Alternatively Spliced Tissue Factor Promotes Plaque Angiogenesis Through the Activation of Hypoxia-Inducible Factor-1α and Vascular Endothelial Growth Factor Signaling

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Background—Alternatively spliced tissue factor (asTF) is a novel isoform of full-length tissue factor, which exhibits angiogenic activity. Although asTF has been detected in human plaques, it is unknown whether its expression in atherosclerosis causes increased neovascularization and an advanced plaque phenotype.

Methods and Results—Carotid (n=10) and coronary (n=8) specimens from patients with stable or unstable angina were classified as complicated or uncomplicated on the basis of plaque morphology. Analysis of asTF expression and cell type–specific expression revealed a strong expression and colocalization of asTF with macrophages and neovessels within complicated, but not uncomplicated, human plaques. Our results showed that the angiogenic activity of asTF is mediated via hypoxia-inducible factor-1α upregulation through integrins and activation of phosphatidylinositol-3-kinase/Akt and mitogen-activated protein kinase pathways. Hypoxia-inducible factor-1α upregulation by asTF also was associated with increased vascular endothelial growth factor expression in primary human endothelial cells, and vascular endothelial growth factor–Trap significantly reduced the angiogenic effect of asTF in vivo. Furthermore, asTF gene transfer significantly increased neointima formation and neovascularization after carotid wire injury in ApoE$^{-/-}$ mice.

Conclusions—The results of this study provide strong evidence that asTF promotes neointima formation and angiogenesis in an experimental model of accelerated atherosclerosis. Here, we demonstrate that the angiogenic effect of asTF is mediated via the activation of the hypoxia-inducible factor-1/vascular endothelial growth factor signaling. This mechanism may be relevant to neovascularization and the progression and associated complications of human atherosclerosis as suggested by the increased expression of asTF in complicated versus uncomplicated human carotid and coronary plaques. **(*Circulation*. 2014;130:1274-1286.)**

Key Words: alternative splicing ■ atherosclerosis ■ hypoxia-inducible factor 1 ■ neovascularization, physiologic procoagulant, tissue factor ■ thromboplastin ■ vascular endothelial growth factors

Atherosclerosis is a leading cause of death and disability worldwide with 17.3 million deaths per year[1–3] and is projected to increase significantly over the next 2 decades.[4] Approximately 75% of acute coronary events and 60% of symptomatic carotid artery disease are caused by rupture of vulnerable plaques.[5–8] Over the last decade, angiogenesis has been the target of intense investigation because it is the critical source of plaque hemorrhage[9] and rupture,[8,10,11] ultimately responsible for the sudden onset of myocardial infarction and stroke.[9,12] Therefore, identifying new angiogenic factors and signaling pathways that can facilitate the development of tailored therapies for plaque stabilization is of great necessity.

Clinical Perspective on p 1286

Tissue factor (TF), also known as full-length TF (fl-TF), is an integral-membrane glycoprotein (47 kDa) that is implicated in tumor growth, angiogenesis, and metastasis[13–15] and is abundantly present in the lipid-rich core of plaques, where it is considered a major determinant of plaque thrombogenicity on rupture.[16]

More recently, a naturally occurring isoform of human fl-TF called alternatively spliced TF (asTF) was described. Through alternative splicing of the primary RNA transcript, the transmembrane and cytoplasmic domains of fl-TF are replaced with a unique COOH-terminal domain

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in asTF.[17] asTF is a non–membrane-anchored molecule of 27 kDa that, while retaining most of the contact sites for coagulation factor VIIa, lacks large portions of an exosite for macromolecular substrates.[18] As a result, asTF has no appreciable procoagulant activity, and it is believed to play a more prominent role in promoting angiogenesis.[19,20] Unlike fl-TF, asTF exhibits angiogenic activity via integrin ligation and activation of focal adhesion kinase (FAK) in a factor VIIa– and protease-activated receptor-2–independent manner.[19,21–23] In addition to its expression in pancreatic, lung, breast, and cervical cancer,[19,21,24] asTF has been detected in human plaques[25,26]; however, limited evidence exists beyond analysis of lipid-rich aortic lesions.[26] Whereas asTF is expressed by monocytes/macrophages and vascular smooth muscle cells,[17,27] the main cellular components of plaques, it is unknown whether asTF expression in atherosclerotic plaques is related to a more advanced phenotype and, if so, what its functional relevance is.

To test the hypothesis that asTF plays a role in atherosclerosis, we analyzed its expression and cellular distribution in complicated and uncomplicated human atherosclerotic plaques. Next, we investigated the integrin signal transduction pathways to identify new angiogenic signaling and effector(s) of asTF. Finally, we evaluated the impact of asTF overexpression on neointima formation and neovascularization in a murine model of accelerated atherosclerosis.

Methods

Please refer to the Methods section in the online-only Data Supplement for a detailed description and Table I in the online-only Data Supplement for the primer sequences used for conventional polymerase chain reaction.

Human Samples

Human carotid specimens (n=10) were obtained from patients undergoing carotid endarterectomy and the Institutional Biorepository/Biospecimen Bank. Their use was approved by the Institutional Review Board of the Icahn School of Medicine at Mount Sinai, and subjects gave written informed consent. Immediately after surgery, carotid plaques were processed by transverse dissection at the site of maximum plaque diameter.[28,29] One cross-sectional half of the entire plaque was fixed in formalin and embedded in paraffin for histopathology. The other half was flash-frozen in liquid nitrogen and stored at −80°C for further analysis. Paraffin blocks of coronary plaques (n=8) were from patients presenting with stable angina (n=4) or acute coronary events (n=4) undergoing percutaneous directional atherectomy. Written informed consent for the analysis of tissue samples was obtained from all patients before revascularization, and their use was approved by the Institutional Review Board of the University of Bonn. All specimens were classified as complicated and uncomplicated according to validated pathological criteria as previously reported.[30–33]

Animals

Eight-week-old male C57Bl6 mice (The Jackson Laboratory, Bar Harbor, ME) were used for the in vivo Matrigel Plug model. ApoE$^{-/-}$ mice (8-week-old male mice on a C57BL/6 background), obtained from The Jackson Laboratory, were used for the in vivo carotid artery wire injury and lentiviral transduction experiments. Animal procedures were approved by the Institutional Animal Care and Use Committee and carried out in compliance with Institutional Standards for Humane Care and Use of Laboratory Animal experiments.

Effect of asTF on Hypoxia-Inducible Factor-1α Expression

Briefly, endothelial cells were treated with asTF (10 nmol/L) or vehicle for 6 to 24 hours, and hypoxia-inducible factor-1α (HIF-1α) protein and mRNA levels were measured by Western blot and polymerase chain reaction, respectively. In additional experiments, endothelial cells were treated with asTF (10 nmol/L), fl-TF (10 nmol/L), or vehicle for 24 to 72 hours, and HIF-1α protein expression was measured by immunofluorescence and Western blot analysis.

Role of Integrin Signaling on HIF-1α Induction by asTF

Endothelial cells were preincubated with blocking antibodies against αv, α6, β3, or β1 integrins (10 μg/mL) for 30 minutes before treatment with asTF (10 nmol/L) for 6 hours, and HIF-1α expression was measured by Western blot of cell lysates. Phosphorylation of FAK and total FAK expression after asTF stimulation of endothelial cells were detected by Western blot analysis. After preincubation with the FAK inhibitor PP2 (10 μmol/L) for 30 minutes, endothelial cells were treated with asTF (10 nmol/L) for 6 hours, and HIF-1α upregulation was measured by Western blot analysis. Endothelial cells were also transduced with Ad.dnAkt or Ad.βgal (multiplicity of infection of 100) and after 24 hours treated for 8 hours with asTF (10 nmol/L), and HIF-1α upregulation was measured by Western blot analysis.

In Vivo Matrigel Plug Assay

Eight-week-old C57Bl6 mice were anesthetized, and 0.5 mL ice-cold Matrigel (growth factors reduced) was injected subcutaneously. Matrigel was supplemented with 10 nmol/L asTF, fl-TF, or PBS (vehicle). Vascular endothelial growth factor (VEGF; 50 ng/mL) was used as positive control. After 10 days, the animals were euthanized, and Matrigel plugs were harvested and processed for immunohistochemical analysis and vessel density (N/mm^2) quantification.[34]

In separate experiments, Matrigel was supplemented with asTF (10 nmol/L) or PBS (vehicle) in either the presence or absence of anti-αv, anti-α_6, anti-β_1, or anti–β_3 integrin subunit blocking antibodies or specific kinase inhibitors. Ten days after injection, Matrigel was harvested for neovessel quantification and HIF-1α immunostaining.

Another series of experiments were designed to test the hypothesis that VEGF is an angiogenic effector of asTF. Twenty-four hours before Matrigel injection, Ad.VEGF-Trap (10^{11} viral particles) or Ad. LacZ (10^{11} viral particles) were systemically injected as we previously reported.[34] Animals from each group (Ad.VEGF-Trap or Ad.LacZ) were then randomized to receive Matrigel supplemented with asTF (10 nmol/L), VEGF (50 ng/mL), or vehicle.

Mouse Model of Carotid Artery Wire Injury and Lentiviral Transduction

ApoE$^{-/-}$ mice (8-week-old mice on a C57BL/6 background) were fed a Western-type diet (Harlan Laboratories) beginning 2 weeks before surgery and continuing throughout the experiment.

Transluminal wire injury of the left common carotid artery was performed as previously described.[35] Immediately after injury, the left common carotid artery was cannulated, and the biclamped segment was incubated with 20 μL lentivirus encoding asTF–green fluorescent protein (GFP; 2×10^6 and 8×10^6 IU per mouse) or GFP (2×10^6 IU per mouse) for 30 minutes.

Statistical Analysis

All experiments were performed in triplicate (unless otherwise specified) from at least 3 independent experiments, and data are shown as mean±SD or median (minimum–maximum) as appropriate. Intra-assay and interassay coefficients of variation were calculated to measure variations of results within 1 experiment and between replicates, respectively. Intra-assay coefficients of variation were <10% and interassay coefficients of variation were <15% for the experiments performed. Normality was assessed with the Kolmogorov-Smirnov

and Shapiro-Wilk tests. When only 2 groups were compared, statistical differences were assessed with unpaired 2-tailed Student *t* tests or Mann-Whitney tests for the comparison of normally or not normally distributed variables, respectively. Otherwise, statistical significance was determined with ANOVA followed by the Bonferroni multiple-comparison test or Kruskal-Wallis test as appropriate. Relationships between variables were determined by the Pearson or Spearman ρ correlation coefficients. Values of $P<0.05$ were considered statistically significant. Computations were performed with IBM SPSS 19.0 statistical software (SPSS Inc, Chicago, IL).

Results

asTF Is Highly Expressed Within Complicated Carotid Plaques and Coronary Lesions From Patients With Acute Coronary Events

Carotid plaques were classified as type IV and V (atheroma or fibroatheroma, respectively; n=5) and type VI (type IV or V plaques complicated by disruption, hemorrhage, or thrombosis; n=5). Coronary specimens were classified as type IV (n=4) and type VI (n=4) primary lesions. As expected,[33] type IV lesions were from patients with stable angina, whereas type VI specimens were from patients with acute coronary events.

Compared with type IV and V, type VI (complicated) carotid lesions showed a significantly (P=0.03) increased macrophage density (13.4±8.6% versus 3.0±3.1% of stained area). Significantly (P=0.02) greater neovascularization was observed in complicated versus uncomplicated carotid plaques (178.7±28.3 versus 82.1±1.3 neovessels per plaque; 28.0±4.8 versus 9.9±0.8 neovessels per mm^2). In addition, complicated lesions showed a significantly (P=0.03) lower vascular smooth muscle cell density (15.8±6.6% versus 29.2±11.0% of stained area) and collagen content (28.0±8.7% versus 42.5±6.2% of stained area) compared with uncomplicated ones (Figure I in the online-only Data Supplement).

Of note, we observed a greater expression of both asTF and TF in complicated versus uncomplicated carotid plaques (Figure 1A and 1C). Similarly, complicated (type VI) and clinically unstable coronary lesions exhibited increased expression of asTF and TF versus uncomplicated (type IV) and clinically stable specimens (Figure 1B and 1C). Quantitatively, asTF mRNA levels (reverse transcription–polymerase chain reaction) increased 2.9-fold and protein levels (Western blot) increased 3.7-fold in complicated versus uncomplicated carotid lesions (Figure 1D).

asTF Is Expressed by Macrophages and Neovessels Within Complicated Carotid and Coronary Plaques

To analyze asTF expression in macrophages and vascular endothelial cells, we performed double-immunofluorescence labeling for asTF and CD68 (macrophages) and asTF and CD31 (vascular endothelial cells) in carotid and coronary

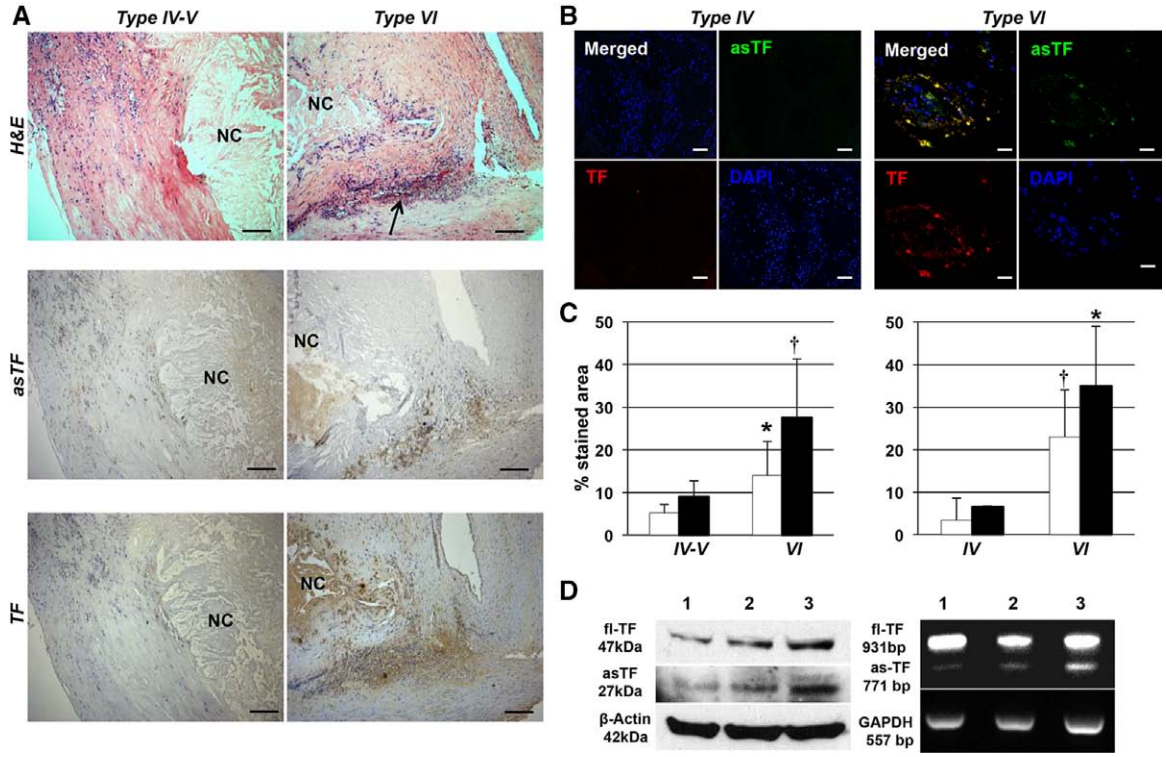

Figure 1. Alternatively spliced tissue factor (asTF) expression is greater in type VI (complicated) lesions than in type IV and V carotid and type IV coronary (uncomplicated) plaques. **A**, Hematoxylin and eosin (H&E)–stained sections (**top**) of type IV and V (**left**; n=5) and type VI (**right**; n=5) carotid plaques. Necrotic core (NC) and hemorrhage (arrow) of type VI lesions (**right**) are clearly visible. asTF (**middle**) and tissue factor (TF; **bottom**) immunostaining pattern of type VI (**left**) and IV and V (**right**) carotid plaques. Magnification, ×100. Scale bar, 100 μm. **B**, Double-immunofluorescence staining for asTF and TF of type IV (**left**; n=4) and VI (**right**; n=4) human coronary lesions. A greater expression of both asTF and TF and their colocalization was clear in type VI vs IV lesions. Magnification, ×400. Scale bar, 20 μm. **C**, Bars show asTF (white bars) and TF (black bars) quantification in type IV and V (n=5) and VI carotid (n=5; **left**) and type IV (n=4) and VI coronary (n=4; **right**) plaques. **P*=0.04 and †*P*=0.02 vs type IV and V carotid or type IV coronary plaques. **D**, Western blot and polymerase chain reaction analysis of type IV and V (lines 1 and 2) and type VI (line 3) carotid plaques shows asTF and full-length TF (fl-TF) protein and mRNA expression in human plaques.

human specimens. In agreement with published results, we observed a greater CD68+ cell density in both complicated carotid and coronary plaques compared with uncomplicated plaques (Figure 2A–2D). Importantly, asTF+ cell density was higher in complicated than in uncomplicated lesions. Furthermore, image analysis revealed a strong colocalization of asTF expression with CD68+ cells in complicated plaques. Notably, a specific fraction of the asTF+/CD68+ subset of all CD68+ cells was significantly higher in complicated versus uncomplicated lesions (Figure 2E and 2F).

We also observed a significantly higher density of CD31+ cells in both complicated carotid and coronary plaques compared with uncomplicated specimens with a marked colocalization of asTF with CD31+ cells in complicated carotid and coronary lesions (Figure IIA–IID in the online-only Data Supplement). Specifically, the percentage of asTF+/CD31+ of all CD31+ cells was significantly higher in complicated than in uncomplicated lesions (Figure IIE and IIF in the online-only Data Supplement).

asTF Promotes Endothelial Cell Proliferation, Migration, and Tube Formation

Proliferation

Proliferation was measured by BrdU incorporation assay. Results showed significantly increased endothelial cell proliferation when exposed to asTF and fl-TF (0.1–10 nmol/L) compared with vehicle (Figure IIIA in the online-only Data Supplement). Increased expression of the proliferation marker Ki67 in cells treated with asTF and fl-TF was also observed (Figure IIIB in the online-only Data Supplement). Cyclin D1 upregulation at 2 and 6 hours was followed by a downregulation at 12 hours in cells treated with asTF (Figure IIIC in the online-only Data Supplement). A strong colocalization of asTF and fl-TF with the proliferation marker Ki67 was seen in complicated plaques (Figure IIID in the online-only Data Supplement).

Migration

To measure migration, we used the Boyden Chamber Assay. A 2-fold increase in cell migration similar to that of fl-TF at 24 hours was observed when asTF (10 nmol/L) was present in the lower compartment (Figure IVA in the online-only Data Supplement). No migration was observed at 6 hours (data not shown). Separate experiments were performed to investigate the effect of coating trans-wells with asTF– on cell migration. These experiments showed that the increased migration induced by the presence of asTF in the lower compartment was further increased when trans-wells were also coated with asTF (Figure IVB in the online-only Data Supplement).

Cytotoxicity and Cell Viability

Significant cytotoxicity was observed in cells exposed to asTF and fl-TF at concentrations of 100 and 200 nmol/L (Figure IVC in the online-only Data Supplement). MTT assay showed an increased viability of cells exposed to asTF and fl-TF at up to 10 nmol/L compared with controls at 24, 48, and 72 hours (Figure IVD in the online-only Data Supplement). On the basis of these findings, the 10-nmol/L concentration was used for all further studies.

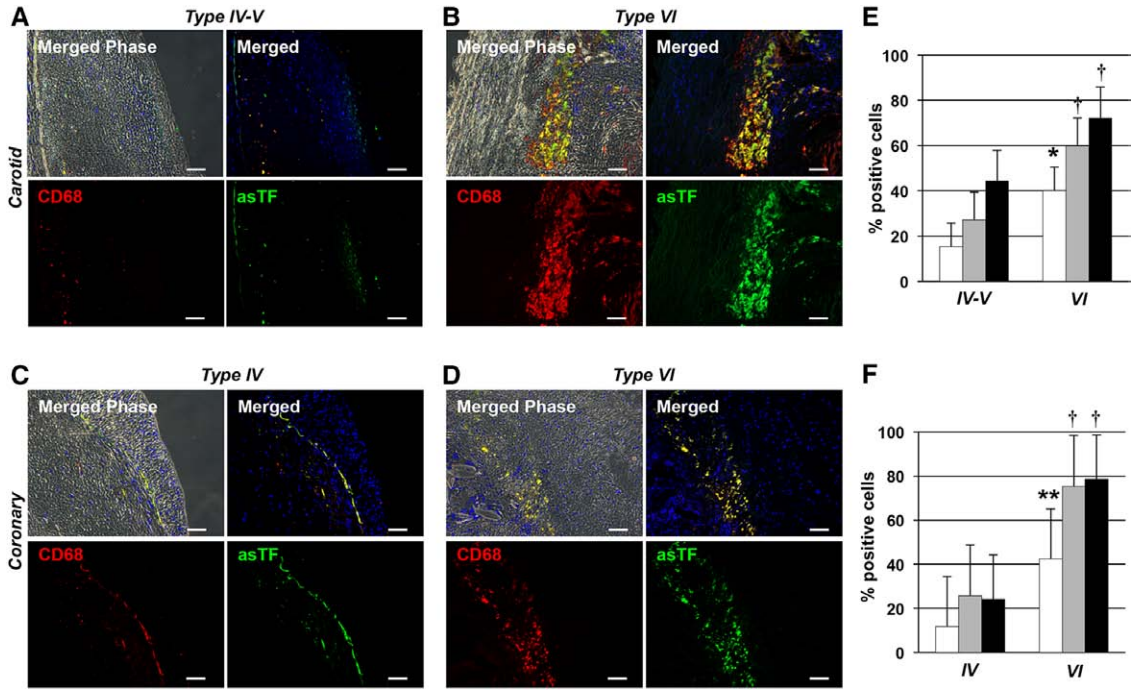

Figure 2. Alternatively spliced tissue factor (asTF) is highly expressed by macrophages within complicated human atherosclerotic plaques. Shown is the pattern of double immunofluorescence of uncomplicated (type IV and V) and complicated (type VI) carotid (**A** and **B**) and coronary (**C** and **D**) plaques. A greater expression of asTF and macrophages (CD68+ cells) was observed in complicated carotid (**B**; n=5) and coronary (**D**; n=4) lesions vs uncomplicated carotid (**A**; n=5) and coronary (**C**; n=4) plaques, respectively. asTF showed a strong colocalization with macrophages (CD68+ cells) in type VI lesions (**B** and **D**). Magnification, ×200. Scale bar, 40 μm. Quantification of asTF/CD68+ double-positive cells (white bars), total asTF+ (grey bars), and total CD68+ (black bars) cells in carotid (**E**) and coronary (**F**) plaques. **P*=0.006, ***P*=0.007, †*P*=0.04 vs type IV and V or type IV lesions.

In Vitro Matrigel Assay

A 2.5-fold increase in capillary-like formation was induced by asTF and fl-TF compared with vehicle. This effect was similar to that of VEGF, which was used as a positive control (Figure VA and VB in the online-only Data Supplement). Protease-activated receptor-2 inhibition did not significantly affect asTF-induced angiogenesis (Figure VC in the online-only Data Supplement). In contrast, no capillary formation was observed when asTF and fl-TF were denatured by boiling at 95°C for 5 minutes, demonstrating that the observed angiogenic activity was protein specific and not dependent on endotoxin contamination (Figure IVE in the online-only Data Supplement).

In Vivo Matrigel Assay

The angiogenic effects of asTF and fl-TF were also investigated with the in vivo Matrigel assay. The results of these experiments confirmed significantly higher neovessel formation induced by asTF, fl-TF, and VEGF compared with vehicle. However, asTF exhibits greater angiogenic activity compared with fl-TF and VEGF (Figure VD in the online-only Data Supplement).

asTF Induces HIF-1α Upregulation

Because the angiogenic activity of asTF is mediated via binding to integrins[19] and integrin signal transduction pathways have been implicated in HIF-1α expression in tumors,[36,37] we investigated the possibility that asTF promotes HIF-1α expression. A significant HIF-1α protein upregulation was detected in endothelial cells treated with asTF but not with fl-TF. This increase was dose dependent (Figure 3A) and was apparent as early as 6 hours with a maximum expression at 12 to 24 hours (Figure 3B). In contrast, no effect was observed on HIF-1β (Figure 3C). For the first 24 hours, the increase in HIF-1α was restricted to the cytoplasm, followed by a progressive translocation to the nucleus of the cells (48 and 72 hours; Figure 3D). No significant induction was observed when cells were treated with fl-TF (Figure 3E and 3F). Significant upregulation of HIF-1α induced by asTF was confirmed in

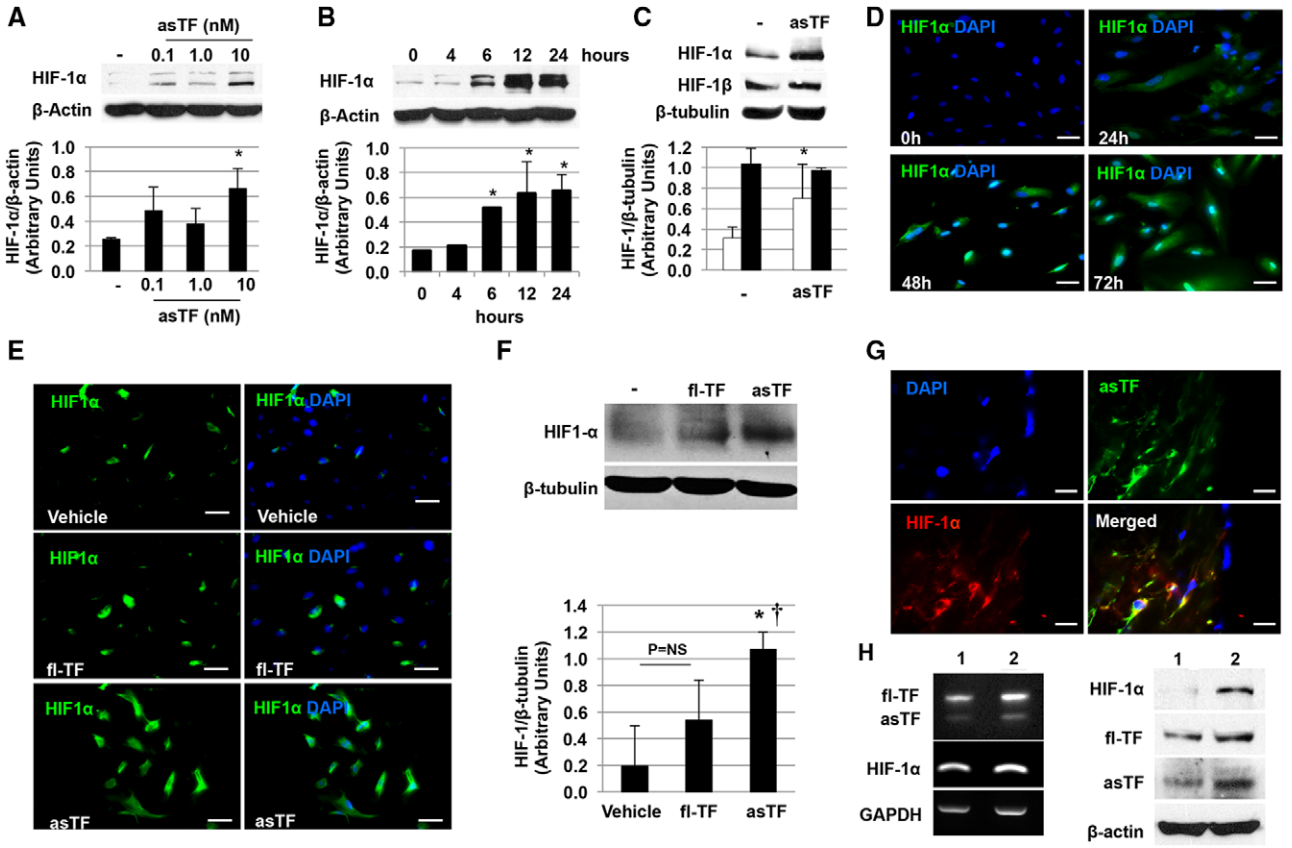

Figure 3. Alternatively spliced tissue factor (asTF) induces hypoxia-inducible factor-1α (HIF-1α) upregulation. **A**, Western blot analysis of endothelial cell lysates shows an asTF dose-dependent increase in HIF-1α vs control (vehicle). **B**, Time-course studies showed an increase in HIF-1α as early as 6 hours with a peak at 12 24 hours vs control (vehicle). **C**, Unlike HIF-1α (white bars), no significant effect of asTF on HIF-1β (black bars) was observed. **D**, Immunofluorescence staining showing the time course of HIF-1α expression (Alexa Fluor 488) in endothelial cells treated with asTF (10 nmol/L) vs control (vehicle). DAPI was used as counterstaining. **E**, Upregulation of HIF-1α was also observed in human aortic endothelial cells (HAECs) treated with asTF (10 nmol/L) for 24 hours (**left**). No significant effect was observed when HAECs were treated with full-length TF (fl-TF; **right**). Magnification, ×200. Scale bar, 50 μm. **F**, Similar results were confirmed by Western blot analysis of cell lysates treated with either fl-TF (10 nmol/L) or asTF (10 nmol/L) for 24 hours. **P*=0.02 vs vehicle; † *P*=0.04 vs fl-TF. **G**, Immunofluorescence staining showing colocalization of HIF-1α (Alexa Fluor 594) and asTF (Alexa Fluor 488) in complicated (type VI) carotid plaques. DAPI was used as counterstaining. Magnification, ×1000. Scale bar, 10 μm. **H**, asTF mRNA was identified by polymerase chain reaction in carotid plaques. Sample 1 is from type IV and V plaques (n=5); sample 2 is from type VI lesions (n=5). An increase in HIF-1α expression was observed in sample 2 also characterized by a higher asTF gene expression. Western blot analysis of the same samples confirmed the findings at the protein level. Data from in vitro experiments are the average of triplicates of single experiments that were repeated 3 times.

vivo, as shown by the greater percentage of HIF-1α+ cells detected in plugs containing asTF versus vehicle (Figure 4). A positive linear correlation between HIF-1α and neovessel density (r=0.29, P=0.036) was also found. Increased HIF-1α expression was observed in complicated human lesions that expressed high levels of asTF. Of note, a strong colocalization between HIF-1α and asTF was observed in complicated plaques (Figure 3G and 3H).

To elucidate whether the increased levels of HIF-1α induced by asTF were the result of increased transcription, we first measured HIF-1α mRNA levels by reverse transcription–polymerase chain reaction. We observed a dose-dependent upregulation (4-fold increase) of HIF-1α mRNA, which appeared to plateau at 6 to 24 hours (Figure 5A).

To investigate whether asTF affects HIF-1α degradation, endothelial cells treated with asTF were incubated with the proteasome inhibitor MG132 and the protein synthesis inhibitor cycloheximide. Cycloheximide treatment abolished asTF-induced HIF-1α expression, and as expected, MG132 alone increased HIF-1α accumulation as a result of reduced proteasomal degradation. In agreement with this interpretation, the addition of MG132 to asTF-treated cells resulted in a drastic accumulation of polyubiquinated forms of HIF-1α (Figure 5B).

HIF-1α Induction by asTF Occurs via Integrin-Mediated Activation of Mitogen-Activated Protein Kinase and Phosphatidylinositol-3-Kinase/Akt Signal Pathways

Next, we investigated whether the induction of HIF-1α depends on integrins. The blockade of α_6, β_3, and β_1 integrins significantly attenuated the upregulation of HIF-1α by asTF (Figure 5C), yet no significant effect was observed after α_v integrin inhibition. In vivo results showed that when asTF was coadministered with α_6, α_v, β_3, or β_1 integrin–neutralizing antibodies, neovessel formation was significantly reduced (Figure VI in the online-only Data Supplement). Furthermore, lower HIF-1α+ cell density in the plugs was observed when asTF was coadministered with antibodies against α_6 and β_3 integrins. A trend (P=0.05) toward a reduced HIF-1α+ cell density was also observed when β_1-neutralizing antibody was coadministered with asTF. In line with the in vitro data, no significant reduction in HIF-1α induction by asTF was observed in the presence of α_v integrin blockade (Figure 4). In addition, endostatin alone, which was used as a positive control, showed no effect on either neovessel formation or HIF-1α basal expression (Figure 4 and Figure VI in the online-only Data Supplement). When endostatin was coadministered with asTF, a significant inhibition of asTF-induced angiogenesis

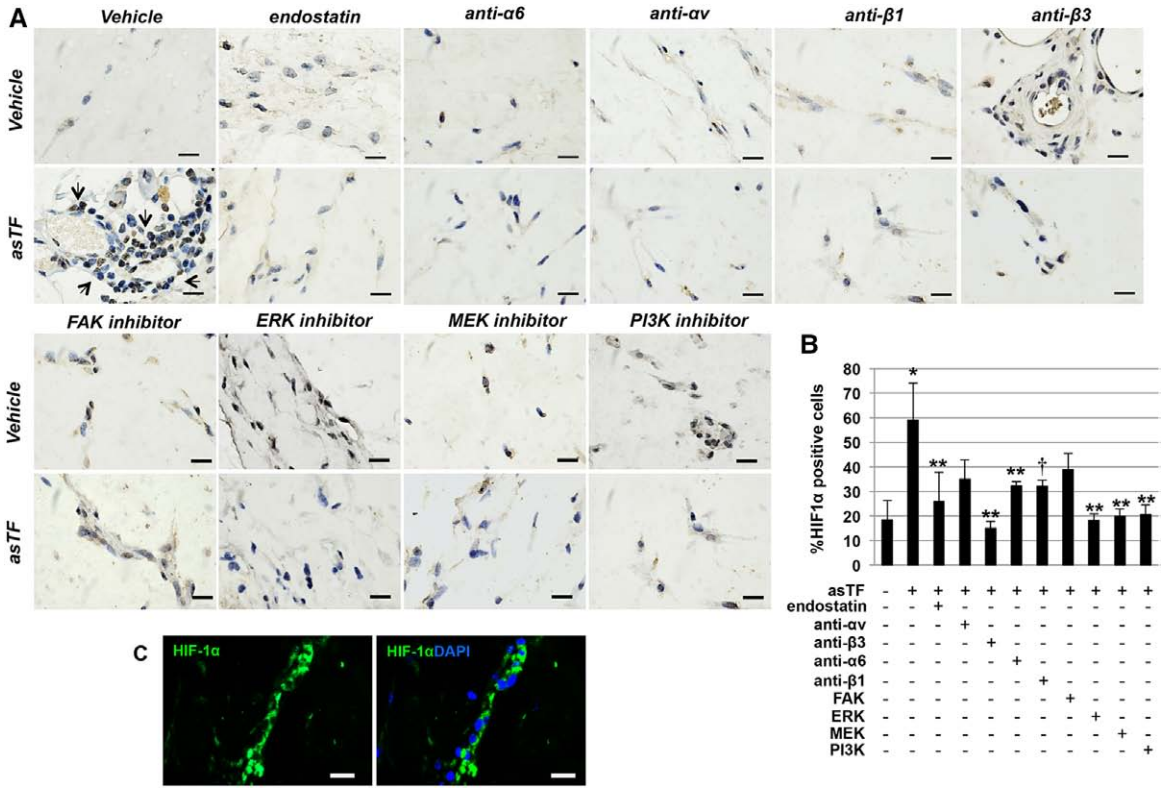

Figure 4. Alternatively spliced tissue factor (asTF) induces hypoxia-inducible factor-1α (HIF-1α) expression in vivo, and this increase is inhibited by specific integrin blocking antibodies and intracellular kinases inhibitors. **A**, HIF-1α staining of sections of Matrigel plugs shows an increase in HIF-1α–positive cells in plugs containing asTF (10 nmol/L). HIF-1α expression induced by asTF in vivo was significantly inhibited when specific neutralizing antibodies and kinase inhibitors were coadministered with asTF in the preparation. Magnification, ×1000. Scale bar, 10 μm. **B**, Bars show the percentage of HIF-1α–positive cells in the plugs. **C**, Immunofluorescence staining showing HIF-1α (Alexa Fluor 488)–positive cells forming a tube-like structure in a Matrigel plug admixed with asTF (10 nmol/L). DAPI was used as counterstaining for nuclei. Magnification ×1000. Scale bar=10 μm (n=2 plugs per mouse; n=3 mice per group). ERK indicates extracellular-signal-regulated kinase; FAK, focal adhesion kinase; MEK, mitogen-activated protein kinase kinase; and PI3K, phosphatidylinositol-3-kinase. *P<0.0001 vs control; **P<0.01, † P=0.058 vs asTF.

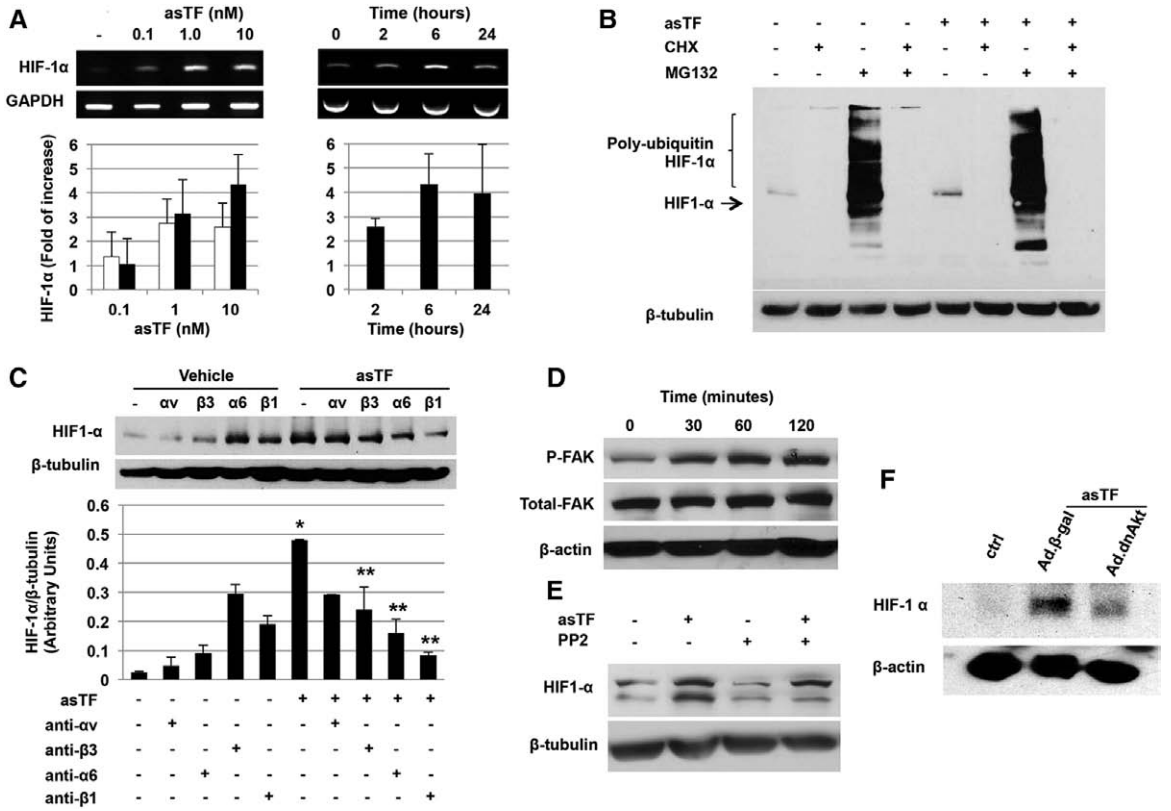

Figure 5. Mechanisms involved in alternatively spliced tissue factor (asTF)–induced hypoxia-inducible factor-1α (HIF-1α) upregulation. **A**, Dose-dependent upregulation of HIF-1α mRNA by asTF was detected (**left**) at 2 hours (white bars) and 6 hours (black bars). Time-course analysis of HIF-1α expression in the presence of asTF (10 nmol/L) shows a plateau at 6 hours (**right**). **B**, asTF enhanced protein synthesis of HIF-1α with no effect on degradation. **C**, Representative immunoblot (**top**) shows inhibition of asTF-induced HIF-1α by integrin blockade. Bars (**bottom**) show quantification of 3 separate experiments. *P<0.01 vs vehicle. **P<0.01 vs asTF. **D**, Focal adhesion kinase (FAK) activation by asTF was detected in endothelial cells stimulated with asTF. **E**, FAK inhibition by PP2 resulted in partial downregulation of asTF-induced HIF-1α. **F**, HIF-1α expression was inhibited in cells transduced with an adenovirus encoding dominant-negative Akt (Ad.dnAkt) but not with Ad.βgal. Briefly, 24 hours after Ad.dnAkt or Ad.βgal (100 multiplicity of infection), human aortic endothelial cells were treated with asTF (10 nmol/L) for 8 hours, and HIF-1α expression was measured by Western blot. Data are the average of triplicates from single experiments that were independently repeated 3 times.

and HIF-1α upregulation in vivo was observed (Figure 4 and Figure VI in the online-only Data Supplement). To determine the contribution of FAK to angiogenesis and HIF-1α expression by asTF, we first analyzed the phosphorylation status of effect of FAK by immunoblotting and found a 3.5-fold increase in FAK Y397 phosphorylation (P=0.01 versus baseline) in endothelial cells treated with asTF (Figure 5D). When asTF was coadministered with FAK inhibitor, neovessel formation in vivo was significantly reduced (Figure VI in the online-only Data Supplement); however, FAK inhibition did not significantly inhibit asTF-induced HIF-1α expression under our in vitro (Figure 5E) and in vivo (Figure 4) experimental conditions.

The contribution of mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathways to HIF-1α induced by asTF was also investigated. To elucidate the role of MAPK pathways, extracellular signal-regulated kinases 1/2 and mitogen-activated protein kinase kinase 1/2 inhibitors were used. Inhibitors alone did not significantly affect either neovessel formation or HIF-1α expression (Figure 4 and Figure VI in the online-only Data Supplement), but inhibitors significantly inhibited asTF-induced angiogenesis and HIF-1α upregulation when coadministered with asTF (Figure 4 and Figure VI in the online-only Data Supplement). To determine whether Akt activation plays a role in the control HIF-1α expression induced by asTF, endothelial cells were first transduced with an adenovirus encoding a dominant-negative Akt mutant and then treated with asTF. Of note, HIF-1α expression was significantly reduced in dominant-negative Akt mutant–transduced cells compared with Ad.βGal (Figure 5F), suggesting a role of Akt pathway in HIF-1α upregulation.

asTF Angiogenic Activity Is Mediated via the Expression of VEGF

Next, we tested the hypothesis that HIF-1α upregulation by asTF results in transactivation of VEGF. To this end, we investigated the effect of asTF on the gene expression of VEGF-A isoforms. The results of these experiments confirmed a significant increase in HIF-1α mRNA and revealed that the level of the $VEGF_{165}$ isoform was significantly increased in endothelial cells that were stimulated with asTF (Figure 6A). A significant increase in the soluble $VEGF_{165}$ protein was also observed in the conditioned media of cells treated with asTF (Figure 6B).

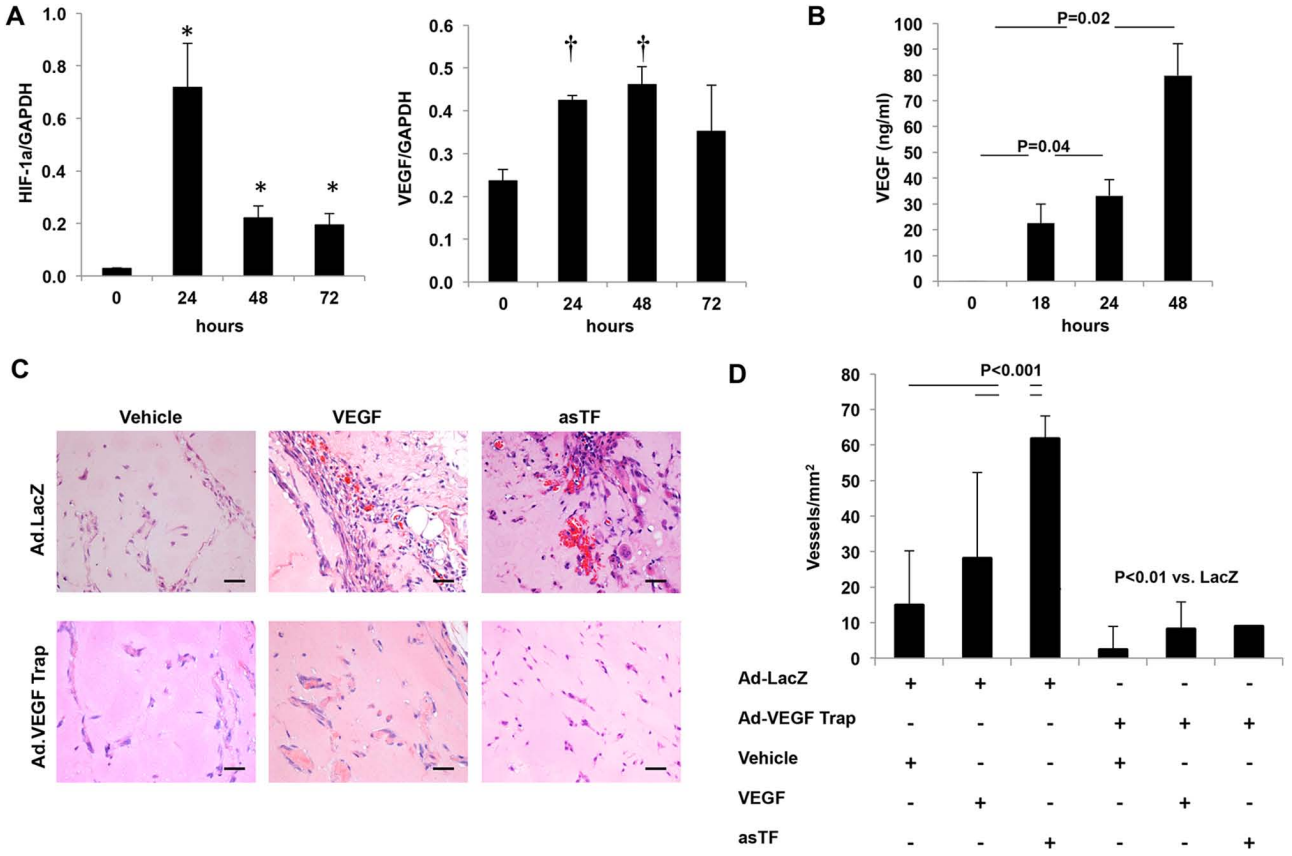

Figure 6. The angiogenic effect of alternatively spliced tissue factor (asTF) is mediated via hypoxia-inducible factor-1α (HIF-1α)–mediated activation of vascular endothelial growth factor (VEGF). **A**, Time course of the expression of HIF-1α and the VEGF-A isoform 165 in endothelial cells treated with asTF (10 nmol/L). No significant upregulation of the other VEGF-A isoforms (121, 189, 206) was observed (data not shown). **B**, A significant increase in the expression of the VEGF-A isoform 165 was observed in conditioned media from cells treated with asTF (10 nmol/L) for up to 72 hours by ELISA. **C**, Hematoxylin and eosin–stained sections of Matrigel plugs supplemented with asTF (10 nmol/L), VEGF (50 ng/mL), or PBS (vehicle). Twenty-four hours before Matrigel injection, mice were systemically administered either Ad.VEGF-$Trap_{R1R2}$ (10^{11} viral particles) or Ad.LacZ (10^{11} viral particles). Magnification, ×400. Scale bar, 20 μm. **D**, As expected, quantitative analysis showed a significant inhibition of VEGF-induced angiogenesis. A similar inhibition was observed when the angiogenic effect of asTF was tested in mice injected with Ad.VEGF-$Trap_{R1R2}$ vs Ad.LacZ. *P=0.03; †P<0.02. In **A** and **B**, data are the average of triplicates from single experiments that were independently repeated 3 times. **C** and **D**, n=2 plugs per animal; n=5 animals per group.

The contribution of VEGF to the angiogenic activity of asTF was investigated in vivo with an adenovirus vector encoding the receptor chimera VEGF-$Trap_{R1R2}$. As expected, the angiogenic activity of VEGF was completely abolished in mice overexpressing VEGF-Trap compared with LacZ controls. Remarkably, the angiogenic activity of asTF was inhibited in mice overexpressing VEGF-Trap (Figure 6C and 6D), confirming the role of VEGF in asTF-induced neoangiogenesis.

asTF Overexpression Promotes Neointima Formation and Angiogenesis in Response to Arterial Injury in $ApoE^{-/-}$ Mice

To test the hypothesis that asTF contributes to the progression of atherosclerosis and to plaque angiogenesis, left carotid artery injury, with or without asTF gene transfer, was performed in $ApoE^{-/-}$ mice kept on a Western-type diet starting 2 weeks before surgery. As expected, 4 weeks after arterial wire injury, histological analysis revealed the presence of complex neointima lesions in all injured arteries. Results showing efficient transduction of HEK293T cells with generated lentivirus encoding for GFP or asTF-GFP are presented in Figure VIIA in the online-only Data Supplement (Figure VIIB in the online-only Data Supplement gives details on asTF and spliced sites of fl-TF exons). Efficient transduction was confirmed in vivo by the expression of GFP in the injured carotids transduced with lentivirus (Figure VIII in the online-only Data Supplement). Remarkably, neointima thickness was significantly greater in asTF-transduced/injured carotid arteries. No significant difference in medial area was observed between groups (Figure 7A). Importantly, an increased number of neovessels/plaque and increased neovessel density were observed in asTF-transduced lesions versus nontransduced lesions (Figure 7B).

Discussion

The results of this study provide strong evidence that asTF promotes neointima formation and angiogenesis in an experimental model of accelerated atherosclerosis. Here, we show that the angiogenic effect of asTF is mediated via the activation of the HIF-1/VEGF signaling. HIF-1α upregulation occurs through integrin and activation of the MAPK and PI3K/Akt pathways (Figure 8). This mechanism may be relevant to neovascularization and the progression and complications of human atherosclerosis as suggested by the higher expression

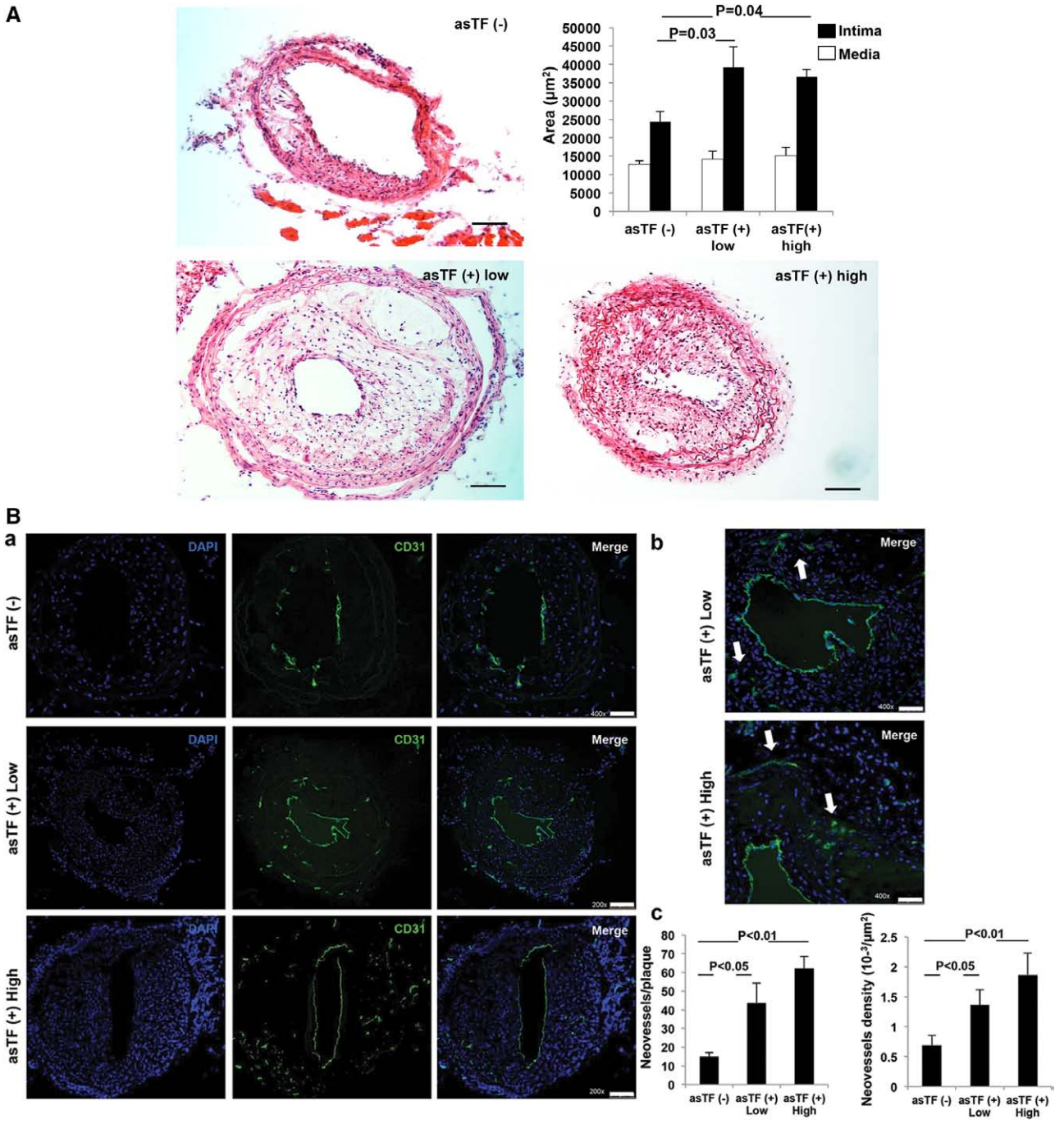

Figure 7. Effect of alternatively spliced tissue factor (asTF) on neointima formation and neovascularization in an experimental model of accelerated atherosclerosis. **A**, Bright-field microscopic photographs of cross sections of the left common carotid artery (LCCA) harvested from ApoE$^{-/-}$ mice 4 weeks after injury and stained with hematoxylin and eosin. LCCAs were infected with a lentivirus encoding asTF; asTF(+) low (n=4) and asTF(+) high (n=4) doses. Injured but nontransduced LCCA, asTF (-), were used as controls (n=5). Magnification, ×200. Scale bar, 40 μm. Quantitative analysis of neointima (black bars) and media (white bars) formation in the injured LCCAs of ApoE$^{-/-}$ mice overexpressing asTF (asTF^{+} low and asTF^{-} high, 2×10^{6} and 8×10^{6} IU, respectively) showed increased neointima formation vs controls (asTF^{-}). No difference was observed in media formation among groups. **B**, **a**, Representative photographs of LCCA cross sections immunostained for CD31 (Alexa 488). Photographs clearly show increased neovascularization in arteries injured and transduced with asTF (asTF^{+} low and high) vs nontransduced controls (asTF^{-}). Magnification for asTF^{-} is ×400 (scale bar, 50 μm) and for asTF+ is ×200 (scale bar, 100 μm). Higher magnification (×400) of asTF^{+} low and high is shown for comparison with asTF^{-}. **b**, Quantification of neovessel formation shown as neovessels per plaque (**left**) and neovessel density (**right**).

of asTF in complicated versus uncomplicated human carotid and coronary plaques.

Cellular distribution analysis of human plaques revealed that asTF was highly expressed within macrophage-rich and highly vascularized areas of complicated coronary and carotid lesions. Because monocytes/macrophage infiltration has been implicated in plaque angiogenesis[38] and vulnerability[39] and asTF appears to increase monocyte adhesion to

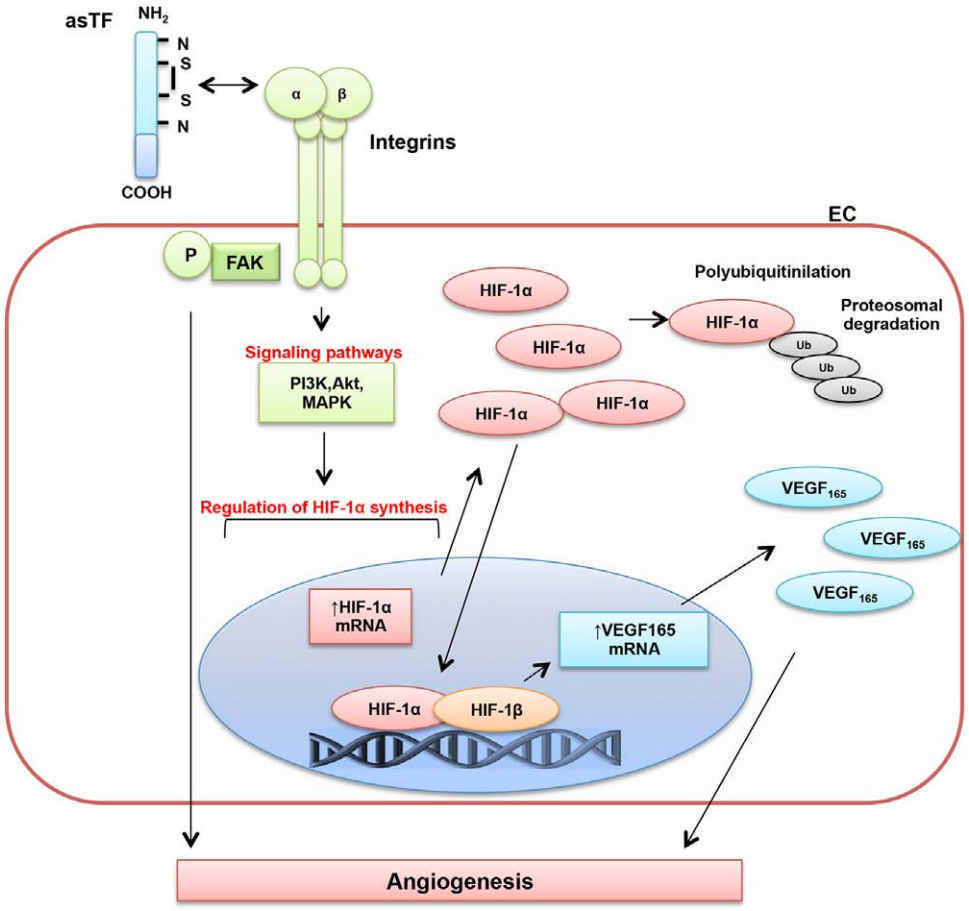

Figure 8. Schematic representation of alternatively spliced tissue factor (asTF) signaling pathway. EC indicates endothelial cell; ERK, extracellular-signal-regulated kinase; HIF-1α, hypoxia-inducible factor-1α (inducible); HIF-1β, hypoxia-inducible factor-1β (constitutive); MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; P-FAK, phosphorylated focal adhesion kinase; PI3K, phosphatidylinositol-3-kinase; Ub, ubiquitin; and $VEGF_{165}$, vascular endothelial growth factor isoform 165 (soluble).

endothelial cells,[26] it was not surprising to observe that the number of $asTF^+/CD68^+$ and $asTF^+/CD31^+$ cells was higher in complicated versus uncomplicated plaques. In particular, asTF appeared to be expressed by specific subsets of macrophages and endothelial cells identified in complicated lesions.

These observations are consistent with the involvement of asTF in the angiogenic switch of unstable atherosclerotic plaques. In light of the observed higher expression of asTF in plaques from patients with acute coronary events compared with lesions from patients with stable angina, it is logical to speculate that asTF may contribute to the onset of coronary events.

Remarkably, asTF influences all critical phases involved in the formation of new blood vessels such as the migration, proliferation, and differentiation of endothelial cells into capillaries.[40] Increased proliferation of endothelial cells treated with asTF involves the expression cyclin D1, showing an early upregulation followed by downregulation, reflecting the typical cyclin D1 rise early in G1 followed by its rapid decline in the G1/S phase when DNA replication occurs.[41] Colocalization of asTF with the proliferation marker Ki67 was observed in complicated plaques highly expressing asTF, suggesting that asTF promotes cell proliferation in human atherosclerosis.

asTF increases endothelial cell migration via both chemotaxis and haptotaxis as demonstrated by directional migration toward a gradient of soluble asTF, an effect that was further increased when inserts were coated with asTF.

Others could observe only the haptotatic activity and could not detect any proliferative effect of asTF on endothelial cells[19]; however, in these studies, different approaches and higher concentrations of asTF (100 nmol/L), which we found to be cytotoxic, were used.

We observed a greater angiogenic potency of asTF compared with fl-TF in vivo. Of note, this effect was observed when equipotent concentrations of TF isoforms and VEGF were used when their activities were tested in vitro. Although suggestive of a greater angiogenic potency of asTF compared with fl-TF, the pathophysiological relevance of these findings is difficult to extrapolate to atherosclerotic disease. In fact, the relative expression of fl-TF and asTF is in favor of fl-TF in human atherosclerotic plaques, and it remains undetermined how much asTF is expressed in vivo. Moreover, in our studies, we used relipidated fl-TF to mimic the effect of circulating fl-TF,[42,43] an approach that does not reflect the proteome complexity of circulating microparticles carrying fl-TF in humans.

Despite these limitations, our findings highlight the possibility that asTF activates specific signaling pathways. In

separate experiments, we observed that asTF but not fl-TF induces a significant upregulation of HIF-1α. A positive correlation between HIF-1α+ cells and neovessel density in vivo further confirmed the hypothesis that the angiogenic activity of asTF is mediated via HIF-1α activation. In light of the observation that HIF-1α protein upregulation occurs only after 6 hours of asTF stimulation, it was not surprising that we did not detect a significant difference in the angiogenic activity of asTF compared with fl-TF in vitro. In fact, capillary formation in vitro was measured 6 hours after treatments, whereas the in vivo Matrigel plug assay was examined after 10 days.

The observed significant dose-dependent upregulation of HIF-1α mRNA suggests that asTF regulates HIF-1α at the transcriptional level. In fact, regulation of HIF-1α protein degradation, which under normoxic conditions occurs via its hydroxylation, binding to pVHL, polyubiquitylation, and ultimately proteasomal degradation, is the most recognized mechanism for HIF-1α stabilization, resulting in HIF-1 heterodimer formation.[44] Interestingly, the detection of polyubiquinated forms of HIF-1α demonstrates that asTF does not interfere with polyubiquitination, a required step for proteasomal degradation. Of note, the inhibition of HIF-1α protein synthesis by cycloheximide suggests that asTF may increase de novo protein synthesis via increased HIF-1α mRNA transcription.

In previous studies, asTF was found to oligomerize integrins and to activate downstream signaling to promote endothelial cell migration and capillary formation in vitro.[19] However, in vitro conditions may not reflect the complexity of physiological interactions (ie, blood flow, interactions with cells and matrixes) occurring in vivo. Here, we report that the downstream signaling events activated by asTF through integrin activation are relevant for the angiogenic activity of asTF in vivo. A critical role of the β_3, α_6, and β_1 integrins in mediating the angiogenic activity of asTF was demonstrated by the significant reduction in neovessels formation induced by asTF in vivo on integrin inhibition. We have also demonstrated that HIF-1α upregulation induced by asTF is mediated via integrin activation. Specifically, it was observed that β_3, β_1, and α_6 integrin blockade was associated with a significant reduction in HIF-1α upregulation by asTF. In contrast, no effect was observed in the presence of α_v blockade.

In support of the validity of our experimental conditions, the formation of large vessels with anti–β_3-neutralizing antibody alone was expected and in accordance with previous findings revealing paradoxically increased angiogenesis via VEGF receptor-2 upregulation.[45]

Analysis of the downstream signaling of integrins confirmed the activation of FAK induced by asTF.[19] However, whereas FAK inhibition abolished neovessel formation in vivo, it only partially inhibited HIF-1α induction by asTF. Taken together, these findings suggest that, although required for asTF-induced angiogenesis, FAK activation is not essential for asTF-induced HIF-1α expression. A possible explanation is the essential role of FAK in modulating early, essential steps (ie, endothelial cell migration and proliferation) for the sprouting of new capillaries[46,47] such that residual HIF-1α expression cannot balance under our experimental conditions.

Because integrins lack kinase activity, it is possible that signaling pathways other than FAK are involved. Our results revealed that MAPK and PI3K/Akt signaling pathways modulate HIF-1α upregulation after asTF stimulation. Integrin-mediated activation of MAPK and PI3K/Akt independently of FAK has been extensively described,[48] and our results suggest that, as in cancer,[36] stimuli other than hypoxia such asTF may activate HIF-1 in atherosclerosis, possibly contributing to disease progression.

Increased expression of HIF-1α was detected in complicated plaques with high levels of asTF, findings that support the pathophysiological importance of the proposed mechanism in the progression and complications of atherosclerotic disease.

On asTF stimulation of endothelial cells, HIF-1α translocates from the cytoplasm to the nucleus. Here, HIF-1α dimerizes with HIF-1β, becoming transcriptionally active and directly activating the expression of a number of proangiogenic factors, the best characterized of which is VEGF, thereby promoting the formation of new vessels.[49] Our results demonstrate that asTF induces gene and expression of the secreted VEGF-A isoform 165. Of note, the angiogenic effect of asTF was reduced when VEGF signaling was inhibited by gene transfer of VEGF-$Trap_{R1R2}$ in vivo. These observations suggest that the angiogenic activity of asTF is mediated via the activation of HIF-1/VEGF signaling.

Finally, we demonstrated for the first time that asTF plays a critical role in the progression of atherosclerosis and plaque angiogenesis. Our results show a significantly greater neointima thickness in injured carotid arteries that have been transduced with lentivirus encoding asTF compared with injured but untransduced carotid arteries of $ApoE^{-/-}$ mice on a Western-type diet. Remarkably, plaque neovascularization was significantly higher in injured and asTF-transduced arterial vessels.

In previous studies, it was determined that a 50% reduction in TF, in all cells or in hematopoietic cells only in $ApoE^{-/-}$ and $LDLR^{-/-}$mice, respectively, does not affect atherosclerosis development.[50] In our study, we found that asTF overexpression increases neointima formation and lesion neovascularization after arterial injury in $ApoE^{-/-}$ mice fed a Western-type diet. In line with the possibility of a pathophysiological effect of asTF on atherosclerosis progression and complications is the observation that asTF is highly expressed within advanced human carotid and coronary plaques complicated by hemorrhage or disruption.

Conclusions

We have demonstrated for the first time that asTF promotes neointima formation and neovascularization in a model of accelerated atherosclerosis. The angiogenic activity of asTF is mediated via the activation of HIF-1/VEGF signaling through the integrin-mediated activation of MAPK and PI3K/Akt signaling pathways. The potential role of asTF in plaque neovascularization and in the progression and complication of atherosclerosis is further supported by the increased expression of asTF in complicated coronary plaques from patients

with acute coronary events. The identification of asTF as a potent angiogenic factor, acting via the nonhypoxic HIF-1α upregulation in atherosclerosis, makes it an attractive marker of plaque vulnerability and a potential therapeutic target for plaque stabilization and prevention of cardiovascular clinical events.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Phenotypic switching of atherosclerotic plaques from a stable to a vulnerable phenotype plays a key role in the onset of cardiovascular events (ie, acute myocardial infarct and ischemic stroke). Despite the proven efficacy of existing lipid-lowering treatment, statins reduce cardiovascular events by only 30% to 40%. Increased neovascularization has been implicated in plaque hemorrhage and rupture and sudden onset of clinical events. In the present study, we examined the role of the angiogenic factor alternatively spliced tissue factor (asTF) on atherosclerosis. In human carotid and coronary plaques, we showed that asTF is highly expressed within lesions complicated by hemorrhage or disruption versus uncomplicated lesions. Cellular distribution analysis showed that asTF was highly expressed by monocytes/macrophages and endothelial cells within complicated human atherosclerotic plaques. Of note, asTF expression was higher in coronary lesions from patients with acute coronary syndrome versus stable angina. The angiogenic activity of asTF is mediated via the increased expression of hypoxia-inducible factor-1α though integrins and activation of the mitogen-activated protein kinase and phosphatidylinositol-3-kinase/Akt pathways. As a consequence of hypoxia-inducible factor-1 activation, vascular endothelial growth factor signaling appears involved in the angiogenic effect of asTF. Importantly, local gene transfer of asTF in a murine model of carotid injury in $ApoE^{-/-}$ mice on Western diet resulted in increased neointima formation and neovascularization. The identification of asTF as a potent angiogenic factor driving neointima neovascularization improves our understanding of the angiogenic factors and mechanisms involved in plaque angiogenesis and may contribute to the development of tailored therapies for atherosclerotic plaque stabilization.