Vascular Remodeling in Mice Lacking the Cytoplasmic Domain of Tissue Factor

Ilka Ott, Cornelia Michaelis, Maren Schuermann, Birgit Steppich, Isabell Seitz, Mieke Dewerchin, Dietlind Zohnhofer, Rainer Wessely, Martina Rudelius, Albert Schömig, Peter Carmeliet

Abstract—Tissue factor (TF), the cell surface receptor for the serine protease FVIIa supports cell migration by interaction with the cytoskeleton. Intracellular signaling pathways depend on the cytoplasmic domain of TF to modify cell migration and may alter vascular remodeling. Vascular remodeling was analyzed in a femoral artery injury and a blood flow cessation model in mice with a targeted deletion of the 18 carboxy-terminal intracellular amino acids of TF (TF<sup>Δct</sup>) and compared with TF wild-type mice (TF<sup>wt/wt</sup>). Morphometric analysis revealed a decrease in the intima/media ratio after vascular injury in arteries from TF<sup>Δct</sup> mice compared with TF<sup>wt/wt</sup> mice (femoral artery injury: 2.4±0.3 TF<sup>Δct</sup> versus 0.6±0.3 TF<sup>wt/wt</sup>, n=9 to 10, P=0.002; carotis ligation: 0.45±0.11 TF<sup>Δct</sup> versus 0.22±0.03 TF<sup>wt/wt</sup>, n=12 to 14, P=0.09). This was caused by an increase in the media by 54% (P=0.04) in the femoral artery model and by 32% (P=0.03) after carotis ligation and was associated with an increased number of proliferating cells. Isolated aortic smooth muscle cells (SMCs) of TF<sup>Δct</sup> mice showed an increased migratory response toward the TF ligand active site-inhibited FVIIa that was abolished in TF<sup>Δct</sup> SMC. In contrast, the unstimulated proliferation rate was increased in TF<sup>Δct</sup> SMC compared with TF<sup>wt</sup> SMCs. Thus, retention of SMCs attributable to a migratory defect and increased proliferation results in thickening of the media and in decrease in neointima formation after arterial injury. TF cytoplasmic domain signaling alters vascular remodeling and, thereby, may play a role in the development of restenosis, atherosclerotic disease, and neovascularization. (Circ Res. 2005;97:293-298.)

Key Words: arterial injury ■ tissue factor ■ smooth muscle cells ■ restenosis

Tissue factor (TF) is a transmembrane glycoprotein that initiates the clotting cascade and is considered to be a major regulator of coagulation and hemostasis. Previous studies have shown that TF is important in mediating acute arterial thrombosis and intimal hyperplasia in several animal models.1–4 Previous studies suggest an additional role for TF in tumor metastasis, angiogenesis, and cell migration.5–9 After binding TF FVIIa is activated to FVIIa, a serine protease. The complex TF–FVIIa induces gene transcription and activates signaling cascades.10–14 Independent of the proteolytic activity TF ligand binding supports cell spreading and migration.5,15 Furthermore, phosphorylation of the cytoplasmic domain serves as a regulator for angiogenic responses.7–9 Because mice lacking the cytoplasmic domain of TF exhibit normal development we sought to investigate if deletion of the cytoplasmic domain of TF in mice alters vascular responses to arterial injury.

Materials and Methods

Mouse Injury Model

Mice with a deletion of 18 amino acids of the cytoplasmic domain of TF (TF<sup>Δct</sup>) were generated by Cre-lox recombination technique on a MF1/129S/v/Swiss strain background.16 These animals displayed normal embryonic and postnatal development, fertility, and coagulation parameters. Mice were bred and housed under specific pathogen-free conditions. All studies were approved by the institutional Animal Care and Use Committee (Bayerisches Wissenschaftsministerium, München, Germany). Mice underwent carotid ligation and femoral-artery wire injury model as described.17,18 Before surgery, all mice were anesthetized by intraperitoneal injection with a solution composed of ketamine (80 mg/kg body weight) and xylazine (5 mg/kg body weight) for carotid ligation we used a midline neck incision, the right external carotid artery was looped proximally and tied off distally. Additional silk ties were looped round the common and internal carotid arteries for temporary vascular control during the procedure. For the femoral artery injury model a 0.014-in flexible angioplasty guide wire was introduced into the right femoral artery and endothelial denudation injury was performed using wire withdrawal injury and 3 passes along the artery. After removal of the wire the arteriotomy was closed and normal blood flow restored. At the time of euthanasia (14 days for carotid ligation and 28 days for femoral wire injury), the animals were reanesthetized, and after an overdosage of pentobarbital (210 mg/kg IP) in situ perfusion fixation was achieved with phosphate-buffered paraformaldehyde (4%, 0.1 mol/L sodium phosphate buffer, pH 7.3). Both injured right and uninjured left arteries were excised and embedded in paraffin. Serial sections (2 μm thick) were cut at...
50 μm intervals for hematoxylin-eosin and elastica van Gieson staining for histomorphometry and for immunohistological staining.

Quantitative Histomorphometry
Histomorphometric analysis was performed on serial Elastica van Gieson stained cross sections. Digitized images of the vessels were analyzed using the image analysis software Sigma Pro. The total vessel area, the area within the internal elastic interna, the area within the internal elastic lamina, as well as, the lumen area were determined by planimetry, and the lumen area, plaque area, medial area, intima-to-media (I/M) ratio, and overall vessel area were calculated.

Immunohistochemistry
Slides were exposed to heat-induced epitope retrieval using target retrieval buffer (DAKO) according to the manufacturer’s protocol. Sections were incubated with primary antibody Ki67 (PharMingen, 1:100 dilution) for 60 minutes followed by 30 minutes incubation with secondary rabbit anti-mouse immunoglobulins (LINK, Chemmate, DAKO, Hamburg, Germany). In a third step slides were incubated with APAAP immunocomplex (Chemate, DAKO) for 30 minutes. Ki67 positive cells were visualized by a red chromogen (Fast Red, DAKO) and counterstained with hematoxylin. The number of Ki67 positive cells was counted in the media and in the neointima as a measure for SMC proliferation.

Cell Culture
Mouse vascular SMCs were cultured from the aortas according to a method previously described. Briefly, connective tissue of the aorta was removed and endothelial cells were stripped by gently scraping the lumen side with a scalpel blade. The vessel was cut into 1- to 2-mm sections and plated in a culture dish supplied with DMEM (Invitrogen, Carlsbad, Calif) containing 20% FBS and 100 U/mL penicillin and 100 μg/mL streptomycin. The explants were incubated in a humidified incubator at 37°C with 5% CO2. Initial migration of SMCs was observed between 5 and 6 days. Experiments were conducted on SMCs after 3 to 5 passages in culture. Cultures were characterized by immunofluorescent staining with SMC-actin (Sigma) and CD31 (Serotech).

Cell Migration Assays
For detection of cell migration, in vitro wounds were created by scraping cell monolayers. Therefore, SMC were grown on fibronectin-coated 6-cm wells. After injury of the monolayer, the cells were gently washed and stimulated with 500 nM FPR-FVIIa (Novo Nordisk, Denmark). SMC migration was quantified after 2 hours by counting the number of cells with a spindle-shaped tail migrating from the edge of the injured monolayer toward the center of the wound before and after injury with a computer-assisted microscope (Zeiss) at 3 distinct positions. Migration toward fibronectin was analyzed with precoated Boyden chambers (QCM-FN Chemicon, International). After serum starvation 4×10^4 cells/well were added to the upper compartment of the chamber. After incubation for 18 hours at 37°C cells were removed from the upper side of the membrane. The remaining cells on the membrane were stained (Cell Stain, Chemicon) and the dye was eluted with extraction buffer for measurement of the absorbance at 570 nm. Migration on uncoated membranes was subtracted from the data.

SMC Proliferation
Primary SMC cultures were seeded on 6-well plates, trypsinized and counted after 24, 48, and 72 hours. Each experiment was performed using 2 different SMC preparations of TF^+/+ and TF^−/− mice. To analyze cell proliferation we used BrDU ELISA (Roche) according to the manufacturer’s instructions.

Statistical Analysis
Data are reported as mean±SEM. Student t test or ANOVA as appropriate were used to determine statistical significance between the groups. P<0.05 was considered significant.

Results
Vascular Remodeling in TF^+/+ and TF^−/− Mice
To determine the role of the cytoplasmic domain of TF on vascular remodeling and neointima formation we explored 2 different models of arterial injury. After transluminal wire injury which disrupts both the internal elastic lamina and the media the intima/media ratio was decreased from 2.4±0.3 in TF^+/+ mice (n=9) to 0.6±0.3 in TF^−/− mice (n=10, P=0.002; Figure 1A). This was attributable to an increase in the medial area of 54% (P=0.04) whereas the decrease in the neointimal area of 38% did not reach statistical significance (P=0.09; Figure 1B and 1C). No significant changes in the luminal or cross-sectional area were found between TF^+/+ mice and TF^−/− mice (data not shown).

After ligation of the carotid artery, which induces minimal focal mechanical trauma to the internal elastic lamina, similar results were obtained. The medial area increased by 32% in TF^−/− mice (n=14) compared with TF^+/+ mice (n=12 P=0.03; Figure 2A), whereas no significant changes were observed in the neointimal area (Figure 2B). The reduction in intima/media ratio in TF^−/− mice did not reach statistical significance (P=0.09; Figure 2B). Luminal or cross-sectional area was similar in TF^+/+ mice and TF^−/− mice (data not shown). These changes were not attributable to constitutive differences in the vessel wall diameters because the uninjured carotid and femoral arteries showed no differences in medial area between TF^+/+ mice and TF^−/− mice (data not shown).

Migration of SMC From TF^+/+ and TF^−/− Mice
Because we recently identified a coagulation-independent and ligand-dependent role for TF in cell migration,3 we analyzed the migratory response of aortic SMC isolated from TF^+/+ and TF^−/− mice toward TF ligand. To exclude interfering effects by activation of protease activated receptors (PARs) we used active site inhibited FPR-FVIIa. SMCs from TF^+/+ showed a significant increase in migration in the presence of FPR-FVIIa. This increase was abolished in SMCs lacking the cytoplasmic domain of TF (Figure 3A and 3B). Furthermore, no changes in TF-independent migration toward fibronectin in a Boyden chamber migration assay were found (TF^+/+ SMC: 0.19±0.2 mOD; TF^−/− SMC: 2.1±0.3 mOD). Thus, the cytoplasmic domain of TF seems necessary only for TF-dependent migration. Furthermore, the proteolytic activity of TF ligand FVIIa is not necessary for the enhancement of migration induced by the cytoplasmic domain of TF. Because differences in the migratory response may be attributable to altered integrin expression, we analyzed β1, β3, and α3-integrin expression using quantitative PCR (TaqMan). However no significant differences in integrin expression were observed (online Figure I available at http://circres.ahajournals.org). We also did not find significant changes in surface expression of β1, β3, and α3 in human coronary smooth muscle cells after stimulation with FPR-FVIIa using flow cytometry (data not shown). These results suggest that neither the cytoplasmic domain, nor TF ligand interactions alter expression of integrins β1, β3, and α3 and, thereby, contribute to enhanced migratory responses.
Isolated primary aortic cells contained more than 90% SMC-actin positive cells (Figure 3C) with no contaminating endothelial cells because no cells stained positive for CD31.14

**Proliferation of SMC From TF<sup>wt/wt</sup> and TF<sup>ΔcΔct</sup> Mice**

To determine whether the enhanced media formation in TF<sup>ΔcΔct</sup> mice was attributable to an augmented proliferation of SMCs we analyzed the number of proliferating cells in the neointima and media by immunohistochemical staining for Ki67 in the neointima and media. In TF<sup>ΔcΔct</sup> mice we found an increase in the number of proliferating cells in the media whereas no changes were observed in the neointima compared with wild-type (WT) mice after wire injury (Figure 4). Because the number of cells in the neointima and the media were comparable in TF<sup>ΔcΔct</sup> and TF<sup>wt/wt</sup> mice, lack of migration per se may be the cause of limited neointima in the TF<sup>ΔcΔct</sup> mice (237±36 versus 297±80). In the carotid ligation model no proliferating cells were found in the vessel wall. In vitro experiments revealed an increase in cell density.
and BrDU incorporation rate in aortic SMC isolated from TF<sup>Δct/Δct</sup> mice (Figure 5) as a measure for an enhanced constitutive proliferative capacity of TF<sup>Δct/Δct</sup> SMCs compared with TF<sup>wt/wt</sup> SMCs. Addition of FFR-FVIIa, however, did not alter SMC proliferation assessed by BrDU incorporation (data not shown). Thus, lack of the cytoplasmic domain of TF enhances the proliferation of SMCs independently of TF ligand interactions.

**Discussion**

Major findings of our study are as follows: (1) Media formation is enhanced whereas neointima formation is reduced in mice lacking the cytoplasmic domain of TF. (2) Enhancement of migration by FFR-FVIIa required the cytoplasmic domain of TF in aortic SMCs. (3) Lack of the cytoplasmic domain of TF enhance the proliferative rate of SMCs independent of TF ligand interaction.

The formation of neointima and remodeling of the media after injury are known to involve SMC migration, proliferation and cell death in 3 steps: replication in the media, migration of SMCs from the media to the intima and proliferation within the neointima. Multiple growth factors have been implicated driving these processes. Although the initial response to the vascular insult is not affected by the cytoplasmic domain of TF, the second and the third steps were affected. These data suggest that migration of the SMCs from the media to the neointima is impaired, involving TF ligand interactions and resulting in an increase in media formation with a decrease in neointima formation even though we observed an increased proliferative capacity of the SMCs lacking the cytoplasmic domain of TF.

Our results are supported by recent studies in low TF mice that suggest a role for TF in intimal hyperplasia by inhibition of migration. However, these studies did not focus on the role of TF-mediated signaling via its cytoplasmic domain but evaluated the net effect of decreased FVII activation and TF signaling attributable to less TF expression. In contrast, our study only addresses the role of TF signaling via its cytoplasmic domain. Activation of PAR-2 is known to stimulate cell migration, thus, the observed result may be attributable to a decreased activation of PAR-2. Our data from different models of arterial injury suggest a defective migration of TF<sup>Δct/Δct</sup> SMC toward TF ligand paired with an enhanced constitutive proliferative potential of TF<sup>Δct/Δct</sup> SMC resulting in an enhanced media formation and, thereby, decreased neointima formation in TF<sup>Δct/Δct</sup> mice. The fact that retention of SMCs in the media was not associated with a decrease in neointima may be attributable to blood-borne progenitor cells contributing to neointima formation by other mechanisms than SMC migration.

Recent studies have shown that interaction of the extracellular domain of TF inhibits migration by interaction with β1 integrins. This effect was dependent on the cytoplasmic domain of TF because phosphorylation of the cytoplasmic domain abolished the inhibitory role of TF. FVIIa can bridge between TF and immobilized extracellular inhibitors to sup-
transport cell spreading and migration. Furthermore, TF cytoplasmic domain deleted mice exhibit enhanced angiogenesis. Immunochemical studies found an association of phosphorylated TF with neoangiogenesis in diabetic retinopathy, as evidence for TF phosphorylation to release suppressive functions of the cytoplasmic domain of TF in vivo. Because TF cytoplasmic domain is unphosphorylated and palmitoylation suppresses agonist-induced phosphorylation by activation of PAR-2, regulation of phosphorylation of the cytoplasmic domain of TF may be crucial for TF signaling. As a result, phosphorylation of the cytoplasmic domain of TF switches off negative regulation of angiogenesis and migration. In concordance with the inhibitory function of the cytoplasmic domain of TF on angiogenesis and migration our data suggest that constitutive lack of the cytoplasmic domain of TF also inhibits proliferation because deletion of TF cytoplasmic domain increased SMC proliferation. In contrast, addition of the TF ligand FFR-FVIIa stimulates cell migration only in TF*wt* mice. If phosphorylation of the cytoplasmic domain of TF plays a crucial role in mediating the inhibitory function of TF on migration this may suggest that FFR-FVIIa enhances signals that phosphorylate the cytoplasmic domain of TF such as activation of PKC-α or decrease in palmitoylation and, thereby, enhances migratory responses. The role of TF in cell migration is further supported by the association of TF with cytoskeletal structures and by activation of MAPK p38 and GTPase Rac by FFR-FVIIa after ligand binding independently of the proteolytic activity of FVIIa. Because different pools of TF might be involved, signaling may require higher concentrations of FVIIa than those necessary for initiation of coagulation. At the site of a ruptured thrombosed plaque these mechanisms may contribute to the migratory capacity of the surrounding cells and, thereby, contribute to vascular remodeling. The fact that proliferation was not altered by FFR-FVIIa, however, suggests different mechanisms for regulation of proliferative responses. Thus, further studies need to clarify the mechanisms of TF cytoplasmic domain and its phosphorylation status on the signaling events that modify cellular responses.

As TF*Δc* mice display a normal phenotype but altered vascular remodeling after arterial injury the signal transduction via the cytoplasmic tail of TF may be only important under certain conditions. Under physiological conditions TF antigen is not detected in the intima or media of uninjured vessels. Vascular injury, however, induces inflammatory mediators that, in turn, stimulate TF expression early in the media and subsequently in the neointima. In addition, cellular TF is induced by activation of immediate early genes by inflammatory mediators. Thus, the regulatory function of TF after arterial injury may be the result of an increased TF expression in a proinflammatory environment.

Considering the relevance of the observed findings for patients with atherosclerotic disease it has to be considered that murine models of vascular injury involve normal arteries without underlying atherosclerotic disease. Because in humans TF is expressed in atherosclerotic lesions and contribute to arterial thrombosis the observed effects may even be underscored.

In summary, our results demonstrate a role for the cytoplasmic domain of TF in vascular remodeling in vivo by inhibition of TF ligand–induced migratory responses and so far unknown enhancement of proliferative responses.

**Acknowledgments**

The study was supported in part by grants from the Wilhelm Sander Stiftung, and the Else Kröner Fresenius Stiftung. We thank B. Campbell, A. Stobbe, R. Hegenloh, and C. Bauer for invaluable technical assistance and W. Erhardt and T. Brill for support with the animal procedures.

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_Circ Res._ 2005;97:293-298; originally published online July 14, 2005;
doi: 10.1161/01.RES.0000177533.48483.12

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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Transcript levels of various integrins in mouse aortal SMCs were quantified by TaqMan® Quantitative RT-PCR. As a control mouse fibroblasts NIH3T3 were used. Total RNA was extracted with RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. 300 ng total RNA was subsequently reverse transcribed using Omniscript Reverse Transcriptase (Omniscript RT Kit, Qiagen, Hilden, Germany). PCR of 30 ng cDNA was performed in a final volume of 25 µl. The PCR mixture contained 1x TaqMan Universal PCR Master Mix, 900 nmol/l of each primer and 250 nmol/l probe corresponding to 1x Assay-on-Demand (Applied Biosystems, Darmstadt, Germany). Assay-on-Demands containing primers and 6-carboxy-fluorescein (FAM)-labeled probes for murine integrin α3 (Mm00442890_m1), integrin β3 (Mm00443980_m1), integrin β1 (Mm01253227_m1) and GAPDH (Mm99999915_g1) were purchased from Applied Biosystems. The PCR protocol included 2 minutes at 50°C and 10 minutes at 95°C for enzyme activation, then 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute. Real-time fluorescence detection was performed and analyzed with the ABI PRISM 7700 Sequence Detector (Applied Biosystems). Values were normalized on GAPDH. The relative expression levels compared to NIH3T3 cells were calculated using the comparative Ct method (Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^(-ΔΔCt) method. Methods 2001;25:402–408).