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Alternatively spliced tissue factor: discovery, insights, clinical implications

Ramprasad Srinivasan, Vladimir Y. Bogdanov

Division of Hematology/Oncology, Department of Internal Medicine, University of Cincinnati College of Medicine

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1. ABSTRACT

Following the molecular cloning of human Tissue Factor (TF) in mid-1980's, great strides have been made in the understanding of TF biology, TF's crucial roles in the initiation of blood coagulation and embryonic development, and TF's contribution to the pathobiology of various disease states. The 21st century brought about a rather unexpected turn in the "TF journey" – a few years back it was reported that the TF gene produces not one, but two proteins with distinct structural and functional characteristics. The so-called "full-length TF" (flTF) – a much-studied integral membrane glycoprotein long presumed to be, and experimentally handled as "the TF" in hundreds of laboratories around the world – is now known to be one of the two TF forms naturally occurring in humans as well as mice. The other, recently discovered form is termed alternatively spliced TF (asTF) which, unlike flTF, lacks a transmembrane domain and can thus be secreted. In this review, we summarize the literature on asTF by discussing asTF's biologic roles as they are currently understood, tackling a number of questions pertaining to asTF's evident and proposed biologic properties, and briefly covering the emerging field of regulated TF pre-mRNA processing.

2. INTRODUCTION

The number of biologically active proteins in higher eukaryotes is estimated to exceed 100,000 whereas it was determined that the human haploid genome comprises only 23,000 genes (1). At the present time, this phenomenon is being largely attributed to the process termed alternative pre-mRNA splicing. A very large number of structurally and functionally diverse, biologically active proteins generated via alternative pre-mRNA splicing have been discovered to date, and many of them were found to participate in various biologic processes that concern normal physiology as well as pathologic conditions. Full-length Tissue Factor (flTF) is a 47 kDa transmembrane glycoprotein that serves as the principal physiologic trigger in the initiation stage of blood coagulation. Differential splicing of Tissue Factor (TF) pre-mRNA results in the biosynthesis of flTF as well as a protein termed alternatively spliced Tissue Factor (asTF). While several TF mRNA variants have been identified (2), asTF mRNA is the only protein-coding TF mRNA generated via alternative pre-mRNA splicing (3). In this review, we focus on the discovery of asTF, its structure and biologic activity, its relevance to certain (patho)physiologic processes, as well as the recent information pertaining to

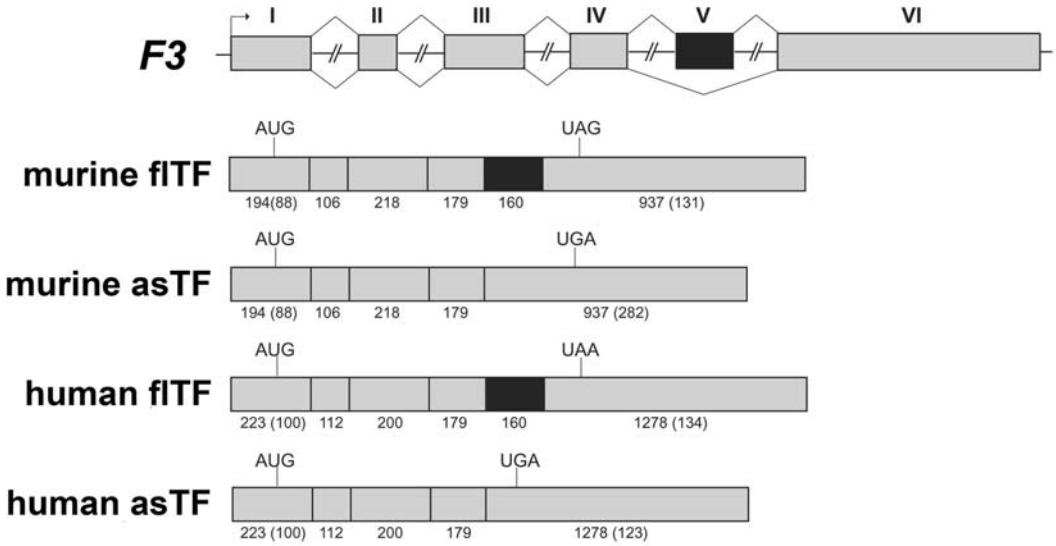

Figure 1. Intron-exon structure of the TF gene (identical in human and mouse) and the resultant exonic structure of murine and human flTF and asTF. Exons are identified by Roman numerals. Numbers under the exons correspond to their lengths (in nt); for the 1st and the 6th exons, the length of the exonic region utilized in the ORF is indicated in parentheses. Adapted from ref. 7.

the regulatory pathways engaged in the governance of the flTF/asTF mRNA ratio.

In 2003, Bogdanov *et al* described for the first time an alternatively spliced, soluble, naturally occurring protein variant of TF – alternatively spliced human Tissue Factor (asHTF, thereafter termed asTF). While studying the effects of granulocyte colony-stimulating factor (G-CSF) on TF transcription in HL-60 cells using conventional RT-PCR, they observed a second, smaller amplicon whose size was smaller than that of the expected amplicon (4). RT-PCR was then carried out to amplify the entire TF open reading frame (ORF), which confirmed the existence of two distinct protein-coding mRNA species generated by the TF gene (*F3*). The smaller ORF was subcloned and sequenced, revealing a previously unknown TF ORF in which exon 5 was absent and exon 4 was spliced directly to exon 6. In 2001, Guo *et al* reported an RT-PCR amplicon lacking TF exon 5 in acute promyelocytic leukemia cells; the authors failed to deduce that such an amplicon may be derived from an mRNA that encodes a distinct form of the TF protein (5). The alternative splicing event (exon 5 exclusion) yields a complete codon at the end of exon 4 and an incomplete codon at the beginning of exon 6; as a result, a frameshift is created in the asTF mRNA. Hence, asTF features a unique 40 amino acid C-terminus distinct from that of flTF. The amino acid residues 1-166 of the mature asTF peptide are identical to those in the flTF extracellular domain, and the residues 167-206 comprise the unique C-terminus. asTF lacks the transmembrane region, thus rendering asTF soluble. The lysine doublet at the positions 165-166 was shown to be crucial for the enzymatic activity of the TF-FVIIa complex (6). Importantly, this lysine doublet is also present in asTF, which constitutes a significant sub-fraction of TF that circulates in plasma. Evaluation of seven specimens of human plasma revealed that asTF may comprise upward of 30% of total plasma TF as for the six of these specimens, the results were 61±59 pg/ml using a commercial anti-TF antibody and 18±16 pg/ml using a specific anti-asTF antibody; for the seventh specimen, the results were 672 pg/ml and 400 pg/ml, respectively (4). Immunostaining of spontaneously formed arterial thrombi revealed extensive co-localization of flTF and asTF; interestingly, asTF rapidly accumulated in platelet-rich experimental thrombi formed under shear in normal human blood, suggesting that asTF may participate in thrombogenesis. Biochemical solubility and co-factor activity of asTF was then confirmed via expression and purification of recombinant asTF in the bacterial cytosol: in the absence of phosholipids, asTF exhibited no procoagulant activity, yet it shortened clotting times when added, along with negatively charged phospholipid vesicles, to recalcified human plasma (4).

3. OF MICE AND MEN: THE ASTF PERSPECTIVE

3.1. Murine asTF: structure, expression, functional characterization

Following the discovery of human asTF protein, Bogdanov *et al* also identified and characterized the murine homolog of asTF (7). Like human asTF, murine asTF (masTF) mRNA lacks exon 5 which creates a frameshift, resulting in a unique C-terminus that lacks a transmembrane domain. (Figure 1) Immunostaining of murine tissues using a panel of custom polyclonal antibodies specific for either murine flTF or masTF revealed robust levels of masTF protein in several tissues including kidney, brain, heart, lung, and spleen, whereas only minimal staining for masTF in blood vessels was observed. The tissue distribution pattern of masTF was similar to that of flTF, suggesting that asTF levels reflect the overall TF levels under normal physiologic conditions (7). Analysis of an expressed sequence tag clone from a commercial library indicated that the 3' UTR of masTF and

flTF mRNAs are identical: they contain AU rich elements and hence it is likely that flTF and masTF mRNAs have similar properties regarding half-life/stability. Interestingly, the 3' UTR of human asTF mRNA was also found to match that of human flTF mRNA (4), yet the half-life/stability of these mRNAs may not be identical (8). Procoagulant activity of masTF required the presence of negatively charged phospholipids and, like human asTF, masTF incorporates in arterial thrombi (7). The accumulation of masTF in thrombi suggests that masTF protein comprises a component of the circulating TF pool that participates in thrombogenesis (9-13). It is notable that, in a follow-up study, Brüggemann *et al* observed systemic upregulation of circulating masTF, alongside flTF, in mice intranasally challenged with streptococcus: the experimental thrombi examined in this study were also found to contain robust levels of masTF as well as flTF, which went up sharply after the bacteria were administered (14). Bogdanov *et al* demonstrated that like human asTF, masTF is soluble and can be secreted – they generated recombinant masTF using an inducible expression system that secretes the protein to the bacterial periplasmic space, from which masTF was extracted by osmotic shock without any detergent present; masTF's coagulant activity, which required phospholipids, was ~3% when compared to that of flTF (7), prompting some investigators to propose a scenario whereby masTF may, perhaps, act as an inhibitor of coagulation in certain settings (14). At the present time, the role of (m)asTF in normal hemostasis and/or embryonic development is poorly understood, and appropriately designed mouse models thus comprise an indispensable tool to conduct such studies. In particular, knockout and/or overexpression approaches that are "genetically tailored" to study asTF function in health and disease would provide valuable new insights into asTF biology; such models are currently being developed in our laboratory. Below, we briefly summarize the presently available data on murine models of TF, and provide explanation as to why some of these models may hold substantial value in ongoing and future research focusing on asTF.

3.2. TF knockout models: landmark studies

Shortly after the murine TF gene (Cf-3) was cloned (15), it was established that TF expression is crucial for survival as well as organogenesis in developing embryos: mice lacking both functional Cf-3 copies fail to develop to term. This was convincingly demonstrated in the genetically distinct TF knockout mouse models independently developed and reported by three groups. In all three models, the embryos did not survive past E10.5. Bugge *et al* observed that the post-E9.5 embryos were pale, edematous, and exhibited signs of retarded growth, whereas their organogenesis appeared normal; the lethality of TF-/- embryos was thus attributed to defective hemostasis, leading to the hemorrhage from extra-embryonic as well as embryonic vessels (16). Similar observations were made by Toomey *et al* who attributed the embryonic lethality of TF null mice to circulatory failure caused by defects in yolk sac vessel development and vitello-embryonic circulation (17). Importantly and rather fortuitously in retrospect, the third TF knockout study, by Carmeliet *et al*, reported the lack of large vitelline vessels and irregular, enlarged capillaries in the yolk sac of TF null mice; these findings were attributed to a defect in mesenchymal cell/pericyte accumulation and/or function, hence raising the possibility that TF expression plays an important role in the formation of the vasculature (18).

While the three above studies examining the effects of complete "pan-TF" deficiency were instrumental and highly significant, they were designed and carried out under the assumption that flTF protein is the sole product of the Cf-3 gene. At the present time, it is not known whether some of the effects seen in any of the three TF knockout models were solely the result of flTF deficiency, solely the result of masTF deficiency, or the result of combined flTF / masTF deficiency; in this regard, we note that the expression of flTF is seen much earlier than that of masTF in the course of embryonic development, particularly in the heart: E7.5 embryos express flTF, but not masTF, in the mesoderm and in E9.5 embryos, staining for flTF in cardiomyocytes is more widespread than that for masTF, which indicates that the two TF proteins may play distinct, yet perhaps partially overlapping, roles in organogenesis (7).

3.3. Genetically modified TF models: current state of the art

Embryonic lethality of TF knockout mice fueled efforts to try alternative genetic modeling approaches to study TF biology using murine models. In 1998, Nigel Mackman and colleagues generated a modified murine genotype that rescued the embryonic lethality caused by "pan-TF" deficiency (19). This mouse model was generated by crossing the transgenic mice expressing human flTF from a mini-gene cassette driven by the human TF promoter onto TF-/- background to ultimately obtain murine TF-null, human flTF expressing mice. The human flTF mini-gene expressed flTF protein whose coagulant activity was ~1% when compared to wild type TF levels; thus, these mice were termed "low TF mice" (hereafter, we refer to them as "low flTF mice"). Importantly, low flTF mice are viable, which indicates that even very low levels of flTF are sufficient to avoid embryonic lethality. However, the low flTF mice have increased bleeding times and shorter life spans compared to wild type mice, and develop hemosiderin deposits and fibrosis of the heart (20), indicating that compromised hemostasis (most likely due to flTF deficiency) and/or faulty organogenesis (most likely due to flTF deficiency as well as the complete absence of masTF expression, particularly in the heart) may be chiefly responsible. In this regard, low flTF mice hold significant potential value in asTF research as examination of asTF's biologic properties on "low flTF" background is likely to be facilitated by the fact that hemostatic as well as non-hemostatic flTF functions are likely to be greatly attenuated in these mice.

Following the generation of low flTF mice, other genetically modified models were developed and characterized. One such model expresses human TF from a human chromosome vector (HCV) containing the complete human TF gene and its 5'/3' UTR (21); this mini-chromosome was crossed onto the TF-null background so

that the HCV is the only source of TF in these mice. Unlike the "low human flTF" mice, these animals were found to constitutively express heightened levels of human TF protein in all examined tissues except, somewhat curiously, the heart (albeit the nature of the detection reagents used in the study precludes one from discerning whether human asTF was at all expressed in any of the examined tissues). Another "humanized TF" mouse was produced by knocking in human flTF cDNA into the murine TF locus, thereby achieving regulation of human flTF production by the endogenous murine TF promoter (22); these mice, termed "TFKI," were found to exhibit levels of human flTF expression similar to those of murine flTF in wild type mice. In humans as well as mice, TF is expressed in many tissues and cell types including monocytic and vascular smooth muscle cells; the reader is advised to consult a stellar review that thoroughly covers this subject (23), but it is pertinent to note that the role and/or (patho)physiological significance of constitutive and inducible TF expression in a number of tissues is yet to be completely understood, with the issue of TF expression in endothelial cells (EC) *in vivo* being one of the most controversial subjects in the field (24). Recently, TF-floxed mice were generated by Pawlinski and colleagues (25) which, when crossed with mice expressing cell type/tissue specific Cre recombinase, yield a cell type/tissue specific TF knockout phenotype. Using this model, TF knockout was achieved in monocyte/macrophages and neutrophils using the TF floxed/LysMCre approach, and in EC/non-hematopoietic cells using the TF floxed/Tie-2 Cre approach. The overall TF levels in these models were decreased by ~95%. Using these models, it was determined that plasma thrombin-antithrombin (TAT) levels are reduced in the LPS induced TF-myeloid knockout mice (TF floxed/LysMCre), as well as in TF-EC/non-hematopoietic knockout mice (TF floxed/Tie-2 Cre); on the other hand, TF knockout exclusively in EC did not have any effect of TAT, indicating that, in this experimental setting, the coagulation cascade is initiated *in vivo* by TF-expressing monocytes and yet-to-be-identified non-monocytic cells (25). We note that in other experimental settings – e.g., "non-LPS" vascular challenge such as chemically induced injury of the vessel wall – smooth muscle cell derived TF is the major contributor to thrombin generation and fibrin formation (26). Although the cell type/tissue specific TF knockout strategy employed in the very elegant and insightful study by Pawlinski *et al* does not allow selective evaluation of the effects of flTF depletion vs masTF depletion, it opens the road for next-generation flox-based strategies to target the expression of a specific TF isoform produced by the Cf-3 gene.

As we mentioned earlier, human and murine forms of recombinant asTF exhibit low pro-coagulant activity *in vitro* whose manifestation requires the presence of negatively charged phospholipids. In this regard, we point out that a genetically modified TF mouse with an intermediate genotype, reported in 2005, constitutively expressed a truncated non-membrane bound mutant form of the flTF protein that yielded severe thrombophilia: expression of this soluble flTF mutant, even at extremely low circulating concentrations, caused massive systemic intravascular coagulation resulting in high mortality, demonstrating that circulating soluble TF protein containing the entire ectodomain can elicit thrombosis *in vivo* (27). In the following section, we discuss the present body of evidence attesting to asTF's likely involvement in the pathobiology of disease states highlighted by vascular aberrations.

4. ASTF IN DISEASE

4.1. asTF and cardiovascular disorders

The link between TF and cardiovascular disease has been extensively characterized. TF is not expressed under normal states in EC and non-stimulated monocytes express very low levels of TF (28); however, monocytes/macrophages within atherosclerotic plaques express high amounts of TF. The expression of TF was documented in foam cells, vascular smooth muscle cells, and EC in pathological settings (29, 30). Increased monocyte infiltration promotes plaque destabilization leading to plaque rupture, causing intra-plaque TF to come in contact with circulating FVII(a) thereby initiating thrombogenesis that can result in myocardial infarction and stroke (31). asTF protein is detectable, alongside flTF, not only in spontaneously formed arterial thrombi (4), but also in atherosclerotic lesions (32). (Figure 2A) TNFα and IL-6 stimulate EC to secrete native asTF in the culture media (8). Szotowski *et al* tested asTF-containing media free of microparticles and found that it acquires procoagulant activity in the presence of phospholipids; notably, removal of asTF by immunoprecipitation using a specific anti-asTF antibody resulted in the decrease of this coagulant activity, confirming that the procoagulant activity was contributed by asTF (8), which agrees with the observation of Livnat *et al* who determined that soluble TF present in microparticle free human plasma contributes to thrombin generation induced by recombinant FVIIa (33). The findings of Szotowski *et al* pertaining to the coagulant activity of native asTF were confirmed in the course of a study examining the effects of anti-oxidative treatment on cytokine-stimulated EC (34). However, it was also suggested that asTF is not procoagulant: asTF cDNA was transfected into HEK293 cells and MiaPaCa-2 cells, neither of which expresses endogenous TF, and no procoagulant activity was detected (35, 36). We note that the overexpressed asTF in these two studies completely failed to secrete into the media, indicating that the cellular machinery of these cells, which express neither native flTF nor native asTF, does not enable asTF protein's secretion when asTF is produced via transient transfection of intronless asTF cDNA. While the requirements for effective asTF secretion are not known, the possibilities include the elements of the protein "quality control" system in the endoplasmic reticulum that deals specifically with N-linked glycans (37), the necessity of intron excision for effective intra-cytoplasmic targeting of asTF mRNA (38) or, perhaps more globally, the concomitant expression of flTF: asTF appears together with flTF in all biologic settings examined thus far, including adult heart tissue (4, 7, 8, 39). Moreover, crude static assays (35, 36) may have failed to detect asTF activity. Clearly, more sophisticated

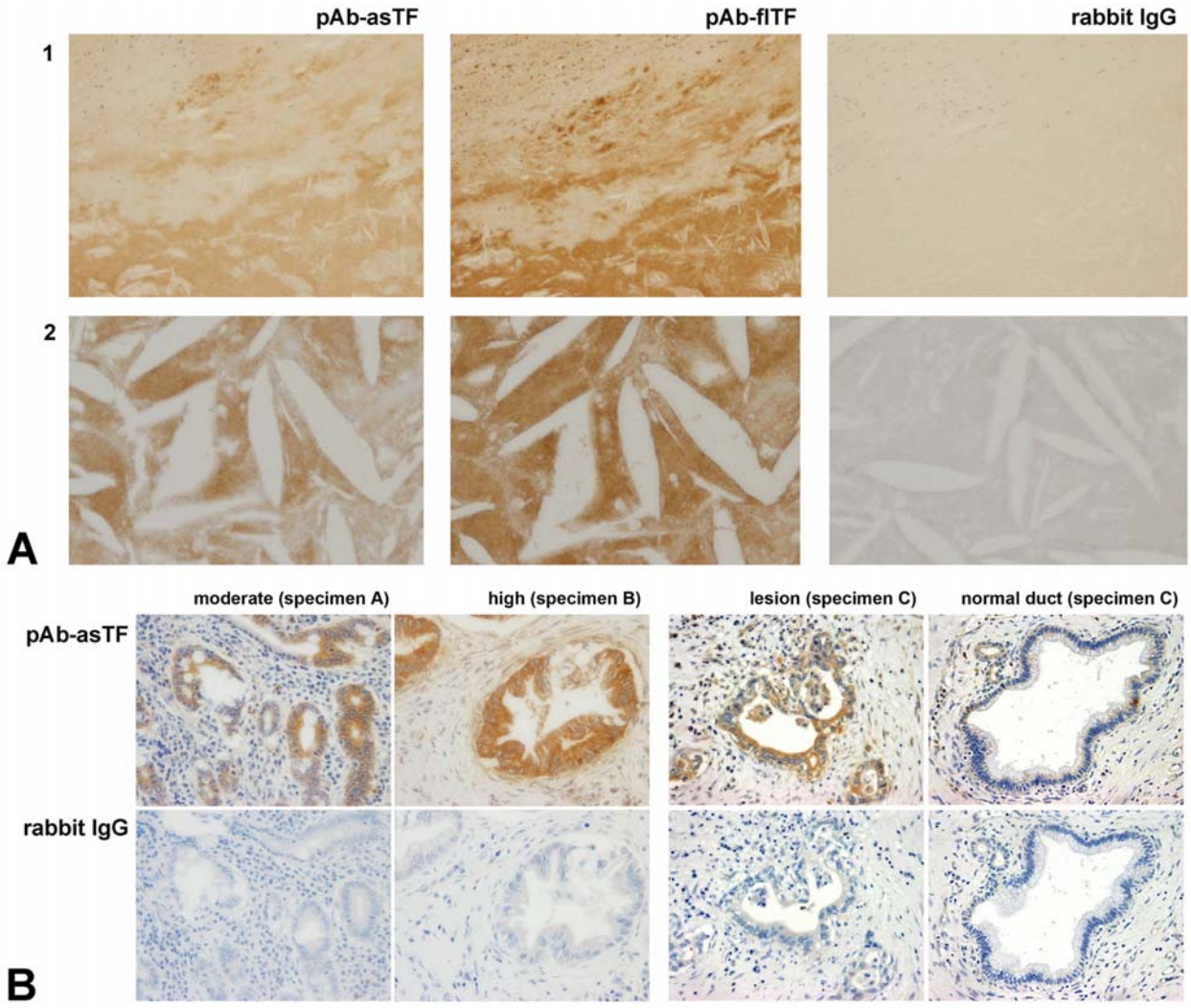

Figure 2. A. Co-localization of asTF and flTF proteins in human aortic plaques (specimen 1, the border between the plaque and the vascular wall is shown, magnification 20X; specimen 2, the acellular core, magnification 40X). B. Staining for asTF in PDAC lesions (n = 5, representative images are shown). Left composite panel, the observed range of asTF expression (specimen A – moderate, specimen B - high, magnification – 60X); right composite panel, non-malignant ducts stain very weakly for asTF, magnification – 40X.

research is needed to rigorously evaluate the possible contribution of plasma asTF to the total TF potential in circulation. In human plasma, two non-cell-bound TF forms are present: i) microparticle/exosome associated flTF and ii) asTF. The subject of microparticle-associated flTF and its pathophysiological significance in such disease states as Type 2 diabetes is discussed in detail elsewhere (28). In this review, we draw the reader's attention to the experimental evidence suggesting that asTF may, in fact, contribute to thrombogenesis. Susan Zawaski studied the significance of asTF in thrombogenesis on biomaterials (vascular grafts); she found that inhibition of asTF's activity increased the patency of small diameter grafts, and that asTF levels in the blood of patients on hemodialysis positively correlated with access thrombosis, which indicates that circulating asTF supports thrombus propagation (40). We recently examined the levels of total TF activity in a cohort of patients with stable coronary artery disease undergoing angioplasty and determined that the soluble TF species in the plasma of these patients comprise a substantially higher fraction of total active TF when compared to that found in the plasma of healthy individuals, which not only confirms that potentially active TF is always present in plasma (4, 10, 41), but also demonstrates that flTF-bearing microparticles do not represent the sole source of pro-coagulant plasma TF (42). This underscores the need for large-scale studies to re-evaluate and precisely dissect the role(s) of potentially active circulating TF forms in thrombogenesis, and strengthens the notion that asTF, a soluble protein whose half life in circulation may exceed that of microparticle-associated flTF, may hold promise as an early marker of disturbed hemostatic balance (8).

4.2. asTF and cancer

The connection between cancer and thrombosis is well established. Patients suffering from various forms of solid cancer associated with thromboembolic events have poor prognosis (43). Concerning the non-coagulant implications of heightened *F3* expression in cancer tissues, it is well documented that flTF is functionally linked to cancer pathobiology and appears to promote tumor growth and metastasis chiefly through the activation of PAR-2 receptors in its vicinity on cell membranes (44). Since its discovery, asTF was detected in a large number of cancer cell lines and several types of solid cancer: the levels of asTF and flTF are increased in squamous cell carcinoma of the lung (45), and asTF was detected, along with flTF, in 6 out of 8 pancreatic cancer cell lines that were examined (46). In 2008, Goldin-Lang *et al* evaluated tissue specimens obtained from patients with lung adenocarcinoma (LAC) for flTF mRNA, asTF mRNA, and asTF protein expression. asTF was abundantly expressed in cancer cells, compared to low expression levels in normal lung tissue (47). asTF mRNA was elevated ~7 fold in LAC samples compared to controls, compared to only ~3.5 fold elevation of flTF mRNA; asTF mRNA levels, but not flTF mRNA levels, were significantly associated with stages IIIA/IIIB compared with stage I, while flTF mRNA levels revealed no such association; of note, total levels of TF protein in the plasma of LAC patients were significantly higher than those in the plasma of healthy subjects (47). Later studies, reported by another group, revealed that asTF mRNA is a strong and independent outcome predictor in non-small-cell lung cancer (48). Hobbs *et al* compared pro-angiogenic potentials of human flTF and asTF in a subcutaneous model of pancreatic ductal adenocarcinoma (PDAC): athymic mice were injected with MiaPaCa-2 cells stably transfected with flTF, asTF, and empty vector (49). Importantly, the constitutive expression of the synthetic asTF cDNA, which contained a leader peptide sequence highly effective in MiaPaCa-2 cells, yielded effective secretion of the asTF protein in this study, as opposed to the study discussed earlier (36). One hundred percent of asTF expressing cells grew tumors, compared to only 18% of flTF expressing cells; moreover, asTF expressing tumors were ~4.5 fold larger, suggesting that human asTF promotes primary tumor growth. By PCNA staining, asTF expressing tumors had an increased number of proliferating tumor cells; hematoxylin/eosin staining showed that asTF expressing tumors had abundant vascular leakage commonly observed in highly angiogenic tumors, which was not observed in flTF tumors; CD34 staining was performed to assess tumor associated microvessels and again, asTF expressing tumors had more microvessels compared to flTF tumors (49). While this indicates that human asTF is pro-angiogenic *in vivo*, the molecular mechanisms underlying this effect were not investigated. To determine whether asTF protein is present in bona fide PDAC lesions, we stained serial sections of formalin fixed, paraffin embedded specimens of moderately differentiated PDAC (n=5) with our custom polyclonal antibody specific for asTF (4): asTF was detectable in most PDAC lesions (80-90%) at the levels ranging from moderate to high, whereas normal ductal epithelium exhibited extremely weak staining for asTF. (Figure 2B) In the next chapter, we discuss recent findings that add to the list of asTF's biologic properties and shed more light on the key molecular mechanics of asTF-induced neovascularization.

5. THE ASTF-INTEGRIN AXIS

5.1. Angiogenesis

In a recent study dissecting the role of asTF in angiogenesis, matrigel supplemented with recombinant human asTF was injected subcutaneously in mice, revealing extensive invading neovascularization (50). asTF-induced angiogenesis was comparable to VEGF, which indicates that asTF is a strong pro-angiogenic factor. *Ex-vivo* experiments employing aortic matrigel implants produced similar results, confirming that human asTF promotes angiogenesis at concentrations as low as 1 nM (50). Surprisingly, inhibition of FVIIa and/or hirudin did not affect neovascularization elicited by asTF, indicating that asTF's vessel building function does not involve limited proteolysis. Whereas the flTF/FVIIa/(Xa) complex cleaves PAR receptors to promote angiogenesis, asTF does not require PAR-mediated signaling to elicit neovascularization: asTF-induced sprouting was observed in PAR-deficient mice (50). Aside from activating PAR-mediated signaling, flTF is known to interact with integrins: specifically, $\alpha 3\beta 1$ and $\alpha 6\beta 1$ control flTF/FVIIa signaling via PAR-2 (51). van den Berg *et al* observed that human EC avidly bound to asTF-coated wells, which suggests that asTF is recognized by integrins on EC surfaces. Cell adhesion assays carried out in the presence of integrin-blocking antibodies helped determine that asTF binds $\alpha 6\beta 1$ and $\alpha v\beta 3$ integrins, which activates several integrin-linked kinases including FAK, MAPK, p38 MAPK, and PI3K/Akt. Interestingly, ligation of $\alpha v\beta 3$ was shown to be responsible for EC migration, whereas ligation of $\alpha 6\beta 1$, but not $\alpha v\beta 3$, was necessary for capillary formation (50). Importantly, intratumoral levels of asTF protein in a set of cervical cancer specimens were in the 10-100 nM range, vastly exceeding those found in the systemic circulation of healthy individuals (4); these findings indicate that the observed potentiation of vessel formation elicited by asTF may be particularly relevant in tumor microenvironments characterized by heightened *F3* expression.

5.2. Endothelium-monocyte interactions

TF-positive leukocytes are the hallmark of intravascular thrombosis associated with cancer (52) and inflammation (53). Monocytes are the major source of TF in systemic circulation. Expression of the *F3* gene in monocytes can be triggered by various stimuli, such as LPS and CRP (54, 55). Extracts of human atherosclerotic plaques contain significant amounts of TF, and are capable of inducing TF pro-coagulant activity in human monocytes (56). LPS-stimulated and fibronectin-adherent monocytes produce high levels of flTF and asTF mRNA (57). The infiltration of leukocytes, including the monocyte/macrophage population, is seen in many types of solid tumors and comprises an important component of tumor pathobiology (58). Transmigration of monocytes is known to induce TF expression, resulting in the acceleration of the inflammatory processes and focal

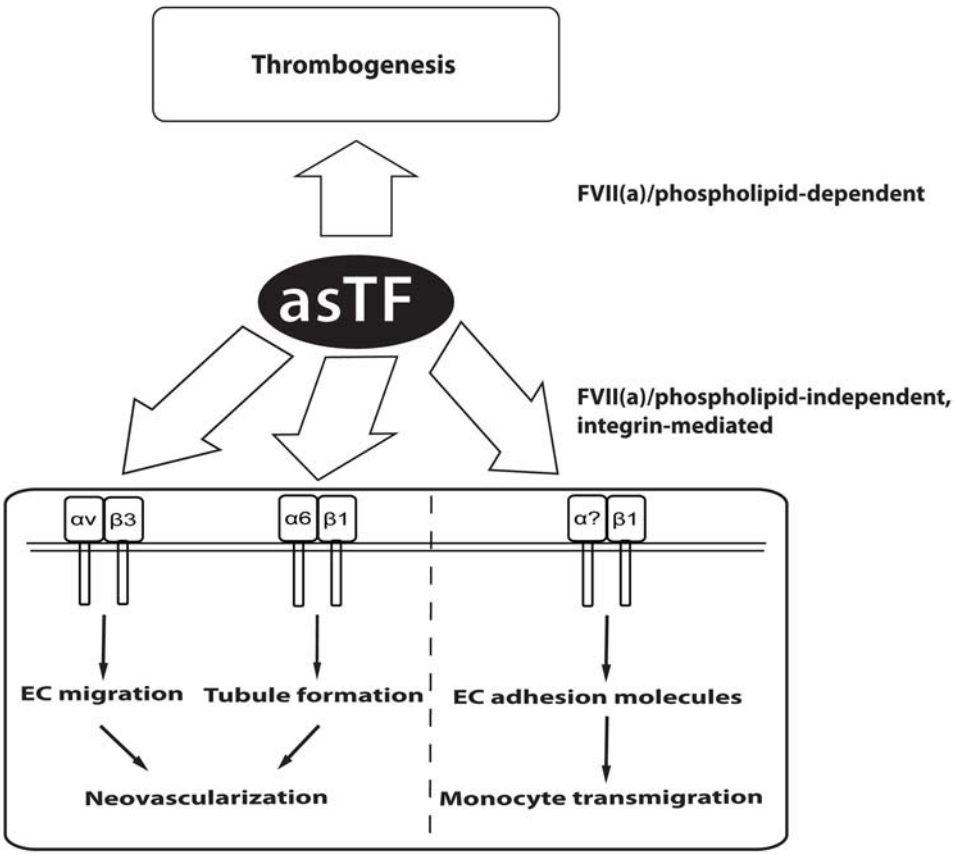

Figure 3. Schematic representation of the FVII(a)/phospholipid-dependent and FVII(a)/phospholipid-independent, integrin-mediated functional properties of human asTF. Black arrows denote upregulation.

increase in the procoagulant potential (59). Tumor cells secrete chemokines such as CCL2/MCP-1 that attract monocytes to the tumor site, promote their emigration into the tumor, and trigger monocytes to produce multiple pro-angiogenic mediators such as VEGF, FGF2, TNFα, and MMP-2, thus facilitating tumor cell migration, invasion, and metastasis. The recently reported experimental evidence suggests that VEGFR-positive hematopoietic cells are involved in "priming" the site of metastases even before the arrival of tumor cells, by modulating the site's microenvironment so that it becomes suitable for tumor cell adhesion and growth; such sites are known as "premetastatic niches" (60). Thus, abrogation of EC-monocyte interactions may hold potential in preventing metastasis. Rather analogously, in the cardiovascular pathobiology setting, monocytes emigrate to the core of the atherosclerotic plaque and, upon their differentiation into macrophages/foam cells, comprise a rich source of TF that can trigger arterial thrombosis upon plaque rupture (61). In the course of an ongoing study, we tested whether human asTF promotes interactions between monocytes and microvascular endothelial cells (MVEC) and found that, upon ligation of β1 integrins, asTF upregulates a group of genes in human cardiac and retinal MVEC essential for monocyte chemotaxis and adhesion; specifically chemotaxis molecules such as CCL2/MCP-1 and adhesion molecules such as VCAM-1, ICAM-1, and E-selectin were upregulated, causing an increase in MVEC-monocyte interactions in the orbital shear assay; moreover, asTF promoted monocyte transmigration en masse across the MVEC monolayers in a transwell assay carried out under MCP-1/CCL2 gradient (Srinivasan *et al*, manuscript in preparation). These findings broaden the scope and the significance of non-proteolytic, integrin-linked signaling cascades activated by asTF – the cell agonist whose functions likely play a major role in the contexts of tumor progression as well as atherogenesis. (Figure 3)

6. REGULATION OF HUMAN ASTF BIOSYNTHESIS: RECENT FINDINGS

It is important to understand the molecular mechanisms governing the regulated processing of TF pre-mRNA in human monocytes, which serve as the major source of asTF in systemic circulation (4): identification of the regulatory checkpoints essential for asTF's biosynthesis could prove useful in modulating TF pre-mRNA splicing as a treatment strategy in asTF-associated disease states. Serine-arginine rich or, as they are also known, "splicing regulator" (SR) proteins are the major spliceosomal components whose activity may result in either enhanced inclusion or, alternatively, enhanced exclusion of a cassette exon (62). The exon 5 of the *F3* gene contains a variety of putative binding sites for SR proteins, known as exonic

splicing enhancers (ESE) (63). Our laboratory recently published two studies examining the regulated TF pre-mRNA splicing in human monocytic cells. We designed and generated a TF splicing reporter construct termed pGL-hTF, whereby we inserted an *F3* genomic segment comprising the region exon 4-exon 6 into the CMV promoter-driven GFP ORF and transiently expressed it in THP-1 cells. Splicing of the pre-mRNA generated by pGL-hTF in THP-1 cells yields two mRNA species, the longer one featuring the exonic composition of flTF mRNA and the shorter – asTF mRNA. Four SR proteins, namely ASF/SF2, SC35, SRp55, and SRp40 were found to be expressed in abundance in THP-1 cells. Site-directed mutagenesis of the putative ESE for the above four SR proteins in the human TF exon 5 revealed that ASF/SF2 and SRp55 facilitate exon 5 inclusion, thereby promoting flTF mRNA production, which is antagonized by SC35 and SRp40 that bind to specific ESE in TF exon 5 that overlap the ESE for ASF/SF2 and SRp55, thereby facilitating TF exon 5 exclusion and, consequently, production of asTF mRNA (64, 65). We note that in a study examining the regulation of TF pre-mRNA splicing in TNFα-stimulated human umbilical vein endothelial cells (HUVEC), it was determined that the PI3K/Akt pathway modulates the phosphorylation status of SRp55 and SF2/ASF, as well as SRp75 – the SR protein that plays a major role in regulated TF pre-mRNA splicing in human EC (66, 67). Cdc-2 like kinases and DNA topoisomerase I are known to phosphorylate SR proteins, which modulates their activity thereby affecting constitutive as well as alternative pre-mRNA splicing (68, 69). TNFα induces asTF expression in HUVEC ~14 fold, and this phenomenon appears to be regulated by Cdc-2 kinase as well as DNA topoisomerase I, inasmuch as inhibition of the former resulted in the decreased expression of asTF, whereas the inhibition of the latter resulted in the increased expression of asTF (67). Thus, it is possible that PI3K/Akt is involved in effecting Cdc-2 kinases and DNA topoisomerase I to regulate the phosphorylation of the relevant SR proteins that govern the expression of TF splice variants in the vasculature and, possibly, solid tissues.

7. PERSPECTIVE

Discovery and characterization of human and murine asTF proteins opened a new, post-genomic chapter in the field of TF research. With the present knowledge at hand, it is now certain that co- and post-transcriptional regulation of *F3* expression must be re-visited on several levels as it will help answer many outstanding questions, e.g., is it advantageous to selectively target asTF in certain pathological states linked to TF, and will the approaches of targeting asTF on the (pre-)mRNA level be more efficacious than those targeting the protein itself? Is pre-mRNA splicing plasticity in any way linked to plaque remodeling – the process most recently shown to involve extensive intraplaque biosynthesis of many coagulation proteins, including TF (70)? To which extent is alternative TF pre-mRNA processing affected by systemic splicing aberrations in cancer (71)? We see that in the immediate future, certain issues must receive our primary attention, i.e., is non-proteolytic biologic activity of murine asTF analogous to that of human asTF? Do soluble forms of TF exist in species other than human and mouse and if so, do they possess biologic activity and what are the mechanisms that enable their biosynthesis? There is much hope – and likelihood – that the answers are forthcoming but without any doubt, they are guaranteed to bring up yet another set of questions pertaining to the scientifically fascinating and highly clinically relevant subject of TF biology in health and disease.

8. ACKNOWLEDGEMENTS

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Send correspondence to: Vladimir Y. Bogdanov, Division of Hematology/Oncology, University of Cincinnati College of Medicine, 3125 Eden Avenue, Cincinnati, OH 45267, Tel: 513-558-6276, Fax: 513-558-6703, E-mail: vladimir.bogdanov@uc.edu

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