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Tissue Factor–Factor VIIa complex induces cytokine expression in coronary artery smooth muscle cells

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ABSTRACT

Objective: Within atherosclerotic lesions Tissue Factor (TF)–Factor VIIa (FVIIa) not only contributes to thrombotic events but also alters vascular remodeling through enhancement of migration. Moreover, the TF–FVIIa–FXa complex activates protease-activated receptors (PAR). TF/FVIIa/PAR-2 signaling has also been shown to promote proliferation and metastasis of tumor cells. Since coagulation factors promote inflammation which plays a major role during atherosclerosis as well as tumor metastasis this study sought to investigate the effects of FVIIa on the inflammatory response in vascular cells.
Methods/results: FVIIa induces interleukin-8 (IL-8) and IL-6 in primary smooth muscle cells (SMC), which was correlated to the expression of TF and PAR-2 as shown by immunoassay and qRT-PCR. The effect was dose-dependent and required TF, the proteolytic activity of FVIIa and PAR-2. Secondary effects of downstream coagulation factors were excluded. No proinflammatory FVIIa effect was observed in endothelial cells (EC) and mononuclear cells (MNC), expressing either TF or PAR-2. In atherosclerotic lesions mRNA expression of PAR-1, PAR-2 and IL-8 was elevated compared to healthy vessels indicating a role for PAR-1 and PAR-2 signaling in atherosclerosis.
Conclusion: In addition to the procoagulant and promigratory role of the TF–FVIIa complex we identify a proinflammatory role of FVIIa in human SMC dependent on expression of TF and PAR-2 that provides yet another link between coagulation and inflammation.

1. Introduction

Tissue Factor (TF), a 47 kDa transmembrane glycoprotein and member of the class II cytokine receptor family, is the main initiator of blood coagulation and serves as co-factor for plasma clotting factor VIIa (FVIIa). The TF–FVIIa complex catalyzes the activation of factor X and IX, finally leading to the generation of thrombin and the formation of a fibrin clot. In healthy blood vessels TF is abundantly expressed only in the adventitia. After vascular injury, TF is rapidly induced in the smooth muscle cell (SMC) of the media and accumulates in the SMC of the developing neointima [1]. Within atherosclerotic lesions TF is highly expressed in lipid areas, macrophages and smooth muscle cells [2] and displays high procoagulant activity, which suggests that TF plays a role in determining plaque thrombogenicity. In addition to hemostasis TF contributes to cell migration and remodeling after vascular injury [3]. Furthermore, TF expression has been reported in a number of cancers, such as glioma, pancreatic cancer, non-small-cell lung cancer, colorectal cancer, ovarian cancer, prostate cancer, hepatocellular cancer, and breast cancer [4]. TF expression in tumors not only correlates with the incidence of thrombosis [5] but also promotes metastasis [6], tumor progression and tumor angiogenesis [7].

TF-mediated intracellular signal transduction has not been completely elucidated so far. On one hand TF serves as a docking station for FVIIa and, therefore, promotes the generation of downstream coagulation factors possibly inducing intracellular signal transduction, on the other hand there is evidence for direct signaling through the cytoplasmic domain of TF following TF–FVIIa complex formation [8]. Several studies suggest a signaling mechanism of the TF–FVIIa complex via a second receptor type, a protease-activated receptor (PAR). In this model, TF-bound FVIIa proteolytically activates PAR-2 and, to a lesser extent, PAR-1, two G-protein coupled receptors, and thereby evokes intracellular signaling cascades [9].

Interleukin-8 (IL-8), a CXC cytokine, is not only a neutrophil and lymphocyte chemoattractant but also a monocyte agonist and can be detected in macrophage-rich atherosclerotic plaques [10]. Mice lacking the murine IL-8 receptor homologous to human CXCR2 displayed less accumulation of macrophages and had smaller atherosclerotic lesions [11]. Furthermore, IL-8 is reported to mediate the arrest of monocytes on the endothelium of atherosclerosis-prone vessel under flow conditions [10]. These data

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suggest a role for IL-8 in the trafficking of monocytes into the intima and thus revealed the contribution of IL-8 to the pathogenesis of atherosclerosis. In addition, members of the CXC chemokine family have also been observed as tumor cell products thought to contribute to the growth and progression of a large variety of tumor cells [12].

IL-6 secreted from vascular smooth muscle cells contributes to inflammation by inducing B-cell differentiation, T-cell activation and synthesis of acute phase proteins such as C-reactive protein (CRP) and fibrinogen in the liver [13]. Furthermore, IL-6 increases PDGF-dependent smooth muscle cell proliferation [14].

It has been shown that TF–FVIIa induced IL-8 in human keratinocytes [15] and in breast cancer cells, where an involvement of PAR-2 was reported [9]. In the present study we investigated the role of FVII-induced cytokine release in vascular cells and atherosclerotic lesions by analyzing IL-8 and IL-6 expression following TF–FVIIa complex formation in primary smooth muscle cells (SMC), endothelial cells (EC) and mononuclear cells (MNC). Our results demonstrate that FVIIa induces IL-8 release in SMC, but not in EC and MNC, which correlates with the distinct surface receptor distribution of TF and PAR-2. Furthermore we found increased mRNA expression of PAR-1, PAR-2 and IL-8 in human atherectomy samples pointing out the relevance of PARs in atherosclerosis.

2. Methods

2.1. Reagents

FVIIa and FFR-FVIIa were a generous gift from Novo Nordisk (Bagsvaerd, Denmark) and used in a concentration of 500 nM or as indicated. PAR-1 agonist SFLLRN (200 μM) and PAR-2 agonist SLIGKV (200 μM) were from Bachem (Weil am Rhein, Germany). The inhibitory monoclonal mouse antibody 6B4 against human TF was a kind gift from Wolfram Ruf, Scripps Research Institute, La Jolla, USA, and was used in a concentration of 50 μg/ml.

FXa was inhibited by 1 μM MC53255 (Morphochem AG, Munich, Germany), thrombin by 100 nM Lepirudin (Refludan, Aventis Pharma, Bad Soden, Germany).

2.2. Cell culture and RNA interference (RNAi) targeting PAR-2

Coronary artery smooth muscle cells (SMC) were purchased from Clonetics (San Diego, USA), human umbilical vein endothelial cells (EC) from Promocell (Heidelberg, Germany) and peripheral blood mononuclear cells (MNC) were isolated by Ficoll gradient centrifugation. SMC were cultured in SmGM-2 (Cambrex, Verviers, Belgium), EC in EGM-2 (Cambrex) and MNC in RPMI-1640 (Gibco, Paisley, Scotland, UK) containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (Sigma) and 1% L-glutamine (Sigma). Prior to stimulation cells were starved in basal medium without FBS for 24 h (SMC) or 2 h (EC, MNC). To determine mRNA levels cells were stimulated for 2 h and for analysis of cytokine release cells were stimulated for 16 h in serum-free medium. For down-regulation of PAR-2 by RNAi 2.5×10^5 SMCs were transfected with 2.5 μg annealed siRNA (PAR-2 sense: 5′-GGAAGAAGCCUUAUUGGUAtt-3′, fluorescein-labeled non-silencing siRNA (siCtr) sense: 5′-UUCUCCGAACGUGUCACGUdTdT-3′, Ambion, Austin, Texas) and 7.5 μl RNAiFect (Qiagen). Experiments were performed 48 h after transfection.

2.3. Human atherectomy samples

Human atherectomies were obtained from carotid atherectomy samples ($n = 28$) and healthy vessels from the external pudendal artery ($n = 8$). The institutional ethics committee approved the study protocol and all subjects gave informed consent. Samples were homogenized in 600 μl RLT-Buffer (Qiagen, Hilden, Germany) containing 1% (v/v) β-mercaptoethanol. Total RNA was isolated using RNeasy Mini Kit (Qiagen).

2.4. Immunoassay

For cytokine release assays cells were seeded in 96-well plates and supernatants were analyzed using ELISA immunoassays (human IL-8, IL-6, R&D Systems, Wiesbaden, Germany).

2.5. RNA extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells according to the manufacturer's instructions (RNeasy Mini Kit, Qiagen) and subsequently reverse transcribed by Omniscript Reverse Transcriptase (Omniscript RT Kit, Qiagen) using random primers (Invitrogen, Karlsruhe, Germany).

Transcript levels were detected by qRT-PCR (TaqMan, ABI PRISM 7700, Applied Biosystems, Darmstadt, Germany) using commercially available primers and probes in a final volume of 25 μl. The PCR mixture contained 1× TaqMan Universal PCR Master Mix, 900 nM of each primer and 250 nM of a 6-carboxy-fluorescein (FAM)-labeled probe (Applied Biosystems). Assays-on-Demand containing specific primers and probe for IL-8 (Hs00174103_m1), PAR-1 (Hs00169258_m1), PAR-2 (Hs00608346_m1), TF (Hs00175225_m1) and GAPDH (Hs99999905_m1) were from Applied Biosystems. Fold increase was calculated using the $\Delta\Delta C_t$-method by normalization on GAPDH. For absolute quantification C_t-values were analyzed based on standard curves of pre-quantified PCR products specific for each target mRNA. mRNA copies were normalized on GAPDH copies present in the sample.

2.6. Statistics

Statistical analysis was performed using Student's *t*-test. Values were considered as significant when $p < 0.05$ and marked with an asterisk.

3. Results

3.1. FVIIa increases IL-8 and IL-6 secretion in SMC, but not in EC or MNC

Primary vascular SMC, EC and MNC were analyzed for cytokine release as response to FVIIa. In the same experiments cells were treated with agonistic peptides for PAR-1 and PAR-2 corresponding to the tethered ligand sequence of the receptor. Interestingly, FVIIa-induced IL-8 secretion only in SMC (Fig. 1A), but not in EC (Fig. 1B) or MNC (Fig. 1C). Similarly treatment with PAR-2 agonistic peptide SLIGKV (PAR-2 AP) led to significantly increased IL-8 secretion in SMC (Fig. 1A) and to a much lesser extent in EC (Fig. 1B), but not in MNC (Fig. 1C). PAR-1 AP SFLLRN up-regulated IL-8 release in all three cell types (Fig. 1A–C).

Consistent with the results obtained for IL-8, FVIIa increased IL-6 release in SMC (Fig. 2A), but not in EC (Fig. 2B) or MNC (Fig. 2C). Accordingly, PAR-2 agonist stimulated IL-6 expression in SMC and sparsely in EC, but not in MNC (Fig. 2A–C). The PAR-1 agonist elicited IL-6 secretion in SMC, EC and MNC. Comparable results were found for monocyte chemoattractant-1 (MCP-1) and IL-1β (data not shown).