Coagulation proteases and human cancer

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Abstract
Tumours are capable of activating blood coagulation through the expression of procoagulant molecules such as tissue factor, cancer procoagulant and hepsin. Initiation of the clotting cascade results in the generation of the activated serine proteases factor VIIa, factor Xa and thrombin. These proteases act via protease-activated receptors and tissue factor to alter gene expression, thereby modulating tumour cell growth, invasion, metastasis and angiogenesis.

Introduction
Since Trousseau's observation in 1872 [1] that thrombosis frequently complicates cancer, much cellular, histological and clinical research has confirmed this phenomenon. Indeed, a recent population-based study has shown that patients with the diagnosis of venous thromboembolic disease have a 3-fold increased risk above that of a non-thrombosis control population for the diagnosis of overt cancer within the next 6–12 months [2]. In the past decade, research has focused on trying to explain how tumours are able to activate blood coagulation, and the consequences of such activation, with the generation of coagulation serine proteases, on tumour growth, invasion, metastasis and angiogenesis. In this regard, tissue factor (TF), the physiological initiator of coagulation, has come to be seen as a key determinant of the coagulation/cancer interaction.

TF
The human TF molecule is a single chain, 263 amino acid, 47 kDa transmembrane glycoprotein whose primary sequence indicates structural similarity with the cytokine receptor family [3]. It is made up of three domains: extracellular (219 residues), transmembrane (23 residues) and cytoplasmic (21 residues) [4]. It acts as the principal surface receptor and cofactor for the activated coagulation protease factor VIIa (FVIIa) as well as a receptor for the zymogen precursor of FVIIa, factor VII. The coagulation cascade is triggered by the binding of FVIIa to TF, which creates a complex for the activation of the protease factor X, and its conversion to factor Xa (FXa). The procoagulant activity of TF is dependent upon the integrity of its extracellular domain alone: tumour cells transfected with a TF construct that lacks a cytoplasmic domain do not show an alteration in surface procoagulant activity [5]. Generation of FXa by the TF–FVIIa complex triggers the proteolytic conversion of prothrombin to thrombin. This key step in the coagulation cascade is effected by the catalytic activity of the 'prothrombinase' complex which consists of FXa and the non-enzymic cofactor factor Va in a 1:1 stoichiometric complex that is assembled in the presence of calcium ions upon an appropriate phospholipid membrane. In the case of physiological haemostasis, this would usually be the activated platelet membrane, but other cell surfaces including those of tumour cells [6] have been shown to provide a suitable surface for generation of prothrombinase complexes and activation of thrombin. This serine protease is primarily responsible for the enzymic conversion of fibrinogen into fibrin at sites of vascular injury, though it is known to have a number of other cell-mediated functions, that are dependent upon expression of protease-activated receptors (PARs).

TF is expressed constitutively on the adventitia of uninjured blood vessels and other extra-vascular tissues. Its anatomical distribution suggests a protective role in initiating rapid activation of coagulation through its procoagulant function. However, under certain conditions, including the localized presence of cytokines, growth factors [4] and endotoxins [7], vascular endothelial cells (VECs) and fibroblasts have been demonstrated to start de novo TF synthesis and expression. TF is seldom expressed in normal...
epithelial tissue but its expression has been confirmed on malignant cells, particularly those with enhanced metastatic potential. The degree of TF expression has been shown to correlate with the histological tumour grade for pancreatic cancer cells [8], with increased TF expression in more undifferentiated tumours. Colorectal cancer cell lines express higher TF activity than do their parental lines [9]. TF expression has been demonstrated in tumour-associated VECs of patients with invasive breast cancer [10]. Recent experiments have confirmed that TF expression alters the cancer cell’s phenotype, rather than its expression being an incidental finding. Transfection of tumour cells with the TF gene in sense and antisense orientations has shown that overexpression of the TF gene (and consequently overexpression of the TF antigen) is associated with enhanced procoagulant activity and increased in vitro tumour cell invasion and primary tumour growth [11]. Expression of TF also alters the metastatic potential of tumour cell lines in vivo [12]. Other factors that have been shown to enhance procoagulant activity of tumour cells include the proteases hepsin and cancer procoagulant (CP). Hepsin, a type II transmembrane serine protease, is physiologically expressed on the surface of hepatocytes, but has been found to be overexpressed in several tumour cell lines, including ovarian [13] and prostatic [14,15] carcinomas. In vitro studies suggest that it is required for growth and maintenance of normal morphology in human hepatocytes [16], although mice with a homozygous deficiency for hepsin appear to have normal growth and fertility phenotypes [17]. Hepsin has also been shown to initiate blood coagulation by activating FVIIa independent of TF. Cells transfected with the hepsin gene were able to activate FVII to FVIIa in a time- and calcium-dependent manner [18], which eventually resulted in thrombin generation. Hepsin was also able to catalyse the generation of low levels of FXa and thrombin in the absence of FVII [18]. CP is a cystine protease whose only known enzymic property is the calcium- and vitamin K-dependent conversion of FX to FXa [19], which occurs independently of FVIIa. It has been identified in many tumour cell lines, but never in normal tissue apart from human chorion-amnion [20]. The levels of CP expression appear to correlate with a malignant phenotype. CP concentrations in metastatic melanoma cell lines have been shown to be significantly higher than those found in the primary tumour [21].

Control of TF expression

As is the case for any protein, the expression of TF is subject to regulation at a number of levels: (i) gene transcription to form mRNA, (ii) translation, (iii) post-translational phosphorylation, disulphide bond formation, glycosylation and insertion through the cell membrane, and (iv) downregulation and metabolism. A number of factors have been implicated in increasing TF mRNA synthesis. These include serum growth factors, thrombin and fibrin. The human TF gene is located on chromosome 1 and has a promoter region with a variety of transcription factor binding sites, which mediate responses to factors including the early growth response factor (Egr-1). Egr-1, an 80 kDa nuclear phosphoprotein transcription factor, contains three zinc-finger DNA-binding domains, and is rapidly activated in various cell types in response to a variety of stimuli. When serum growth factors bind to their receptors on cells, signal transduction via the mitogen-activated protein kinase (MAPK) pathway causes phosphorylation of signalling proteins such as Elk-1. By binding with a serum response factor to a serum response element on the Egr-1 promoter region, this signalling protein will induce de novo expression of Egr-1. Egr-1 then binds to sites within the serum response region of the TF gene to mediate TF mRNA induction. Growth factors such as platelet-derived growth factor, fibroblast growth factor, transforming growth factor-β and epidermal growth factor upregulate the transcription of TF mRNA in human fibroblasts and epithelial cells [4], and in tumour cells lines such as HeLa [22] by this mechanism. Experimental evidence has also shown that endothelial cell expression of TF can be subject to control by growth factors such as vascular endothelial growth factor (VEGF) [23], mediated through the tyrosine kinase receptors of VEGF: Fms-like tyrosine kinase (SLT1) (fli-1) and flk-1/kinase insert domain-containing receptor (KDR) [24]. Treatment of the cells with VEGF results in accumulation of Egr-1 in the nucleus and subsequent binding of Egr-1 to its recognition site in the TF promoter region. Indeed, transfection of endothelial cells with an Egr-1 expression plasmid mimics the upregulation of TF expression seen upon treating the cells with VEGF [25]. Thrombin-mediated activation of TF expression has also been demonstrated. Thrombin can affect tumour cells by inducing proliferation, adhesion and migration in vitro. Supraphysiological concentrations of thrombin (at least
8.8 nM) will induce TF mRNA expression in VECs [26]. More recently, it has been shown that stimulation of SW-480 colon adenocarcinoma cells with thrombin will induce TF mRNA expression and TF procoagulant activity within 4-6 h, and that this effect is appreciable at a thrombin concentration of 1 IU/ml [27]. Another downstream component of the coagulation cascade which has been shown to regulate endothelial cell TF expression is fibrin. In VECs, there was a time- and dose-dependent upregulation of TF mRNA and antigen expression as well as TF procoagulant activity following incubation with fibrin [7]. Thus, it would appear that positive feedback loops may play a significant role in propagating and facilitating sustained TF expression. This may be of particular relevance in the peritumoral environment, although the precise impact of TF/FVIIa signalling in tumour cells is unclear.

Less is known about control of TF translation and post-translational modifications, but it appears that differential glycosylation of the extracellular domain of TF reduces its expression by 59-98% in mutant glycosylation-defective cells compared with that observed in wild type cells [28].

Downregulation and inhibition of TF expression are equally significant in modulating tumour cell procoagulant activity. Tissue factor pathway inhibitor (TFPI) is the only protease inhibitor that is known to downregulate TF procoagulant activity at physiologically significant rates. TFPI molecules are produced by endothelial and tumour cells and the majority remain associated with the cell surface, although TFPI can be detected circulating in plasma. Endogenous TFPI can be released from cell membranes by treatment with phospholipase, indicating that TFPI is a glycosylphosphatidylinositol (GPI)-anchored protein, or is bound to a GPI-linked receptor [29]. Structurally, TFPI is a trivalent Kunitz-type serine protease inhibitor, which in association with FXa regulates TF function by producing a quaternary complex with TF and FVIIa. The active site of the FVIIa–TF complex binds to the first Kunitz domain, and the active site of FXa binds to the second. These quaternary complexes, which markedly decrease the capacity of FVIIa to dissociate from TF, are internalized via glycosphingolipid-rich microdomains [29]. Inside the cell, the complexes dissociate after approx. 12 h and the TF and TFPI are rapidly recycled, although approx. 75% of the internalized FVIIa is degraded [30]. TFPI also binds to thrombospondin (TSP), a 450 kDa protein that is secreted by endothelial cells, platelets and tumour cells, which has an affinity for extracellular matrix proteins and cell surfaces. The binding of TSP to TFPI enhances its ability to inhibit the FVIIa–TF-mediated generation of FXa [31]. Interestingly, VEGF has been shown to play a role in TF downregulation as well as in its induction in VECs. It is postulated that VEGF can activate two different pathways, one via MAPK p38 and extracellular signal-related kinases ERK1/2 leading to accumulation of Egr-1 and upregulated TF expression, and a second via receptor dimerization with autophosphorylation of VEGF receptor domains, and attachment of the signalling enzyme phosphatidylinositol 3-kinase to generate a docking site for protein kinase B. The downstream signalling mediated by protein kinase B results in downregulation of TF expression [32]; however, it has been suggested that this mechanism is predominantly seen in the context of inflammation, whereas in neoplasia the MAPK pathway is of principal importance.

**Cellular effects of TF expression**

Characterization of the TF molecule has shown it to bear structural similarity to a class II cytokine receptor [3]. The capacity of TF to mediate an intracellular signal upon binding to its ligand, FVIIa, was demonstrated by Rottingen et al. [33]. A calcium flux was induced in J82 cells (a human bladder carcinoma cell line) and human endothelial cells upon binding of FVIIa to TF. Further experiments by the same researchers showed that this phenomenon occurs in cell lines in which TF is both expressed constitutively and can be induced, the cytoplasmic tail of the TF molecule being crucial in mediating such signalling events [33]. By transfecting either full-length TF cDNA, an extracellular domain mutant ‘TF cDNA or TF cDNA with a truncated cytoplasmic tail into a melanoma cell line that produced VEGF but expressed low TF levels, Abe et al. [34] showed that only the cytoplasmic domain was necessary for production of VEGF. Those cells expressing truncated TF synthesized normal levels of this molecule, but no VEGF. On the other hand, those cells expressing the extracellular domain mutant TF produced the same levels of VEGF as cell lines with complete TF, despite having markedly reduced procoagulant function. The TF cytoplasmic domain also has an important function.
with regard to mediating cytoskeletal reorganization. Experimental work has shown that extracellular ligation of the TF molecule results in the interaction of its cytoplasmic tail with actin-binding protein 280 (ABP-280). The ABP-280 ligand recruitment is associated with reorganization of actin filaments [35]. It is thought that TFPI and its homologous Kunitz-type inhibitor TFPI-2, which are associated with the extracellular matrix, interact with the TF–FVIIa complex and thereby influence migratory and adhesive properties of tumour cells. The TF cytoplasmic domain does not have an essential cell-signalling role in all cell types, though. Sorensen et al. [36] studied the stimulation of signal transduction in response to FVIIa in baby hamster kidney cells transfected with TF. Results indicated that the proteolytic activity of FVIIa was mandatory for there to be any intracellular effect, and that FVIIa binding to TF led to p44/p42 MAPK activation, but that the cytoplasmic domain of TF was not essential for this signalling. It was postulated that transmembrane signalling might be mediated by a PAR adjacent to TF in the case of baby hamster kidney cells.

TF, therefore, has cellular effects that are independent of blood coagulation. These can confer a metastatic phenotype, as demonstrated in an animal model by Zhang et al. [37]. By injecting nude mice with sarcoma cells transfected to overexpress or underexpress TF, the overexpression of TF was found to promote in vivo growth even in the presence of warfarin (to exclude any coagulation effect as a cause of preferential growth). The mechanism of this effect is likely to be via the release of growth and angiogenesis factors in cells expressing TF. When VECs were incubated with the supernatants from the sarcoma cells described above, proliferation was found to be most pronounced in cells incubated with supernatant from cell lines that overexpressed TF. Nuclear analysis of the sarcoma cells that overexpressed TF confirmed that they did indeed also express increased levels of VEGF mRNA compared with wild-type cells [37]. Similarly, overexpression of TF in a transfected Mia PaCa-2 human pancreatic adenocarcinoma cell line resulted in increased invasion in vitro (using a Matrigel assay) and in vivo (by assessing primary tumour growth in nude mice) [11]. In addition to inducing the expression of VEGF, the TF pathway can also upregulate the expression of the urokinase-type plasminogen activator receptor (uPAR), a key factor in the plasminogen activation system that promotes the production of matrix-lysing enzymes, and which therefore may have a role in promoting tumour cell migration and metastasis [38]. The increase in uPAR mRNA levels only occurred when TF-expressing cells were treated with FVIIa, and the effect was neutralized if anti-TF monoclonal antibodies or active-site inactivated FVIIa was used, and downstream coagulation inhibitors had no effect [39]. More recently, the advent of microarray analysis has confirmed that the specific binding of FVIIa to TF can induce the upregulation of a wide variety of genes: in a keratinocyte cell line expressing TF, addition of FVIIa resulted in significant upregulation of 24 genes [40]. These genes coded for transcription factors such as Egr-1, c-Fos and c-Myc, growth factors such as fibroblast growth factor-5 and connective tissue growth factor (CTGF), proinflammatory cytokines such as interleukin-1 and interleukin-8, and proteins that affect cellular migration and reorganization such as uPAR and collagenases. In another experiment, it was shown using cDNA microarrays that the growth factor-inducible immediate early genes Cyr-61 and CTGF were upregulated by stimulating fibroblasts with FVIIa [41]. The products of these genes promote cell adhesion, stimulate cell migration and promote DNA synthesis in both fibroblasts and endothelial cells in response to growth factors [42]. Although there is as yet little confirmatory experimental evidence, it is likely that similar effects occur in malignant cell lines.

**Cellular effects of downstream coagulation proteases**

In addition to the effect of FVIIa–TF on the biological activity of tumour cells, there is much evidence that the downstream coagulation proteases FXa and thrombin also have cellular effects in addition to their role in blood clotting. As described above, FXa is generated by activation of zymogen FX when the latter is bound to FVIIa–TF; this in turn converts prothrombin into active thrombin via generation of the prothrombinase complex. Thrombin has a number of cellular effects, which are triggered by binding to PARs. PARs are G-protein-coupled receptors, of which four subtypes have been identified in humans: PAR-1, -3 and -4 can be activated by thrombin, and PAR-2 can be activated by trypsin, tryptase, FVIIa and FXa, but not by thrombin. PAR-1, the prototype of the family, a receptor with seven transmembrane domains, which is activated by
cleavage of a specific site at the N-terminus of its extracellular domain. This results in the generation of a tethered ligand that binds to a site within the molecule itself to effect transmembrane signalling. When thrombin binds to PAR-1, proteolytic cleavage of this site is essential for signalling to take place. Synthetic PAR-activating peptides act by mimicking the tethered ligand sequence and binding to the active site within the receptor molecule. Once PAR-1 is activated, the ‘agonist’ ligand revealed by the receptor cleavage remains tethered to the active site until PAR-1 is uncoupled from signalling and is internalized by phosphorylation-dependent mechanisms [43]. Having been internalized, activated PAR-1 is not recycled, but is delivered to lysosomes for degradation. In order to maintain responsiveness to thrombin, endothelial cells and fibroblasts have an intracellular pool of preformed PAR-1 which can be delivered to the cell surface. Other cell types, such as megakaryocytic cells, do not store intracellular PARs and synthesize this receptor de novo as required.

The importance of the role of thrombin and its receptor in cancer behaviour has been known for some time, though understanding remains incomplete. The presence of PAR-1 has been demonstrated on many tumour cells, and it appears to be preferentially expressed in highly metastatic cell lines [44]. While cells that over-express PAR-1 have been shown to be invasive in vitro, this phenotype becomes yet more pronounced on ligand activation of PAR-1 [45]. The binding of thrombin to its receptor has been shown to have a number of cellular effects: TF mRNA and a procoagulant phenotype are induced in colon adenocarcinoma cells in a protein kinase C-dependent pathway [27], the expression of urokinase-type plasminogen activator is enhanced in prostatic carcinoma cells [46], progelatinase A is activated in VECs [47] and VEGF receptor (KDR and flt-1) expression is upregulated with subsequent sensitization of cells to the mitogenic activity of VEGF via the transduction mechanisms of protein kinase C and MAPK pathways [48].

FXa is also known to have cell signal-inducing effects independent of downstream proteases such as thrombin. This is thought to occur via a number of different receptors: effector protease receptor (EPR)-1 (EPR-1), an approx. 65 kDa protein that is similar structurally to the light chain of factor Va, has been identified on monocytes and endothelial cells where it acts as a cofactor for FXa in the conversion of prothrombin into thrombin in the absence of factor Va. The binding of FXa to EPR-1 on VECs is associated with signal transduction which is dependent on FXa catalytic activity. As EPR-1 itself does not contain proteolysis-sensitive sites, it has been suggested that FXa binding to EPR-1 instigates a chain of receptor activation resulting in the proteolytic cleavage of PAR-2 and subsequent intracellular calcium signalling [49]. Experimental work on cell signalling in keratinocytes has also

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**Table 1**

**Summary of established effects of proteases and receptors on tumour biology**

<table>
<thead>
<tr>
<th>Protease/receptor</th>
<th>Effect</th>
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<tbody>
<tr>
<td>TF</td>
<td>Overexpression leads to:</td>
</tr>
<tr>
<td></td>
<td>Increased histological grade of Ca pancreas [8]</td>
</tr>
<tr>
<td></td>
<td>Increased metastatic potential of tumours [9,12]</td>
</tr>
<tr>
<td></td>
<td>Increased tumour cell invasion in vitro [11]</td>
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<tr>
<td></td>
<td>Increased tumour growth in vivo [11,12,37]</td>
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<tr>
<td>TF/FVIIa</td>
<td>Increased expression of uPAR in SW979 cells [39]</td>
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<td></td>
<td>Induced calcium flux in J82 cells [33]</td>
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<td></td>
<td>VEGF production in melanoma cells [34]</td>
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<td>Cytoskeletal reorganization in J82 cells [35]</td>
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<td></td>
<td>Induces TF expression in SW480 Ca colon cells [27]</td>
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<tr>
<td></td>
<td>Enhanced uPA expression in Ca prostate cells [46]</td>
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<tr>
<td></td>
<td>Enhanced invasiveness of Ca breast cells [45]</td>
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<td></td>
<td>Expression of Cyr-61 and CTGF in HeLa cells [51]</td>
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<tr>
<td>Thrombin/PAR-1</td>
<td>Induces TF expression in SW480 Ca colon cells [27]</td>
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<td>Expression of Cyr-61 and CTGF in HeLa cells [51]</td>
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<tr>
<td>FXa/PAR-1</td>
<td>Expression of Cyr-61 and CTGF in HeLa cells [51]</td>
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implicated PAR-2 as the key component of a signal transduction pathway. Here, incubation of cells with both FVIIa and FXa results in an elevation of cytosolic calcium, phosphorylation of ERK1/2 and MAPK p38, and upregulation of transcription of Egr-1 [50]. However, PAR-1 has also been identified as a candidate receptor for the cellular effects of FXa. Recent microarray studies on gene induction in the HeLa cell line, which expresses PAR-1 but not PARs 2–4, have shown that both FXa and thrombin induce nuclear factor xB binding activity, as well as expression of both the angiogenesis-promoting gene Cyr61 and CTGF, via MAPK phosphorylation [51]. The activity of FXa is dependent on its proteolytic activity, and is independent of downstream thrombin activation. (Table 1).

**Summary**

The key factors of the extrinsic cascade of blood coagulation, FVIIa, FXa and thrombin, have a dual role by promoting blood clotting and by effecting intracellular signalling and therefore altering gene expression. These signalling pathways are dependent on the cell surface receptors TF and the PARs. The regulation of expression of such receptors, proteases and gene products is complex and multifactorial. Many positive feedback loops serve to propagate the formation of a thrombus in the peritumoral environment and to promote localized angiogenesis. Experimental evidence strongly suggests that greater cellular expression of TF and PARs correlates with more aggressive tumour behaviour. The direct targeting of these receptors with, for example, monoclonal antibodies or inactivating peptides, or the inhibition of activated coagulation proteases may, therefore, be developed as potentially useful therapeutic strategies. There is already retrospective clinical evidence that antithrombotic therapy may affect the incidence of certain cancers in a sample population [52]. Clinical trials are ongoing to assess the efficacy of low-molecular-mass heparins in improving survival in advanced malignancy.

**References**


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Urokinase-type plasminogen activator: a potent marker of metastatic potential in human cancers

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Abstract

Urokinase-type plasminogen activator (uPA) is a serine protease that is causally involved in cancer progression, especially invasion and metastasis. Multiple studies have shown that breast cancer patients whose primary cancer contains high levels of uPA have a significantly worse outcome than patients with low levels. As a prognostic marker for breast cancer the information supplied by uPA is both independent of traditionally used factors and significant in the important subgroup of axillary-node patients. Paradoxically, high levels of plasminogen activator inhibitor-1 (PAI-1), an endogenous inhibitor of uPA, also predict for aggressive disease. Recently, the prognostic impact of both uPA and PAI-1 in axillary node-negative breast cancer was confirmed using two different Level 1 Evidence studies, i.e. in both a randomized prospective trial and a pooled analysis. Therefore, uPA and PAI-1 appear to have fulfilled all the criteria for the routine assessment of prognosis in newly diagnosed breast cancer patients.

Introduction

Metastasis, or the spread of malignant cells from a primary tumour to a distant site, is the main cause of death in patients with cancer. Metastasis is