblast-conditioned media were centrifuged at 14,000 rpm for 15 min, filtered through 0.22 μM filters and stored at −20 °C until use.

2.3. Western blot

Cells were lysed in Laemmli lysis buffer, incubated for 5 min at 95 °C, and whole cell lysates were separated by 10% SDS-PAGE. After electrophoresis, proteins were transferred onto immobilon-P PVDF membranes (Millipore, Billerica, MA). Membranes were incubated overnight at 4 °C with primary antibodies to β-actin, MMP-9, MMP-2, ICAM-1, VEGF-A, fibronectin (all Santa Cruz, CA) and p42/44 MAP Kinase (total and phosphor-specific; Cell Signaling Technology (Beverly, MA)) in TBS with 0.1% Tween (TBS-T)/1% BSA. All secondary HRP-conjugated antibodies were from DakoCytomation (Glostrup, Denmark). Blots were imaged using Lumilight Plus ECL substrate (Roche, Basel, Switzerland) on a GeneGnome imager (Syngene, Cambridge, UK).

2.4. Viability assay (MTT)

10^4 cells per well were seeded onto 96-well plates in DMEM supplemented with 1% FCS, and were treated with FXa (174 nM) or PBS as control. To determine the effect of the conditioned media (CMs) on cell viability, cells were seeded directly in the different CMs. Cell viability was determined at the indicated intervals using a MTT assay as described previously [51].

2.5. Anoikis

To induce anoikis, cells were plated in the different CMs on culture plates coated with polyHEMA (10 mg/ml). At the indicated time points, cells were harvested and viability was determined by MTT assays.

2.6. Wound scratch assay

Wound scratch assays were performed as described previously [21]. Briefly, cells were plated in six-well plates and maintained in DMEM supplemented with 10% FCS. After the cells reached 80 to 90% confluence, a wound was created in the center of the cell monolayer by a sterile plastic pipette tip. Immediately thereafter, the cells were washed with PBS to remove floating cellular debris and re-incubated for an additional 24 h with CM or CM FXa. The ability of cells to proliferate and migrate into the wound area was assessed after 24 h by comparing the zero and 24 h phase-contrast micrographs of at least 3 marked points along the wounded area at each plate. The percentage of non-recovered wound area was calculated by dividing the non recovered area after 24 h by the initial wound area at zero time.

2.7. Endothelial cell morphogenesis assay

Murine endothelial cells ($5*10^4$) were resuspended in 0.3 ml of the indicated medium, and plated on 24-well plates pre-coated with growth factor-reduced (GFR) Matrigel (0.15 ml). After 16 h at 37 °C/5% CO_2, each well was examined for endothelial cells alignment using a phase contrast microscope. For quantification of endothelial tube formation, the number of tubes was counted in each well by blindly scoring (by two independent investigators) at least 7 different fields were by two observers.

2.8. RNA isolation and MLPA procedure

After 0–8 h of the indicated treatment, total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA) according to manufacturer's recommendation. Next, 100 ng total RNA was used for the MLPA reaction as described previously [52,53]. Briefly, MLPA is a multiplex ligation-dependent amplification procedure (RT-MLPA), which accurately