Factor VIIa-induced signal transduction; possible explanations for tissue factor-associated events

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General Introduction

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1. TF in coagulation

Tissue Factor (TF), also known as thromboplastin, is a 47 kDa transmembrane glycoprotein, found on the surface of various cells, and is the principal initiator of the extrinsic coagulation cascade. Consisting of 295 amino acid residues in total, the major part of TF comprises the 219 amino acid extracellular region. In addition, TF contains a 29 amino acid hydrophobic transmembrane region and a C-terminal intracellular tail of 21 amino acids. Structurally, TF shares a high degree of homology with the interferon class of receptors (1) and the fact that the intracellular part of TF contains two putative phosphorylation sites, suggests a role for this protein in intracellular processes.

As mentioned, TF is a key player in blood coagulation (e.g. (2,3)); as a consequence of the disruption of the vessel wall, TF-expressing cells located in the underlying cell layers will be exposed to the bloodstream. Upon binding of activated factor VIIa (FVIIa), a coagulation factor circulating at low levels within the bloodstream, to TF the so-formed complex initiates

![Diagram of TF activation]

1. initiation

![Figure 1: Tissue factor as the activator of the Extrinsic Pathway. Upon vessel rupture, the transmembrane TF protein binds to activated factor FVII (FVIIa). This complex proteolytically activates FX, leading to formation of thrombin from its precursor prothrombin (IIa and II respectively). Finally, thrombin activation will trigger fibrin deposition, thus creating a blood clot. Alternatively, FIX, normally activated by the intrinsic pathway, is converted to FIXa by the TF/FVIIa complex, leading to subsequent formation of FXa, thrombin and fibrin deposition.](image-url)
the extrinsic coagulation pathway; the TF:FVIIa complex proteolytically cleaves FX to FXa which in turn converts prothrombin to thrombin (fig.1). As a last step, thrombin will induce the formation of fibrin from fibrinogen thereby initiating the formation of a blood clot. It is now generally recognized, however, that the extrinsic coagulation pathway operates in close harmony with the intrinsic pathway. Whereas the intrinsic pathway was generally believed to be activated through exposure of blood to negatively charged surfaces it is now evident that triggering of this cascade occurs through TF-mediated conversion of FIX to FIXa. Tissue factor is widely distributed in many cell types. The role of constitutively expressed tissue factor in extravascular tissue, e.g. fibroblasts and smooth muscle cells as a hemostatic “envelope” outside the vasculature, poised to activate coagulation upon vascular injury is well established (4-6). Tissue factor is not normally expressed in blood vessels but intravascular cells, i.e. leukocytes and endothelial cells, may respond to extracellular stimuli or as a response to injury by expression of tissue factor (7,8). However, as will be discussed later, the physiological importance of TF expression in each of these intravascular cell types is unknown but may cause severe deregulation of physiological processes like hemostasis.

Next to cell-type dependent distribution, TF/FVIIa activity is also regulated by a negative feedback loop; TF/FVIIa-induced cleavage of FXa triggers the expression of a Kunitz-type plasma protease inhibitor, known as tissue factor pathway inhibitor (TFPI). In addition to direct inhibition of FXa, TFPI is capable of forming a quaternary complex with FXa, TF and FVIIa, thereby inactivating this whole complex (9).

In recent years, TF has been shown to play an important role in biological processes independent of the clotting cascade. As already stated above, TF and its natural ligand FVII/FVIIa are important players in inflammation, but also in processes such as neoangiogenesis, tumorigenesis and intracellular signaling. In this chapter we will summarize the pleiotropic effects of TF and finally we will discuss potential links between the physiological processes, which TF affects and the underlying signaling cascades.

2. Tissue factor in (patho)physiology

Tissue factor in sepsis and inflammation- Bacterial sepsis causes severe problems, associated with coagulopathy. One of the coagulatory disorders that is subject of extensive research is ‘disseminated intravascular coagulopathy’ or DIC; activation of the cytokine network, for example upon the invasion of micro-organisms, leads to widespread activation of
blood coagulation, resulting in fibrin formation, and consumption of clotting factors which predisposes to bleeding. In addition, the generation of multiple proteolytically active enzymes of the clotting cascade enhances inflammatory activity, which will worsen the systemic inflammatory syndrome that accompanies DIC. In contrast to earlier work, which pointed towards contact phase triggered activation of coagulation, the induction of coagulation is recently shown to be directly and solely related to tissue factor (TF) activity (10,11)). Disturbance of complex formation between TF and FVII/FVIIa has been shown to reduce sepsis-induced DIC in many studies, using different approaches.

There are basically two distinct approaches to investigate the role of the coagulation pathway in sepsis, i.e. experimental endotoxemia and sepsis models. Experimental endotoxemia relies on the intravenous injection of a low dose of endotoxin (lipopolysaccharide, LPS) to human volunteers and/or chimpanzees, resulting in TF-dependent coagulation (12,13) (fig. 2). The LPS-induced activation of the TF system and subsequent activation of coagulation appears to be mediated by proinflammatory cytokines like TNFα, IL-1 and IL-6. TNFα administration to healthy volunteers elicited rapid activation of coagulation which was similar to that evoked by LPS (14). Whereas interventions with TNFα specific monoclonal antibodies proved unsuc-

Figure 2: Tissue factor as a key player in sepsis models. Bacterial organisms or their membrane components, like LPS, induce TF expression on intravascular cells, such as endothelial cells and monocytes. As a consequence, FVIIa binds to TF resulting in intravascular coagulation. Combined action of LPS and the coagulation pathway will trigger enhanced cytokine release, giving rise to increased inflammatory reactions. In addition, the FVIIa/TF complex itself might also contribute to the rise in cytokine production. In various studies, anticoagulant strategies, such as TF antibodies, TFPI and active site-blocked FVIIa have proven potent inhibitors of DIC-induced cytokine production.
cesful in preventing LPS-induced coagulation activation (15-17), monoclonal IL-6 antibodies do completely block this activation (18). In addition, IL-1 receptor antagonist also attenuates the activation of coagulation (19) by either a direct mechanism or by inhibiting IL-1 induced cytokines.

The alternative approach assessing the role of coagulation factors in sepsis involves lethal or sublethal challenge of animals with LPS or live bacteria. Sepsis-induced coagulation has especially been well documented in baboons. For instance, administration of 500 micrograms/kg of either anti-TF immunoglobulin G (IgG) or anti-TF Fab fragments to baboons has shown to result in the attenuation of coagulopathy and protected against LD100 infusion with Escherichia coli (20). Administering tissue factor pathway inhibitor (TFPI) to baboons already infused with LD100 E. coli also turned out to be highly protective (21). Moreover, whereas the E. coli-injected baboons showed a mean survival time of 39.9 hours, E. coli/TFPI treated baboons survived for the full duration of the experiment (7 days). Consistent with the decreased serum levels of markers of hypoxia, acidosis and cell injury, TFPI provided morphologic protection to the organs from pathological changes. Interestingly, administration of TFPI also attenuated LPS-induced IL-6 production, whereas TNFα levels were not decreased after TFPI treatment.

The observation that TFPI binds to LPS in vitro, thereby preventing LPS from binding to its receptor CD14 (22), suggests that the TFPI-induced attenuation of IL-6 release might be in part direct and thus coagulation independent. Strong arguments against this hypothesis come from intervention studies with active site-inhibited FVIIa (DEGR-FVIIa) (23). Inactive FVIIa diminishes both the IL-6 and IL-8 responses in baboons injected with LD100 E. coli, whereas the LPS-induced TNFα response was insensitive to DEGR FVIIa. In addition, like TFPI, DEGR-FVIIa rescues baboons from the lethal injection of E. coli. Despite convincing evidence on the involvement of TF and FVIIa in inflammation, the involvement of coagulation in these responses remains obscure.

The involvement of TF in inflammatory systems has also been studied on a cellular level; during inflammation, mononuclear phagocytes will cross the lymphatic endothelium in the basal-to-apical direction (reverse migration). Randolph et al. (24) showed that this process is dependent on the expression of TF on the surfaces of these cells. TF does not have chemotactic functions but rather adheres to an unknown binding site exposed on the endothelium, suggesting that mononuclear phagocytes use TF as an adhesive protein to exit the site of inflammation.
Proof for another TF-associated inflammatory event comes from studies addressing the ability of TF/FVIIa complexation to induce proinflammatory effects in macrophages (25). In TF-expressing human monocyte-derived macrophages, administration of FVIIa leads to production of reactive oxygen species (ROS). These ROS are potent killers of intracellular pathogens and, when released extracellularly, are effectors of inflammatory tissue injury. The production of ROS by FVIIa proves to be very specific since inactivated FVIIa, FXa and thrombin are all unable to produce these proinflammatory mediators. Interestingly, treatment of macrophages with a TF-antibody inhibits both ROS production and attenuate activation of peritoneal macrophages in vivo.

Overall, the above-discussed data strongly suggest a direct role of TF and FVIIa in sepsis and inflammation by both extracellular and intracellular mechanisms.

**Tissue factor in embryonic vasculogenesis**—It is generally accepted that TF is the primary initiator of blood coagulation, serving as a cofactor for FVIIa. Interestingly, it does not only play a role in maintaining blood vessel integrity but appears to play a pivotal role in the development of these vessels as well. This notion comes, among others, from experiments with heterozygous TF deficient (TF+/-) mice; in a 129/Sv X NIH Black Swiss background, TF deficiency causes catastrophic hemorrhaging into the yolk sac cavity between embryonic days (E) 8.5 and 9.5. No TF/- embryos survived beyond E10.5 (26), being the stage at which the extra-embryonic circulatory system is generated by vasculogenesis. This indicates that TF plays an indispensable role in establishing and/or maintaining vascular integrity in the developing embryo at a time when embryonic and extra-embryonic vasculatures are fusing and blood circulation begins. TF deficiency in C57BL/6 X 129/Sv mice causes abnormalities of vascular pericytes, resulting in defective yolk sac vessel development and subsequent embryo wasting by E10.5 (27). As a result of these studies it is generally believed that TF plays an essential role in the regulation of blood vessel development in early embryogenesis. Recent observations however, shed a completely different light on the role of TF in embryogenesis and vascular development. Two populations of TF/- embryos in the 129/Sv X C57BL/6 background are observed (28). One dies at midgestation and another dies at birth. Before birth these TF/- embryos look normal and one TF/- embryo delivered by Cesarean section lived four weeks before dying of a massive right cerebral hemorrhage. These results suggest that TF is not essential for normal cell proliferation but is instead necessary to survive major challenges to embryonic vascular integrity at midgestation and at birth. Analysis of the vasculature of TF/- embryos revealed that endothelial cell differentiation is not disturbed, as
concluded from the presence of endothelial cell markers. Therefore it is hypothesized that the absence of TF does not lead to defective angiogenesis through deregulated endothelial cell differentiation, but rather has a direct effect on vessel structure (29). TF-dependent fibrin deposition is hypothesized to play a role, thereby providing an extracellular matrix component that regulates migration of endothelial cells to the developing vasculature. Another attractive theory might be TF-dependent expression of vascular endothelial growth factor (VEGF) in the embryo. VEGF plays an essential role in vascular development, and just like TF/-/mice, VEGF/-/mice die as a consequence of under-developed blood vessels. However, heterozygous VEGF deficient mice die as well, pointing out the critical importance of VEGF for embryonic development.

**TF in tumor angiogenesis and metastasis**- In addition to the development of the embryonic vasculature by vasculogenesis, TF is also involved in post-natal angiogenesis, a form of vascularization highly associated with the process of tumorigenesis. A tumor consists of a heterogeneous cell mass that requires oxygen and other factors that facilitate cell growth and survival. Therefore, tumor growth will cease in the absence of tumor-localized vessel development. Targeting TF itself has proven a successful approach, at least in experimental models; transflecting TF antisense oligonucleotides into murine tumor cells or expressing a cytoplasmic domain-deleted, although still procoagulant-active TF mutant, drastically reduces tumorigenic potential and vascularization (30,31). Abe et al (32) demonstrated that deletion of the TF cytoplasmic domain in human melanoma tumor cells results in decreased expression of VEGF, indicating a role for the intracellular tail in VEGF production and tumor angiogenesis. Extracellular domain mutants expressed in human melanoma cells excluded the involvement of the extracellular domain and thus of the sequential activation of FVIIa, FXa and thrombin in tumorigenesis. In contrast, in human fibroblasts FVIIa-induced VEGF production appears mainly linked to the proteolytic activity of the TF-FVIIa complex (33), resulting in the generation of thrombin (34). Therefore, it is well conceivable that TF-dependent tumorigenesis and vascularization are dependent on both intracellular signaling, elicited by TF's cytoplasmic tail and the generation of thrombin by the TF/FVIIa/FXa complex. The latter may be accomplished by thrombin-induced upregulation of adhesive receptors and changes in cell-cell junction organization in endothelial cells (35). Furthermore, thrombin-induced cleavage of the so-called protease-activated receptors (PARs) generates a mitogenic signal in tumor cells and might enhance tumor cell survival (36).
Recently, new candidates as important players in TF/FVIIa-mediated tumor angiogenesis are identified (37). The administration of FVIIa to human fibroblasts leads to a FXa and thrombin-independent increase in mRNA-expression of Cyr61 and CTGF, which are genes encoding the extra-cellular matrix proteins Cyr61 and connective tissue growth factor. Whereas the Cyr61 protein acts as a ligand to integrin αvβ3 (38), a cell adhesion molecule implicated in cellular processes like angiogenesis and tumorigenesis, CTGF has strong mitogenic potential. Therefore one might argue that FVIIa/TF-mediated tumor angiogenesis is accomplished through the expression of Cyr61 and CTGF. Although the Cyr61/CTGF mechanism provides an attractive and plausible explanation for FVIIa/TF-dependent tumor angiogenesis, the role of TF/FVIIa in tumor angiogenesis is long from resolved.

Next to tumor angiogenesis, the role of TF in tumor growth extends to tumor metastasis; expression of tissue factor by melanoma cells promotes efficient hematogenous metastasis in severe combined immunodeficient (SCID) mice (39). Moreover, the process of metastasis is heavily impaired by inhibition of TF receptor function and the consequent reduction in local protease generation (36,39). Furthermore, metastatic cells have been shown to express a 100-fold higher level of TF, adding to the evidence already pointing towards a role for TF in cancer. Inhibition of FVIIa, the natural ligand for TF, using active site inhibited FVIIa, drastically inhibits TF-driven metastasis as well, pointing out the requirement for FVIIa proteolytic activity in metastasis (40).

**Tissue factor in atherosclerosis**- Atherosclerosis is the primary cause of heart disease in the western world and is a major cause of death. This progressive disease is characterised by the accumulation of lipids and fibrous elements in the large arteries (for an excellent review, see (41)). The accretion of lipoprotein particles in the intima of early lesions is followed by monocyte adherence to the surface of the endothelium. These monocytes transmigrate through the endothelial monolayer into the intima where they differentiate into macrophages, which take up lipoproteins turning into so-called ‘foam cells’. Subsequent foam cell death and secretion of fibrous elements from accumulated SMC’s eventually lead to plaque formation. Advanced lesions can block blood flow, however, the most important clinical complication is an acute occlusion as a consequence of thrombus formation, resulting in myocardial infarction and stroke. Plaque rupture has been recognised to be the main cause of thrombus formation. Pathological studies have identified TF as the major candidate molecule responsible for the thrombogenicity of ruptured plaques. Here, we summarise TF’s role in atherosclerosis.
In situ hybridization and immunohistochemistry showed that cells within the atherosclerotic plaque express TF (4,5). Specifically, TF is localized in vascular SMC’s, the extracellular matrix, foam cells, monocytes and even in endothelial cells overlying atherosclerotic plaques (42). In vitro studies focusing on the underlying mechanism of TF expression in atherosclerotic plaques have elucidated an important role of low-density lipoproteins (LDL). Oxidized LDL up-regulates TF in endothelial cells (43), whereas acetyl LDL induces TF expression in monocytes and macrophages (44,45). Moreover, growth factors like platelet derived growth factor (PDGF), thrombin and vascular endothelial growth factor (VEGF) may induce TF transcription. However, a large pool of TF remains intracellular. It merely appears to be the combination of growth factors that determines the intracellular / surface ratio. The latter suggests a delicate mechanism controlling the thrombogenicity of atherosclerotic plaques. Recently, the immunoregulatory signaling molecule CD40 has been identified in atherosclerosis associated cell types (46). As it is known that CD40 ligation induces TF expression, it is very well conceivable that CD40 is a major player in the thrombogenicity of atherosclerotic plaques (47).

Evidence for a role of TF in atherosclerosis comes from the observation that both TF antigen and activity are frequently found in atherectomy specimens. The presence of TF is demonstrated in both cellular and acellular areas of the plaque (48,49). Ex-vivo experiments revealed that the amount of TF expressed in the atherosclerotic plaque is most abundant in the lipid-rich, acellular core. Correspondingly, thrombus-forming potential within the lipid core, when exposed to mildly heparinized blood, is six times increased compared to the cellular part of the plaque (50). The amount of TF expressed within the plaque appears to have important implications for the occurrence of thrombosis; TF activity is significantly higher within atherosclerotic plaques from unstable coronary syndrome patients than that of stable syndrome patients (51).

Another intriguing aspect associated with atherosclerosis is an apparent discrepancy in the occurrence of a thrombotic event. Only approximately 50 % of TF-positive lesions leads to a thrombus, indicating that in many cases atherosclerotic plaques do not lead to thrombus formation. The mechanism responsible remains to be elucidated and is one of the best-kept secrets in atherosclerosis. Better established is the fact that plaque rupture-induced thrombosis is dependent on TF activity, supporting the hypothesis that upon plaque rupture the coagulation cascade is indeed initiated via the TF pathway.

The appreciation of TF being the main initiator of thrombus formation after plaque rupture has led to the development of new treatment strategies in atherosclerosis aiming at decreased
thrombosis through direct interference with TF activity. In animal models, TFPI, monoclonal antibodies directed against TF and active site inhibited FVIIa are very successful in reducing arterial thrombosis (52-54). As an alternative approach, the inhibition of TF expression seems very promising; especially cAMP concentration-increasing agents, such as prostacyclin and its analogues (55), are effective, most likely through interference with NF-kB-dependent TF expression. Cytokines like IL-4, IL-10 and IL-13 have been reported to inhibit LPS- and IL-1-induced TF expression and are therefore additional candidate anti-thrombotic agents (56,57). Finally, n-3 fatty acids (58) and statins (59) strongly inhibit TF expression in blood cells, thereby potentially lowering the incidence of thromboembolic complications.

3. Tissue factor as a signal transducer

TF and Protease-activated Receptors- In the previous paragraphs of this chapter, we summarised current knowledge about the involvement of TF in physiology beyond its established coagulant activity. As evident from the above, the involvement of TF in inflammation, angiogenesis, tumorigenesis and atherosclerosis is indisputable, however the underlying mechanisms remain poorly understood. Realising that physiological responses are largely dependent on the intracellular state of the cells involved, it is of tremendous importance to deepen our knowledge concerning FVIIa/TF-induced signal transduction pathways. This prompted many researchers to investigate the potential of FVIIa and TF to induce signal transduction. As already mentioned, striking homology has been observed between TF and the interferon γ-type receptors both in their secondary structure (amino acid composition, (1)) as well as in their tertiary structure (crystallographic structures, (60,61)), suggesting the possibility of FVIIa-induced signal transduction via its receptor TF.

A number of observations appear to validate the view of TF acting as the direct receptor prior to signal transduction. As already mentioned, the TF cytoplasmic tail has two potential phosphorylation sites, and these serine residues are readily phosphorylated upon PKC activation, thus creating potential docking sites for important signaling proteins. Second, in yeast two-hybrid studies the cytoplasmic tail has shown to contain a high affinity binding site for a protein known as actin-binding protein (ABP)-280 (62). Third, the cytoplasmic tail appears essential for the generation of calcium transients and for tumorigenic TF-effects, such as the generation of VEGF (see above).

Another model for the initiation of TF dependent signaling has been put forward; in this model TF merely serves as a 'platform' for FVIIa. After binding of FVIIa to TF, the complex
proteolytically cleaves another transmembrane protein, leading to cellular responses. In this model, not TF, but rather another protein might function as the actual receptor. Observations supporting this model comprise the FVIIa-induced TF cytoplasmic tail-independent activation of signal transduction and the necessity of a proteolytically active FVIIa for intracellular signaling, as discussed below (63). The nature of the putative TF/FVIIa target is still subject of debate. A role in this respect has been ascribed to the protease-activated receptors, a class of receptors that needs proteolytical processing for activation. PAR1, 3 and 4 are exclusively activated by thrombin, whereas PAR1 and PAR2 have been shown to be activated by FXa. Recently a role for PAR2 in FVIIa-induced signaling has been shown as well; in KOLF fibroblasts, only the combined expression of TF and PAR2 leads to FVIIa-induced calcium transients, suggesting a role for this PAR in TF/FVIIa-signaling (64). In contradiction with that, Petersen’s group found no role for PARs at all, and therefore predict a role for another transmembrane protein, perhaps an unknown PAR (65).

A new vision on FVIIa-dependent Protease-activated Receptor activation was provided by Riewald and Ruf (66); although FVIIa:TF and FXa were shown to separately induce signaling, the combination of these coagulation factors, immobilized in a ternary complex by using a Nematode Anti-Coagulant Protein C2 backbone, elicited signaling at lower concentrations than that triggered by the individual coagulation factors. Although FVIIa proteolytic activity in this complex was inhibited, FXa efficiently activated both PAR-1 and PAR-2. Thus, TF:FVIIa appears to induce signaling both via proteolytic activation of PAR-2 at higher concentrations and via FXa-mediated PAR-1 and -2 activation at lower concentrations, likely by functioning as a docking site for FXa.

Both the ‘TF receptor’ model and the PAR model of FVIIa:TF signaling are supported by sound experimental data, therefore it is hypothesized that TF signal transduction can occur through two pathways. First, TF functions as a true receptor, leading to calcium transients, ROS production and VEGF production. Alternatively, TF activates other transmembrane targets leading to activation of signal transduction independent of TF’s cytoplasmic domain. Whether the alternative TF signaling pathways serve a delicate regulatory mechanism remains highly speculative.

**FVIIa:TF-induced calcium signaling** - The first observation confirming a role for FVIIa and TF in signal transduction came from Rottingen et al., (67) who showed that FVIIa induced transient cytosolic calcium signals in J82 cells, transfected COS-1 cells, Madin-Darby Canine Kidney cells and human endothelial cells induced to synthesize TF (fig.3). This response is
critically dependent on the proteolytic activity of FVIIa and pre-incubation of cells with the phosphatidyl inositol-specific phospholipase C inhibitor U73122, but not tyrosine kinase inhibitors, abrogated FVIIa-induced calcium oscillations (68). These data suggest that the FVIIa/TF interaction triggers the classical PLC/calcium pathway independent of tyrosine phosphorylation, suggesting PLCβ activity via a heterotrimeric G-protein rather than that of a receptor tyrosine kinase-stimulated PLCγ.

Recently, Camerer (64) and co-workers have demonstrated that in lung fibroblasts and *Xenopus* oocytes, only the combined expression of PAR-2 and TF could mediate FVIIa-induced calcium transients and phosphoinositide hydrolysis. Absence of the TF cytoplasmic domain did not influence these outcomes, ruling out a role for this domain in FVIIa-induced calcium signaling in these cell types.

Some recent observations, however, have made interpretation of these data rather difficult. Using the myoblastoma cell type U937, Cunningham et al found that FVIIa was able to induce PLC activity and calcium signaling and that these signals were highly dependent on the cytoplasmic tail of TF (25). Finally, in Baby Hamster Kidney cells stably transfected with TF (BHK TF), FVIIa does not induce calcium signals (65). From these data it becomes clear that FVIIa:TF-induced signaling shows large variety and is absolutely dependent on the cell type used, as will also be clear from the following paragraphs.

**FVIIa:TF-induced MAP kinase signaling**- Apart from calcium signaling, the interaction of FVIIa with TF has initially been shown to cause numerous intracellular processes, such as transcription of poly(A) polymerase in human fibroblasts and tyrosine phosphorylation in monocytes (69,70). The first report on FVIIa/TF dependent kinase activation (71) describes the transient activation of the pro-mitogenic p42/p44 MAP kinase. The activation of MAP kinase was shown to be dependent on the activation of the upstream MAP kinase kinase MEK since the MEK inhibitor PD98059 abolished this signaling. Furthermore, functional FVIIa was absolutely required for this effect, since FVIIa that was blocked in its active site, did not induce MAP kinase activation. Sørensen et al. (63) showed that deletion of the TF cytoplasmic tail does not abolish FVIIa-induced MAP kinase activation in BHK TF cells and even complexation of soluble TF/FVIIa is sufficient to result in phosphorylation of this signaling mediator.

Activation of p42/p44 MAP kinase has been extensively characterized in various other cell types, such as the spontaneously immortalized keratinocyte HaCaT, primary embryonic...
mouse fibroblasts and Madin Darby Canine Kidney cells (72, 73, 65). Therefore, activation of this kinase appears to be a major event in FVIIa:TF-induced signaling.

A physiological role for this kinase in FVIIa:TF-signaling has been suggested to be activation of transcription factors, resulting in gene transcription (see below). Proof for a mitogenic function of FVIIa-induced activation of this kinase is non-existent, since FVIIa does not appear to have any mitogenic effects at physiological concentrations (74,75).

**Figure 3: FVIIa:TF-induced signal transduction.** Upon FVIIa:TF complex formation, PAR-2 or a still unknown PAR is proteolytically activated. Subsequently, depending on the cell type, events such as calcium signaling, activation of the MAP kinase pathways and nuclear translocation of transcription factors such as NF-kB takes place. This will eventually result in the upregulation of a set of mRNAs. Alternatively, the cytoplasmatic tail may bind proteins such as ABP-280 upon phosphorylation. The FVIIa:TF complex may also serve as a scaffold for FXa, targeting PAR-1 and PAR-2. This will again lead to activation of MAP kinase pathways and transcription. Note that the pathways and genes shown in this figure, are those described in the literature, although each specific interaction might result in activation of additional pathways and genes.

Next to p42/p44 MAP kinase, the MAP kinase family consists of at least two more major isoforms, being p38 MAP kinase and c-Jun N-terminal kinase (JNK), also termed Stress-activated kinase (SAP kinase). Both kinases play a key role in inflammation and stress, but their role in intracellular signaling appears to be more diverse. Activation of both kinases upon FVIIa-stimulation has been reported in HaCaT cells, and similar to FVIIa-induced p42/p44 MAP kinase activation, p38 MAP kinase and JNK activation is highly dependent on FVIIa proteolytic activity (72). The physiological relevance of FVIIa/TF-induced p38 MAP
kinase signaling is still unknown, but it may turn out essential for TF-associated inflammation, angiogenesis and tumorigenesis, via activation of gene transcription.

**TF as an extra-cellular substrate adhesion molecule**- In addition to a function as a receptor, TF is also implicated in substrate adherence. As already discussed, TF-expressing granulocytes appear to bind to endothelial cells, using TF as a ligand-binding protein. Furthermore, J82 bladder carcinoma cells bind to coverslips coated with FVIIa, a process that was shown to be competitively inhibited by free TF extracellular domain. Cells respond to this adherence with cell spreading and cortical actin polymerization (62). Finally, spreading of J82 cells on FVIIa-coated coverslips induced phosphorylation of Focal Adhesion Kinase (FAK) to levels comparable to cells adherent to fibronectin. Since FAK is involved in focal adhesion complex formation and thus adherence, transient phosphorylation of this kinase upon FVIIa:TF complexation would support the hypothesis that TF is an adhesion molecule.

**FVIIa:TF-induced gene and protein expression**- As already discussed, FVIIa:TF induces activation of various MAP kinase family members. These kinases are well-known mediators of gene transcription via the phosphorylation of transcription factors. Therefore, it is no surprise that cells respond to FVIIa-stimulation with upregulation of a specific set of genes (Figure 3). Genes regulated by FVIIa can be divided into several categories; growth factors, cytokines transcriptional regulators and genes regulating cell organization and motility (76,77). Especially the growth factor genes attract substantial attention; FVIIa may not induce proliferation directly, but it could induce paracrine effects, leading to proliferation of cells, other than those targeted by FVIIa. In HaCaT cells FVIIa leads to upregulation of Fibroblast Growth Factor-5 (FGF-5), heparin-based Epidermal Growth Factor (hbEGF) and Connective Tissue Growth Factor (CTGF) mRNA, whereas in lung fibroblasts, FVIIa has been demonstrated to enhance CTGF and Cyr61 mRNA expression. The latter two genes encode proteins that function as growth factors and extracellular matrix proteins, facilitating the process of angiogenesis and therefore attract major interest. Genes encoding proteins that mediate cell organization and motility include collagenase-1, collagenase-3 and RhoE. Although a role for these genes in TF-associated angiogenesis and metastasis remains speculative, it is well known that the proteins encoded by these genes facilitate cell detachment from the extracellular matrix and cell migration, processes that are required for both angiogenesis and metastasis.
Finally, as mentioned, FVIIa stimulates upregulation of *IL-1β*, *IL-8*, *MIP2a* and *LIF*, encoding cytokines. These small molecules act as messengers in the regulation of inflammatory processes and could form an explanation for TF's role in inflammation and sepsis.

Up to now, only one of the above described FVIIa:TF-induced gene transcripts has been characterized on a protein level; in HaCaT cells, administration of FVIIa leads to a dose- and time-dependent upregulation of IL-8 protein.

### 4. Aim and outline of this thesis

It is indisputable that TF, apart from initiating the blood coagulation cascade, is an important player in a variety of pathophysiological events. The evidence for a role of TF in inflammation, angiogenesis, tumor metastasis and atherosclerosis is comprehensive and compelling. The appreciation that TF exerts its effects in a coagulation independent manner, highlights the potential significance of TF dependent intracellular signaling in physiology. However, despite growing knowledge concerning TF’s role in signal transduction, a number of highly relevant questions remain unanswered, such as 'How does TF influence inflammation?', ‘Why is TF indispensable for angiogenesis?’ or ‘What is the mechanism behind TF-mediated metastasis?’.

Therefore, we asked ourselves the next questions;

1) What is the nature of the cellular events induced by FVIIa:TF? Do these events include pathophysiologically relevant processes such as cell motility, protein synthesis or cell survival?

2) Which molecular signal transduction cascades lead to these FVIIa-induced phenomena and do cytoskeletal alterations play a role in FVIIa:TF signaling?

3) How can cellular events and the intracellular events that lie at their basis explain the involvement of TF in the numerous processes that are described above?

In our opinion, therefore, our efforts should be focused on the integration of TF-dependent signal transduction pathways in physiology. In chapter 2, we sought to characterize FVIIa-induced signaling in a fibroblast cell line, since this type of cells is an ubiquitous TF-expressing cell line. Since changes in the cytoskeleton lie at the basis of cell movement, a
process that associated with processes such as angiogenesis and metastasis, we also addressed the question whether FVIIa stimulation leads to cytoskeletal rearrangements. In **Chapter 3**, we further explored FVIIa-signaling in a keratinocyte cell line and in a cell type that does normally not express TF, transfected with the gene encoding TF. In this chapter we also characterized potential differences in FVIIa:TF signaling. As discussed, FVIIa:TF mediate various physiological and pathological processes. These processes often depend on specific events such as cell proliferation and the synthesis of proteins. Therefore, in **Chapter 4** we tested whether FVIIa:TF interaction results in proliferation and protein synthesis. We have also determined the activational state of signal transduction components underlying these possible FVIIa:TF-induced events. FVIIa and TF play a large but still unclear role in inflammation. Therefore in **Chapter 5**, we have explored the activation of signal transducers that are typically associated with inflammatory action; Jaks and STATs. In **Chapter 6**, we have explored possible mechanisms other than proliferation that lead to FVIIa:TF-induced metastasis, such as induction of cell survival and adhesion independence. The role of the various parts of TF in physiology is discussed in **Chapter 7**, in which we have performed an *in-silico* analysis. Finally, in **Chapter 8**, we have attempted to find a solution for the often laborious work that accompanies the screening of activational states of signal transduction pathways, after stimulation of cells with agents such prostanoids or FVIIa.
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Chapter 1


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General introduction


Chapter 1


