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Tissue Factor in Cancer Progression and Angiogenesis

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Abstract

Constitutive expression of tissue factor (TF) by cancer cells triggers local and systemic activation of the coagulation cascade and is a major cause of cancer associated thrombosis. Primary breast cancer biopsies show a marked upregulation of TF and protease activated receptor (PAR) 2, as well as increased TF cytoplasmic domain phosphorylation that is correlated with cancer relapse. TF signaling involving PAR2 and integrins has multiple effects on angiogenesis and tumor progression. The non-coagulant, alternatively spliced form of TF retains an integrin-binding site and, upon deposition into the tumor stroma, stimulates angiogenesis by ligating endothelial integrins $\alpha_v\beta_3$ and $\alpha_6\beta_1$. On tumor cells, full-length TF is constitutively associated with laminin-binding β_1 integrins that support TF-VIIa-PAR2 signaling leading to upregulation of pro-angiogenic and immune modulatory cytokines and growth factors. Deficiency of PAR2, but not of the thrombin receptor PAR1, delays spontaneous breast cancer development and the angiogenic switch in mice. In addition, human xenograft breast cancer growth and angiogenesis is suppressed by selective antibody inhibition of TF-VIIa-PAR2 signaling, but not by blocking TF initiated coagulation. Thus, interruption of TF signaling represents a potential anti-angiogenic strategy that does not carry an increased risk of bleeding associated with prolonged inhibition of the TF coagulation pathway.

Keywords

Protease activated receptor; factor VIIa; tissue factor; thrombin

The TF coagulation pathway in cancer

A prothrombotic state characterizes advanced malignancies and thromboembolic disease is a significant cause of death for cancer patients [1]. Tissue factor (TF), the initiator of the coagulation cascade, is shed on tumor-derived microparticles into the circulation [2]. Moreover, tumor cells that ectopically synthesize TF's protease ligand VIIa shed a functional TF-VIIa complex [3], which may significantly increase the procoagulant activity of tumor-derived microparticles to promote the hypercoagulable state of cancer patients. TF expressed by tumor cells also triggers multiple pathways that directly support tumor progression. TF promotes intravasation [4] and downstream coagulation activation orchestrates thrombin-, platelet- and fibrin-dependent cancer cell survival pathways important for metastatic tumor dissemination [5]. Host and tumor cell TF further play partially overlapping roles to enhance tumor growth and to shape the tumor microenvironment [6]. While this redundancy has been explained by the release of tumor and host-derived procoagulant TF microparticles into the tumor stroma [7], direct TF signaling pathways recently emerged as dominant regulators of angiogenesis and primary

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tumor growth. Here, we briefly summarize progress in understanding these pathways and potential therapeutic implications.

TF-integrin interactions in tumor growth

Several studies have found increased levels of TF expression in primary colorectal, breast and pancreatic cancer and correlated TF levels with aggressive cancer phenotypes [8]. In addition, alternatively spliced mRNA of TF was detected in pancreatic, hepatocellular and leukemia cancer cell lines [9]. Overexpression of alternatively spliced TF (asTF) that lacks a transmembrane domain promotes angiogenesis and tumor growth by incompletely understood mechanisms [10]. Although asTF retains most of the energetically important contacts for coagulation factor VIIa, asTF lacks large portions of an exosite for macromolecular substrates. Therefore, asTF has no appreciable procoagulant activity. In addition to its protease ligands, the extracellular domain of TF binds several β1 integrins as well as αvβ3 and TF regulates integrin function in cell migration through TF cytoplasmic domain signaling [11]. Remarkably, asTF preserves integrin binding activity and serves as a ligand for endothelial cell-expressed integrins. Association of asTF with extracellular matrices ligates endothelial αvβ3 and α6β1 to promote vascular sprouting *in vitro* and angiogenesis *in vivo* [12].

However, increased expression of full-length TF also enhances tumor growth in several experimental models [13]. In addition to the integrin interactions in *trans* that are common to all forms of TF, cancer cell full-length TF is constitutively associated with the integrins α3β1 and α6β1 in *cis*. Moreover, integrin ligation on tumor cells supports TF-VIIa proteolytic signaling through PAR2 [14]. Further studies are required to fully understand how asTF and full length TF cooperate to regulate endothelial integrins in tumor angiogenesis. While asTF does not support proteolytic signaling of VIIa, it is important to point out that full length TF can initiate proteolytic signaling in the extravascular space, because VIIa is ectopically expressed in cancer cells by hypoxia- or constitutive histone acetyltransferase-dependent pathways [15,16].

Tumor cell TF-VIIa-PAR2 signaling

PAR2 cleavage by tumor cell TF-VIIa triggers canonical 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 the modulation of the tumor microenvironment. Specifically, breast cancer cell TF-VIIa-PAR2 signaling induces pro-angiogenic factors such as VEGF [17], 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) [18]. Although some of these genes are also induced by PAR1 signaling in breast cancer cells, TF-VIIa-PAR2 appears to be a more potent stimulus for the upregulation of the immune and angiogenesis regulators CXCL1, IL8, GM-CSF and M-CSF.

Other aspects of TF-PAR2 signaling influence tumor cell migration with important implications for metastasis. PAR2 recruits the intracellular adaptor β-arrestin [19]. β-arrestins are best known for their role in receptor internalization [20], but recruitment of β-arrestin to PAR2 targets activated ERK to pseudopodia of migrating cells [21] and promotes breast cancer migration through the cofilin pathway that is crucial for cytoskeleton reorganization [22,23]. Notably, PAR2 also contributes to thrombin-PAR1 signaling-dependent tumor cell metastasis [24].

Considering these tumor promoting pathways, we asked if TF-PAR2 signaling supports breast cancer growth *in vivo*. In an aggressive model of breast carcinoma, we blocked the

cleavage of PAR2 or selectively targeted TF-VIIa coagulant or signaling functions with a unique pair of monoclonal antibodies [14,25]. Inhibition of TF-initiated coagulation had marginal effects on xenograft growth in immune-deficient mice, but tumor growth was suppressed upon blockade of either PAR2 or specifically the signaling function of TF-VIIa. In the orthotopic microenvironment of the mammary fat pad, tumor-induced angiogenesis was reduced upon selective blockade of TF-VIIa signaling, consistent with the crucial role of proangiogenic TF-VIIa-PAR2 signaling previously established by *in vitro* studies.

Genetic evidence for TF-PAR2 signaling in tumor development

Tumor cell lines maintained under tissue culture conditions have inherent limitations in studying the complexity of tumor progression *in vivo*. Spontaneous genetic tumor models provide additional opportunities by allowing the simultaneous evaluation of signaling pathways in host and tumor cells and the study of tumor progression in immune competent hosts. The mammary tumor virus (MMTV) promoter-driven expression of the Polyoma Middle T antigen (PyMT) results in spontaneous development of breast cancer in mice that mimics important aspects of human breast cancer progression. In addition, the early stages of tumor progression in the PyMT model are highly dependent on tumor cell-derived angiogenic regulators. Considering that TF can initiate thrombin-mediated PAR1 signaling as well as direct signaling through PAR2, we used the PyMT model as an unbiased approach to study contributions of PARs to spontaneous breast cancer development.

PAR1-deficienty did not delay PyMT breast cancer development [26], which was unexpected because PAR1 has previously been shown to be upregulated in human breast cancer samples [27]. Tumor cells isolated from PAR1−/− mice were thrombin insensitive, excluding the compensatory upregulation of other thrombin receptors. In contrast to PAR1-deficiency, a significant delay in the transition from adenomas to invasive carcinoma was observed in PAR2−/− mice [26]. Vascularized tumors appeared later in PAR2−/− mice relative to wild-type, consistent with a role for PAR2 signaling in promoting the angiogenic switch. Levels of the TF-VIIa-induced cytokine CXCL1 were significantly reduced in early tumors of PAR2−/− relative to wild-type mice, indicating a possible mechanism by which PAR2 signaling regulates the angiogenic switch. Macrophages were also less abundant in early tumors of PAR2−/− mice, providing initial evidence that the recruitment of proangiogenic immune cells is also dependent on PAR2 signaling.

PAR2−/− tumor cell lines established from these mice grew slower than a similar wild-type line when transplanted into either wild-type or PAR2-deficient hosts [26]. Furthermore, reconstitution of PAR2 in PAR2−/− PyMT cells improves tumor growth, confirming that tumor cell, rather than host PAR2 signaling supports breast cancer progression. Consistent with previous data in xenograft models [28], deletion of the TF cytoplasmic domain results in a similar delay of tumor development in the PyMT model (Schaffner et al., unpublished), providing evidence that TF is an active signaling partner in this proangiogenic pathway.

Correlation of PAR2 expression with TF cytoplasmic domain phosphorylation in invasive breast cancer

These data in experimental tumor models indicated that TF-PAR2 signaling is crucial for breast cancer progression. PAR2 activation leads to TF phosphorylation and increased TF phosphorylation has been observed in neovascular eye diseases [29,30]. We asked whether deregulated tumor cell PAR2 signaling is associated with increased phosphorylation of the TF cytoplasmic domain. Indeed, wild-type PyMT tumors or human breast cancer xenografts propagated in mice showed increased phosphorylation of TF [31]. Notably, phosphorylation of tumor cell TF was not seen in breast carcinomas from PAR2−/− mice, indicating that TF

phosphorylation can be used as a surrogate marker for upregulated PAR2 signaling in tumor progression.

This notion was further substantiated by the analysis of clinical breast cancer samples. Biopsies of newly diagnosed invasive breast cancer showed marked increases of TF, PAR2 and TF phosphorylation that were in sharp contrast to non-invasive ductal carcinoma *in situ*. Upregulation of the TF-PAR2 signaling pathway was correlated with increased expression of VEGF, confirming the link to tumor angiogenesis. Importantly, recurrence of breast cancer was observed only in patients that stained positive for phosphorylated TF. Thus, TF phosphorylation may be a useful biomarker to identify patients that can benefit from therapeutic interventions in TF signaling pathways.

Therapeutic opportunities in TF initiated signaling

These basic studies show that TF-PAR2 signaling contributes to tumor angiogenesis. The experiments with inhibitory antibodies that selectively ablate TF-VIIa signaling or coagulant activities provide further proof of principle evidence that a blockade of direct TF signaling is sufficient to achieve substantial attenuation of tumor growth. Other inhibitors of TF that more broadly neutralize the TF-VIIa complex have similar anti-tumor and anti-angiogenic activities [32,33], but their anticoagulant effects raise significant safety concerns in regard to bleeding complications. Indeed, reduced levels of TF in mice [34] or prolonged inhibition of the coagulant limb of the TF pathway [35] are both associated with spontaneous hemorrhage. Considering these concerns, inhibition of TF-VIIa signaling by either TF-directed antibody [14] or antagonists of PAR2 [36] appears to be a preferred strategy that can find broader acceptance as a clinical anti-angiogenic cancer therapy.

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WR has pending patent applications on the use of antibodies described in this review.

Abbreviations

PAR	protease activated receptor
TF	tissue factor
PyMT	Polyoma Middle T antigen

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