The role of blood coagulation in cancer, inflammation and embryonic development

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Alternatively spliced tissue factor in mice: induction by *Streptococcus Pneumoniae*

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Abstract

Alternatively spliced tissue factor (asTF) has recently been described in humans to be a soluble isoform of tissue factor (TF), which is present in blood. However, the existence of asTF is raising controversy in the field of haemostasis because active TF within the bloodstream would lead to massive intravascular thrombosis. Therefore the existence and biological role of asTF has been disputed ever since its discovery.

The objective of this study was to prove or refute the significance of asTF in (patho)physiology.

We investigated whether asTF is present in mice. To assess the potential involvement of asTF in (patho)physiology, we determined the effect of \textit{Streptococcus Pneumoniae} infection and acute arterial thrombosis on asTF levels in mice.

We show that mice also express a soluble TF variant lacking exon 5 and thus the transmembrane region. This murine asTF contains a novel C-terminus of 94 amino acids leading to a protein of 291 amino acids. The C-terminus of murine asTF shares strong homology with human asTF, is 54 amino acids longer and has no significant homology with any other protein in the NCBI database. Murine asTF is expressed in lung tissue, in which it is induced by \textit{S. Pneumoniae} infection. Furthermore, murine asTF is present in plasma and can be found throughout arterial blood clots induced by FeCl$_3$.

The finding that mice produce asTF, which is induced by \textit{Streptococcus Pneumoniae} and is omnipresent in blood clots, strongly suggest an important role for asTF in (patho)physiology.
Introduction

In the traditional view of blood coagulation, vascular injury leads to the exposure of extravascular membrane bound tissue factor (TF) to the blood stream thereby initiating blood clot formation. Essential in this model of haemostasis is that TF is normally not in contact with blood as it resides in the adventitial lining of blood vessels. Recently, however, a soluble TF variant, which is derived by alternative splicing of the TF mRNA, has been described in humans [1]. This alternatively spliced tissue factor (asTF) lacks the transmembrane domain, possesses a unique 3' peptide sequence, is present in plasma and seems biologically active, albeit in high concentrations.

Since its first description, several groups proceeded to unravel the physiological importance of this soluble TF variant. To this end, it has been shown that asTF is upregulated in human squamous-cell carcinoma which might lead to an increased risk of acute thrombotic events and poor prognosis of patients suffering from squamous-cell carcinoma [2]. On the contrary, HCT116 colorectal carcinoma and A431 squamous-cell carcinoma cells mainly shed TF containing microparticles but not the asTF isoform into the culture medium [3]. Recently, asTF was shown to be present in cardiomyocytes of the right ventricular septum and the expression level of asTF was down-regulated in the myocardium of dilated cardiomyopathy (DCM) patients [4]. Whether down-regulation of asTF impairs the structural integrity of the myocardial muscle thereby leading to DCM remains elusive. Finally, islets of Langerhans from the pancreas, intended for islet transplantation in diabetes type 1 express asTF, next to full-length TF [5]. As consequence of TF expression an instant blood mediated inflammatory reaction (IBMIR) after clinical islet transplantation may occur.

The biological procoagulant activity of asTF, as suggested initially [1], has been disputed ever since its discovery [6] mainly because active TF within the bloodstream would lead to massive intravascular thrombosis. Indeed, only microparticle associated full-length TF obtained from patients with coronary artery disease was shown to exhibit procoagulative properties, whereas soluble TF had procoagulant activity [7]. Further controversy as to the biological activity of asTF was raised by observations that asTF expressed and released from endothelial cells in response to inflammatory cytokines has procoagulant activity [8].

In order to prove or refute the significance of asTF, we hypothesized that if it were physiologically relevant it should be present in other species as well. In this study, therefore, we investigated whether asTF is present in mice. Moreover, to assess the potential involvement of asTF in (patho)physiology, we determined the effect of Streptococcus Pneumoniae infection and acute arterial thrombosis on asTF levels in mice.

Materials and methods

Animal experiments

All animal experiments were approved by the Committee on Use and Care of Animals of the Academic Medical Center, Amsterdam, The Netherlands.
Induction of pneumonia

Pneumococcal pneumonia was induced as described previously [9,10]. Briefly, S. pneumoniae, serotype 3, obtained from American Type Culture Collection (ATCC 6303; Rockville, MD), were grown for 6 hours to midlogarithmic phase at 37°C using Todd-Hewitt broth (Difco, Detroit, MI), harvested by centrifugation at 1500xg for 15 minutes, and washed twice in sterile isotonic saline. Bacteria were then resuspended in sterile isotonic saline at approximately 1·10⁷ colony forming units (CFU)/ml, as determined by plating serial 10-fold dilutions on sheep-blood agar plates. Eight weeks old C57/Bl6 mice were lightly anesthesized by inhalation of isoflurane (Abott, Queensborough, Kent, UK), and 50 μl of bacterial suspension was inoculated intranasally, corresponding with 5·10⁵ CFU S. pneumoniae.

Preparation of lung homogenates

Twenty hours after inoculation, mice were anesthesized by intraperitoneal injection with Hypnorm® (Janssen Pharmaceutica, Beerse, Belgium) and midazolam (Roche, Mijdrecht, The Netherlands), and blood was collected from the inferior caval vene. Whole lungs were harvested and homogenized at 4°C in 5 volumes of sterile isotonic saline with a tissue homogenizer (Biospect Products, Bartlesville, UK).

RNA isolation

Total RNA was isolated from snap frozen tissue using guanidine isothiocyanate (Trizol®; Gibco, Carlsbad, CA, USA)/chloroform extraction followed by precipitation with 2-propanol as described previously [11].

RT-PCR

Full-length and alternatively spliced TF mRNA levels were measured using quantitative real-time PCR using Light Cycler technology (Roche Molecular Biochemicals, Alameda, CA, USA) with SYBR Green II detection using standard software for quantification as recommended by the manufacturer. Primers for TF (forward: 5’-GGAAAGGCTCA AGCACAGGAAA-3’ and reverse: 5’-CAGGAAACTCTTTCCATTGCTCTGTG-3’) and asTF (forward: 5’-GCACGGGGAAAGAAACACTCATCATTG-3’ and reverse: 5’-CTCCGCAACAGTGCCGTGCAGGCAG-3’) were chosen based on the murine TF mRNA sequence (Genbank accession number NM_010171). Amplification products were also analyzed on a 2% agarose gel. Expression levels were normalized to the expression level of GAPDH mRNA (forward: 5’-CTCATGACCACAGTCCATGC-3’ and reverse: 5’-CACATTGGGGGTAGGAACAC-3’).

Induction of asTF antibodies in rabbits

New Zealand White female rabbits (Harlan, SPF) were used to develop rabbit anti-mouse asTF antibodies. Before injection of the Freund’s Complete Adjuvant mixture, pre-immune serum was taken. The rabbits were immunized with a mixture of 500 μl Freund’s Complete Adjuvant and two different synthetic murine asTF peptides (peptide 1: CXX-RRAWHRGKAEEANAH and
peptide 2: CXX-ASAERTERDRKGRT) coupled to the carrier hemocyanin keyhole limpet (KLH) by subcutaneous injection (2x 0.5 ml) on the back and intramuscular injection in the hind legs (2x 0.5 ml). After 8 weeks test-serum was taken and the rabbits were boosted after 3 months with the peptide-KLH complex in presence of Freund’s Incomplete Adjuvant.

**Arterial thrombosis**

Mice were anesthetized by intraperitoneal injection of 70 μl/kg body weight FFM mixture (Fentanyl (0.315 mg/ml)- Fluanisone (10 mg/ml) (Janssen Pharmaceutical, Beerse, Belgium), Midazolam (5 mg/ml) (Roche, Mijdrecht, The Netherlands)). Body temperature was monitored with a rectal probe and maintained at 37°C ± 1°C via a heating pad and a halogen-heating lamp. During anesthesia, oxygen (1 L/min) was supplemented via a tube placed at the nose of the mouse. Acute arterial thrombosis was induced by application of ferric chloride (25% FeCl₃) to the left carotid artery, as described [12] resulting in the formation of platelet and fibrin-rich thrombi [13]. At the end of the arterial thrombosis experiment, the left carotid arteries were formalin-fixed, embedded in paraffin and sectioned. For immunohistochemical analysis paraffin sections of 4 μm were deparaffinized and rehydrated. Thrombus composition was evaluated using haematoxylin-eosin staining. For detection of asTF expression, sections were incubated with 1.5 % H₂O₂ in PBS for 20 minutes and then blocked with TENG-T (10mM Tris, 5 mM EDTA, 0.15 M NaCl, 0.25% gelatin, 0.05% (vol/vol) Tween-20%, pH 8.0) for 30 minutes at room temperature. Thereafter sections were washed and incubated with primary antibodies against asTF overnight at 4°C. After washing, goat anti-rabbit (DAKO A/S, Glostrup, Denmark) was used as the secondary antibody in a 1:250 dilution for 1 hour at room temperature. Next, the sections were incubated with SABC complex (DAKO A/S) for one hour at room temperature, and washed. Enzyme activity was detected with AEC (Sigma Chemical Co., St. Louis, MO, USA) after incubation for 5 minutes.

**Western Blotting**

Samples were resuspended in SDS-PAGE sample buffer and incubated for 5 minutes at 95°C, after which they were loaded onto SDS-PAGE and subsequently transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore Corp, Bedford, MA, USA) membrane. The membranes were blocked with Tris-buffered saline (TBS), supplemented with 0.1% tween-20 (wash buffer) and 1% low-fat milk powder, and incubated with primary antibodies over night at 4°C, diluted 1:1000 in wash buffer. Subsequently, the membranes were incubated with a horse radish peroxidase-conjugated secondary antibody in wash buffer, containing 1% low-fat milk powder. The bands were visualized, using Lumilight plus® ECL substrate and a chemiluminescence detector with a cooled CCD-camera (GeneGenome) from Syngene (Cambridge, UK).
Results

As in humans asTF is mainly expressed in lung tissue, we performed RT-PCR on murine cDNA collected from lung homogenates, using primers in exons 4 and 6 spanning the proposed alternatively spliced region. Indeed, a specific PCR product lacking exon 5 was detected (data not shown). As shown in figure 1a, the novel C-terminus of murine asTF is 94 amino acids long leading to a protein of 291 amino acids (as compared to the 294 amino acid full length TF). As shown in figure 1b, the C-terminus of murine asTF shares strong homology with human asTF (51% versus 55% homology between full-length murine and human TF) but is 54 amino acids longer. Homology searches with the C-terminus of murine asTF using the NCBI database did not result in any significant homologies.

TF plays a crucial role in several (patho)physiological processes, including inflammation and infection [14]. To assess the potential importance of asTF in inflammatory disease, mice were intranasally infected with *S. pneumoniae*. As shown in figure 2, asTF mRNA levels in lung homogenates of infected mice were about two-fold increased compared to uninfected

**Figure 1:** Alignment of murine asTF with full-length murine TF (A) or human asTF (B). The novel C-terminus of asTF resulting from alternative splicing of exon 5 is underlined, whereas homologous amino acids are indicated by asterixes. The alignment was performed using ClustalW.
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controls. *S. pneumoniae* infection also induced full length TF mRNA levels about two-fold, indicating that no differential upregulation of the splice variant took place (figure 2).

To confirm the presence of asTF in mice, we raised and validated two rabbit polyclonal antibodies against the unique C-terminal amino acid sequence of asTF. As shown in figure 3a, using the antibody raised against peptide-1 we were able to detect asTF in murine plasma. Competition experiments with peptide-1 reduced detection of plasma asTF, whereas competition with peptide-2 did not impair detection (figure 3b). As shown in figure 4, *S. pneumoniae* infection induced circulating asTF protein levels, thereby confirming the mRNA data.

To further investigate the potential biological importance of murine asTF, we induced vessel injury and subsequent acute arterial thrombosis in mice by application of ferric chloride (25% FeCl₃) to the left carotid artery [12]. As shown in figure 5, immunohistochemical staining

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**Figure 2:** Both asTF and full-length TF mRNA levels are induced by *S. pneumoniae* (s.p.) infection. Mean ± SEM (n=8) of asTF, p=0.0013 (A) and full-length (tTF), p=0.014 (B) TF mRNA levels and of the ratio between asTF and tTF, p=0.63 (C) levels in lung 20 hours after *S. pneumoniae* infection.

**Figure 3:** Murine plasma contains asTF. A) Western blot of three independent plasma samples probed with either pre-immune serum or immune serum raised against asTF specific peptide 1. B) Pre-incubation of murine plasma with asTF specific peptide 1 (10 μg) blocks asTF detection whereas pre-incubation with asTF specific peptide 2 (10 μg) does not block asTF detection using peptide 1.
of the platelet and fibrin-rich thrombus for asTF showed profound staining throughout the blood clot. Again this suggests an important role of asTF in (patho)physiology.

**Discussion**

Alternatively spliced human tissue factor (asHTF) has recently been described to be a soluble isoform of TF, which is present in blood [1]. However, the existence of (active) asTF is raising controversy in the field of haemostasis because active TF within the bloodstream would lead to massive intravascular thrombosis. Therefore the existence and biological role of asTF has been disputed ever since its discovery [6, 15]. In the current study, we show that mice also express a soluble TF variant lacking exon 5 and thus the transmembrane region. This murine asTF is expressed in lung tissue, in which it is induced by *S. Pneumoniae* infection. Furthermore, murine asTF is present in plasma and can be found throughout arterial blood clots induced by FeCl₃.
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Originally asTF was shown to be present in human tissues, plasma and to be incorporated into thrombi. More recently, endothelial and epithelial cells as well as leukocytes and several cancer cell lines [8, 16] were documented as possible sources for asTF. However, whether the alternatively spliced TF variant is of biological relevance or is just a coincidental and irrelevant byproduct remains a matter of debate. Our observations that mice express a similar alternatively spliced TF variant (lacking exon 5) argues against the notion that it is just a coincidental byproduct. In addition, the fact that mice express asTF facilitates future research into its physiological relevance using mice as model system.

TF is known to play an important role in inflammatory disease and inhibition of TF prevents lethal disease [17-20]. The potential role of asTF in inflammation remains elusive. Our data, showing that asTF levels increase after intranasal inhalation of S. Pneumoniae, suggest that asTF might play a role in inflammatory processes. Indeed, endothelial cells treated with pro-inflammatory cytokines express and secrete functional (i.e. coagulant) asTF [8]. However, in lungs of infected mice full length TF is induced to a similar extent as asTF (figure 2). In addition, pro-inflammatory cytokines also induce full length TF in endothelial cells [21, 22], however the relative induction of full length versus asTF is not yet known. Although asTF is not differentially regulated during infection, the induction by infectious and inflammatory agents indicates that asTF should be taken into account in models studying the cross talk between coagulation and inflammation.

Human asTF has been shown to be present in arterial thrombi, but as already mentioned its intravascular presence raises controversy. Our data confirm the presence of asTF in arterial thrombi, thus establishing a potential role in coagulation related (patho)physiology. Soluble TF is a potent activator of coagulation [23], although other data do not confirm these observations [7], and thus one would expect asTF to cause massive intravascular thrombosis. However, both the human and our mice experiments argue against asTF induced massive and lethal thrombosis suggesting regulatory mechanisms. It could be that asTF is scavenged from circulating FXa (as the interaction between TF/FVIIa and FXa needs phospholipids) or it could be that under physiological circumstances asTF has limited procoagulant activity compared to full length TF [1, 3] thereby even preventing massive thrombosis. In this view, asTF behaves as an inhibitor of coagulation rather than an activator [6].

Although the presence of soluble TF in blood remains puzzling, we believe that our mouse data are in favour of an important biological role. Obviously, this biological role, be it stimulating thrombus growth [1] or inhibiting this process [6] needs further exploration. The relevance of asTF can be best obtained using knock-out animals for asTF or using mice that only express the splicing variant in state the art of models of blood coagulation, infection, atherosclerosis and/or tumor growth. The finding that mice produce asTF which is omnipresent in blood clots is only a first important step towards the definitive answers.
References


