Atherosclerosis in Mice Is Not Affected by a Reduction in Tissue Factor Expression

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Objective—To determine whether tissue factor (TF) contributes to the progression of atherosclerotic lesions in mice.

Methods and Results—We determined the effect of a 50% reduction of TF levels in all cells on atherosclerosis in apolipoprotein E-deficient (apoE$^{-/-}$) mice. No differences were observed in the extent of atherosclerosis in apoE$^{-/-}$/TF$^{+/+}$ and apoE$^{-/-}$/TF$^{+/-}$ mice fed regular chow for 34 weeks. Atherosclerosis could not be analyzed in apoE$^{-/-}$ mice expressing low levels of TF because of premature death of these mice. Macrophages are a major source of TF in atherosclerotic plaques. Therefore, in a second series of experiments, we investigated the effect on atherosclerosis of selectively reducing hematopoietic cell-derived TF by transplanting bone marrow from mice expressing low levels of TF into low-density lipoprotein receptor deficient (LDLR$^{-/-}$) mice. Atherosclerosis within the arterial tree and aortic root were similar in LDLR$^{-/-}$ mice with low-TF bone marrow compared with control bone marrow (TF$^{+/+}$ or TF$^{+/-}$) after 4 and 16 weeks on an atherogenic diet. Furthermore, the cellular composition of the aortic root lesions was similar between the 2 groups.

Conclusions—Our data indicate that either a 50% reduction of TF in all cells or a selective reduction in hematopoietic cell-derived TF does not affect the development of atherosclerotic lesions in mice. (***Arterioscler Thromb Vasc Biol.* 2006;26:555-562.)**

Key Words: atherosclerosis ■ macrophages ■ mice ■ tissue factor

Atherosclerosis is a major cause of death in Western counties.[1,2] Rupture of atherosclerotic plaques induces the formation of a platelet-rich thrombus and vessel occlusion, which leads to myocardial infarction and stroke.

Tissue factor (TF) is the primary cellular initiator of the coagulation cascade and is constitutively expressed by extravascular cells.[3] After vessel injury, plasma factor VII/VIIa (FVII/VIIa) binds to TF to form a TF:FVIIa complex, which activates FX and FIX, leading to thrombin generation, fibrin deposition, and platelet activation.[4] The activity of the TF:FVIIa complex is regulated by tissue factor pathway inhibitor (TFPI).[5]

High levels of TF are observed in human atherosclerotic plaques.[6,7,8] Similarly, TF is expressed in rabbit atherosclerotic lesions.[9,10] Macrophages within the atherosclerotic lesions appear to be the major source of TF, although vascular smooth muscle cells (VSMCs) also express TF.[7] TF expression has been found to increase with the progression of human atherosclerotic lesions.[11] For instance, coronary atheroma from patients with unstable angina contained more functional TF than atheroma from patients with stable angina.[12] Furthermore, higher levels of TF activity are observed in plaques with thrombi.[8] These data support the idea that TF plays an important role in the formation of an occlusive thrombus after plaque rupture.

At present, it is unclear if TF plays a role in the progression of atherosclerosis. TF may contribute to plaque progression by enhancing the generation of thrombin and fibrin, as well as by increasing platelet activation.[13,14] Furthermore, TF has been shown to enhance both macrophage migration and VSMC proliferation and migration in vitro.[15–18] Finally, TF-dependent generation of coagulation proteases, such as FVIIa, FXa, and thrombin, may increase proinflammatory signaling within atherosclerotic plaques by activation of protease activated receptors (PARs).[19] Macrophages within atherosclerotic vessels coexpress TF and FVII.[20] Interestingly, the administration of statins to rabbit and mouse models of hyperlipidemia reduced macrophage accumulation, inflammation and TF expression.[21–23]

Original received July 27, 2005; final version accepted December 7, 2005.

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This abstract was presented at the ATVB (AHA) conference, Washington DC, April 2005.

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Arterioscler Thromb Vasc Biol. **is available at http://www.atvbaha.org** **DOI: 10.1161/01.ATV.0000202028.62414.3c**

The role of TFPI in atherosclerosis was investigated by examining the effect of reducing TFPI levels by 50% in apoE$^{-/-}$ mice.[24] TFPI$^{+/-}$ mice have increased TF activity. The apoE$^{-/-}$/TFPI$^{+/-}$ mice exhibited a selective increase in atherosclerosis in carotid and iliac arteries compared with apoE$^{-/-}$ mice expressing wild-type levels of TFPI,[24] suggesting a role for TFPI in atherosclerosis.

In the present study, we examined if TF plays a role in the progression of the atherosclerosis by examining the effect of a 50% global reduction of TF expression or by selectively reducing TF expression in hematopoietic cells. We used mice deficient in either apolipoprotein E (apoE$^{-/-}$) or the low-density lipoprotein receptor (LDLR$^{-/-}$) and mice expressing 100%, 50%, or ≈1% of TF levels (low-TF mice).[25,26] Reducing TF levels of apoE$^{-/-}$ mice by 50% in all cells did not alter the amount of atherosclerotic lesions. Similarly, selectively reducing TF levels in hematopoietic cells in LDLR$^{-/-}$ mice did not affect the percentage surface area of aortic lesions or aortic root lesion size and cellular composition.

Methods

Mice

All studies were approved by The Scripps Research Institute and University of Michigan Animal Care and Use Committees and comply with National Institute of Health Guidelines.

The apoE-deficient (apoE$^{-/-}$) mice on a C57Bl/6J background were purchased from Jackson Laboratories (Bar Harbor, Me). Low-TF mice express very low levels of human (h) TF (≈1% relative to mouse (m) TF) and completely lack mouse TF (mTF$^{-/-}$/hTF^{+}).[25] The apoE$^{-/-}$ mice were bred in-house with low-TF mice on a C57Bl/6J background to eventually generate apoE$^{-/-}$/TF$^{+/+}$, apoE$^{-/-}$/TF$^{+/-}$, and apoE$^{-/-}$/low-TF mice. For some experiments, apoE$^{-/-}$ mice were bred with TF$^{+/-}$ mice[26] (a kind gift from Dr G. Broze) to generate apoE$^{-/-}$/TF$^{+/+}$ and apoE$^{-/-}$/TF$^{+/-}$. Mice were fed ad libitum either a standard mouse chow (regular chow) or, when stated, an atherogenic diet (15.8% fat, 1.25% cholesterol, and no cholic acid; No. 94059, Harlan, Teklad).

LDLR-deficient (LDLR$^{-/-}$) mice, backcrossed onto a C57Bl/6J background, were purchased from Jackson Laboratories and bred in-house. The mice were weaned at 4 weeks and fed ad libitum either a standard mouse chow diet (regular chow; Purina 7012, Harlan, Teklad) or an atherogenic diet for 4 weeks (21.2% fat, 1.25% cholesterol; No. 96121, Harlan, Teklad) and 16 weeks (15.8% fat, 1.25% cholesterol, and no cholic acid; No. 94059, Harlan, Teklad).

apoE$^{-/-}$ and LDLR$^{-/-}$ mice are different models of atherosclerosis. apoE$^{-/-}$ mice have high very-low-density lipoprotein levels, whereas LDLR$^{-/-}$ mice have high LDL. In bone marrow transplantation experiments, macrophage apoE will reduce the hypercholesteremia and protect the mice from atherosclerosis.[27] Therefore, we used LDLR$^{-/-}$ mice for the bone marrow transplantation experiments because macrophage LDL receptor does not alter lipid levels.[27]

Irradiation and Bone Marrow Transplantation

LDLR$^{-/-}$ mice (8 weeks old) were subjected to 13 Gy (1300 rad) γ-irradiation from a cesium 137 irradiator (Gammacell 40; Atomic Energy of Canada, Mississauga, ON, Canada) to ablate endogenous bone marrow-derived cells and stem cells. All irradiated mice were injected with 2×10^6 bone marrow cells from control (either mTF$^{+/+}$ or mTF$^{+/-}$/hTF^{+}) or low-TF mice via the retro-orbital sinus. Mice were allowed to recover for 4 weeks before being fed an atherogenic diet.

Genotyping of DNA From Bone Marrow Recipient Mice

Blood was collected after euthanasia and DNA was prepared from peripheral blood mononuclear cells. Analysis of the levels of the wild-type (WT) and low-TF allele was used to demonstrate bone marrow reconstitution. The WT mouse (mTF) allele was detected by polymerase chain reaction (PCR) using a forward primer, 5′-ATGAGGAGCTGTGTTAAAGGGTCGCAGAA, and a reverse primer, 5′-TGCAGTAAATGCACGTGTCTGCCAT, that are located upstream of exon 1 of the mTF allele (559bp). The mutant TF allele was detected using the mTF forward primer, 5′-CAAGATGGATTGCACGCAGGTTCTCC, in conjunction with a reverse primer, 5′-CACGAGGAAGCGGTCAGCCCATTCG, that is located within the neo cassette (700 bp).

TF Activity

Functional TF activity in homogenized uninjured carotid arteries was determined using a previously described chromogenic assay.[28] Briefly, 30 μL of carotid artery homogenate was combined with 90 μL of a reaction mixture containing 3 nmol/L human FVIIa (Haemtech), 100 nM human FX (Haemtech), 8.3 mmol/L $CaCl_2$, and 0.33 mmol/L Spectrozyme FXa (American Diagnostica) in Tris-buffered saline, pH 7.4, with 1 mg/mL bovine serum albumin. After 45 minutes of incubation at 37°C, the optical density was measured at 405 nm and the TF activity was expressed in pmol/L as determined by reference to a standard curve of recombinant human relipidated TF (rTF) (American Diagnostica). This analysis provides data on the relative levels of TF activity in the carotid arteries of WT and TF$^{+/-}$ mice and does not accurately measure TF levels because of the inefficiency of human FVIIa binding to murine TF.[29]

The procoagulant activity of peritoneal macrophages was determined using a single-stage clotting assay. Peritoneal macrophages, elicited by thioglycollate, were collected from TF$^{+/+}$, TF$^{+/-}$/hTF^{+} (TF$^{+/-}$) and low-TF mice and 2×10^6 cells were plated per well of a 6-well plate. Cells were treated with lipopolysaccharide (LPS) (1 μg/mL; *Escherichia coli* serotype OB111; Sigma) or vehicle for 6 hours, then scraped and stored at −80°C. The cells were subsequently lysed with N-octyl-β,D-Glucopyranoside and diluted in 25 mmol/L Hepes-saline, pH 7.4, before combining with mouse pool plasma (Sigma) in a Start 4 clotting machine (Diagnostica Stago). The reaction was initiated by the addition of 20 mmol/L $CaCl_2$ in 25 mmol/L Hepes-saline, pH 7.4, and the time to clot was determined. TF activity expressed in relative units/1×10^6 cells was calculated by reference to a standard curve of mouse brain extract.

Analysis of Atherosclerosis

Assessment of the atherosclerotic lesions was performed in 2 different ways.

Method 1

After euthanasia, the vasculature of the mice was perfused with phosphate-buffered saline (PBS), pH 7.4, followed by formal sucrose (4% paraformaldehyde and 5% sucrose in PBS, pH 7.4). The aorta from the proximal ascending aorta to the bifurcation of the iliac artery was dissected and stained with Sudan IV. The aortas were photographed and images digitized. The total arterial surface area and total lesion area were determined using Adobe Photoshop 5.0.2 and NIH Scion Image Software. The extent of lesion development was reported as percentage of the total area of a given artery that was occupied by atherosclerotic lesions.

Lesions of the aortic root were analyzed as previously described.[30,31] The proximal aortic root and adjoining portion of the heart from LDLR$^{-/-}$ mice was removed, immersed in formal sucrose for 6 hours and subsequently embedded in OCT and stored at −70°C until sectioning. Serial sections (10 μm in thickness) were cut through a 250-μm segment of the aortic root, where all 3 valve leaflets are present. For each mouse, 4 sections separated by 40 μm were examined. Each section was stained with oil red O, counterstained with Gill's Hematoxylin #1 (Fisher Scientific) and images were digitized. Total lesion cross-sectional area, including the

intima, lipid cores, and fibrotic components, was calculated for each cross-section and mean cross-sectional areas were calculated for each animal.

Method 2

After euthanasia, the vasculature of the mice was perfused with PBS followed with zinc formalin. The aorta and its major branches (left and right distal carotid, brachiocephalic, left and right iliac, left and right subclavian arteries) were dissected and stained with Sudan IV. The images were analyzed and the extent of lesion development was reported as percentage of the total area of a given artery that was occupied by atherosclerotic lesions using Image-Pro Plus software (Media Cybernectics, Marietta, Ga).

Immunohistochemistry

Serial frozen tissue sections of aortic root lesions were stained immunohistochemically with MOMA-2 and α-actin to identify macrophages and smooth muscle cells, respectively, and with anti-TF antibody to detect the cellular location of TF expression. Sections were fixed and quenched in acetone/0.3% H_2O_2 for 30 minutes and rehydrated in either distilled water or PBS/0.1% Tween-20 for 10 minutes. All sections were blocked in 1% bovine serum albumin/PBS for 30 minutes. Sections were incubated with primary antibody and incubated overnight at 4°C at the following dilutions: rat anti-mouse MOMA-2 (Serotec) at 1:25 and rabbit anti-human TF polyclonal antibody, a generous gift from Dr T. Edgington, at 1:1000. RTU-HRP–labeled mouse anti-human α-actin (DAKO) was used as directed by manufacturers' instructions and incubated for 2 hours at room temperature. Nonspecific IgG was used as a negative control. Sections were washed 3 times in PBS/0.1% Tween-20 for 5 minutes. MOMA-2 was detected using horse radish peroxidase (HRP) conjugated rabbit anti-rat IgG at 1:200 (BioJackson) and the antigen/antibody complexes were visualized with a 30-minute exposure to Vectastain ABC Elite solution (Vector Laboratories). TF was visualized using DAKO envision plus HRP-labeled polymer (DAKO). All sections were counterstained with Mayer's Hematoxylin. Stained sections were dehydrated and mounted.

Plasma Cholesterol and Triglyceride Levels

Mice were fasted for 6 hours before collection of venous blood from the retro-orbital sinus into a heparinized capillary tube. Plasma was isolated by centrifugation at 3000*g* for 5 minutes at 4°C and stored at −20°C. Enzymatic measurements of total cholesterol and triglyceride levels were performed using the Infinity Cholesterol kit (Thermo Electron Corporation) and Triglyceride GPO (Raichem), respectively.

Statistical Analyses

All results were presented as means±standard error of the mean (SEM). Data were analyzed by Student *t* test or by Mann-Whitney rank sum test for nonparametric data (SigmaStat, v.3.1, SYSTAT 2004). A value of $P<0.05$ was considered significant.

Results

Effect of Reducing TF by 50% on Atherosclerotic Lesion Formation in the Vasculature of ApoE$^{-/-}$ Mice

A previous study showed that a 50% reduction of TFPI increased atherosclerosis in the carotid and iliac arteries,[24] which may be because of increased TF activity. This led us to investigate the effect of reducing TF levels by 50% in apoE$^{-/-}$ mice. apoE$^{-/-}$ mice were bred with TF$^{+/-}$ mice to generate apoE$^{-/-}$/TF$^{+/+}$ and apoE$^{-/-}$/TF$^{+/-}$ mice. TF activity of carotid artery homogenates obtained from apoE$^{-/-}$/TF$^{+/+}$ or apoE$^{-/-}$/TF$^{+/-}$ mice was measured to confirm that TF levels were functionally reduced in TF$^{+/-}$ mice. The mean TF activity detected in the carotid arteries of apoE$^{-/-}$/TF$^{+/-}$ mice (2.8±0.2 pM, n=8) was approximately half that of apoE$^{-/-}$/TF$^{+/+}$ mice (5.1±0.5 pM, n=6, $P<0.001$).

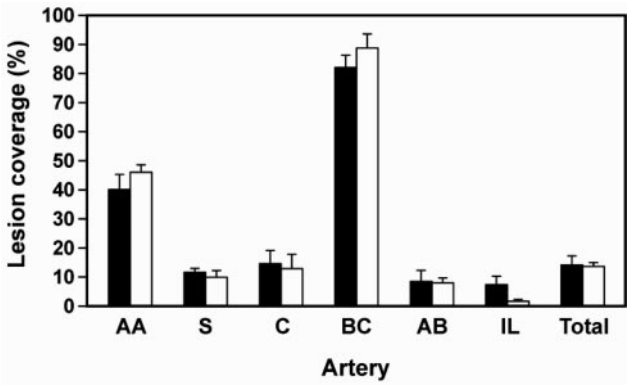

Figure 1. Percentage lesion coverage in the aorta and major arteries of apoE$^{-/-}$/TF$^{+/+}$ (n=5; black bar) and apoE$^{-/-}$/TF$^{+/-}$ (n=6; white bar) mice fed regular chow for 34 weeks. AA, ascending aorta, S, subclavian, C, carotid, BC, brachiocephalic artery, AB, abdominal aorta, IL, iliac artery. Data are expressed as means±SEM.

Next, apoE$^{-/-}$/TF$^{+/+}$ and apoE$^{-/-}$/TF$^{+/-}$ mice were fed regular chow for 34 weeks and plasma cholesterol levels and atherosclerotic lesions in the arterial tree were quantitated. The plasma cholesterol level was not different between apoE$^{-/-}$/TF$^{+/+}$ and apoE$^{-/-}$/TF$^{+/-}$ mice (mean±SEM: 683±69 mg/dL versus 795±56 mg/dL; n=5 to 9). Whole-mount arterial trees were stained with Sudan IV and the percentage lesion coverage of the arterial tree was determined in the 2 groups (Figure 1). Atherosclerotic lesions had developed, albeit to different extents, in all the regions of the arterial tree that were examined. No significant differences were observed in lesion development between the apoE$^{-/-}$/TF$^{+/+}$ and apoE$^{-/-}$/TF$^{+/-}$ mice groups (Figure 1). apoE$^{-/-}$ mice were also bred with low-TF mice to generate apoE$^{-/-}$/TF$^{+/-}$/hTF^{+} (apoE$^{-/-}$/TF$^{+/-}$) and apoE$^{-/-}$/TF$^{-/-}$/hTF^{+} (apoE$^{-/-}$/low-TF) mice. However, we observed a very high mortality rate in apoE$^{-/-}$/low-TF mice fed an atherogenic diet that precluded analysis of the atherosclerotic lesions at 16 weeks. All of these mice (7/7) died by 12 weeks.

Effect of Reducing Hematopoietic Cell TF on Atherosclerotic Lesion Development in LDLR$^{-/-}$ Mice

TF is primarily expressed by macrophages in atherosclerotic lesions.[7] In addition, monocytes and macrophages are the major hematopoietic cell type that can express TF. Therefore, we used a genetic approach to determine the effect of reducing TF in hematopoietic cells on the development of atherosclerosis. Figure 2 shows the levels of TF activity in peritoneal macrophages isolated from TF$^{+/+}$, TF$^{+/-}$, and low-TF mice. Unstimulated low-TF macrophages expressed 21-fold and 43-fold lower levels of TF compared with TF$^{+/-}$ and TF$^{+/+}$, respectively. Similar, but slightly less dramatic differences were observed using LPS stimulated peritoneal macrophages (Figure 2). These results indicated that there was a significant reduction in TF in peritoneal macrophages from low-TF mice compared with either TF$^{+/-}$ or WT mice.

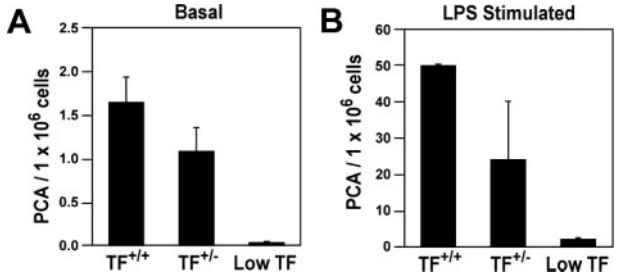

Figure 2. Procoagulant activity of peritoneal macrophages obtained from $TF^{+/+}$, $TF^{+/-}$, and low-TF mice under (A) basal conditions and (B) stimulated with LPS (1 μg/mL) for 6 hours. Data are presented as mean±SEM.

Therefore, both WT and $TF^{+/-}$ bone marrow were used as controls in our experiments.

Bone marrow from either control or low-TF mice was transplanted into $LDLR^{-/-}$ recipients to determine the contribution of hematopoietic cell-derived TF on atherosclerotic lesion progression. Bone marrow reconstitution in recipient mice was determined by PCR analysis of peripheral blood cell DNA from the $LDLR^{-/-}$ mice transplanted with either $TF^{+/-}$ or low-TF bone marrow. The wild-type mouse TF allele was detected in bone marrow from $LDLR^{-/-}$ mice transplanted with $TF^{+/-}$ bone marrow, but not in mice transplanted with low-TF bone marrow (Figure 3A). This demonstrated efficient irradiation and bone marrow reconstitution in these mice.

We performed 3 independent bone marrow experiments, one with $TF^{+/+}$ bone marrow as a control (experiment 1) and 2 with $TF^{+/-}$ bone marrow as a control (experiments 2 and 3). In experiment 2, we analyzed plasma triglyceride levels in the 2 groups of mice. Triglyceride levels were not altered by bone marrow transplantation or by the atherogenic diet and were similar in the $LDLR^{-/-}/TF^{+/-}$ and $LDLR^{-/-}$/low-TF groups (Figure 3B) (mean concentration [mg/dL±SEM]: 0 weeks, 55±3 versus 59±3; postdiet, 67±5 versus 60±4). In experiments 2 and 3, we analyzed cholesterol levels in the 2 groups. In experiment 2, plasma cholesterol levels were determined before initiating the atherogenic diet and after 16 weeks on an atherogenic diet (Figure 3C). As expected, plasma cholesterol dramatically increased after 16 weeks on an atherogenic diet. A small difference in cholesterol levels was observed at 16 weeks between the $LDLR^{-/-}/TF^{+/-}$ and $LDLR^{-/-}$/low-TF groups (mean concentration [mg/dL]±SEM: 0 weeks, 284±14 versus 253±6; 16 weeks, 917±36 versus 1064±41; $P=0.02$). However, no differences in cholesterol levels were observed in experiment 3 at any time point (Figure 3C) (mean concentration [mg/dL]±SEM at 16 weeks for the control and low-TF groups were 1170±108 and 1361±83, respectively).

We investigated whether reducing TF in hematopoietic cells affects early atherosclerotic lesion development (experiment 1). Analysis of lesion development in $LDLR^{-/-}/TF^{+/+}$ and $LDLR^{-/-}$/low-TF transplanted mice was performed after

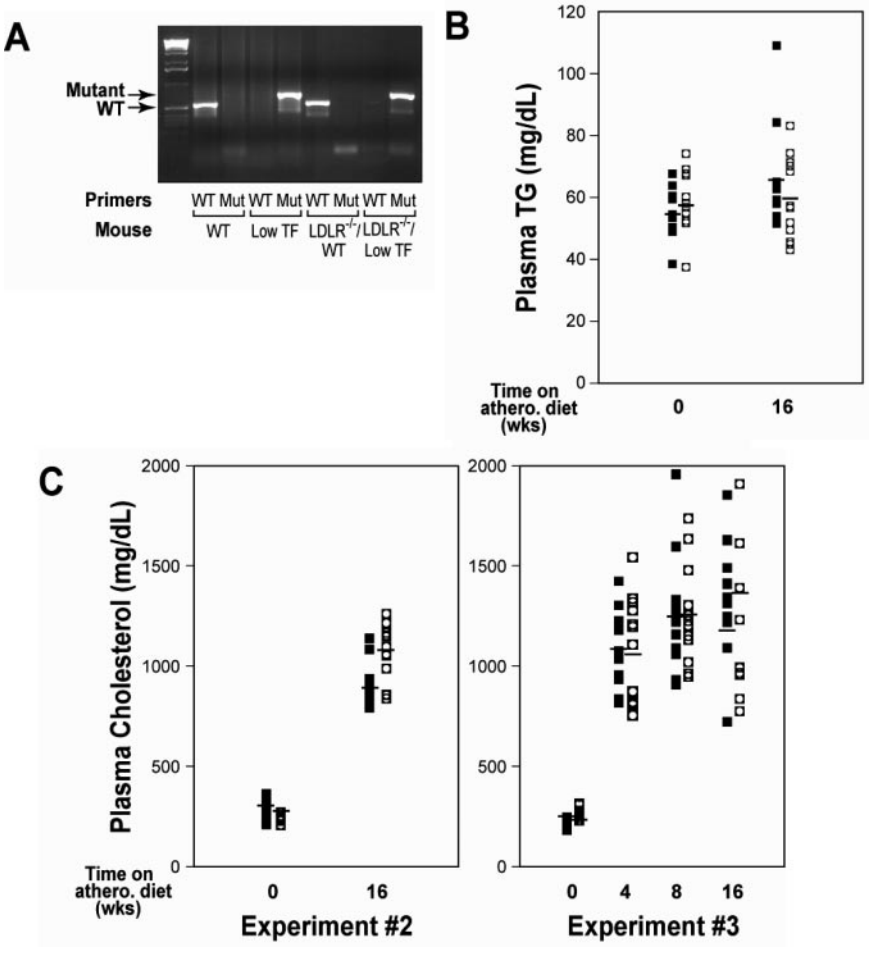

Figure 3. A, PCR of peripheral blood cell DNA from $LDLR^{-/-}$ mice transplanted with bone marrow from WT or low-TF mice demonstrating efficient ablation and reconstitution with donor bone marrow. B, In experiment 2, plasma triglyceride (TG) levels were determined before initiating the atherogenic diet regime and after 16 weeks on an atherogenic diet. C, In experiments 2 and 3, cholesterol levels were determined at various times. Black squares represent $LDLR^{-/-}/TF^{+/-}$ mice and white squares represent $LDLR^{-/-}$/low-TF mice. Horizontal bars represent the means of the plasma triglyceride or cholesterol levels.

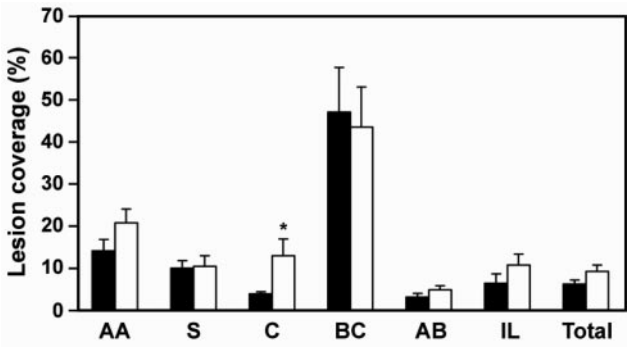

Figure 4. Percentage lesion coverage in the aorta and major arteries of LDLR$^{-/-}$/TF$^{+/+}$ (n=7; black bar) and LDLR$^{-/-}$/low-TF (n=8; white bar) mice fed an atherogenic diet for 4 weeks. AA, ascending aorta, S, subclavian, C, carotid, BC, brachiocephalic artery, AB, abdominal aorta, IL, iliac artery. Data are expressed as means±SEM. A statistically significant difference was observed in the carotid artery (*P<0.05). Male mice were used for this experiment.

4 weeks on an atherogenic diet. At 4 weeks, whole-mount arterial trees were stained with Sudan IV and the percentage lesion coverage was determined in the aorta and its major arteries (Figure 4). No significant differences in percentage lesion coverage between the LDLR$^{-/-}$/TF$^{+/-}$ and LDLR$^{-/-}$/low-TF groups were observed in any vessel except the carotid artery, which exhibited an increase in the low-TF group compared with the control group (P=0.009).

Next, we investigated whether reducing TF in hematopoietic cells affects atherosclerotic lesion development after 16 weeks on an atherogenic diet (experiments 2 and 3). We analyzed the percentage lesion area in the aorta in concert with analysis of the aortic root (heart valve) lesion size and morphology in the LDLR$^{-/-}$/TF$^{+/-}$ and LDLR$^{-/-}$/low-TF mice. Lesions were observed at the aortic arch, thoracic and abdominal regions of the aorta and this distribution was similar in the 2 groups (Figure 5A). In experiment 3, the percentage lesion coverage was not significantly different (P=0.4) between the LDLR$^{-/-}$/TF$^{+/-}$ and LDLR$^{-/-}$/low-TF groups (Figure 5B) (mean %±SEM: 8.5±0.8 versus 9.6±0.9). Similar results were observed in experiment 2 (mean %±SEM: LDLR$^{-/-}$/TF$^{+/-}$ 6.1±0.3, n=10, versus LDLR$^{-/-}$/low-TF 6.9±0.4; n=12).

Lesion area in the aortic root was also determined in the 2 groups. Serial transverse sections of the aortic root were stained with oil red O to allow quantification of the extent of the atherosclerotic lesions (Figure 5C). Lipid-laden lesions were observed within the vessel wall as well as on the heart valves. However, only vessel wall lesions were quantified because the heart valves are often damaged during the dissection/fixing process. In experiment 3, the area of the lesions was not affected by a reduction of TF in hematopoietic cells in LDLR$^{-/-}$ mice (Figure 5D) (mean area [$\mu M^2 \times 10^5$]±SEM: LDLR$^{-/-}$/TF$^{+/-}$ 5.7±0.5 versus LDLR$^{-/-}$/low-TF 6.4±0.5). Similar results were observed in experiment 2 (mean area [$\mu M^2 \times 10^5$]±SEM: LDLR$^{-/-}$/TF$^{+/-}$ 5.5±0.9, n=10, versus LDLR$^{-/-}$/low-TF 8.2±1.8, n=12).

Because there was no difference in the gross morphology and size of the lesions from the 2 groups, we analyzed the cellular composition in the aortic root lesions. Serial sections were stained with MOMA-2 and smooth muscle α-actin to detect macrophages and smooth muscle cells, respectively (Figure 6A). A nonspecific IgG served as a negative control (Figure 6A). The lesions from both groups showed a high degree of MOMA-2–positive macrophages in the center of the lesion, whereas α-actin–positive smooth muscle cells were predominantly located in the region of a fibrous cap or

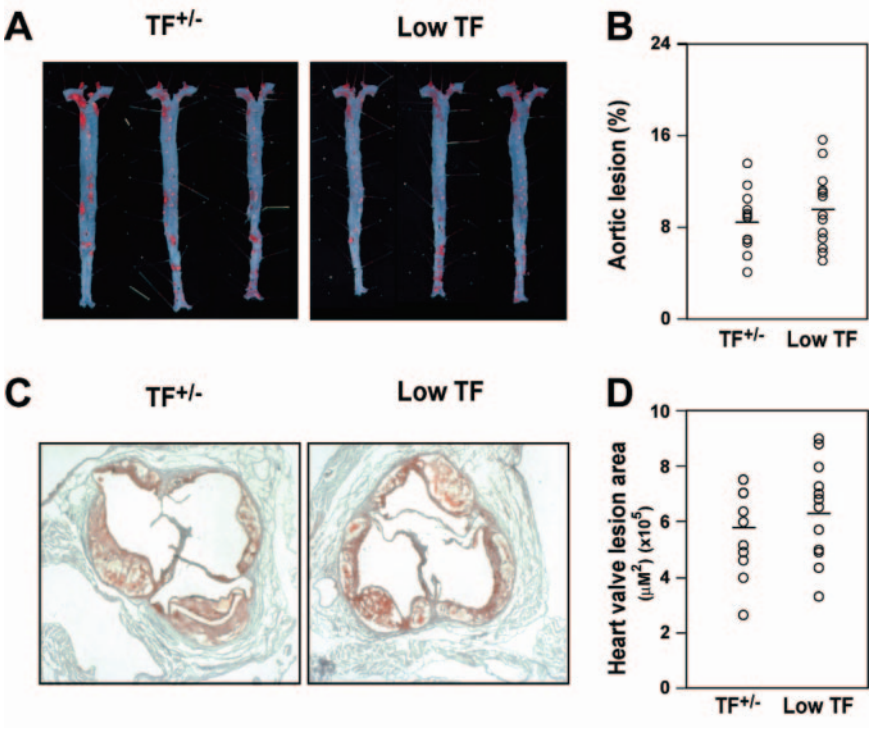

Figure 5. Atherosclerosis in LDLR$^{-/-}$ transplanted mice fed an atherogenic diet for 16 weeks. A, Whole-mount aortas stained with Sudan IV from 3 representative LDLR$^{-/-}$/TF$^{+/-}$ and LDLR$^{-/-}$/low-TF mice. B, Percentage aortic lesion area in LDLR$^{-/-}$/TF$^{+/-}$ (TF$^{+/-}$; n=12) or LDLR$^{-/-}$/low-TF (low-TF: n=13). Horizontal bars represent the mean percentage aortic lesion. Female mice were used for this experiment. C, Representative sections of heart valve lesion from LDLR$^{-/-}$/TF$^{+/-}$ (TF$^{+/-}$) and LDLR$^{-/-}$/low-TF (low-TF) stained to demonstrate lipid accumulation (oil red O). Images are representative of the 2 groups. D, Heart valve lesion area in LDLR$^{-/-}$/TF$^{+/-}$ (TF$^{+/-}$; n=11) or LDLR$^{-/-}$/low-TF (low-TF; n=12). Horizontal bars represent the mean area. Female mice were used for this experiment.

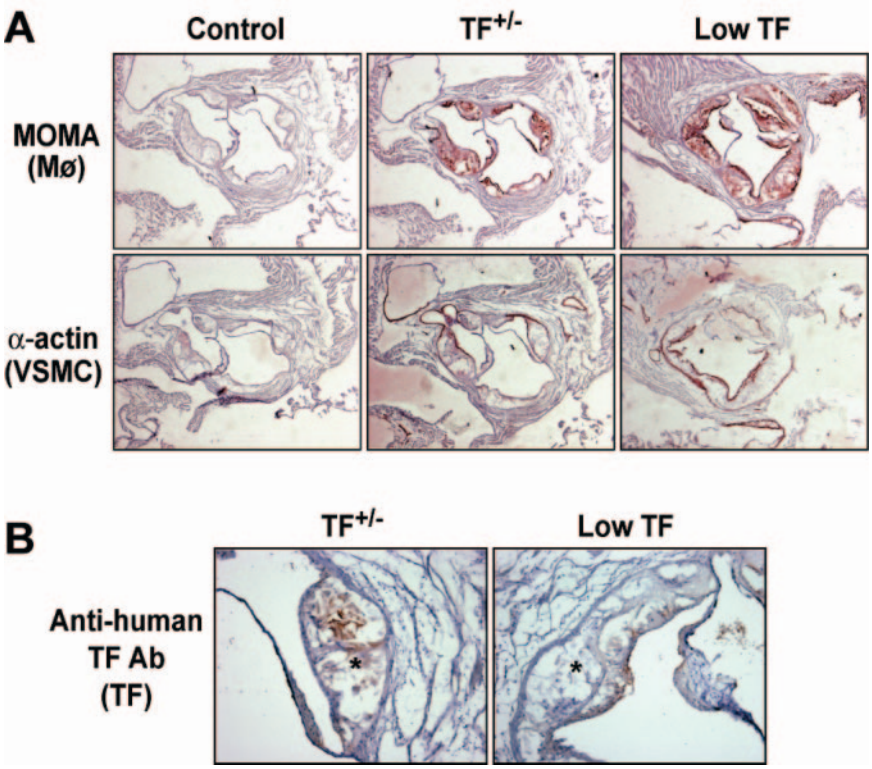

Figure 6. A, Cellular composition of heart valve lesions from LDLR$^{-/-}$/TF$^{+/-}$ and LDLR$^{-/-}$/low-TF mice. Control panels: primary antibody was substituted with a nonspecific IgG. Central and right panels demonstrate lesions from LDLR$^{-/-}$/TF$^{+/-}$ (TF$^{+/-}$) and LDLR$^{-/-}$/low-TF (low-TF) stained for macrophages (MOMA-2) and vascular smooth muscle cells (α-actin). Magnification $\times$40. B, Lesions from LDLR$^{-/-}$/TF$^{\pm}$ (TF$^{+/-}$) and LDLR$^{-/-}$/low-TF (low-TF) stained for TF (anti-human TF antibody). Magnification $\times$100. Images are representative of the 2 groups. *Center of the lesion. Female mice were used for this experiment.

beneath the lesion in the media region of the vessel wall. No differences in the cellular composition were observed between the LDLR$^{-/-}$/TF$^{+/-}$ and LDLR$^{-/-}$/low-TF mice.

Aortic root lesions were also stained with an anti-human TF polyclonal antibody that cross-reacts with mouse TF. In the LDLR$^{-/-}$/TF$^{+/-}$ group, TF was predominantly expressed by macrophages in the center of the lesions, as well as in VSMCs in the fibrous cap (Figure 6B). In contrast, TF staining in the LDLR$^{-/-}$/low-TF group was predominantly in VSMCs with low levels in the macrophages, suggesting that macrophages within the lesion were predominantly derived from the transplanted low-TF bone marrow (Figure 6B).

Discussion

This study assessed whether TF expression contributed to the progression of atherosclerosis in mice. First, we reduced TF expression by 50% in all cells but observed no effect on atherosclerosis in apoE$^{-/-}$ mice after 34 weeks on a regular chow diet. Second, we used bone marrow transplantation to selectively reduce TF expression in hematopoietic cells. LDLR$^{-/-}$ mice receiving low-TF bone marrow had similar levels of atherosclerosis to LDLR$^{-/-}$ mice that received control bone marrow after 4 and 16 weeks on an atherogenic diet. These data suggest that the level of TF expression, particularly in macrophages, does not influence the development of atherosclerosis in mice. Our results are consistent with a previous study showing that genetically depleting fibrinogen did not affect atherosclerosis in apoE$^{-/-}$ mice.[32] In addition, treatment of mice with anti-coagulant warfarin did not reduce atherosclerosis.[33] However, a recent study demonstrated a reduction of lesion size in FVIII-deficient apoE$^{-/-}$ mice after 8 weeks on an atherogenic diet and, to a lesser extent, at 16 weeks.[34] These studies suggest that the role of coagulation in the development of atherosclerotic lesions is complex and may play a more prominent role in early lesion development in mice.

TF is primarily expressed by macrophages in atherosclerotic lesions.[7] Furthermore macrophages are the first TF-expressing cell type to infiltrate, proliferate, and accumulate in early as well as advanced lesions.[1] Therefore, in this study we investigated whether hematopoietic cell TF plays a role in atherosclerotic lesion progression in LDLR$^{-/-}$ mice by transplanting LDLR$^{-/-}$ mice with bone marrow from either control or low-TF mice. In one experiment, we observed a significant difference in cholesterol levels between the 2 groups. However, no differences in cholesterol levels were observed in a second experiment. This indicates that there are no consistent differences in cholesterol levels between the 2 groups. We did not observe any differences in the lesion surface area in the aorta or most of the major arterial branches in the 2 groups at 4 or 16 weeks on an atherogenic diet except for a significant increase in lesion area in the carotid artery in the low-TF group at 4 weeks. The reason for this difference is not clear. One limitation of the study is that only female mice were used in the experiments in which the mice were fed a high-fat diet for 16 weeks. Interestingly, the degree of atherosclerosis in the aorta and aortic root of the low-TF group was consistently higher, although was not statistically significant than the control. These results suggest that TF may be protective in atherosclerosis by stabilizing the plaque. Indeed, in support of this notion, dramatic reduction in TF expression in apoE$^{-/-}$ mice leads to premature death (see below).

The cellular composition of a lesion may determine its stability and propensity to plaque disruption in humans.[35] We hypothesized that reducing macrophage derived TF in LDLR$^{-/-}$ mice may alter the cellular composition of the aortic root lesions. Infiltration and proliferation of inflammatory cells, such as monocytes, as well as VSMCs, may be influenced by TF expression via local generation of coagulation proteases and the activation of PARs. For instance, TF/FVIIa enhances the migration of macrophages and VSMCs in vitro and fibrin matrices provide a support for the migration of inflammatory cells and VSMC.[15,17,36–38] However, we observed a similar distribution and content of MOMA-2–positive macrophages and α-actin–positive VSMCs within lesions of LDLR$^{-/-}$ mice with either low-TF or control bone marrow. TF expression in macrophages in the LDLR$^{-/-}$/low-TF group was dramatically reduced compared with the control group. This is consistent with our study showing that bone marrow derived macrophages from low-TF mice express very low levels of TF.[28] In contrast, TF expression by VSMCs in the fibrous cap and vessel wall was similar in both groups of LDLR$^{-/-}$ mice. Our data do not support a role for macrophage TF on the development of atherosclerosis or the composition of the lesion. However, our studies do not exclude a role of VSMC-derived TF in this process. Recently, we generated mice that have a floxed TF gene. Breeding of these mice with mice that express Cre recombinase under the control of a VSMC-specific promoter will allow us to investigate the effect of deleting TF, specifically in VSMCs, on the development of atherosclerotic plaques.

A recent study showed that apoE$^{-/-}$ mice with a 50% reduction of TFPI had increased levels of atherosclerotic lesions in the carotid and iliac arteries compared with controls.[24] This increase in lesion size could be because of an increase in TF activity or TF-independent effects of TFPI. Our data showing that a 50% reduction in TF did not affect atherosclerosis in apoE$^{-/-}$ mice suggest that these results may not be caused by an increase in TF activity. However, it is difficult to compare the studies because one increases TF activity and the other decreases TF activity. It is also possible that TFPI might affect atherosclerosis in a TF-independent manner. For instance, the presence of TFPI may inhibit the activation of the endothelium and reduce atherosclerosis.

Analysis of atherosclerotic lesions in apoE$^{-/-}$/low-TF mice fed an atherogenic diet was not possible because of the premature death of these mice. All 7 apoE$^{-/-}$/low-TF mice died by 12 weeks. At present, we do not know the cause of sudden death. Low-TF mice are prone to fatal lung hemorrhages, but this occurs at a much lower frequency than observed for the apoE$^{-/-}$/low-TF mice.[39] A recent study reported a high rate of sudden death of apoE$^{-/-}$ mice fed an atherogenic diet over a 24-week period.[40] The frequency of plaque rupture in the brachiocephalic artery was similar in the survivors and the nonsurvivors. However, some of the mice had myocardial infarctions, suggesting that plaque stability in the coronary circulation may cause sudden death. We speculate that low levels of TF may further destabilize atherosclerotic plaques in apoE$^{-/-}$ mice fed a high-fat diet, thus increasing the frequency of myocardial infarctions and sudden death. Further studies are needed to analyze the cause of death of the apoE$^{-/-}$/low-TF mice.

A major distinction between the lesions in atherosclerosis-prone mice and humans is the formation of mural thrombi secondary to plaque fissure in the latter.[41,42] Although our study suggests that TF in mice is not involved in progression of atherosclerosis, indirect evidence from studies of atherosclerosis in humans suggest that TF may play an important role in human vascular disease.[8,11,12] Therefore, our studies do not exclude a role for TF in the progression of atherosclerotic lesions in humans.

Acknowledgments

We thank M. Szeto for technical assistance and C. Johnson for preparing the manuscript. This is manuscript number 17781-IMM from The Scripps Research Institute, La Jolla, California.

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JOURNAL OF THE AMERICAN HEART ASSOCIATION

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Arterioscler Thromb Vasc Biol. 2006;26:555-562; originally published online December 29, 2005;
doi: 10.1161/01.ATV.0000202028.62414.3c
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 1079-5642. Online ISSN: 1524-4636