Tissue factor isoforms and cancer

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Universiteit Leiden

The handle http://hdl.handle.net/1887/21936 holds various files of this Leiden University dissertation.

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Title: Tissue factor isoforms and cancer
Issue Date: 2013-10-08

Chapter 3 - Alternatively spliced tissue factor: a crippled protein in coagulation or a key player in non-hemostatic processes?

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Summary

Full-length tissue factor (flTF) initiates coagulation, but also exerts non-hemostatic functions such as inflammation and angiogenesis through protease activated receptors (PARs). In 2003 a soluble variant of flTF was described which results from alternative splicing. Since its discovery the role of Alternatively Spliced Tissue Factor (asTF) in coagulation has been debated. asTF may have pro-coagulant properties but due to structural differences when compared to flTF, asTF coagulant function may be relatively low. Nevertheless, similar to flTF, asTF appears to have non-hemostatic properties; asTF expression in tumors correlates with increased tumor size, vessel number and poor survival in some cancer types, and drives tumor growth in animal models. Interestingly, unlike flTF, asTF does not promote angiogenesis through activating PARs but rather via integrin ligation. flTF is a critical determinant in cardiovascular disease but little is known about asTF in cardiovascular disease. asTF is produced by monocytes and macrophages, thus macrophage-derived asTF may contribute to atherosclerotic disease. In conclusion, unraveling asTF's non-hemostatic properties may generate new insights in the pathophysiology and diagnostics of cancer and cardiovascular disease.

Introduction

Full-length tissue factor (flTF) is a 47 kDa transmembrane glycoprotein and is considered to be the principal initiator of coagulation. flTF is encoded by a mature mRNA transcript that consists of 6 exons, in which exon 1 encodes the N-terminal signal sequence, exons 2-5 the extracellular domain (AA 1-219) and exon 6 the transmembrane region (AA 220-244) and the cytoplasmic tail (AA 245-263). Expression of flTF is normally limited to subendothelial tissues, but upon endothelial disruption, flTF becomes exposed to blood-borne factor VII (VII) and then a proteolytically active flTF:VIIa complex is formed. Subsequently, factor X (X) binds and when activated, Xa leaves the flTF:VIIa complex and then converts prothrombin into thrombin. Finally, fibrin fibers are generated, which together with activated platelets, form a hemostatic plug. The pattern of flTF-expression thus serves as a hemostatic envelop surrounding the vasculature[1].

It was long thought that under physiological conditions flTF is not in contact with the blood. However, in the late nineties circulating TF antigen in blood was first detected[2]. Further studies revealed that levels of circulating flTF antigen, either on the surface of microparticles or on activated monocytes, are higher in plasma from patients suffering from diseases such as coronary artery disease, sepsis, cancer or sickle cell disease[3-6]. In addition to these circulating forms of TF, Bogdanov described an alternatively spliced soluble isoform of flTF (asTF) in 2003[7]. This TF isoform results from alternative splicing of

the flTF transcript, in which exon 5 is omitted. As a result, a shift of the open reading frame occurs and the transmembrane and cytoplasmic domains are replaced with a unique 40 amino acid C-terminal domain, rendering asTF soluble. Later, the same group identified murine asTF in which exon 5 is also omitted as a result from alternative splicing[8]. Since its discovery the role of asTF in coagulation has been a matter of debate. Some reports attribute procoagulant function to asTF and find asTF present in thrombi, but others exclude such a role for asTF, making the role of asTF in physiology unclear. More recently, asTF was shown to be expressed in tumor tissues and to have angiogenic properties, suggesting a role in tumor angiogenesis. Although the majority of the research on asTF in (patho)physiology has focused on cancer, it is becoming clear that asTF might also impact on cardiovascular disease. In this review we will discuss the current literature on asTF considering its synthesis and its role in (patho)physiology.

TF pre-mRNA splicing

In biology the process of pre-mRNA splicing is a carefully regulated process and alternative splicing increases the proteome's abilities to adapt to environmental changes. Serine/arginine rich proteins (SR proteins) regulate constitutive and alternative splicing of pre-mRNA depending on their phosphorylation status by binding to exonic splicing enhancer (ESE) motifs. In silico analysis revealed that exon 5 of the TF gene contains ESE motifs that bind SF2/ASF and SRp55, which are SR proteins that are abundantly expressed in monocytic cell lines and monocyte-enriched peripheral blood mononuclear cells. Use of a minigene reporter system that was transiently expressed in monocytic cells, led to the identification of five ESE motifs in exon 5 that bind SF2/ASF and one ESE motif binding to SRp55. Selective mutagenesis of these ESE motifs weakened the SR protein-RNA binding, which further supported that SF2/ASF and SRp55 regulate TF pre-mRNA splicing in monocytes[9]. Further analysis of the monocytic THP-1 cell line demonstrated the presence of two other SR proteins, SC35 and SRp40, both having binding sites in exon 5. However, in stead of exon inclusion, SC35 and SRp40 were found to promote exon exclusion by competing with SF2/ASF and SRp55[10]. A delicate balancing of SR protein function underlies TF pre-mRNA splicing; however, the mechanism how SR protein phosphorylation status is regulated in monocytes is still unknown.

As in monocytic cells, TF pre-mRNA processing has also been studied in endothelial cells and furthermore, upstream signaling events in SR protein phosphorylation have been characterized. Human umbilical vein endothelial cells (HUVECs) are shown to express and secrete asTF after stimulation with TNF-α leading to pro-coagulant activity of the culture medium after addition of phopholipids[11]. The stimulus with TNF-α was found to affect the phosphorylation status of SR proteins that are present in HUVECs, SRp75, SRp55 and

SF2/ASF. After pharmacological interference with Cdc2-like kinase, the phosphorylation status of the SR proteins, SRp75, SRp55, and SF2/ASF was diminished in TNFα-stimulated cells. In a similar approach, inhibition of DNA topoisomerase I down-regulated phosphorylation of SRp55 and SF2/ASF, while leaving SRp75 unaffected. Experiments in which SRp75 and SF2/ASF were directly targeted with siRNA revealed that these SR proteins predominantly regulate the balancing between flTF and asTF mRNA. Whereas silencing of SF2/ASF up-regulated asTF mRNA levels, those of flTF mRNA were diminished; silencing of SRp75 left asTF mRNA levels unaffected, but lowered flTF mRNA levels. Changes at the mRNA level of these experiments indeed resulted in a diminished pro-coagulant activity of the HUVECs indicating the importance of TF pre-mRNA splicing in coagulant activity of stimulated HUVECs[12]. In addition to Cdc2-like kinases and DNA topoisomerase I, the PI3K/PKB (Akt) pathway, but not the NFκB pathway, was reported to alter phosphorylation status of SRp55, SRp75 and SF2/ASF in endothelial cells upon TNF-α stimulation[13]. The effect of SR proteins on exon 5 inclusion or exclusion in endothelial cells and monocytes are summarized in table 1.

	Exon 5 inclusion	Exon 5 exclusion
Monocytes	SF2/ASF	SC 35
	SRp55	SRp40
Endothelial cells	SF2/ASF	Unknown
	SRp55	
	SRp75	

Table 1. Overview of SR proteins determining exon 5 inclusion or exclusion in TF pre-mRNA splicing in monocytes and endothelial cells.

The studies on TF pre-mRNA splicing in endothelial cells and monocytes provide a better understanding of vascular wall thrombogenicity and lead to the plausible theory that pathological conditions could promote asTF synthesis in endothelial cells and monocytes. Whether this could lead to a pro-thrombotic state and how TF pre-mRNA splicing might be altered in e.g. cancer are questions that remain unanswered.

asTF in Coagulation

As mentioned before, asTF differs significantly from flTF such that a major part of the TF extracellular domain is replaced by a new C-terminus, which largely impacts on coagulant function. Nevertheless, Bogdanov and co-workers reported asTF to have pro-coagulant activity in presence of phospholipids. In addition, they showed asTF to be incorporated at the growing edge of *in vivo* thrombi. This led to the theory that asTF has its main physiological role in propagating thrombus formation[7]. Szotowski and colleagues

proposed that endothelial cells may act as a source for asTF after stimulation with IL-6 and TNF-α. Furthermore, asTF produced by HUVECs supported generation of activated factor X (Xa) in presence of phospholipids, which supports the theory that pathologic conditions trigger asTF production in endothelial cells hereby leading to a pro-thrombotic state[11].

However, several studies by others failed to show pro-coagulant activity of asTF[14-16]. Censarek and Hobbs used cells that were transfected with an asTF-expression construct, an in vitro set-up that differs from cytokine-stimulated HUVECs and that therefore may account for their findings. In addition, Böing found asTF to be retained in cells thereby limiting the bioavailability of asTF; however, in her experiments HUVECs were stimulated with IL-1α which may be a rather mild stimulus compared to TNF-α used in previous experiments. Nevertheless, these data seriously question the pro-coagulant activity of asTF. Despite the presence of the functional the 165-166 lysine doublet in asTF, which is critical for binding to VIIa, the affinity of asTF for VIIa is most likely diminished when compared to that of soluble flTF, consisting of the complete extracellular domain (AA 1-218). Furthermore, due to absence of the AA residues encoded by exon 5, asTF might lack a proper X docking domain explaining its hampered properties in promoting blood coagulation. Moreover, it is unclear how a proposed asTF:VIIa complex can associate with the phospholipid surface which is essential for an efficient conversion of X into Xa[17]. As reviewed before, the contradictory findings on coagulant activity of asTF may be due to differences in experimental set-up and therefore more work is needed to elucidate the role of asTF in coagulation[18].

asTF and Cancer

In multiple types of cancer high tumoral flTF-expression and thrombotic complications are described and the role of the TF:VIIa complex in cancerous processes has since long been recognized[19-21]. Knockdown of flTF in colorectal cancer cells xenografted in mice resulted in impaired tumor angiogenesis and consequently tumor growth[22]. It is currently thought that flTF together with its ligand factor VIIa, regulates the angiogenic switch in tumors through activation of Protease-activated Receptor-2 (PAR-2). TF:VIIa signaling produces expression of VEGF, IL-8 and metalloproteinases in vitro and CXCL-1 *in vivo*[23]. Tumor cell signaling by the flTF-VIIa-PAR-2 axis is negatively regulated by the flTF cytoplasmic tail, but phosphorylation of this domain, which results from activation of PAR-2, reverses this inhibition[24]. In addition, flTF physically associates with α3β1 and α6β1 integrins and disruption of this complex using a flTF monoclonal antibody, downregulates pro-angiogenic signaling and resulting tumor growth *in vivo*[23;25]. Importantly, flTF can exist in an reduced and oxidized form, and whereas the reduced signaling form appears to be involved in PAR-2-dependent tumor angiogenesis, the oxidized form influences metastasis

via activation of the coagulation cascade and activation of PAR-1[23;26]. Thus, flTF influences various stages in the cancerous process, depending on its interaction with other proteins and its redox state.

Despite this prominent role of TF:VIIa signaling in cancer, it is becoming increasingly clear that asTF might be an equally important modulator in cancerous processes. Several groups found asTF to be expressed in both tumor cell lines and tissue specimens from cancer patients. 8 out of 9 pancreatic cancer cell lines were reported to express asTF and pro-coagulant properties of the culture medium were attributed to presence of asTF as well as shedding of flTF-bearing microparticles[27;28]. However, experiments in colorectal cancer cell lines indicated that microparticle-bound flTF rather than asTF may contribute to the pro-thrombotic state in cancer patients[29].

Although a causal role of asTF in cancer-associated thrombosis has not been established yet, asTF expression was shown to relate to clinical outcome in non-small cell lung carcinoma (NSCLC). Lower asTF mRNA levels were found in NSCLC patients with grade Ia disease when compared to patients with a more advanced staging and in concordance with these findings, tumoral asTF expression related to a poorer prognosis in NSCLC in another study[30;31]. In contrast to NSCLC, in oesophageal cancer, asTF mRNA levels were comparable with healthy controls, whereas flTF expression was increased in tumor samples[32]. Further, our group detected intratumoral levels of asTF ranging from 0-75 nM in cervical cancer specimens and found asTF to be differentially expressed but whether this relates to clinical outcome is still unclear[33]. Apparently, flTF and asTF expression patterns may differ amongst tumor types and how asTF expression relates to clinical outcome and whether asTF may be used as a blood-borne marker for staging cancer remains to be investigated.
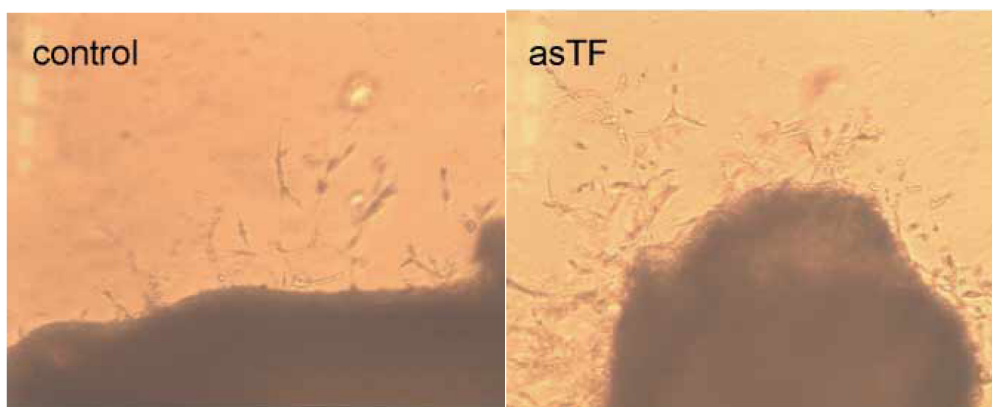
Figure 1. asTF promotes angiogenesis *ex vivo*. asTF angiogenic properties were tested in an aortic ring assay . Aortic segments from C57Bl6 mice were embedded in matrigel supplemented with solvent control (upper panel) or asTF (lower panel). Pictures are shown of outgrowing sprouts on day 5.

Despite presence of asTF mRNA and protein in tumors, the mechanism how asTF affects cancer is still elusive. Hobbs and colleagues set out to investigate how tumoral asTF expression influences tumor biology by means of an *in vivo* model. They used the pancreatic cancer cell line MiaPaca2, which does not express endogenous TF to generate cells that selectively express flTF or asTF expression by means of stable transfection. The flTF-expression construct conferred pro-coagulant TF activity to the culture medium but, in line with earlier observations, the asTF-expression construct failed to do so. When cells were injected subcutaneously to assess tumor growth, flTF expression rendered smaller tumors when compared to cells that did not express TF, but asTF expression increased tumor size. Immunohistochemical analyses of the tumors revealed that asTF-expressing tumors display more tumor vessels and vessel leakage, a feature of tumor angiogenesis. However, these experiments failed to assess how asTF expression results in increased angiogenesis. One option is that asTF drives tumor growth, resulting in tumor cell-dependent angiogenesis through secretion of pro-angiogenic factors. Alternatively, asTF may directly impact on the formation of blood vessels which subsequently drives tumor expansion. To uncover the exact role of asTF in tumor angiogenesis our group set out to characterize the effect of recombinant purified asTF on *in vivo* and *ex vivo* angiogenesis. Using a matrigel plug assay in which mice were injected subcutaneously with matrigel containing either 100 nM recombinant human asTF, 50 ng/ml recombinant murine VEGF or buffer control, we could show that asTF induces an angiogenic response comparable to that evoked by VEGF. In line with these findings we found that human recombinant asTF was able to significantly promote angiogenesis at levels as low as 1 nM in a murine aortic ring assay (Figure 1). Since flTF exerts its angiogenic effect through binding to VIIa and subsequent PAR2 signaling, we first investigated whether asTF could facilitate PAR2 signaling in endothelial cells. To this end, endothelial cells that were adenovirally transduced in order to express PAR2 were stimulated with asTF alone, asTF in combination with VIIa or a PAR2 agonist as positive controls. Whereas the PAR2 agonist elicited a potent phosphorylation of p44/p42 MAPK, asTF alone and the combination of asTF and VIIa failed to do so, which led us to conclude that asTF-induced angiogenesis is not PAR2-driven. Then we dissected the mechanism of asTF-induced angiogenesis into stages of endothelial cell adhesion, migration and capillary formation. All three processes could be triggered with asTF and were sensitive to blockade with specific antibodies to integrin subunits. Whereas asTF-induced endothelial cell migration depended on integrin αVβ3, capillary formation could be abrogated by integrin α6β1 blockade. Interestingly, asTF was shown to ligate different integrins than flTF, indicating that asTF may have a different role in non-hemostatic processes than flTF.

After binding to asTF coatings, endothelial cells displayed phosphorylation of Focal Adhesion Kinase (FAK), PI3 kinase, p38 MAP kinase, p44/42 MAP kinase and Akt. By use of specific kinase inhibitors we found that asTF-induced cell migration depended on PI3 kinase and p38 MAP kinase, but not on p44/42 MAP kinase. Use of an Akt inhibitor only modestly inhibited asTF-induced andothelial cell migration which indicates that both Akt-dependent and independent pathways are involved in asTF-induced endothelial cell migration. In a similar approach we identified PI3 kinase and p44/42 MAP kinase, but not the promigratory p38 MAP kinase to be crucial in asTF-induced capillary formation. In support of that, cells allowed to form capillaries on matrigel supplemented with asTF showed higher levels of p42/p44 and Akt, but not p38 phosphorylation[33].

So far it is known that asTF is expressed in several tumor types and that its expression may relate to clinical outcome. In addition, the intratumoral asTF concentration is sufficient to promote angiogenesis through integrin ligation independent from PAR2-signaling. However, more research is needed to determine the relationship between tumoral asTF expression and circulating asTF levels in cancer patients on the one hand and clinical and tumor type characteristics on the other. In addition, further studies are needed to elucidate the mechanisms that trigger asTF production in tumors.

Cardiovascular disease

Apart from inflammation and cancer, flTF also influences cardiovacular disease. Levels of flTF expression are lower in patients with dilated cardiomyopathy (DCM) hereby influencing cell-to-cell contacts and myocardial contractility. In parallel with flTF expression, asTF mRNA levels were lowered in these patients as well, but the pathophysiological relevance of asTF lowering in DCM patients is still unclear[34].

Diabetes greatly influences the biology of cardiovascular disease and flTF in pancreatic islet cells has been proposed to be an intermediate between coagulation and cardiovascular disease. During prolonged hyperglycemia alpha and beta cells in the islets of Langerhans express higher levels of flTF and they secrete microparticle-bound flTF and asTF. Whether islet cell-derived circulating TF indeed contributes to atherothrombosis is unclear. Furthermore, whether asTF can influence the endocrine pancreatic microenvironment is even more speculative[35].

Lipid rich necrotic cores of atherosclerotic plaques contain flTF which takes part in atherothrombus formation after plaque rupture. Furthermore, high levels of the circulating TF antigen are present during both acute and chronic phases of atherosclerotic disease[36;37]. However, to what extent asTF contributes to circulating TF antigen levels in

cardiovascular disease is unknown. The same applies to the role of asTF in atherosclerotic plaques. Activated monocytes have been found to be bona-fide producers of asTF 10, making a role for asTF in cardiovascular disease plausible. A critical step in the formation of an atherosclerotic plaque is the invasion of monocytes into the subendothelium, followed by their transformation into foam cells which eventually undergo necrosis. As a consequence, monocyte-derived asTF may become available in atherosclerotic plaques to either stimulate vasa vasorum formation, an event that has been shown to disrupt the integrity of the atherosclerotic plaque[38]. In preliminary experiments we stained atherosclerotic plaque material for asTF and we found inflammatory infiltrates to stain positive for asTF (Figure 2), confirming that asTF might play such a role. However, to establish a relationship between asTF and plaque angiogenesis, extensive analysis of human atherosclerotic plaque material for asTF expression and angiogenic markers will prove vital.
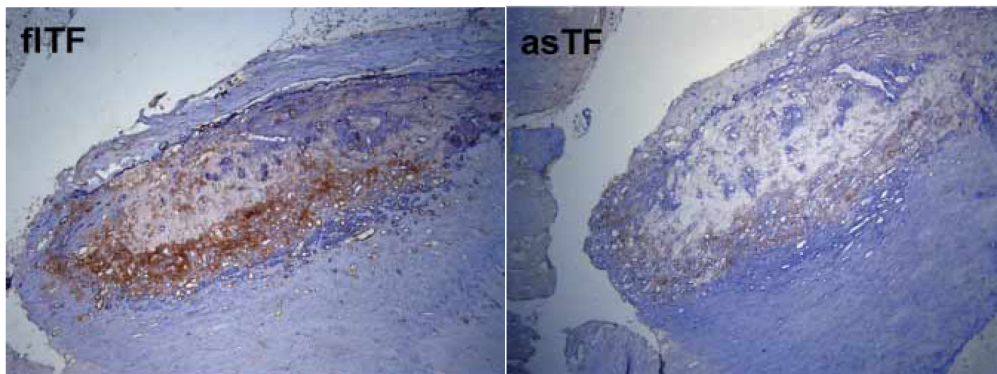
Figure 2. asTF is present in inflammatory infiltrates in atherosclerotic plaques. Paraffin embedded specimens from atherosclerotic plaques on microscope slides were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with 0.3% H2O2 in methanol. For flTF stainings, slides were subjected to antigen retrieval by cooking for 10 minutes in sodium citrate buffer. Antigen retrieval was omitted for asTF stainings. After blocking with 10% normal goat serum in PBS/BSA 1%, slides were incubated with an asTF-specific antibody at a concentration of 1 µg/ml or flTF antibody at a concentration of 10 µg/ml. After appropriate washing steps and incubation with a secondary antibody, immunostaining was performed with 3,3'-Diaminobenzidine (DAB) peroxidase. Sections were counterstained with hematoxylin.

Directions of future research

Despite the work that has been done on asTF function, the role of asTF in (patho)physiology is still unclear. Primary cell cultures and cell lines have been helpful for identification of asTF mRNA in cancer and the study of the splicing mechanism of TF pre-mRNA. However, the mechanism of alternative splicing in cancer is still unclear and cancer cell lines offer the possibility to study this mechanism. However, cell culture has some serious limitations considering the study of (patho)physiologic mechanisms. Lack of proper

triggers or the interplay between different cell types might explain the problems that are encountered by researchers when trying to have asTF secreted in culture media from cells in culture.

Therefore *in vivo* models may be helpful to obtain more insight in the functions of asTF. Our group showed that murine cell lines as well as mice respond to human recombinant asTF in experiments on angiogenesis which justifies the study of human asTF in a murine system. However, the field would benefit from a functional characterization of murine asTF in order to enable the use of valuable *in vivo* models that could be performed in a complete syngeneic approach. Moreover, the generation of murine models on asTF might prove valuable. Although mice have been generated that express low levels of TF and essentially are knock-outs for asTF, it is hard to interpret how the loss of asTF affects the phenotype of these mice[39].

Another direction of future research that may improve our understanding of the relevance of asTF in pathology is determining the relationship between asTF expression and clinical data in cancer. At present the relation between asTF expression and clinical staging is only investigated in non-small cell lung cancer and esophageal cancer on the mRNA level. It is possible that asTF has different effects in different types of cancer, which necessitates the study of asTF in other cancers, also focusing on protein level. Associations found in such studies will give important clues to direct future mechanistic studies. Furthermore, the detection of circulating asTF protein in plasma samples from these patients will be helpful and may even serve as a biomarker for certain types of cancer. As in cancer, asTF may have biomarker properties for patients suffering from cardiovascular disease, however, the role of asTF in cardiovascular disease should be clarified first.

Conclusion

Since its discovery the (patho)physiologic role of asTF has been unclear. Compared to flTF, asTF seems to have only weak pro-coagulant properties. Use of different in vitro approaches hampers a clear interpretation of the experiments done so far on the role of asTF in coagulation. In addition, future studies may elucidate which triggers are necessary for asTF secretion from cells. Although debated as a cripple protein in blood coagulation, asTF seems to have interesting non-hemostatic properties in cancer. Future research is needed on asTF and is likely to provide more insight in the role of TF isoforms in tumor biology and cardiovascular disease. In addition, its properties as a soluble circulating protein may be beneficial for the development of new diagnostic tools.

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