Signaling of the Tissue Factor Coagulation Pathway in Angiogenesis and Cancer
Mattias Belting, Jasimuddin Ahamed, Wolfram Ruf

Abstract—Activation of coagulation precedes or coincides with angiogenesis in wound healing and postischemic tissue regeneration. Advanced cancer is associated with a hypercoagulable state, and tissue factor expression by cancer cells has received widespread attention because of its significant contribution to the pathogenesis of cancer progression and metastasis. Our recent work demonstrates that tissue factor–mediated cellular signaling is relevant to cancer angiogenesis. Here we review the molecular mechanisms of tissue factor pathways in angiogenesis and tumorigenesis with emphasis on the intriguing role for tissue factor cytoplasmic domain signaling. (Arterioscler Thromb Vasc Biol. 2005;25:0-0.)

Key Words:

Accumulating evidence has transformed our view of tissue factor (TF) from being a relatively simple protease coreceptor in the initiation phase of blood coagulation into a multi-facetted transmembrane signaling receptor involved in the regulation of angiogenesis, tumor growth, metastasis, and inflammation. TF-initiated coagulation generates thrombin, and this protease signals through the activation of a unique class of G protein-coupled protease activated receptors (PAR) 1, 3, and 4.1 Signaling activities of TF may occur before or even independently of thrombin and fibrin generation, platelet activation, and blood clot formation.2 These TF-dependent, upstream signaling events are also mediated by the PAR family of G-protein–coupled receptors, in particular PAR1 and PAR2. The TF-VIIa complex activates PAR2 and the product of initiation of coagulation, Xa, while still assembled in the transient ternary TF-VIIa-Xa complex, signals through PAR1 or PAR2. Both upstream PAR-mediated signaling and downstream coagulation events may thus contribute to cancer biology. In vivo, thrombin is generally considered to be the physiological activator of PARs in tumor biology and angiogenesis. We have recently provided in vivo evidence that PAR2, which is not cleaved by thrombin, is linked to TF-dependent angiogenesis.3 Thus, broader roles of TF-initiated coagulation emerge in the regulation of cancer progression and angiogenesis. In this brief review, we will address pathways by which TF supports metastasis, tumor growth, and angiogenesis. The accumulating data indicate that TF expressed by tumors cells as well as host cells initiates direct or indirect signaling events that support tumor development by distinct mechanisms (Figure 1).

Coagulation and Metastasis
Metastasis is the result of multiple pathways that act in concert to provide tumor cells with the capacity to migrate, gain access to the blood stream or lymphatic vessels, and ultimately home to distant sites. Preclinical studies have clearly established that inhibition of platelet function correlates with decreased metastasis and tumor growth. Platelets facilitate metastasis by promoting tumor cell survival at the site of metastasis.4 This involves protection of tumor cells from natural killer cell attack, increased tumor cell association with leukocytes altering the adhesion to endothelial cells, as well as the secretion of proangiogenic and tumor cell mitogenic substances from platelet granules (Figure 1A).

Several studies have established a role for fibrinogen as a scaffolding molecule for cell migration and for the binding of promigratory and angiogenic growth factors, most importantly vascular endothelial growth factor (VEGF).5 However, challenging the general view that fibrinogen contributes to tumor stroma formation and angiogenesis, there was no noticeable difference in tumor growth and vessel density when comparing wild-type and fibrinogen-deficient mice.6 Moreover, granulation tissue formation and angiogenesis during wound healing, ie, normal tissue regeneration, appear to be little influenced by fibrinogen deficiency. However, fibrinogen plays an important role in tumor cell dissemination to establish lymph node as well as hematogenous metastases to the lung, which in part involves stabilizing the platelet thrombus that encases and protects arrested tumor cells.7 Fibrin generation in hematogenous metastasis is the result of coagulation activation by tumor cell–expressed TF that leads to thrombin generation.8 Is the sole role of TF in...
metastasis to generate fibrin that stabilizes interactions of the arrested tumor cell in the target organ? Several lines of evidence argue against this simplified concept. Pretreatment of tumor cells with thrombin changes their adhesive behavior and increases pulmonary metastasis. Signaling by thrombin is implicated because metastasis can similarly be enhanced with specific nonproteolytic agonist peptides for the thrombin receptor PAR1.9 The primary target of thrombin-dependent PAR1 signaling is the tumor cell. Host cell thrombin responses appear to be of lesser importance, because tumor cells metastasize with high efficiency in PAR1-deficient mice.10

Thrombin influences at least 2 important pathways in tumor cells. Thrombin-dependent PAR1 signaling induces proliferation of metastatic tumor cells,11 and at low doses thrombin has been shown to enhance survival of tumor cells,12 consistent with findings in other cell types.13 Although at higher doses thrombin can induce apoptosis, one possible function of thrombin is thus modulation of apoptosis during metastasis. So far there is no in vivo data to support this concept. On the other hand, thrombin signaling through PAR1 influences tumor cell motility. In a crosstalk of the PAR1 cytoplasmic tail with α3β1, integrin mediated migration of tumor cells can be enhanced.14 However, thrombin has also been shown to reduce breast cancer motility.15 And coagulation activation is important for tumor cell adhesive spreading, which may counteract motility at sites of metastatic tumor implantation.16 In addition, a potential signaling crosstalk between PAR1 and PAR2 in tumor cell metastasis further complicates the picture. Chemokinetic effects of thrombin on metastatic melanoma cells were not reproduced by stimulation with PAR1 agonist peptides. Intriguingly, combined treatment with PAR1 and PAR2 agonist peptides recapitulated the thrombin effect on migration, a result that was confirmed in prostate cancer cells.17 Because metastasis was enhanced by PAR2 stimulation, thrombin’s prometastatic effects on tumor cells may involve cross-activation of PAR2. The tethered ligand sequence of PAR1 can activate PAR2, and cross-activation of PAR2 by thrombin-cleaved PAR1 has been suggested for endothelial cells.18 Thus, although thrombin signaling is clearly important in metastasis, additional studies are required to fully define the relative importance of underlying signaling pathways in vivo.

Regulation of cell motility is a possible pathway by which TF directly influences tumor cell metastasis. Experimental hematogenous metastasis is dependent on TF-VIIa driven thrombin generation as well as TF cytoplasmic domain signaling.19,20 We have recently demonstrated that the TF cytoplasmic domain is a negative regulator of cell migration mediated by the integrin α3β1.21 Indeed, integrin α3β1 plays an important role in metastatic arrest to patches of laminin-5 exposed between endothelial cells in target organs.22 These in vivo imaging studies of metastatic arrest indicate that spreading of the cells, rather than migration and extravasation across the endothelium, is a key determinant for tumor cell survival during early stages of metastasis that are also dependent on thrombin.23 TF cytoplasmic domain signaling does not interfere with adhesion and spreading on laminin-5, but selectively suppresses cell motility dependent on activated α3β1 integrin. The TF cytoplasmic domain may enhance efficiency of metastasis by stabilizing the crucial spreading of tumor cells on patches of extracellular matrix exposed in target organs. In addition to metastasis, TF cytoplasmic signaling may influence other aspects of tumor cell biology, as discussed below after the following brief summary of our current view of the signaling crosstalk of TF with integrins.

**TF as a Regulator of Integrin Function**

The demonstration of TF ligation--induced cell spreading indicated that TF influences pathways relevant for cell adhesion.24,25 Three lines of evidence support the concept that the TF extracellular domain is involved in an interaction with integrins: (1) Cell spreading on TF ligands is blocked by anti-β1 antibody, (2) purified TF extracellular domain serves as an adhesive ligand for several integrin heterodimers expressed in a heterologous CHO-cell background, and (3) one specific antibody to TF did not support TF-ligation mediated cell spreading, suggesting a defined epitope in the TF extracellular domain that is involved in an interaction with integrins.21 This antibody, 5G9, binds to the macromolecular substrate exosite on TF. Thus, integrin interaction and activation of coagulation by substrate assembly appear to be
mutually exclusive activities of TF (see Figure 2 for molecular models of integrin or substrate in possible conformations associated with TF-VIIa). This may explain how cellular TF can exert "noncoagulant" functions despite being exposed to plasma components, such as during metastasis and tumor cell invasion.

Although we found that the TF extracellular domain interacts with several integrin heterodimers, there was no evidence that this resulted in competition or inhibition of extracellular matrix interaction by these adhesive receptors. Rather, TF specifically suppressed cell migration on certain extracellular matrices. In keratinocytes and melanoma cells, TF inhibited the migration on laminin-5 that is dependent on activation of integrin α3β1. This inhibition required the TF cytoplasmic domain, and mutagenesis showed that integrin function is suppressed when the TF cytoplasmic domain was not phosphorylated. The suppression of migration on laminin-5 can be reversed by blocking the presumed integrin binding site on TF with antibody 5G9. Antibody binding appears to trigger cell signaling that leads to phosphorylation of the TF cytoplasmic domain and subsequent release of integrin inhibition. TF phosphorylation is specifically induced by PAR2 signaling, and protease-driven TF-VIIa activation of PAR2 is sufficient to release integrin inhibition in dependence of phosphorylation of the TF cytoplasmic domain. Thus, TF-VIIa signaling may simultaneously counteract integrin suppression by phosphorylating the TF cytoplasmic domain and trigger promigratory PAR2 activation.

Recently, active site–blocked proteolytically-inactive VIIa was shown to elicit Rac and p38 MAP kinase activation in a pathway that required the TF cytoplasmic domain. Considering the use of supraphysiological, extremely high concentrations of VIIa in this study, we suspect that these experiments measured competition with as yet to be defined extracellular interactions of TF. It should be noted that active site–blocked VIIa did not release the suppression of laminin-5 migration in keratinocytes, indicating that VIIa does not compete with relevant interactions that regulate promigratory functions of integrin α3β1. Thus, one can envision that TF binds simultaneously VIIa as well as integrin through the substrate binding site. In this constellation, TF-VIIa complex signaling may regulate integrin function. It is notable that the TF-VIIa complex is indeed localized at the leading edge of invasive cancer, whereas more central areas of bladder carcinoma only stained for TF. Whether specific integrins are colocalized with TF expressed in cancer cells in vivo is currently unknown.

There is good experimental evidence that TF-VIIa signaling stimulates cell motility. In fibroblasts, TF-VIIa-signaling enhances platelet derived growth factor BB (PDGF-BB)-stimulated migration, suggesting signaling crosstalk between G-coupled receptors (PARs) and tyrosine kinase receptors in migration. Similarly, signaling of the TF-VIIa-Xa complex is promigratory in a breast carcinoma cell line. TF-VIIa signaling is promigratory and proinvasive dependent on PAR2 in breast carcinoma. Cleavage blocking antibodies clearly showed that PAR2 activation, rather than a bystander effect, is responsible for the promigratory effects of TF-VIIa signaling. Notably, this promigratory effect appears to be indirect and mediated by the release of interleukin (IL) 8. In this context, it is notable that IL 8 expression was also induced by TF-VIIa signaling in keratinocytes, indicating that migration of noncancerous epithelia may similarly be regulated by TF-VIIa signaling. Thus, TF-dependent PAR signaling may stimulate both pathological migration (tumor cell invasion) and physiological re-epithelialization (keratinocyte mediated wound healing).

This raises the question whether tumor cells have lost regulatory mechanisms that typically control these pathways under physiological conditions. One possibility is that dephosphorylation of the TF cytoplasmic domain normally acts as a break to suppress integrin-dependent migration, and that deregulated TF phosphorylation contributes to the aggressive behavior of invasive tumor cells. In addition, aggressive cancer cells upregulate PAR1, which can stimulate invasiveness of tumor cells. PAR1 has recently been shown to be cleaved by matrix metalloproteinases derived from tumor-associated stromal cells, raising the intriguing possibility that PAR1 and PAR2 provide redundant pathways to exploit promigratory cues form a dynamically changing tumor microenvironment.

Role of TF-Mediated Signaling in the Regulation of the Tumor Microenvironment

The tumor microenvironment is shaped by a crosstalk of tumor cells with host stromal and vascular cells. Persistent
activation of the coagulant pathway is associated with an increased risk of developing cancer, especially in the digestive tract.34 This correlative evidence suggests a link between TF and tumor development, and in vivo animal models of cancer progression further support this concept. Carcinoma cells frequently express TF. In many cases, eg, in colorectal and breast cancer, the malignant potential of tumor cells correlates with their TF expression level. Expanding on previous studies that showed an enhanced tumor growth in TF transfected cells (reviewed in5,35), a recent report demonstrated in human colorectal cancer cells an upregulation of TF expression on oncogene K-ras activation and a necessary role for TF to support the growth of these tumors in vivo.36 Importantly, siRNA-mediated knock-down of TF did not affect tumor cell proliferation and spheroid formation in vitro. This indicates that (1) TF specifically regulates the interplay of tumor cells with the tumor microenvironment, eg, by generating thrombin, (2) TF initiates host factor-dependent pathways of autocrine signaling, eg, TF-VIIa mediated survival37,38 or other downstream effects of PAR2 signaling, or (3) TF acts as a tumor promoter dependent on trophic effects uniquely present in vivo, eg, specific extracellular matrices or growth factors (Figure 1).

Indeed, TF expression by tumor cells may trigger several pathways that shape the tumor microenvironment. Coagulation activation produces proteolytic fragments of proteases, inhibitors, and extracellular matrix components with potent regulatory effects on angiogenesis.39 The most frequently discussed mechanism by which TF regulates angiogenesis is through altered expression of angiogenic factors, foremost an upregulation of VEGF and a downregulation of thrombospindin.36,40,41 In vivo, TF may drive local thrombin generation and thus indirectly induce VEGF signaling either by paracrine PAR1 signaling in stromal cells or by PAR1 activation of tumor cell in an autocrine manner. Whether VEGF induction is of importance in the context of hypoxia-driven tumor angiogenesis remains uncertain, but thrombin regulates other aspects of signaling in endothelial cells; eg, thrombin upregulates VEGF receptor through PAR1 signaling42 and induces endocytosis of endoglin and transforming growth factor (TGF) β-receptor II.43

Expression of antiangiogenic thrombospindins is suppressed in TF-positive tumor cells, and this may act in concert to enhance the action of proangiogenic growth factors. Whether thrombospindins are regulated directly by TF overexpression in tumor cells is controversial.36,40 Considering the complex signaling crosstalk of TF with integrins, gene expression may be influenced by adhesion to specific extracellular matrices. In this context, the TF cytoplasmic domain may act as a positive regulator of tumor growth under certain conditions,41 although the details of these signaling events are incompletely understood. In addition, direct effects of TF on tumor cell proliferation, apoptosis, and local invasion may be highly dependent on a unique extracellular environment provided by stromal cells. Thus, indirect effects of TF through protease signaling targeting host cells may reciprocally influence direct effects of TF on the tumor cells specifically in the context of the tumor microenvironment.

Coagulation and Angiogenesis

Host cell–expressed TF makes independent contributions to tumor biology, foremost by regulating angiogenesis. Angiogenesis plays a crucial role during development, tissue regeneration in wound healing, and postischemic tissue repair. An important indication that TF plays a role in physiological angiogenesis came from the TF knock-out mouse, showing embryonic lethality around day 10.5 pc as a result of a failure of the vasculature in the yolk sac.44 PAR1 deficiency produces a very similar although only partially lethal developmental phenotype, and PAR1 expression in endothelial cells is sufficient to rescue embryonic lethality.45 It is thus conceivable that TF on vascular wall cells is required to generate thrombin signaling through PAR1 in a paracrine manner to the endothelium. The importance of G-protein–coupled receptor signaling at this stage of development is further highlighted by the similar phenotype of mice lacking Gα13 to which PAR1 couples.46 In normal endothelium of the adult, TF is absent or only present at minute amounts, whereas subendothelial vessel wall cells, ie, fibroblasts and smooth muscle cells, constitutively express TF. Thus, there is no firm proof that TF expression in endothelial cells regulates vascular maintenance in normal physiological condition. In contrast, TF upregulation has been found in the endothelium during pathological conditions of inflammation and tumor progression.47 In animal models, inhibitors of TF-VIIa suppress tumor growth and in vivo angiogenesis, whereas potent anticoagulants that inhibit Xa display no such activity.48 Thrombin can also stimulate angiogenesis, but so far there is no clear genetic evidence that thrombin signaling though PAR1 in endothelial cells drives pathological angiogenesis. Inhibition of tumor growth by TF-specific inhibitors raised the possibility that TF signaling may drive tumor angiogenesis. We approached this question by characterizing angiogenesis in knock-in mice that lack the TF cytoplasmic domain (TFΔCT mice). Tumor angiogenesis was evaluated by transplanting syngeneic tumor to monitor subcutaneous tumor growth. Surprisingly, tumors grew 2-fold faster in these mice relative to wild-type controls, suggesting that the TF cytoplasmic domain in host cells plays a negative regulatory role in tumor angiogenesis.3 TFΔCT mice formed greater tumors as compared with wild-type animals although injected tumor cells expressed high levels of TF, indicating that host cell TF regulated tumor expansion independent of local thrombin formation by tumor cells.

The conclusion of enhanced angiogenesis in these mice was further substantiated ex vivo by demonstrating that aortic ring endothelial cell sprouting was enhanced in TFΔCT as compared with wild-type animals. Enhanced sprouting in this assay was found to be dependent on serum components, and specific protease inhibitors showed that VIIa is a critical serum factor necessary for the specific enhancement of sprouting from TFΔCT aortas. Furthermore, purified VIIa enhanced angiogenesis, however only when PDGF BB was present. There was no synergy with other major proangiogenic growth factors, including VEGF-A, basic fibroblast growth factor, or PDGF-AA. Further studies with PAR2/TFΔCT double knock-out mice provided genetic evidence that the TF-VIIa signaling phenotype of TFΔCT aortas was depen-
dent on PAR2. Importantly, PAR2 deletion per se did not affect angiogenesis, and angiogenesis in the PAR2/TFA\textsuperscript{CT} double knockout was similar to that in wild-type mice. One possible explanation for this finding is that the TF cytoplasmic domain exerts a potent negative regulatory control on PAR2 to prevent PAR2-dependent proangiogenic signaling. Consistent with such a signaling crosstalk between PAR2 and the TF cytoplasmic domain, we found that suppression of sprouting by overexpression of TF could only be achieved in wild-type but not in PAR2-deficient aortas.

Developmental angiogenesis in the retina was also enhanced in TF\textsuperscript{CT} mice and this phenotype was lost in PAR2/TFA\textsuperscript{CT} double knock-out mice, demonstrating that TF cytoplasmic domain signaling and PAR2 are linked in vivo. The organization of the retina vasculature, recruitment of pericytes, and physiological remodeling all appeared normal, suggesting that endothelial sprouting is accelerated on loss of TF cytoplasmic domain. These data are in line with data suggesting that endothelial sprouting is accelerated on loss of PAR2 signaling\textsuperscript{27,29} and antimigratory TF cytoplasmic domain signaling\textsuperscript{21}.

A key question arising from these findings is how TF cytoplasmic domain inhibitory activity is regulated in physiological and pathological angiogenesis. In endothelial cells, the TF cytoplasmic domain is unphosphorylated by default, but activation of PKC-\(\alpha\) triggers phosphorylation. PAR2 but not PAR1 signaling leads to prolonged PKC-\(\alpha\) activation and downstream TF cytoplasmic domain phosphorylation. Activation of phosphatidylocholine-specific phospholipase C upstream of PKC-\(\alpha\) accounts for this unique signaling response of PAR2 signaling\textsuperscript{26}. However, the-ester modification of the cytoplasmic cysteine counteracts TF phosphorylation.\textsuperscript{49} Thus, PAR2 expression and changes in palmitoylation status of TF emerge as key regulators of TF cytoplasmic domain phosphorylation.

We evaluated the role of TF phosphorylation in pathological neovascularization of the eye. Phosphorylated TF was associated with abnormal proliferative neovascularization in retinas of diabetic patients, whereas TF in normal vessel wall and neuronal tissue was not phosphorylated. PAR2 was clearly detectable in pathological vessels, suggesting a concept that TF phosphorylation in the context of PAR2 signaling may switch off the suppressive function of the TF cytoplasmic domain and facilitate PAR2-dependent pathological angiogenesis. Conceivably, TF may support physiological angiogenesis as well, but dephosphorylation of the TF cytoplasmic domain may serve as a break to arrest excessive neovascularization. Indeed, the TF cytoplasmic domain in its unphosphorylated state regulates integrin \(\alpha3\beta1\), which is also the target for antiangiogenic effects of TIMP-2.\textsuperscript{50}

Conclusions and Future Directions
These studies document an important role of the TF cytoplasmic domain as a regulator of TF-dependent signaling pathways. The signaling functions of the TF cytoplasmic domain are controlled by Ser phosphorylation that induces a conformational switch. In the unphosphorylated state the TF cytoplasmic domain suppresses integrin-dependent migration, and phosphorylation of TF can shut off this suppressive pathway. It is presently unknown whether phosphorylated TF acquires new signaling properties. It is a reasonable hypothesis for future studies that the phosphorylation status of TF is a determinant for documented positive and negative effects of TF-mediated signaling in tumor cell biology. Characterization of ligand binding to the phosphorylated and nonphosphorylated TF cytoplasmic domain will be instrumental to understand the signaling complexes that associate with TF. Although PKC-\(\alpha\) is required for TF cytoplasmic domain phosphorylation at Ser\textsuperscript{253} and subsequent phosphorylation of Ser\textsuperscript{259}, the Pro-directed kinase that targets the latter residue remains to be defined. In addition, pathways that influence the palmitoylation of TF represent additional directions of research. It will be important to establish whether phosphorylated TF is a general regulator of activated endothelium in pathological angiogenesis and whether tumor cell TF is phosphorylated, in particular in invasive areas of carcinoma. Considering that tumor cells are known to shed TF into the circulation, it will be of interest whether circulating phosphorylated TF may serve as a diagnostic and/or prognostic marker of tumor progression or pathological angiogenesis in diabetes and cancer.

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