Update on Tumor Cell Procoagulant Factors

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Abstract
Tumor cells produce tissue factor, cancer procoagulant, plasminogen activators and other factors that interact with the coagulation system, the fibrinolytic system and vascular or blood cells such that they can upset the normal homeostasis and balance between activation and inhibition of the coagulation and fibrinolytic systems. These activities play a role in tumor cell growth and metastasis, vascular wall function, and hemostasis. Proteases and their inhibitors are intimately involved in all aspects of the hemostatic, cell proliferation and cellular signalling systems. This review provides a brief examination of recent observations in this complex interaction of cellular and hemostatic factors.

Introduction
An association between blood coagulation and cancer has been recognized since Trousseau \cite{1} in 1865 first observed that idiopathic thrombophlebitis in patients with gastrointestinal symptoms was diagnostic of malignancy. More recently a large body of clinical evidence clearly shows that coagulation is often activated in cancer patients and it contributes to morbidity and mortality \cite{2}. A variety of hemostatic abnormalities are observed in association with malignant diseases. While disseminated intravascular coagulation (DIC), migratory thrombophlebitis and deep vein thrombosis are common, a variety of other thromboembolic disorders may also occur. These include thrombosis of the portal and hepatic veins, arterial thrombosis and pulmonary embolism \cite{2}. Furthermore, subclinical abnormalities of hemostasis have been detected in about 90\% of patients that died of cancer \cite{2}; these abnormalities are often consistent with DIC and may only be detected with newer, more sensitive laboratory tests such as for fibrinopeptide A, fibrin D-dimer, thrombin-antithrombin complexes and prothrombin activation fragment F1+2.

At a molecular level investigators are beginning to unravel the very complex relationships between the expression of factors associated with malignant transformation and the mechanisms of coagulation activation associated with malignancy; this relationship is still not completely understood. First of all, what are the mediators of blood coagulation that are produced by malignant cells? Clearly both direct and indirect mechanisms of coagulation activation exist. That is, coagulation is directly acti-
vated by procoagulant molecules produced by malignant cells and indirectly activated via the inflammatory response which results in the elaboration of tissue factor on activated macrophages and provides a surface for prothrombinase assembly [3]. Furthermore, increased coagulation may occur as a result of diminished activity of the anticoagulant systems such as tissue factor pathway inhibitor (TFPI), the protein C system or antithrombin. Along with this, VEGF produced by tumor cells induces increased permeability of the vascular system (see below), which increases the perfusion of the malignant cells with plasma coagulation factors [2]. Thus, taken together, there is increased procoagulant activity, the presence of all the relevant components of the coagulation system at the tumor site and possibly decreased anticoagulant activity, so hypercoagulation seems a likely consequence of malignancy.

Secondly, what are the results of this coagulation activation? Fibrin deposition around solid tumors is common and appears to be important for tumor growth, protection of the tumor and perhaps angiogenesis. Some procoagulant factors may be involved both directly and indirectly, via thrombin, in mechanisms of metastasis and angiogenesis by virtue of their activation of signaling pathways that stimulate these processes. Fibrin deposition and platelet aggregation have also been implicated in the process of metastasis. Furthermore, abnormal clotting as a result of this activation can contribute directly to morbidity and mortality in cancer patients.

This review will focus on recent advances in the state of knowledge concerning procoagulant molecules produced by cancer cells. We will rely on recent reviews written prior to 1997–1998 about tumor cell procoagulants and coagulation in cancer to provide historic information on the subject [4–6]. Although we will focus on the known substances produced by malignant cells that directly activate blood coagulation, we will briefly review other elements of the coagulation systems as they relate to these molecules. Finally, this review will attempt to integrate the known molecular events of coagulation with the role of procoagulants in malignant disease.

Tissue Factor

Tissue factor (TF) is a transmembrane receptor protein that is the primary initiator of blood coagulation via its complex with factor VIIa (FVIIa), a serine protease. TF is normally present on fibroblasts of the vascular adventitia and at the boundaries of organs, epithelial cells, stromal cells and astrocytes. But TF is not normally expressed in intravascular cells such as vascular endothelium and peripheral blood cells. Therefore, TF effectively forms a hemostatic ‘envelope’ that stimulates coagulation upon disruption of vascular integrity [4]. However, TF is also involved in a variety of processes apart from fibrin-dependent coagulation. This is suggested by the fact that TF is a member of the cytokine receptor superfamily [17], expression of TF is stimulated by a variety of inflammatory agents and cytokines [4], targeted disruption of the murine TF gene results in abnormal yolk sac vasculature and death at embryonic days 9.5–10.5 [8], and binding of FVIIa to TF induces protease receptor-mediated signaling [9]. This is further evidenced by the involvement of TF in cancer metastasis and angiogenesis [10]. We will discuss recent developments in these processes, especially those that relate to the expression of TF on tumor cells. But we will also refer the reader to recent reviews on some of these topics for a more thorough discussion.

Tissue Factor Expression

Transcriptional regulation of TF, both in constitutive sites of expression such as fibroblasts, and in inducible sites of expression such as monocytes and endothelial cells, has been studied extensively [for review, see 11–13]. Expression of TF on cell surfaces is apparently not sufficient for complete activation of the expressed TF. A significant amount of the TF on the surfaces of various cells is in an ‘encrypted’ form that must undergo a transformation before it is available to initiate clotting. This encryption and decryption is not fully understood but it may involve limiting amounts of phosphatidylserine on the outer cell surface membrane and dimerization of TF [11, 13, 14].

TF is expressed in a wide variety of solid tumors and tumor cell lines [15]. Acute promyelocytic leukemia also expresses TF. It has been observed that TF production by the APL cell line NB4, which is ATRA-maturation-inducible, is downregulated by all-trans retinoic acid (ATRA) and As2O3 [16, 17].

Tissue Factor Structure/Function

Mature TF is a 263-residue glycoprotein of 54,000 daltons after removal of a 32-residue propeptide. TF consists of a 219-residue extracellular domain that contains two fibronectin type III modules, a 23-residue transmem-
brane domain and a 21-residue cytoplasmic tail that contains several phosphorylation sites [18]. The crystal structure of the extracellular domain of TF confirmed the presence of two fibronectin type III-like domains and demonstrated that they are arranged end to end in a rigid structure [19, 20]. A crystal structure of this extracellular domain of TF in complex with FVIIa revealed that TF retained its rigid conformation in this complex and imposed a specific conformation on the flexible FVIIa molecule by binding to it in three main areas in the Gla domain, the EGF-1 domain and the EGF-2 and catalytic domains of FVIIa [21]. This complex of TF and FVIIa is somewhat unique among the protease:cofactor complexes in the blood coagulation pathway in that activated FVIIa by itself has very little proteolytic activity towards peptidyl substrates or macromolecular substrates and binding of TF to FVIIa causes an allosteric change in FVIIa that stabilizes the active site of FVIIa [22–24].

Normally blood clotting is initiated upon vascular injury, whereupon TF is exposed to the blood and binds to FVII/FVIIa that is constitutively circulating in the blood. This TF/FVIIa complex then converts factor X to an active serine protease (FXa) and converts factor IX to an active serine protease (FIXa). FXa forms a complex with factor Va on a membrane surface and this complex converts prothrombin to thrombin, the central serine protease of the blood coagulation cascade. FIXa forms a complex with factor VIIIa on a membrane surface, and this complex activates factor X. Thrombin has various functions, including centrally, the cleavage of fibrinogen, resulting in fibrin, and the activation of platelets (fig. 1). Thrombin also activates factors XI, V and VIII for feedback activation of the coagulation cascade as well as protein C, which is the main feedback inhibitor of the coagulation cascade [25]. TFPI inhibits the TF/FVIIa complex when TFPI is in a complex with FXa. Therefore the inhibited complex is a heterotetramer of TF, FVIIa, FXa and TFPI.

The effects of tumor cell TF production are several. Firstly, coagulation is activated resulting in the production of fibrin and platelet activation. The implications of this are discussed later. Apart from activation of coagulation, TF also has effects on angiogenesis and tumor metastasis. For a recent review of these functions of TF see Ruf and Mueller [9, 10]. Recent results have clarified the pathways involved in the process of metastasis but the pathways involved in the stimulation of angiogenesis are not as fully understood.
Tissue Factor Function in Tumor Metastasis

Levels of TF correlate with the metastatic potential of a variety of tumor types including non-small-cell lung cancer [26] and colorectal cancer [27, 28]. TF levels also correlate with prognosis in breast cancer [29]. In experiments in mice antibodies against TF inhibited metastasis of human melanoma cells [30]. Furthermore, overexpression of TF in a pancreatic adenocarcinoma cell line as a result of transfection with a TF-expressing plasmid resulted in increased in vitro tumor invasion and increased primary tumor growth [31] suggesting an increase in metastatic capacity. It is clear now that several signaling processes are involved in metastasis [for a more complete review, see 9]. Briefly, binding of FVIIa to TF on a variety of cell types, including cancer cells, endothelial cells and keratinocytes induces the mitogen-activated protein kinase (MAPK) pathway, as measured by induction of Ca²⁺ transients and MAPK pathway reporter constructs [32, 33]. This induction results in the expression of a variety of genes including egr-1 [34], poly(A)-polymerase [35], cyr61 and CTGF [36] and others [37]. These genes encode proteins with a variety of functions including transcriptional regulation, growth stimulation, mRNA processing and migration stimulation that primarily seem to be involved in cellular proliferation and migration.

Activation of the MAPK pathway was shown to be dependent on the proteolytic activity of FVIIa in complex with TF and also independent of the cytoplasmic domain of TF [38, 39]. Furthermore, thrombin activated by the TF/FVIIa/Xa pathway was responsible for activation of thrombin receptors that induce proliferative signals [40, 41]. Finally, TF/FVIIa and FXa themselves are able to activate the protease-activated receptor 2 (PAR-2), which is not itself activated by thrombin [42]. Thrombin signaling primarily takes place through activation of PAR-1 [43].

However, experiments with recombinant mutant TF with various mutations in the cytoplasmic domain of TF demonstrate that this domain is also important for the process of metastasis, and that phosphorylation of this domain is important [38, 44, 45]. Two-hybrid screening was used to identify actin-binding protein 280 (ABP-280) as a ligand for the TF cytoplasmic domain [46]. ABP-280 is involved in stabilizing the actin cytoskeleton and in cell motility. Finally, it was demonstrated that TFPI associated with extracellular matrix can bind to the TF/FVIIa complex on tumor cells and stimulate adhesion and migration [47].

All these results taken together demonstrate a complex role for the TF/FVIIa complex in tumor metastasis. Firstly, the active proteolytic complex is required for thrombin activation and subsequent signaling via PAR-1. Direct activation of PAR-2 may also play a role but the inhibition of metastasis by thrombin inhibitors suggests that the PAR-1 pathway plays the primary role. Secondly, the phosphorylation of the cytoplasmic domain stimulated by extracellular ligand binding promotes binding to components of the cytoskeleton involved in cell motility, thus promoting cell adhesion and migration. Finally, it appears that the ligand responsible for this intracellular signaling is TFPI that is bound to the extracellular matrix.

Tissue Factor Function in Angiogenesis

Angiogenesis is required for growth and metastasis of malignant tumors [for review, see 48]. Angiogenesis is controlled by a variety of factors, including the proangiogenic VEGF and the antiangiogenic thrombospondin. Zhang et al. [49] demonstrated a connection between TF and the stimulation of angiogenesis in mouse tumor cells. They found that when TF was transfected into meth-A sarcoma cells these cells grew more rapidly, were more vascularized and had increased transcription of VEGF along with decreased transcription of thrombospondin. Levels of TF production by tumors are positively correlated with angiogenesis measured by microvessel density in human non-small-cell lung carcinoma and human prostate carcinoma [50, 51]. TF levels also correlate with levels of VEGF expression and are colocalized with VEGF in lung cancer and breast cancer cells as measured by in situ hybridization and immunohistochemical staining [51, 52].

The mechanism by which TF induces VEGF production is not as well established as the signaling pathways leading to metastasis. Abe et al. [53] found that TF cDNA transfected into a malignant melanoma cell line induced VEGF production and a TF cDNA with a mutant in the extracellular domain that abrogated factor X-activating activity also induced VEGF production, but a mutant TF lacking the cytoplasmic domain did not produce VEGF. Furthermore, in this cell line VEGF production was independent of FVIIa and did not require generation of thrombin. In contrast to this result it has been demonstrated that TF-dependent production of VEGF in human fibroblasts requires catalytically active FVIIa and also involves Xa and thrombin [54, 55], suggesting that signaling via the PAR pathway is likely involved.
Additional complications in the actual effects of TF on angiogenesis are suggested by the fact that VEGF can induce TF production in vascular endothelial cells and monocytes [56]. Furthermore, platelets are carriers of angiogenic growth factors such as VEGF and could be activated via the coagulation pathway initiated by TF. In support of this connection Verheul et al. [57] demonstrated the presence of activated platelets along with increases in VEGF and coagulation factors in soft tissue sarcomas.

Another angiogenic role of TF is seen in embryonic vascular development. The death of TF null mouse embryos at day 9.5–10.5 appears to be due to abnormal vascular development in the yolk sac [8]. Parry and Mackman [58] determined that this abnormal vascular development was rescued by human TF with a deleted cytoplasmic domain, but not by TF with an inactive extracellular domain, thus suggesting that TF/FVIIa-dependent extracellular protease activity is required for embryogenesis. The difference in TF/FVIIa-dependent effects seen in fibroblasts and embryogenesis versus the FVIIa-independent effects of TF seen in a cancer cell line is not clear.

**Cancer Procoagulant**

The other well-documented procoagulant from tumors is cancer procoagulant (CP). CP is a cysteine protease derived from a broad spectrum of malignant and embryonic (amnion-chorion) tissues [see 6 (table 2), 59, 60] that directly activates factor X in the absence of factor VII [61, 62]. CP has not been found in normal, differentiated tissue. CP activates factor X by activating the factor X molecule at a different site than other serine protease activators, FVIIa, Russell’s viper venom or FIXa [63]. CP requires 7 mM Ca2+ for activity and its activity is enhanced by 10–100 μM Mn2+ and Cd2+ but inhibited by Zn2+, Fe2+, Cu2+ and Sn2+ [64]. CP is inhibited by classic cysteine protease inhibitors such as peptidyl diazomethylketones, HgCl2, iodoacetamide, and E-64 [62, 65]. Cysteine proteases are irreversibly inhibited by E-64, an alkylating agent that binds to the active site sulfhydryl [66]. Recently, Mielicki [67] demonstrated that E-64 is a competitive (reversible) inhibitor of CP. Clostripain, a cysteine protease from *Clostridium histolyticum*, has similar calcium requirements and E-64 inhibition characteristics [68]. Thus, CP has some unique enzymatic properties and a complete understanding of the proteolytic nature of CP awaits further research.

In the last few years, since the reviews by Gordon and Mielicki [6], Francis et al. [69] and others [70–72] there have been a few noteworthy advancements in our understanding of CP. Falanga et al. [16] demonstrated that the expression of CP in promyelocytic leukemia cells paralleled their degree of malignant transformation. ATRA was used to induce the PML cells into their normal mature phenotype and during this transformation, their expression of CP ceased; those cells resistant to ATRA maintained their malignant phenotype and their expression of CP. In contrast, TF expression did not parallel these ATRA-induced phenotypic changes; TF was reduced in both the ATRA-sensitive and ATRA-resistant cells. These findings are consistent with the observations that CP is found in malignant but not normal tissues from the same animal [6].

Since CP is expressed by malignant tissue but not normally differentiated tissue, it may be a good tumor marker for the diagnosis and monitoring of cancer in patients. Previous work showed that CP antigen was present in the blood of a high percentage of patients with cancer but not in normal or benign disease controls [73, 74]. Recently, analysis of the CP enzymatic activity in serum samples from breast cancer patients showed that about 75% of early stage (stage I and II) cancer patients have elevated CP activity and normal prothrombin times [75]. Breast cancer patients with later stage disease (stage III and IV) did not have elevated levels of CP activity. There was no correlation between stage of disease and the level of CP activity. There is evidence that the patients produce anti-CP antibodies that may block the procoagulant activity; this may explain the observation.

It is widely accepted that activation of the coagulation system causes thrombin generation, which in turn stimulates platelet activation and adhesion (aggregation) through the PAR system (fig. 1). Thus, the indirect activation of platelets by both TF and CP via thrombin generation was expected. However, Olas et al. [76, 77] demonstrated that CP can induce direct, dose-dependent platelet activation by a mechanism that appears to be similar to that of thrombin. When CP and thrombin are added to platelets simultaneously, the stimulation is not additive, but rather reflects the mean of the stimulation of each agonist alone. It seems logical that these two proteases may activate platelets by the same PAR-1 mechanism, but more research must verify this speculation. If CP can facilitate PAR-mediated cell stimulation, one might ask what other cell types CP can stimulate. Normal compartmentalization of an early stage malignancy would find tumor cells producing CP in the nonvascular tumor com-
partment where there are no platelets or coagulation factors. Of course, this changes as the tumor grows, angiogenesis commences and VEGF induces the increased permeability of vessels such that coagulation factors bathe the malignant cells.

**Other Cancer Cell-Derived Procoagulant Activity**

A less well-studied and characterized tumor cell procoagulant is CCA-1 (cancer cell-derived blood coagulating activity 1) that has been found in LK52 human squamous cell carcinoma cells. This procoagulant is enzymatically different from CP. A monoclonal antibody to CCA-1 inhibits the procoagulant activity and does not cross-react with either TF or CP [78]. In addition to CCA-1, other procoagulant activities have been described [see 6, table 1] but to our knowledge, there is no new information or further verification of these factors.

**Coagulation Activation**

Although the focus of this review is the tumor cell procoagulants, it is impossible to ignore the huge body of literature on the clinical observations of thrombosis in cancer and related clinical studies that lead investigators to look for the mechanism of this hypercoagulation in malignancy. The clinical observations continue to grow and support the relationship first established by Trousseau [1]. In the past 4 years, several studies have been published, using more modern methods for the measurement of analytes to reassess the thrombogenic phenomenon. These studies include the measurement of thrombin-antithrombin complex, fibrinopeptide A, prothrombin activation peptide F1+2, protein S, protein C, activated protein C [79, 80], TFPI, tPA, uPA and urinary TF [81]. The etiology and pathophysiology of the hypercoagulable state associated with neoplasia is still unclear.

The major result of the production of procoagulant molecules in tumors is, of course, activation of coagulation with the end results of fibrin formation and platelet activation [for review, see 2]. One necessary condition of these final steps is that the necessary coagulation factors and fibrinogen gain access to the extravascular space. It is now clear that the production of VEGF, which has also been known as vascular permeability factor, in connection with tumor cells, is capable of permeabilizing vasculature to allow the extravasation of the necessary coagulation factors [2]. Fibrin is deposited in many types of solid tumors, but not in all solid tumors and the patterns and extent of fibrin deposition is characteristic of the tumor type. One study showed that TF and VEGF colocalized in lung and breast cancer cells and also that TF colocalized with fibrin at the interfaces of tumor and host cells. However, though CP was also found in the tumors, it did not colocalize with fibrin [52].

Studies have shown that fibrin deposits around tumors appear to have specific functions and consequences. Mainly, fibrin deposition is critical in the imposition of the initial structure of the tumor. Secondly, over time this fibrin gel transforms into mature vascularized connective tissue via replacement by connective tissue stroma and angiogenesis to produce new blood vessels. Thirdly, fibrin has significant effects on inflammatory cell infiltration into the tumor, resulting in regulation of stroma formation and protection of the tumor from the host immune system.

Platelets and fibrin also appear to have a role in tumor metastasis. It is thought that fibrin deposition and platelet aggregation on blood-borne tumor cells may prevent the normal function of the host immune systems against tumor cells in the circulation. Also these tumor cells with fibrin and platelet thrombi associated may more readily attach to vascular endothelium [2]. However, ultrastructural analysis of the early arrest phase of a metastatic melanoma in mice showed that platelet and fibrin deposition was absent at the interface of the tumor cell and the endothelial membrane, but rather platelets were aggregated on the luminal side of the tumor cell [10]. Though the exact role of platelets and fibrin in these processes is not entirely clear, it is certain that tumor cells do provoke platelet activation and fibrin formation via the various procoagulant pathways discussed above.

Activation of the coagulation system is clearly intimately involved with the processes of metastasis and angiogenesis in malignancies. This activation of coagulation also results in various clinical conditions such as deep vein thrombosis and DIC that can have serious life-threatening consequences. A better understanding of the relationship between coagulation and cancer should lead to improved treatments for both cancer and the associated hemostatic abnormalities.
References


