Tissue Factor and Cancer Metastasis: The Role of Intracellular and Extracellular Signaling Pathways

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Tissue factor (TF) initiates the coagulation cascade but also plays a role in cancer and metastasis. This transmembrane protein is frequently upregulated on tumor cells and cells that show metastatic behavior. Furthermore, it is a significant risk factor for hepatic metastasis in patients suffering from colon cancer. Recently, it has been shown that TF, together with its natural ligand factor VIIa, induces intracellular changes, such as signal transduction cascades, gene transcription, and protein synthesis. Moreover, TF:factor VIIa interaction leads to survival of cells that have been stimulated to undergo apoptosis. Together with TF-dependent processes such as angiogenesis, these intracellular phenomena form a plausible explanation for the influence of TF on metastasis. In this review, we will discuss these phenomena in more detail and hypothesize on their role in TF-driven metastasis.

INTRODUCTION

Cancer is one of the most prevalent diseases in the western world. Tumor growth may lead to invasion and metastasis, the principal causes of death in cancer. Metastasis, the development of tumors at secondary sites remote from the primary site, is believed to be dependent on a number of physiological processes (1,2). Cancer cells need to detach from the primary tumor mass, migrate toward the lymph and blood vessels, penetrate into the vascular lumen, evade the immune system, adhere to the vascular endothelium, infiltrate, survive, and grow in the invaded microenvironment. Metastasis may seem a very inefficient process because only 0.1% of the tumor cells evade all these obstacles. Nevertheless, tumor metastasis usually results in a poor prognosis for the patient. Similarly, invasion of tumors, especially in the brain, is dependent on many of these processes and hard to treat. In the last few years, it has become clear that the processes of cancer metastasis and invasion are highly dependent on components of the blood clotting (or coagulation) cascade. One of the key proteins in coagulation is tissue factor (TF). Together with the proteolytic enzyme factor VIIa, TF sequentially activates factor X and prothrombin, leading to fibrin deposition and formation of a bloodclot. Although the role of TF in coagulation-unrelated events such as inflammation, sepsis, and angiogenesis is extensively investigated, the role of TF in tumor invasion and metastasis has undoubtedly attracted most attention because of its obvious clinical relevance. In this review, we will discuss the links between TF, metastasis, and metastatic behavior of cells. Understanding the role of the coagulation cascade, and particularly TF, will undoubtedly lead to better and more specific therapies against cancer metastasis.

TF AND THE COAGULATION CASCADE

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 (3). Consisting of 263 amino acid residues in total, the major part of TF comprises the 219 amino acid extracellular region. In addition, TF contains a 23 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 (4), and the fact that the intracellular part of TF contains 2 putative phosphorylation sites suggests a role for this protein in intracellular processes.

As mentioned, TF is a key player in blood coagulation (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, the so-formed TF/FVII complex initiates the extrinsic coagulation pathway; the TF/FVIIa complex proteolytically cleaves FX to FXa, which in turn converts prothrombin to thrombin (Figure 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. TF is widely distributed in many cell types. The role of constitutively expressed TF in extravascular tissue, for example fibroblasts and smooth muscle cells as a hemostatic "envelope" outside the vasculature, poised to

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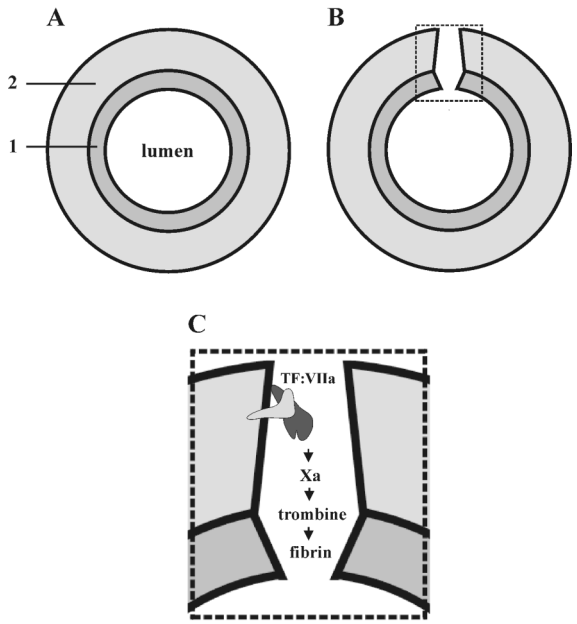

Figure 1. (A) Blood vessels consist of an endothelial cell layer not expressing TF (intima; 1), and TF-containing media and adventitia, indicated by 2. Upon vessel rupture (B), TF comes into contact with the bloodstream and binds the zymogen factor VII. After conversion of FVII to FVIIa, the latter enzyme converts FX to FXa, resulting in subsequent thrombin formation (C) and fibrin deposition.

activate coagulation upon vascular injury is well established (5–7). Tissue factor is not normally expressed in blood vessels, but intravascular cells, such as platelets, leukocytes, and endothelial cells, may respond to extracellular stimuli or as a response to injury by expression of tissue factor (8,9). 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.

In addition 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 (10).

TF AND METASTASIS

TF upregulation has frequently been associated with tumorigenesis, and tumor cells show a high procoagulant activity (11,12). Expression levels of TF in metastatic cells may be up to a 1000-fold higher (13) than in nonmetastatic cells, suggesting a direct role for TF in tumor metastasis. Furthermore, TF expression correlates with the grade of malignancy in human glioma (14) and is highly expressed in metastatic breast carcinoma cells in contrast to nonmetastatic breast carcinoma cells (15). TF expression was shown to be a significant and independent risk factor for hepatic metastasis in patients with colorectal cancer (16,17). TF was detected in the tumors of 57% of the colorectal cancer patients and its expression significantly increased (P = 0.01) in metastatic tumors (88%). A relation between TF detection in cases with lymph node or hematogenous metastasis compared with tumors without metastasis was also observed. Thus, the expression of TF appears to be related with the metastatic potential of colorectal cancer.

The role of TF and other blood-clotting factors has been extensively studied in experimental metastasis models using severe combined immunodeficient (SCID) mice. Already in 1992, Mueller and coworkers demonstrated that inhibition of TF receptor function and consequent reduction in local protease generation, abolished prolonged adherence of tumor cells, resulting in significantly reduced numbers of tumor cells in the lung vasculature of SCID mice that were injected with TF-expressing melanoma cells (13). Using Chinese Hamster Ovary (CHO) cells, transfected with TF, the same researchers showed that the metastatic potential of these cells is not only dependent on TF, but also on FVIIa proteolytic activity, whereas the involvement of FXa was excluded in that model (18). In addition, transfection of CHO cells with TF cytoplasmic domain-deleted mutants resulted in poor metastasis in SCID mice, indicating that, next to FVIIa proteolytic activity, prometastatic functions of TF also depend on the intracellular tail of TF (18).

TF AND ANGIOGENESIS

Metastasis is critically dependent on the formation of new blood vessels. Without development of a local, tumor-supporting vasculature, the secondary tumors will cease to grow. In the last 10 y, TF has been shown to have an enormous impact on both embryonal and tumor vessel formation. In 129/Sv X NIH Black Swiss mice, 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 (19), being the stage at which the extra-embryonic circulatory system is generated. TF deficiency in C57BL/6 × 129/Sv mice causes abnormalities of vascular pericytes, resulting in defective yolk sac vessel development and subsequent embryo wasting by E10.5 (20). As a result of these studies it is generally believed 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 plays an essential role in the regulation of blood vessel development in early embryogenesis.

However, as already mentioned above, TF also has a role in tumor angiogenesis. In non–small-cell lung carcinomas (21) as well as in human prostate carcinomas (22), a significant relationship between TF expression and microvessel density exists. In addition, the significant correlation between TF and vascular endothelial growth factor expression in certain human tumors (21,23,24) implies that TF modifies the angiogenic properties of tumor cells by altering the production of growth regulatory molecules that act on vascular endothelial cells. Importantly, in a murine xenograft model, tumor cells transfected to overexpress TF grew more rapidly and established larger and more vascularized tumors than control transfectants. Antisense TF transfectants grew the slowest and were the least vascularized. Anticoagula-

tion of mice with warfarin did not alter the difference between these tumor lines (25). In agreement, using species-specific antibodies to TF, it was shown that TF was essential for melanoma metastasis in a comparable xenograft model, TF inhibition resulting in significantly reduced numbers of tumor cells retained in the vasculature of the lungs (13). Finally, covalently inactivated FVIIa in these models has antimetastatic properties, emphasizing that proteolytic activity is necessary for the metastatic process (18). Thus the role for TF in angiogenesis is supported by both clinical and experimental work and the notion that TF plays an important role in tumor vascularization has gained widespread acceptance.

So far, the role of downstream coagulation factors in the angiogenic process has remained unclear. However, recently, using specific inhibitors to the TF:FVIIa:FXa complex and to FXa alone, the angiogenic process was shown to be dependent on FVIIa but not FXa (26). Therefore, angiogenesis appears to be fully dependent on TF:FVIIa intracellular function, rather than its role as activator of the coagulation cascade.

TF AND INTRACELLULAR SIGNALING

Metastasis is critically dependent on a number of processes that rely on certain intracellular changes. A cell may start expressing proteins that interfere with cellular adhesion, cell movement, intravasation and extravasation, the immune system, and growth of the secondary tumor. In addition, cytoskeletal reorganization may take place. These processes are generally controlled by a tightly regulated process, called signal transduction, which starts after appropriate stimulation of the cell, for instance the binding of a ligand to its receptor. As will be discussed below, TF and FVIIa induce various signal transduction events that may be involved in TF-dependent metastasis.

Striking homology has been observed between TF and the interferon γ–type receptors both in their secondary structure (amino acid composition [4]) as well as in their tertiary structure (crystallographic structures [27,28]), 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 for signal transduction. As already mentioned, the TF cytoplasmic tail has 2 potential phosphorylation sites, and these serine residues are readily phosphorylated upon protein kinase C (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-280 (29). Third, the cytoplasmic tail appears essential for the generation of calcium transients and for tumorigenic TF effects, such as the generation of vascular endothelial growth factor (30).

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 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 (31). 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 (PARs), a class of receptors that needs proteolytic processing for activation. PAR1, PAR3, and PAR4 are 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 PAR1-deficient lung fibroblasts 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 (32). 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 (33).

A new vision on FVIIa-dependent protease-activated receptor activation was provided by Riewald and Ruf (34); 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 those triggered by the individual coagulation factors. Although FVIIa proteolytic activity in this complex was inhibited, FXa efficiently activated both PAR1 and PAR2. Thus, TF:FVIIa appears to induce signaling both via proteolytic activation of PAR2 at higher concentrations and via FXa-mediated PAR1 and PAR2 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 both pathways.

The 1st observation confirming a role for FVIIa and TF in signal transduction came from Røttingen and others (35), who showed that FVIIa-induced transient cytosolic calcium signals in J82 cells, transfected African Green monkey kidney (COS-1) cells, Madin-Darby canine kidney cells, and human endothelial cells induced to synthesize TF (Figure 2). 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 (36). These data suggest that the FVIIa/TF interaction triggers the classical phospholipase C (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 and coworkers (32) 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 and coworkers 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 (37). Finally, in baby hamster kidney (BHK) cells, stably transfected with TF

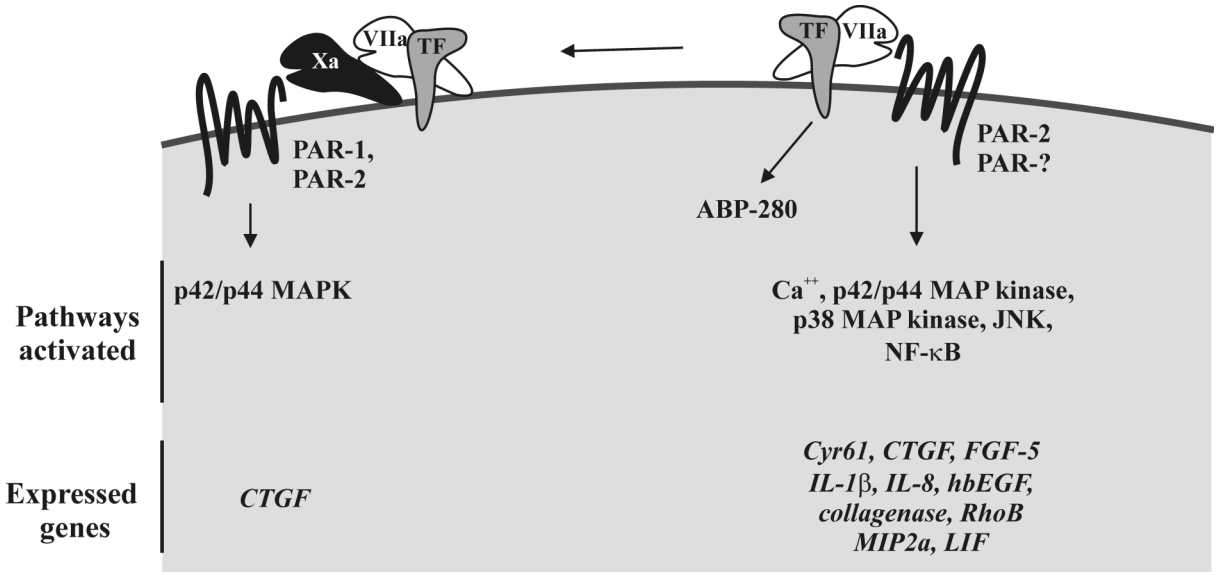

Figure 2. FVIIa:TF-induced signal transduction. Upon FVIIa:TF complex formation, PAR2 or a still unknown PAR is proteolytically activated. Subsequently, depending on the cell type, events such as calcium signaling, activation of the MAP kinase pathways, and nuclear translocation of transcription factors such as NF-κB takes place. This will eventually result in the upregulation of a set of mRNAs. Alternatively, the cytoplasmic tail may bind proteins such as actin-binding protein-280 upon phosphorylation. The FVIIa:TF complex may also serve as a scaffold for FXa, targeting PAR1 and PAR2. 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.

(BHK^{TF}), FVIIa does not induce calcium signals (33). From these data it becomes clear that FVIIa:TF-induced signaling shows large variety and is absolutely dependent on the cell type used.

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 (38,39). The 1st report on FVIIa/TF dependent kinase activation (40) describes the transient activation of the promitogenic p42/p44 MAP kinase. The activation of MAP kinase was shown to be dependent on the activation of the upstream MAP kinase kinase MEK because MEK inhibitor PD98059 abolished this signaling. Furthermore, functional FVIIa was absolutely required for this effect because FVIIa that was blocked in its active site did not induce MAP kinase activation. Sørensen and others (31) 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, A14 fibroblasts, and Madin Darby canine kidney cells (41–43,33), in which MAP kinase activation is mostly dependent on the GTPase Ras and the kinases c-Raf, Src, and PI3-kinase (43,44). 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 nonexistent because FVIIa does not appear to have any mitogenic effects at physiological concentrations (45,46).

Next to p42/p44 MAP kinase, the MAP kinase family consists of at least 2 more major isoforms, being p38 MAP kinase and c-Jun N-terminal kinase, also termed stress-activated 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 c-Jun N-terminal kinase activation is highly dependent on FVIIa proteolytic activity (41,43). The physiological relevance of FVIIa/TF-induced p38 MAP kinase signaling is still unknown, but it may turn out essential for TF-associated angiogenesis, tumorigenesis, and metastasis via activation of gene transcription.

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 (see Figure 2). Genes regulated by FVIIa can be divided into several categories: growth factors, cytokines transcriptional regulators, and genes regulating cell organization and motility (47,48)—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, heparin-based epidermal growth factor, and connective tissue growth factor mRNA, whereas in lung fibroblasts, FVIIa has been demonstrated to enhance connective tissue growth factor and *Cyr61* mRNA expression. The latter 2 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, however, especially IL-1β and IL-8, especially, are strong inducers of angiogenesis.

TF:FVIIa-induced protein synthesis has also recently been described: a physiological concentration of FVIIa induces protein synthesis within 30 min in both HaCaT keratinocytes as well as in BHK^{TF} cells (49). Induction of protein synthesis is regulated through FVIIa-driven activation of the ribosome as well as upregulation of eukaryotic elongation factors eEF1a and eEF2, which facilitate transfer of amino acids to the ribosome. Such mechanisms ultimately lead to FVIIa-induced production of proteins such as IL-8 (50), which may play a role in angiogenesis and metastasis.

TF AND CELL SURVIVAL

Metastatic cells frequently elude apoptosis, either during migration through the blood stream or once arrived at the site of implantation. Strikingly, TF:FVIIa complex formation leads to activation of the anti-apoptotic kinases, MAP kinase and PI-3 kinase, and could therefore, provide such an anti-apoptotic signal in TF-driven metastasis. Indeed, FVIIa appears to be capable of inducing cell survival in BHK and CHO cells overexpressing TF; the complex potently reverses serum starvation-induced apoptotic blebbing, formation of nuclei with chromatin-condensed bodies, DNA degradation, and activation of caspase 3 (51), which are all hallmarks of apoptosis. More recently, it was shown that FVIIa and TF, in addition to inhibiting apoptosis in serum starved cells, also induces cell survival in cells that undergo anoikis (52). Anoikis is a special form of apoptosis that occurs when cells detach from the extracellular matrix and thus lack adhesion signaling. Metastatic cells that travel through the bloodstream have often lost this need for adhesion and anoikis is inhibited. Therefore, it appears that FVIIa:TF signaling can replace adhesion signaling, leading to aberrant cell survival. Treatment of cells with inhibitors such as active site–inhibited FVIIa (FVIIai) efficiently inhibits this process and therefore forms an interesting option for anti–cancer strategies.

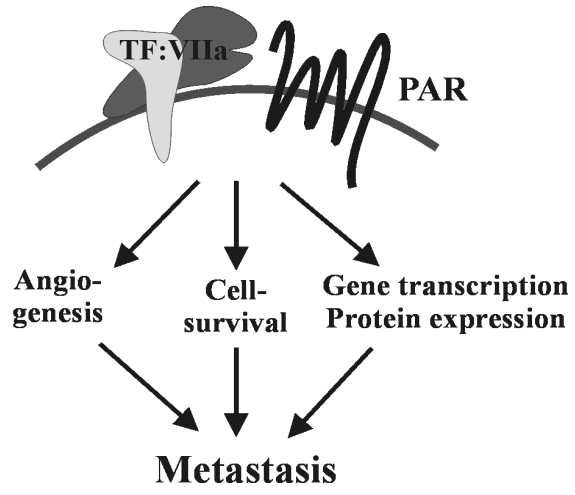

Figure 3. Hypothetical links between TF:FVIIa complex formation and metastasis. TF:FVIIa may influence metastasis through induction of tumor angiogenesis, inhibition of apoptosis and expression of proteins that may facilitate prometastatic events such as matrix detachment and migration.

TF AS AN EXTRACELLULAR SUBSTRATE ADHESION MOLECULE

In addition to function as a receptor, TF is also implicated in substrate adherence. TF-expressing granulocytes appear to bind to endothelial cells, using TF as a ligand-binding protein (53). 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 (29). Finally, spreading of J82 cells on FVIIa-coated coverslips induced phosphorylation of focal adhesion kinase to levels comparable to cells adherent to fibronectin. Because focal adhesion kinase 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 and could potentially be involved in mediating metastatic cell adhesion to the endothelium, prior to extravasation.

CONCLUSIONS

Substantial evidence exists that the primary initiator of the coagulation cascade, TF, is involved in oncogenic processes and specifically tumor metastasis. TF may influence metastasis via a multitude of processes, among which tumor angiogenesis at the site of secondary tumor growth, production of proteins that may create a favorable environment for metastasis, inhibition of apoptosis and anoikis, and possibly by functioning as an adhesion molecule involved in adhesion to the endothelium (Figure 3). Although it is unclear how the downstream coagulation factors FXa and thrombin influence metastasis, an increasing number of studies point out a direct role for TF and FVIIa in this oncogenic process through the activation of signal transduction pathways via the proteolytic

cleavage of PARs. However, it may be that the action of other coagulation factors facilitate TF:FVIIa effects on metastasis. Gaining knowledge on this subject is of extreme importance, especially for the potential clinical use of coagulation cascade inhibitors such as active site–blocked FVIIa, the FXa inhibitor tick anticoagulant protein, the thrombin inhibitor hirudin, and general inhibitors such as heparin or heparin derivates as anticancer agents.

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