Tissue Factor and PAR2 Signaling in the Tumor Microenvironment

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Abstract—Diverse oncogenic transformations result in the constitutive expression of tissue factor (TF) in cancer cells. The local and systemic activation of the coagulation cascade has long been a recognized hallmark for aggressive cancer, but genetic mouse models and new experimental therapeutics have only recently demonstrated crucial roles for TF initiated cell signaling in the pathogenesis of cancer. On tumor cells, the TF-VIIa binary complex mediates activation of protease activated receptor (PAR) 2 and thereby shapes the tumor microenvironment by inducing an array of proangiogenic and immune modulating cytokines, chemokines, and growth factors. PAR2 also uniquely triggers tumor cell migration by G protein–independent pathways through β-arrestin scaffolding. Metastatic tumor cells use additional signaling networks of the coagulation cascade by activating PAR1 through thrombin or the ternary TF-VIIa-Xa signaling complex in the vascular and potentially lymphatic system. Selective antagonists of TF-VIIa-PAR2 signaling may be used as antiangiogenic therapy without increasing the risk of bleeding, whereas coagulation and associated signaling pathways on platelets and other host cells may be targeted for therapeutic benefit in advanced cancer and metastatic disease. (Arterioscler Thromb Vasc Biol. 2009;29:1999-2004.)

Key Words: thrombosis ■ cancer ■ G protein-coupled receptor ■ coagulation signaling

A prothrombotic state is one of the hallmarks of advanced cancer, and thromboembolic disease contributes significantly to the mortality of cancer patients (reviewed in1). Tissue factor (TF), the cellular activator of the coagulation cascade, is central to the hypercoagulable state of cancer patients and responsible for local thrombin generation and fibrin deposition in the tumor stroma. TF also triggers remote thrombotic complications involving procoagulant TF+ microparticles2 with potential contribution from other cancer procoagulants (reviewed in3). TF-dependent coagulation generates thrombin and induces pleiotrophic cellular effects of thrombin on platelets through G protein–coupled protease-activated receptors (PARs)4 as well as thrombin-initiated vascular-protective signaling of the endogenous activated protein C–EPCR–PAR1 pathway.5 Direct signaling by TF-associated proteases are mediated by the binary TF-VIIa enzyme complex that activates PAR2 or the ternary TF-VIIa-Xa coagulation initiation complex in which Xa efficiently cleaves PAR2 as well as PAR1.6 These signaling complexes involve distinct cellular pools of TF.7 Oncogenic mutations of K-ras, upregulation of oncogenic epidermal growth factor receptors (EGFR), or loss of tumor suppressors p53 and PTEN result in constitutive upregulation of TF, and hypoxia can amplify tumor cell TF expression in certain cancers (reviewed in8). Furthermore, hypoxia induces the synthesis of coagulation factor VIIa in various cancer types and ectopically synthesized VII by TF-expressing cells can trigger X activation and tumor cell migration and invasion.9 Potentially, the ectopic synthesis of VIIa may turn on direct TF-VIIa signaling in cancer cells before alterations in vascular barrier function that typically maintains a separation of blood components and TF+ cells located in the extravascular space.

Progression of cancer from noninvasive to invasive disease is critically dependent on hypoxia-induced expression of VEGF that not only promotes vascular hyperpermeability and extravasation of coagulation factors, but also induces TF in the host compartment (reviewed in9). In advanced cancer, tumor associated macrophages, endothelial cells, and myofibroblasts contribute to the pool of TF expressed in the tumor microenvironment (TME). These host cells may further promote tumor progression through direct TF (reviewed in10) or indirect thrombin-mediated coagulation signaling pathways (reviewed in11). Importantly, severe reduction of TF in both host and tumor cells completely aborted the growth of teratomas.12 Because tumor cells can shed TF-positive microparticles, thrombin generation in the TME may be restored, even when the host compartment is largely devoid of TF. Thus, TF and PAR signaling may make different contributions to tumor progression dependent on the tumor type and the stage-specific composition of the TME and different
tumor models may be more sensitive to evaluating thrombin’s effects.14

Several studies have documented that the levels of TF expression in primary colorectal, breast, and pancreatic cancer correlate with aggressive cancer phenotypes and metastatic disease (reviewed in8). Moreover, alternative spliced mRNA of TF was found in pancreatic, hepatocellular, and leukemia cancer cell lines15 and overexpression of alternative spliced truncated TF promotes angiogenesis by incompletely understood mechanisms.16 Expression of full-length TF also increases tumor growth properties in several experimental models (reviewed in17). However, TF expression in clinical and experimental tumors is not uniform. For example, cancer cell subpopulations expressing CD133, a marker for “cancer stem cells,” have higher levels of procoagulant TF, indicating a possible role for TF to locally generate fibrin to establish a tumor stem cell niche.18 In contrast, propagation of tissue culture–adapted breast cancer cells in the orthotopic tumor microenvironment of the mouse mammary pad significantly reduced TF expression levels and essentially abolished the ternary TF-VIIa-Xa signaling response, although these cells acquired a more aggressive growth phenotype that was dependent on TF-VIIa-PAR2 signaling.19 These studies indicate that the functional state of TF, rather than expression level, is a key determinant for tumor promoting activities of the TF pathway.

Only a small fraction of cellular TF contributes to procoagulant activity and a large pool of TF on intact cell surfaces is inert or “encrypted.”7 TF procoagulant activity is suppressed by limiting amounts of procoagulant membrane lipids,20 the chaperone protein Grp78/BIP,21 and by thiol pathways.7,22 Extracellular protein disulfide isomerase (PDI) is associated with TF on epithelial cells and contributes to the regulation of the switch between procoagulant and signaling conformations of TF. TF retains TF-VIIa-PAR2 signaling, but not coagulant function when the allosteric Cys186-Cys209 conformations of TF. TF retains TF-VIIa-PAR2 signaling, although these cells acquired a more aggressive growth phenotype that was dependent on TF-VIIa-PAR2 signaling.19 These studies indicate that the functional state of TF, rather than expression level, is a key determinant for tumor promoting activities of the TF pathway.

TF-VIIa-PAR2 Signaling in Tumor Progression

PAR2 cleavage results in the activation of classical G protein–coupled intracellular signals as well as G protein-independent pathways mediated by the recruitment of the intracellular adaptor protein β-arrestin. G protein-coupled signaling is involved in diverse signaling responses by which tumor cells shape the TME (Figure 1). TF-VIIa-PAR2 signaling of breast cancer cells induces a broad repertoire of proangiogenic factors such as VEGF,23 Cyr61, VEGF-C, CTGF, CXCL1, and IL8, and immune regulators such as granulocyte-macrophage colony stimulating factor (GM-CSF or CSF2) and macrophage colony stimulating factor (M-CSF or CSF1).20 Although some of these genes were also induced by PAR1 signaling in breast cancer cells, TF-VIIa-PAR2 signaling was the major stimulus for upregulation of the immune and angiogenesis regulators CXCL1, IL8, GM-CSF, and M-CSF.

GM-CSF and M-CSF play critical roles in the recruitment and differentiation of myeloid cell populations to the TME. Typically myeloid cells initiate adaptive immune responses, but tumor cells have developed protective mechanisms to prevent CD8 T and natural killer cell–dependent tumor killing. Myeloid populations in the TME are heterogeneous and include macrophages polarized to an immune suppressive M2 phenotype as well as immature dendritic cell populations that develop under the influence of GM-CSF.27 One of the key mechanisms of myeloid suppressor cell–mediated immune suppression is the release of arginase that reduces available pools of Arg required for T cell activation. In addition, myeloid suppressor cells and macrophages promote angiogenesis. The broad proangiogenic and potentially immune suppressive effects of TF-VIIa-PAR2 signaling are consistent with repeatedly documented roles of TF to act as a tumor promoter in vivo, without altering tumor cell proliferation in vitro. The emerging role of M-CSF as a regulator of lymphangiogenesis28 also points to potential roles of TF-VIIa-PAR2 signaling in changing the TME to facilitate the exit of tumor cells for metastatic tumor dissemination through lymphatic routes.

However, TF-PAR2 signaling also influences other aspects of tumor cell behavior, and some of these may play important roles in metastasis. Breast cancer cell TF-VIIa-PAR2 signaling induces antiapoptotic proteins, such as Birc3,26 and prevents apoptosis and death after the loss of cell adhesion (anoikis).29,30 Gα12/13 proteins promote breast cancer migration through activation of the Rho pathway,31 and PAR2 recruits the intracellular adaptor β-arrestin that acts as a scaffolding molecule for activated ERK.32 Although β-arrestins regulate receptor internalization,33 activated PAR2 recruits β-arrestin and ERK to pseudopodia of migrating cells.34 PAR2-dependent recruitment of β-arrestins promotes breast cancer migration35 and leads to dephosphorylation of cofilin by activating a phosphatase (chronophin) and
inhibiting LIM kinase. The activation of the cofillin pathway severs actin filaments required for cytoskeleton reorganization and is crucial for breast cancer invasion and metastasis.

TF-VIIa-PAR2-dependent breast cancer migration is also in part dependent on the secretion of chemokines, such as IL8, and conversely the induction of chemokines is dependent on the adhesion of tumor cells to specific extracellular matrices.

In noncancerous epithelial cells, TF regulates integrin α3β1-dependent migration on laminin 5 and binding of VIIa promotes the association of TF with integrins α3β1 and α6β1. The association of TF with these integrins is constitutive in cancer cells and crucial for TF-VIIa-PAR2 signaling. The constitutive association of TF with integrins may facilitate the escape of tumor cells from controlling cues of the extracellular environment. PAR1 signaling is also deregulated in tumor cells, and deregulated trafficking of PAR1 leads to increased transactivation of the EGF and ErbB2 receptors and tumor cell invasion, in particular when breast cancer cell PAR1 is activated by matrix metalloprotease (MMP) 1. In melanoma cells, the prometastatic activities of PAR1 are dependent on PAR2, pointing to important cooperative effects of PAR signaling in promoting tumor progression.

Mouse Models Support a Crucial Role of TF-VIIa-PAR2 Signaling in Regulating the TME

Transgenic animals provide excellent tools to evaluate the contributions of host and tumor cell factors to tumor progression. Hematogenous metastasis models in syngeneic or immune-deficient mice have been instrumental to demonstrate that the hemostatic system and platelets in particular play key roles in successful homing and tumor cell survival at distant sites (reviewed in ). Loss of the platelet thrombin receptors PAR4 and GP Ibα results in significantly reduced tumor cell metastasis to the lungs. In contrast to these pronounced contributions of thrombin pathways, syngeneic tumor growth and metastasis were normal in mice deficient in PAR1 that, unlike in humans, is not expressed on mouse platelets but only on other host cells. However, transplanted tumor models poorly measure tumor cell contributions as well as complex and compensatory roles of other cell types in the TME during early stages of tumor development.

Genetic mouse models of spontaneous tumor development are powerful tools to simultaneously evaluate the role of receptors in host and tumor cells and can be used as versatile tools to study tumor progression in immune competent hosts. The mouse mammary tumor virus (MMTV) promoter-driven expression of the Polyoma Middle T antigen (PyMT) results in spontaneous development of breast cancer that typically appears with complete penetrance in each mammary gland. Tumors of the PyMT model mimic important aspects of human breast cancer. In the hyperplasia and early adenoma stage, tumors are estrogen (ER) and progesterone receptor (PR)-positive and do not break through interstitial barriers of mammary gland acini. Loss of ER and PR expression and upregulation of the epidermal growth factor receptor ErbB2 and cyclin D1 mark the transition from adenoma to invasive carcinoma that spontaneously metastasize to the lungs.

The transition from adenoma to invasive carcinoma in this model is dependent on the angiogenic switch, characterized by an infiltration of the adenomas with neovascularature. In mice lacking M-CSF (CSF1), macrophages are inefficiently recruited to developing adenomas, resulting in delayed tumor progression. Macrophages infiltrate the TME during the angiogenic switch and, remarkably, are in close contact with tumor cells that escape into the blood stream. Mammary epithelial cell-specific deletion of the hypoxia-induced factor (HIF) 1α that is crucial for VEGF induction also delays the angiogenic switch in this breast cancer model. Thus, the early stages of tumor progression in the PyMT model are highly dependent on tumor cell-derived angiogenic regulators.

We used the PyMT model as an unbiased approach to study contributions of PARs to spontaneous breast cancer development. PAR1-deficiency did not impair tumor progression in this model, which was unexpected because PAR1 has previously been shown to be upregulated in human breast cancer samples. Tumor cell isolated from PAR1−/− mice lost all thrombin signaling, excluding the compensatory upregulation of other thrombin receptors. A limited role for PAR1 in breast cancer progression is also suggested from data demonstrating that overexpression of PAR1 in the mammary gland is insufficient to promote breast cancer development. However, PAR1 may make very specific contributions to tumor progression (eg, in the context of upregulation of the EGF receptor family member ErbB2). Excellent tumor models are available for further studies of potential tumor-promoting roles of PAR1, which is also widely expressed in other cancer types.

In contrast to PAR1-deficiency, there was a significant delay in the transition from adenomas to invasive carcinoma in PAR2−/− mice. Highly vascularized tumors appeared later in PAR2−/− mice relative to wild-type, consistent with a role for PAR2 signaling in the angiogenic switch. We focused on the TF-VIIa–induced ELR CXC-type chemokine CXCL1 that binds CXCR2 and has proangiogenic activities on endothelial cells. Levels of CXCL1 were significantly reduced in early tumors of PAR2−/− relative to wild-type mice, implicating this axis as an important component of the pathway by which TF-VIIa-PAR2 signaling regulates the angiogenic switch. Macrophages were also less abundant in early tumors of PAR2−/− mice, providing initial evidence that the recruitment of myeloid cells is another pathway that is dependent on PAR2 signaling. We succeeded to establish PAR2−/− tumor cell lines that grew slower relative to a similar wild-type line when transplanted into either wild-type or PAR2-deficient mice. This indicated that tumor cell, rather than host PAR2 signaling, makes the major contribution to breast cancer progression. This notion is further supported by data demonstrating improved growth properties of PAR2−/− PyMT cells on transduction of PAR2 (unpublished data, Schaffner and Ruf, 2009). The PyMT model mimics important aspects of human breast cancer. In the hyperplasia and early adenoma stage, tumors are estrogen (ER) and progesterone receptor (PR)-positive and do not break through interstitial barriers of mammary gland acini. Loss of ER and PR expression and upregulation of the epidermal growth factor receptor ErbB2 and cyclin D1 mark the transition from adenoma to invasive carcinoma that spontaneously metastasize to the lungs.

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model should also provide additional insight into the role of the TF cytoplasmic domain in tumor progression and metastasis, a topic that remains controversial in the literature.\textsuperscript{58–62}

**Thrombin Signaling Dominates in Metastatic Tumor Dissemination**

For metastatic homing at distant sites, tumor cells are dependent on invasion and migration to exit the TME and on mechanisms to survive in a rapidly changing environment that they encounter in the lymphatic compartment and blood stream. A role for TF in the extravasation from the TME was recently uncovered in a model of spontaneous metastasis from the chicken chorioallantoic membrane (CAM).\textsuperscript{63} Tumor cells selected for more efficient metastasis in this assay showed increased TF expression, detected by cell surface proteomic profiling. Blocking the TF substrate interaction and coagulation with a selective antibody decreased spontaneous metastasis of these highly metastatic cells from the CAM to the chicken embryo. While TF-VIIa binary complex signaling predominated in shaping the TME for optimal tumor growth, these data indicated that TF ternary complex regulated cell migration\textsuperscript{64} or thrombin pathways become the determining mechanisms, as tumors progress to metastatic disease. Consistently, inhibiting thrombin has a profound effect of spontaneous tumor cell metastasis.\textsuperscript{14}

This notion is further supported by experiments in the hematogenous metastasis assay in which blocking TF signaling has no effect on initial homing and survival of tumor cells. In contrast, inhibition of the ternary complex leads to rapid loss of viable tumor cells at metastatic sites and profoundly attenuates late stage metastatic disease.\textsuperscript{19, 65} In this setting, thrombin is crucial not only for the generation of a protective platelet and fibrin-rich envelope that protects the metastatic tumor cell from rapid clearance by natural killer cells, but also for the exposure of subendothelial matrix and tumor cell adhesion.\textsuperscript{66, 67} Thrombin is known to have endothelial barrier disruptive effects, but these are counterbalanced by signaling of the endogenous protein C (PC) pathway. Endothelial cell PC receptor (EPCR)/aPC–PAR1 signaling is barrier protective in vivo by crossactivating sphingosine 1 phosphate receptor 1.\textsuperscript{1, 5} Intriguingly, overexpression of EPCR attenuates hematogenous metastasis,\textsuperscript{68} pointing to novel control mechanisms at the vascular interface that can prevent metastasis.

**Therapeutic Opportunities in TF Initiated Signaling Pathways**

Thus, tumor cells rely on TF to accomplish environment specific tasks (ie, use TF signaling to induce the angiogenic switch and TF coagulation to accomplish successful metastatic homing). These data suggest that appropriate targeting of the TF pathway can prevent tumor progression both in early and late stages of disease. Blocking TF-VIIa-PAR2 signaling on tumor cells by a selective antibody is sufficient to attenuate angiogenesis and tumor growth.\textsuperscript{19} Profound reductions in tumor growth\textsuperscript{69} and spontaneous colorectal cancer development\textsuperscript{70} were also observed with NAPc2, a nematode-derived inhibitor that blocks TF-VIIa. NAPc2 has a complex TFPI-like inhibitory mechanism\textsuperscript{71} that blocks coagulation but holds nascent product Xa in an active conformation to cleave PAR1 or PAR2,\textsuperscript{6} making the interpretation of its antitumor effects less straightforward. The tumor growth suppressive activities of NAPc2 may result from tumor cell effects and indicate that binary TF-VIIa, rather than TF-VIIa-Xa ternary complex signaling promotes tumor progression. Alternatively, inhibition of local thrombin generation may in complex ways synergize with sustained ternary complex signaling or loss of binary signaling to promote tumor cell apoptosis. Consistent with efficient thrombin blockade NAPc2 blocks metastasis, and NAPc2 may be thus effective in both the early and late stages of tumor progression. NAPc2 has antiangiogenic effects in matrigel plug assays and retinal neovascularization,\textsuperscript{47, 69} indicating additional targets on the host compartment. Despite these impressive effects, potential concerns of bleeding remain if the coagulant limb of the TF pathway is blocked efficiently.\textsuperscript{72}

Although selective inhibition of TF-VIIa signaling appears to be feasible either by TF-directed antibody\textsuperscript{19} or potentially by antagonists of PAR2,\textsuperscript{73} it is currently unclear whether targeting the signaling of thrombin can prevent tumor progression, metastasis and ultimately prolong survival. Antagonists of PAR1 may not only block the important contributions of platelets to metastatic disease, but also attenuate directly tumor cell and host proangiogenic signaling in tumor progression.\textsuperscript{12} However, PAR2 may support alternative prometastatic pathways that can results in an escape from PAR1 antagonistic therapy.\textsuperscript{45}

Our experiments in the PyMT model found no major contributions of PAR2 to late-stage tumor growth. These data and similar findings with HIF1α-deficient tumor cells indicate that the genetic instability and dynamic adaptations of tumor cells results in compensatory mechanisms to escape rate limiting early proangiogenic pathways. The increased production of thrombin in the hyperpermeable TME may represent one such pathway. It is worth exploring whether inhibition of thrombin provides additive or synergistic effects with TF-VIIa-PAR2 blockade to optimally suppress the multiple contributions of the coagulation cascade to cancer progression. Another important area for further research is to understand whether blockade of direct TF signaling provides additional benefits with antiangiogenic therapy. The established animal models and prototypic inhibitors of the TF pathway will be instrumental in addressing these important questions. The continuing basic research and general interest in this area will identify potential innovative avenues to interrupt these pathway for improved cancer therapy.

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References


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