Expression of Human Tissue Factor Pathway Inhibitor on Vascular Smooth Muscle Cells Inhibits Secretion of Macrophage Migration Inhibitory Factor and Attenuates Atherosclerosis in ApoE−/− Mice

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Background—Tissue factor (TF) and coagulation proteases are involved in promoting atherosclerosis, but the molecular and cellular bases for their involvement are unknown.

Methods and Results—We generated a new strain (ApX4) of apolipoprotein E–deficient mice expressing a membrane-tethered human tissue factor pathway inhibitor fusion protein on smooth muscle actin–positive cells, including vascular smooth muscle cells (SMCs). ApX4 mice developed little atherosclerosis on either a normal chow or high-fat diet. Lipid levels were similar to those in parental ApoE−/− mice, and there was no detectable difference in systemic (circulating) tissue factor pathway inhibitor levels or activity. The small lipid-rich lesions that developed had markedly reduced leukocyte infiltrates, and in contrast to ApoE−/− mice, SMCs did not express macrophage migratory inhibitory factor (MIF), including at sites distant from atheromatous lesions. Low levels of circulating MIF in ApX4 mice normalized to levels seen in ApoE−/− mice after injection of an inhibitory anti–human tissue factor pathway inhibitor antibody, which also led to MIF expression by tissue factor–positive medial SMCs. MIF production by SMCs in ApoE−/− mice in vitro and in vivo was shown to be dependent on tissue factor and protease-activated receptor signaling, which were inhibited in ApX4 mice.

Conclusions—Our data indicate that tissue factor plays a hitherto unreported role in the generation of MIF by SMCs in atherosclerosis-prone ApoE−/− mice, inhibition of which significantly prevents the development of atherosclerosis, through inhibition of leukocyte recruitment. These data significantly enhance our understanding of the pathophysiology of this important pathology and suggest new potential translational strategies to prevent atheroma formation. (Circulation. 2015;131:1350-1360. 10.1161/CIRCULATIONAHA.114.013423.)

Key Words: anticoagulants ◼ atherosclerosis ◼ inflammation ◼ muscle, smooth

Atherosclerosis leading to coronary artery, peripheral vascular, and cerebrovascular diseases is a chronic inflammatory disease that is the principal cause of death in the Western world. Early steps in atherogenesis include infiltration of the arterial wall by very-low-density lipoprotein and low-density lipoprotein, which activates endothelial cells and smooth muscle cells (SMCs)² to upregulate leukocyte adhesion molecules and to secrete chemokines such as CCL2 and macrophage migration inhibitory factor (MIF),¹ in turn promoting the infiltration of leukocytes and lymphocytes into the subendothelial space, an event that begins the process of atheroma formation.

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Coagulation proteases play a role in atherosclerosis: They catalyze fibrin formation and signaling through...
protease-activated receptors (PARs). Increased activity of tissue factor (TF), a 47-kDa cell membrane-bound glycoprotein that initiates the serine protease cascade, is seen in the neointima and underlying media of atherosclerotic plaques, and TF is expressed by endothelial cells, monocytes/macrophages, and SMCs. However, the cellular basis for how coagulation proteases act to promote atherogenesis has not been established.

We have previously defined the role that coagulation proteases play in the development of arteriosclerosis after endoluminal injury or allogeneic transplantation using a strain of transgenic mice expressing a membrane-tethered human TF pathway inhibitor (hTFPI) fusion protein on α-smooth muscle actin (SMA)–positive cells (α-TFPI-Tg mice). Here, we crossed α-TFPI-Tg mice with apolipoprotein E–deficient (ApoE−/−) mice to generate a new strain (ApX4) to explore how inhibition of TF on SMCs influences the development of atherosclerosis.

Methods

An expanded methods section is available in the Materials section in the online-only Data Supplement.

Generation, Breeding, and Diets of Mice

α-TFPI-Tg mice were crossed with ApoE−/− mice (The Jackson Laboratory, Bar Harbor, ME). First-generation offspring were crossed to obtain ApoE−/− mice expressing hTFPI. Founder mice were mated to generate the new strain (ApX4). Baseline phenotype was determined at 20 weeks after a normal chow diet (NCD). Some animals were fed a high-fat diet (HFD) for 6 or 12 weeks (35% fat, 1.25% cholesterol, and 0.5% cholic acid; Special Diets Services, Essex, UK), starting at 6 weeks of age. Mice were housed in a temperature-controlled specific pathogen-free environment at 22°C to 24°C, and all procedures were approved by the UK Home Office.

Aortic Transplantation

A sleeve anastomosis technique was used for aortic transplantation. Simply, a 5-mm segment of the infrarenal donor (MIF−/− mice, C57BL/6 [Harlan Olac, Bicester, UK] mice, α-TFPI-Tg mice, or mice deficient in PAR-1 or -2 [The Jackson Laboratory]) aorta, flushed with 300 μL saline containing 50 U heparin, was transplanted into ApoE−/− recipients. Blood flow was confirmed by direct inspection after clamp removal.

Northern Blot Analysis

Total RNA was isolated from frozen tissues with the RNeasy RNA extraction kit (Qiagen, Valencia, CA) after tissue homogenization in TRIZol (Invitrogen, Carlsbad, CA) by use of a TissueLyser (Qiagen). RNA quality and quantity were determined by absorbance at 260 and 280 nm with the use of a NanoDrop 1000 spectrophotometer (Thermo Scientific). Northern blot was performed with a DIG Northern Starter kit (Roche Diagnostics, Mannheim, Germany), following the manufacturer’s protocols. A mouse GAPDH cDNA fragment (Sigma, Dorset, UK) was used as control probe.

Serum and Plasma Assays

Human TFPI and mouse TFPI were detected with specific ELISA kits (Quantikine, R&D Systems, Abingdon, UK; and Antibodies-online Inc., Atlanta, GA, respectively). Plasma hTFPI activity was estimated with a chromogenic assay (Actichrome, American Diagnostica Inc, Stamford, CT). Total cholesterol, high-density lipoprotein, low-density lipoprotein, and triacylglycerol were determined with commercial kits (Cell Biolabs, Cambridge, UK; and Abcam, Cambridge, UK) according to the manufacturer’s protocols. Plasma tumor necrosis factor-α, interferon-γ, and MIF were measured by ELISA according to the manufacturer’s instructions (R&D Systems), as were thrombin antithrombin complexes (Dickinson, San Jose, CA) and D-dimers (Diagnostica Stago).

Table. Characteristics of ApoE−/− Mice With or Without hTFPI Fusion Protein Transgene (n=10 per Group)

<table>
<thead>
<tr>
<th></th>
<th>Fed HFD, 6–12 wk old</th>
<th>Fed HFD, 6–18 wk old</th>
<th>Fed NCD, Aged 0–20 wk old</th>
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<tbody>
<tr>
<td>Body weight, g</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before HFD</td>
<td>21.8±2.15</td>
<td>20.4±1.89</td>
<td>20.8±1.48</td>
</tr>
<tr>
<td>After HFD</td>
<td>28.3±2.00</td>
<td>28.2±2.09</td>
<td>28.0±1.94</td>
</tr>
<tr>
<td>After NCD</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Mouse TFPI, ng/mL</td>
<td>39.5±7.25</td>
<td>48.1±20.27</td>
<td>40.6±19.97</td>
</tr>
<tr>
<td>Human TFPI, pg/mL</td>
<td>47.8±29.14</td>
<td>57.2±33.41</td>
<td>45.3±40.67</td>
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<tr>
<td>Human TFPI activity, U/mL</td>
<td>0.29±0.30</td>
<td>0.55±0.88</td>
<td>0.26±0.03</td>
</tr>
<tr>
<td>Plasma cholesterol, mmol/L</td>
<td>35.5±6.71</td>
<td>35.4±6.32</td>
<td>35.0±5.68</td>
</tr>
<tr>
<td>Plasma triglycerides, mmol/L</td>
<td>1.57±0.47</td>
<td>1.55±0.35</td>
<td>1.53±0.28</td>
</tr>
<tr>
<td>HDL, mmol/L</td>
<td>1.58±0.24</td>
<td>1.44±0.25</td>
<td>1.53±0.28</td>
</tr>
<tr>
<td>LDL, mmol/L</td>
<td>31.3±8.13</td>
<td>31.8±6.76</td>
<td>31.5±5.55</td>
</tr>
<tr>
<td>Mouse TAT, ng/mL</td>
<td>28.8±4.99</td>
<td>28.5±4.36</td>
<td>29.4±4.05</td>
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<tr>
<td>D-dimer, ng/mL</td>
<td>16.2±3.31</td>
<td>15.6±3.09</td>
<td>15.5±3.84</td>
</tr>
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P Values are mean±SD. ApoE indicates apolipoprotein E; HDL, high-density lipoprotein; HFD, high-fat diet; hTFPI, human tissue factor pathway inhibitor; LDL, low-density lipoprotein; NCD, normal chow diet; TAT, thrombin antithrombin; and TFPI, tissue factor pathway inhibitor.
Morphometric Analysis

Hearts were embedded in paraffin and sectioned through the aortic root before staining with elasin/van Gieson with the Accustain Elastin Stain kit (Sigma) for evaluation on an Olympus U-ULH microscope (Olympus Optical Co Ltd, Tokyo, Japan). Atheroma area is expressed as a percentage of total aortic root area that was determined with Image-Pro Plus TM software version 4.0 (Media Cybernetics, Silver Spring, MD). At least 3 random sections were examined from each of 10 mice. Longitudinally opened descending aortas were prepared as previously described and evaluated for the extent of en face atherosclerosis after Oil Red O staining.

Immunohistochemistry

Sections were prepared and examined as previously described. Briefly, tissues were snap-frozen in dry ice, embedded in optimum cutting temperature compound (VWR International, Dorset, UK), sectioned at 5-μm thickness, and fixed in methanol at –20°C. Frozen sections were immersed in 1% BSA-PBS for 30 minutes and then incubated overnight at 4°C with the antibodies defined in the online-only Data Supplement. Stained sections were mounted in Vectashield with DAPI (Vector Laboratories Inc, Burlingame, CA). Sections were directly captured and examined by a Leica DMIRBE confocal microscope equipped with Leica digital camera AG and a confocal laser scanning system with excitation lines at 405, 488, 543, and 560 nm at magnifications x10/0.40CS and x20/0.70IMM (Leica, Planapno, Wetzlar, Germany). Images were processed with associated Leica-TCS-NT software. All immunohistochemistry was performed at 22°C. The staining area was expressed as the ratio of the total lesion area, calculated with Image-Pro Plus TM, version 4.0. All quantification was performed by members of the team blinded to the identity of the sections. Average measurements were derived from at least 6 random sections from each sample.

Culture of Primary SMCs

Isolation and culture of SMCs have been described previously. Briefly, mouse aortas with adventitia removed were washed with Dulbecco modified Eagle medium (Gibco BRL, Paisley, UK), minced, and digested in collagenase I (10 mg/mL), elastase (0.125 mg/mL), soybean trypsin inhibitor (0.25 mg/mL), bovine albumin (2.0 mg/mL), and HEPES (15 mmol/L; all from Sigma) at 37°C for 45 minutes. After passage through a cell strainer and centrifugation, the resulting SMCs were cultured in Dulbecco modified Eagle medium supplemented with 10% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin until subconfluent at 37°C in a humidified 5% CO2 incubator.

SMC-MIF Release Assay In Vitro and Factor IX, Factor X, and Thrombin Generation Assays

SMCs (1×10^6 cells per well in a 24-well plate) were serum starved for 24 hours before the addition of thrombin 50 nmol/L, factor Xa (FXa; 0–30 nmol/L; Enzyme Research Laboratories, Swansea, UK), increasing concentrations of PAR-1 and -2 agonists (Peptides International, Louisville, KY), and (red) anti–α-smooth muscle actin (α-SMA; BioTechne AG and a confocal laser scanning system with excitation lines at 405, 488, 543, and 560 nm at magnifications x10/0.40CS and x20/0.70IMM (Leica, Planapno, Wetzlar, Germany). Images were processed with associated Leica-TCS-NT software. All quantification was performed by members of the team blinded to the identity of the sections. Average measurements were derived from at least 6 random sections from each sample.

Figure 1. Baseline characterization of ApX4 mice. A, Polymerase chain reaction analysis of genomic DNA using human tissue factor pathway inhibitor (hTFPI)–specific primers. B, Northern blot analysis of mRNA purified from aortas using a Dig-labeled hTFPI cDNA probe. GAPDH cDNA was used as an internal control. Representative of other tissues as described in text. C and D, Immunohistology of consecutive sections through mouse aorta. Sections stained with DAPI nuclear stain (blue), anti-hTFPI (green), and anti–CD31 (C) or anti–α-smooth muscle actin (α-SMA; D). Yellow indicates colocalization. E, Immunocytofluorescence of isolated medial smooth muscle cells from apolipoprotein-deficient (ApoE−/−) and ApX4 mice. Slides stained with DAPI (blue), anti-hTFPI (green), and anti–α-SMA (red). Yellow indicates colocalization.
RNA Isolation and Quantitative Reverse Transcription–Polymerase Chain Reaction Analysis

Total RNA from SMCs was isolated with TRIzol reagent according to the manufacturer’s instructions (RNeasy Plus Mini Kit, Qiagen, Hilden, Germany). First-strand cDNA was synthesized from 1 μg total RNA by use of the SuperScript III first-strand synthesis system (Invitrogen, Paisley, UK) as recommended by the manufacturer. Quantitative reverse transcription–polymerase chain reaction was performed with SYBR Green Mix (Thermo Scientific) in a quantitative reverse transcription–polymerase chain reaction system (AB7500, Applied Biosystems, Foster City, CA). Details of MIF and β-actin primers, thermal cycling conditions, and quantification of MIF RNA are given in the online-only Data Supplement. Fold increase in MIF was determined with the \(2^{-\Delta\Delta C_t}\) (Livak) method.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism software. The Mann-Whitney test was used for comparison of 2 groups and the Kruskal-Wallis test for ≥ 3 groups. All data are presented as mean±SEM except in the Table, in which data are presented as mean±SD. A value of \(P<0.05\) was considered significant. The pairwise \(P\) values are 2 sided, and because of the nature of the study, there are no adjustments for multiple comparisons.

Results

Baseline Characteristics of ApX4 mice

Transgene frequencies, assessed by polymerase chain reaction (Figure 1A), were as expected at each stage of breeding. ApX4 mice are viable and grow with normal physical characteristics at the same rates as both parental strains (Table). Expression of hTFPI mRNA was found in aortas by Northern analysis (Figure 1B). Fusion protein expression was seen in the tunica media of large (Figure 1C and 1D), medium, and small arteries; arterioles; and larger venules of all major tissues (Figure I in the online-only Data Supplement) and in vitro–cultured aortic SMCs from ApX4 but not ApoE−/− mice (Figure 1E). In mice fed an NCD, there were no significant differences in circulating mouse or human TFPI or in human TFPI activity between ApoE−/− and ApX4 mice (Table). After an HFD, levels of all 3 tended to increase in ApX4 or (ApX4×ApoE−/−) hTFPI+ littermates, although the differences remained statistically insignificant (Table). There were no significant differences in the levels of circulating lipids in ApX4 compared with ApoE−/− mice or in hTFPI-positive or -negative littermates fed either type of diet (Table). Expression of the hTFPI fusion protein had no significant impact on circulating thrombin antithrombin complexes or D-dimers (Table), indicating no major perturbation of baseline systemic clotting or fibrinolysis.

Mild Atherosclerosis in ApX4 Mice Associated With Significant Reduction in Inflammation

ApX4 mice and (ApX4×ApoE−/−) hTFPI+ littermates developed significantly attenuated atherosclerosis after being fed an HFD for 6 or 12 weeks compared with ApoE−/− or negative littermate controls (Figure 2). Obvious atheromatous lesions were also visible in ApoE−/− mice fed an NCD for 20 weeks, but they were almost undetectable in ApX4 mice (Figure II in the online-only Data Supplement). Confocal examination of atheromatous lesions revealed that inflammatory cells expressing CD68, CD11b, or CD3 accounted for a significantly reduced proportion of lesional area in TFPI+ compared with TFPI− mice (Figure 3 and Figure II in the online-only Data Supplement). Analysis of circulating plasma tumor necrosis factor-α and interferon-γ showed that atheroma development in ApX4 mice (fed either type of diet) occurred in the context of significantly reduced systemic inflammation (Figure 4A and 4B).

Specific Inhibition of MIF Expression by SMA+ Cells in ApX4 Mice

Plasma levels of MIF in 20-week-old ApX4 mice fed an NCD were ≈25% those seen in age-matched ApoE−/− mice.
an NCD, MIF was expressed predominantly by α-SMA+ cells (Figure 4E). In contrast, there was little MIF staining within the small plaque areas seen in ApoX4 mice, even though α-SMA+ cells occupied the same proportional area (Figure 4D and 4F). After an HFD, all SMA+ cells in ApoE−/− mice coexpressed MIF, and these accounted for ≈25% of all MIF+ cells within the plaques (Figure 4E), suggesting that expression by infiltrating leukocytes was responsible for the remaining 75%. The area occupied by cells expressing MIF in ApoX4 mice was significantly reduced, consistent with reduced infiltration of leukocytes in these lesions (Figure 4F). Most important, although the proportional area occupied by SMA+ cells in ApoX4 mice fed an HFD for 12 weeks was similar to that seen in ApoE−/− (15±0.5% versus 27±1.8%; P<0.05), none of these cells coexpressed MIF (Figure 4F). These data indicate that hTFPI expression was associated with specific suppression of MIF expression by α-SMA+ cells within atheromatous areas in ApoX4 mice.

Additionally, in ApoX4 mice fed an NCD, there was no MIF expression by SMCs within nonatheromatous areas of the vessel wall compared with ApoE−/− mice, in which all the media stained strongly for MIF (Figure 4D). These data indicate that MIF expression by SMCs was independent of plaque development. Similar findings in medial SMCs were seen in all the mice fed an HFD (Figure III in the online-only Data Supplement). However, in these mice, it was obvious that CD31+ intimal cells distant from atheroma were MIF+ in both hTFPI+ and hTFPI− mice, indicating that endothelial cell expression of MIF was induced under these conditions and was not prevented by expression of the hTFPI on medial SMC.

**Vessel Wall MIF Is Required for the Development of Atherosclerosis**

To define the importance of vessel wall–derived MIF for atheroma development, we developed a congenic aortic transplantation model in ApoE−/− mice. Development of atheroma in the surrounding recipient aorta after an HFD was unaltered by the transplanted section (Figure 5). Aortic transplants from mice completely deficient in MIF were resistant to atheroma development compared with MIF+ littermates, which developed exaggerated atheroma (Figure 5A, 5C, and 5H). Transplants from α-TFPI-Tg mice were also resistant, indicating that expression of hTFPI fusion protein on SMCs had the same functional impact as complete MIF deficiency. Atheroma resistance was associated with absent MIF production by SMCs in the transplanted segments (Figure IV A in the online-only Data Supplement) but not with alterations in plasma level of MIF (Figure IVB in the online-only Data Supplement), indicating the importance of vessel wall rather than systemic MIF for the development of atheroma in this model.

**MIF Secretion by SMCs is TF and PAR Dependent.** To explore how hTFPI influenced MIF expression, SMCs from both ApoE−/− and ApoX4 mice were first shown to be TF expressing (Figure VA–VD in the online-only Data Supplement). In vitro, the generation of factor IXa, FXa, and thrombin was completely dependent on TF and FVIIa (Figure VE–VI in the online-only Data Supplement), and SMCs were unable to convert FXI.
and FXII into their activated forms (Figure VJ in the online-only Data Supplement), indicating no involvement of the intrinsic pathway in the generation of FXa or thrombin in vitro by SMCs. SMCs from ApoE−/− mice were incubated with FXa (Figure 6A), thrombin (Figure 6D), or prothrombin (with FXa, anionic phospholipid, and FVa to promote conversion to thrombin; Figure 6F). MIF was induced under all these conditions. The effect of FXa was partially inhibited by an antagonist to either PAR-1 or PAR-2 (Figure 6B), whereas thrombin was inhibited by a PAR-1 but not a PAR-2 antagonist (Figure 6E). When FXa (Figure 6C) or thrombin (Figure 6F and 6G) was generated in situ, MIF production was completely FVIIa dependent and completely inhibited by an anti-TF antibody. In contrast, cells from ApX4 mice made MIF after exposure to exogenously added thrombin (Figure 6D) but not after addition of prothrombin, even when FXa and FVa or FVIIa, or FX and FVa were provided (Figure 6F and 6G), and ApX4 SMCs were resistant to the effects of exogenously added FXa (Figure 6A) and FXa made in
situ (Figure 6C). PAR-1– and PAR-2–dependent MIF secretion was transcriptionally induced and involved signaling through p42/p44 ERK and nuclear factor-κB pathways (Figure VI in the online-only Data Supplement). PAR-1–induced MIF was also partly dependent on p38 kinase (Figure VI in the online-only Data Supplement). All these data indicate that MIF production by SMCs in vitro is entirely TF and PAR dependent and, absent MIF production from ApX4, is consistent with the known anti-TF inhibitory effects of human TFPI.

Finally, to demonstrate that the SMC-expressed hTFPI was inhibiting MIF expression in vivo, 6-week-old ApX4 mice were injected with an inhibitory anti-hTFPI antibody daily for 3 days. SMCs from these mice started to express MIF, like those from ApoE−/− mice (Figure 7A). This also significantly increased circulating MIF concentrations compared with animals injected with an isotype control to levels similar to those seen in age- and sex-matched ApoE−/− mice (Figure 6H), suggesting that, because hTFPI was expressed only on SMA+ cells, plasma MIF levels were determined by MIF released from SMCs. Additionally, these effects were abolished by coadministration of active site–inhibited FVIIa (but not FXIIa) and mimicked by administration of a PAR-1 but not PAR-2 agonist (Figures 6H and 7A), confirming the in vitro findings.

In ApoE−/− mice, injection of a PAR-1 antagonist, active site–inhibited FVIIa, or FXa and an inhibitory anti-TF antibody significantly reduced circulating MIF levels (Figure 6H), whereas a PAR-2 antagonist and active site–inhibited FXIIa had only a modest impact, indicating that systemic inhibition of TF had a similar impact on circulating MIF as expression of hTFPI on SMCs. Analysis of the arterial walls in these animals revealed changes in the expression of MIF within SMCs that mirrored those seen in plasma (Figure 7B).

All these data indicate that MIF expression by SMC in ApoE−/− mice is completely dependent on the activity of coexpressed TF and associated PAR-1 signaling by FXa and thrombin, inhibition of which by the hTFPI fusion protein in ApX4 mice is associated with a significant reduction in systemic MIF levels, reduced leukocyte recruitment, and marked inhibition of atherosclerosis.

**Discussion**

Previous studies have described how TFPI influences atherogenesis. For instance, mice deficient in TFPI show enhanced atherosclerosis. However, there are 2 potential mechanisms operating in these mice, the first involving loss of the direct effects of TFPI on circulating lipids and the second involving loss of regulation of TF activity and resulting enhanced thrombin generation. To illustrate the first mechanism, transgenic mice overexpressing soluble TFPI were shown to be resistant to atherosclerosis as a result of a direct effect of soluble TFPI on very-low-density lipoprotein metabolism. The importance of the second mechanism is illustrated when ApoE−/− mice are made deficient in HCII, a natural thrombin inhibitor, in that they develop severe atheroma. Additionally, the direct thrombin inhibitor melagatran reduces lesion progression and maintains plaque stability in ApoE−/− mice.

In this study, using a new strain of transgenic mice expressing a membrane-tethered hTFPI fusion protein on SMA+ cells, we provide an explanation of the molecular and cellular bases...
Figure 6. Migratory inhibitory factor (MIF) expression by smooth muscle cells (SMCs) is tissue factor (TF)– and factor (F) VIIa–dependent and inhibited by human TF pathway inhibitor (hTFPI). A through G, In vitro ELISA analysis of MIF secretion into the supernatants by $1\times10^6$ stimulated SMCs. A, ApoE−/− (circles) or ApX4 (squares) SMCs exposed to increasing concentrations of FXa. B, ApoE−/− SMCs exposed to increasing concentrations of FXa without inhibitors (circles) or with the addition of 10 nmol/L protease-activated receptor (PAR)-1 antagonist (squares) or PAR-2 antagonist (triangles). C, MIF made after incubation with 80 nmol/L FX for the indicated amount of time; ApoE−/− SMCs were incubated with FX alone (circles), FX and 10 nmol/L FVIIa (squares), or FX, FVIIa, and 80 μg/mL inhibitory anti-TF antibody (triangles). Diamonds shows MIF made by ApX4 SMCs incubated with 80 nmol/L FX and 10 nmol/L FVIIa. D, ApoE−/− (circles) continued
of this second mechanism and highlight the importance of regulation of TF activity in the evolution of atherosclerosis in ApoE−/− mice. The hTFPI fusion protein used in ApX4 represents a hybrid of the 2 main isoforms of TFPI (α and β; physiological TFPIα and TFPIβ, mouse and human) generated by alternative splicing and differ in their C-terminal structure and cellular localization.23 TFPIα is a secreted protein with 3 tandem Kunitz-type domains (K1–K3) and a basic C-terminus, whereas TFPIβ has K1 and K2 domains and a glycosylphosphatidylinositol anchor membrane attachment. TFPIβ inhibits TF-mediated thrombin generation better than either TFPIα or a soluble truncated form of TFPI (TFPI-160) similar to TFPIβ, suggesting that cell surface association plays an important role in efficient inhibition of TF.24 Conversely, K3 and the C-terminal region of TFPIα significantly enhance FXa inhibitory activity without having direct protease inhibitor activity themselves.24 The fusion protein combines both cell-tethering and K3 effects and therefore acts as a highly efficient inhibitor of TF.

Our results indicate that vessel wall cells in ApX4 mice did not make MIF, which prevented inflammatory cell recruitment into the vessel wall. Resistance to atheroma formation was seen in mice fed either an NCD or an HFD but was more significant in mice fed chow in which MIF expression was almost exclusively restricted to α-SMA+ cells. After a high-cholesterol diet, MIF was also obviously expressed by CD31+ endothelial cells.
and by infiltrating leukocytes within atheromatous plaques, which might explain why plasma MIF levels were higher in ApoX4 mice after an HFD. Importantly, MIF was expressed by SMCs in areas of vessel wall remote from atheromatous lesions and was seen in mice before significant atheroma had developed, consistent with it being involved early in disease pathogenesis. These results therefore indicate a crucial role for TF in the generation of MIF by SMCs in this model, a conclusion that is supported by all our in vitro and in vivo data.

MIF was first described as a T-cell cytokine important for inhibiting macrophage migration in delayed-type hypersensitivity responses but is now known to be secreted by many different cell types and to have chemokine properties, attracting monocytes and T cells and binding, among other ligands, CXC chemokine receptors. MIF expression within atheromatous plaques has been closely associated with progression and instability in human disease. Moreover, deficiency of MIF is known to significantly impair atheroma development in low-density lipoprotein receptor−deficient mice, and inhibition of MIF activity (using an anti-MIF antibody) prevents atherosclerosis in ApoE−/− mice.

In vitro experiments have suggested that MIF secretion and the subsequent development of atherosclerosis in ApoE−/− mice. However, the definition of this novel mechanism, which involves no detectable perturbation of lipid metabolism, enhances our understanding of atheroma development, particularly the role that TF and coagulation proteases play, and may lead to new translational strategies for intervention in human atherosclerosis.

Conclusions
Our data indicate that the proatherogenic stimuli that promote MIF expression by SMCs in vivo act via a TF-dependent mechanism and that inhibition of this can completely inhibit MIF secretion and the subsequent development of atherosclerosis. Like all mouse studies, our findings provide clues to the pathophysiology of human disease but cannot be directly extrapolated into humans. However, the definition of this novel mechanism, which involves no detectable perturbation of lipid metabolism, enhances our understanding of atheroma development, particularly the role that TF and coagulation proteases play, and may lead to new translational strategies for intervention in human atherosclerosis.

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Disclosures
None.

References


38. CLINICAL PERSPECTIVE

Atherosclerosis is a chronic inflammatory disease and the principal cause of death in the West. Mouse models have taught us a great deal about its pathophysiology. This study reveals a novel mechanism in the pathophysiology and suggests new translational preventative strategies. Early steps in atheroma formation include infiltration of arterial walls by lipids, activation of endothelial and smooth muscle cells resulting in chemokine secretion, and recruitment of leukocytes, which initiates atheroma formation. An important chemokine in this process, identified in mice and humans, is macrophage migration inhibitory factor. Similarly, it is already known that tissue factor and thrombin play a role. In this study, we show how both are linked mechanistically and how important they are for atheroma formation. In an atheroma-prone mouse strain, migration inhibitory factor production by medial smooth muscle cells is tissue factor dependent via production of activated serine proteinases and signaling through protease-activated receptors. Moreover migration inhibitory factor production by smooth muscle cells is detectable in young mice before atheroma formation, it is necessary for atherosclerosis to occur, and is relevant in mice fed either a normal or high-fat diet. A new mouse strain, expressing a cell-tethered fusion protein to inhibit the activity of tissue factor on smooth muscle cells, is almost completely resistant to atheroma formation, despite having elevated plasma lipids comparable to parental strains. The small lesions that develop in these mice are almost devoid of infiltrating leukocytes. Novel compounds to locally inhibit serine protease activity, protease-activated receptor signaling, or chemokine receptors may offer potential avenues to prevent or treat atherosclerosis in humans.
Expression of Human Tissue Factor Pathway Inhibitor on Vascular Smooth Muscle Cells Inhibits Secretion of Macrophage Migration Inhibitory Factor and Attenuates Atherosclerosis in ApoE−/− Mice
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SUPPLEMENTAL MATERIAL

Methods

Generation, breeding and diets of mice

α-TFPI-Tg and ApoE<sup>−/−</sup> strains are on a C57BL/6J background. For some experiments, hTFPI+ or hTFPI-negative littermates were generated by crossing ApX4 with ApoE<sup>−/−</sup> mice. Genotypes were determined by polymerase chain reaction (PCR), using the suppliers’ instructions (Bioline, London UK) and previously defined primers. Some animals were injected with 200ng/g of anti-human TFPI antibody (American Diagnostica Inc. Stamford, CT, USA) or isotype control IgG1 10µg/g of PAR-1 or PAR-2 agonists or antagonists (Peptides International. Kentucky, USA), 0.5µg/g of FVIIai, 0.5µg/g of FXai, 0.5µg/g of FXIIai (all from Enzyme Research Laboratories. Swansea, UK), 100µg of anti-TF (American Diagnostica Inc. CT, USA), and PBS as the control by tail vein once a day (o.d.) for three consecutive days prior to phenotypic analysis.

Immunohistochemistry

The following antibodies were used: Rabbit anti-mouse TF (American Diagnostic Inc.) anti-human TFPI (Enzyme Research Laboratories, Swansea, United Kingdom), MIF (Abcam, Cambridge, UK); rat anti-mouse CD68, CD11b (Serotec, Oxford, United Kingdom), CD31 or CD3 (BD Bioscience Pharmingen, Oxford, United Kingdom); mouse anti-SMA conjugated with Cy3 (Sigma). The following anti-IgG FITC or TRITC-conjugated antibodies were used: sheep anti-mouse, goat anti-rat and goat anti-rabbit (all Sigma).

qRT-PCR analysis.
Amplifications were performed using the thermal cycling conditions including enzyme activation at 95°C for 15 min, 40 cycles of denaturation at 95°C for 15 s, and annealing/extension at 55°C for 1 min. All samples were run in triplicate in three independent experiments.

MIF primers: 5'-TGCCCAGAACCAGCAACTACAGTAA-3' (Forward) and 5'-TCGCTACCGGTGGATAAACACAGA-3' (Reverse) were used for qRT-PCR, and β-actin primers 5'-CTGTGGGATCCACGAAACTA-3' (Forward) and 5'-AGTACTTGCGGTCTAGAGGA-3' (Reverse) were used as an endogenous control to normalize gene expression for MIF quantification. The quantity of MIF mRNA was calculated with normalizing the cycle threshold (C_T) value to the C_T of the β-actin in the sample according to the formulas in the manufacturer’s Bulletin (Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR . Applied Biosystems) and the fold increase in MIF was determined using the 2^{-ΔΔCT} (Livak) method.
Supplemental Figure 1

A: Heart
B: Lung
C: Kidney
D: Spleen

ApoE⁻/⁻

hTFPI  Combined

ApX4

hTFPI  Combined
Supplemental Figure 2

A. Lesion area (% of total aorta)

B. ApoE−/− vs. ApX4

C. Lesion area (% of aortic sinus)

D. Immunohistochemical staining of CD68, CD11b, CD3

E. CD68, CD11b, CD3 staining in ApoE−/− and ApX4

F. Area of staining within atheroma (% of lesional area)

P<0.001
Supplemental Figure 3

(ApoE<sup>−/−</sup> x ApX4) littermates

(ApoE<sup>−/−</sup> x ApX4) littermates

100 µm
Supplemental Figure 4

A

<table>
<thead>
<tr>
<th>SMA</th>
<th>MIF</th>
<th>combined</th>
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<tbody>
<tr>
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<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
</tbody>
</table>

B

![Graph](graph.png)

Plasma [MIF] (ng/ml)

- MIF+
- MIF−
- C57BL/6
- α-TFPI-Tg
- PAR-1−
- PAR-2−

MIF+ littermate
Supplemental Figure 5

A  SMA  TF  combined

B  SMA  TF  combined

C

D

E  [FXa] (nM)

F  [FX] (nM)

Anti-TF antibody (µg/ml)

G  [Thrombin] (nM)

H  [Prothrombin] (nM)

Anti-TF antibody (µg/ml)

I  [FXa] (nM)

J  [FXa, FXIa or FXIIa] (nM)

[FX], [FXI] or [FXII] (nM)

FX, FIX or FXII (nM)
SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1 - hTFPI expression in medium and small vessels of various tissues.

Immunohistology analysis of ApoE<sup>−/−</sup> and ApX4 mouse hearts (A), lungs (B), kidneys (C) and spleens (D) with anti-hTFPI mAb (green). All sections stained with DAPI (blue). Sections stained with (red) either anti-CD31 or anti-α-SMA as indicated. Yellow = co-localisation.

Supplemental Figure 2 - Human TFPI fusion protein on α-SMA<sup>+</sup> cells inhibits atheroma development on a normal diet.

Atheromatous lesions were assessed in the whole length of the aorta (A&B) and at the aortic root (C&D) in 20-week-old mice fed a normal Chow diet. Quantitative assessments (A&C) show the area occupied by atheroma as a proportion of the total area examined (n=10 males each group). Analyses performed by members of the team blinded to the mouse strain. B: representative Oil Red O-stained en face preparations of aorta. D: representative light photomicrographs of elastic/van Gieson stained sections from the aortic root.

E&F: Immunohistological analysis of aortic sinus from mice fed a Chow diet for 6 weeks. E: Representative two colour images of sections through area of atheroma stained with DAPI (blue) and (green) anti-CD68, CD11b or CD3 as indicated. L=lumen F: Quantitative analysis of infiltration into aortic root atheromatous lesions. N=10 male mice per group.
Supplemental Figure 3: MIF expression in mice fed a high fat diet for 6 weeks.

Representative three colour immunohistological images of consecutive sections stained with DAPI (blue), anti-MIF (green) and (red) anti-α-SMA (A) or anti-CD31 (B) . L=lumen. Yellow = co-localisation. Arrows in ‘combined’ sections from ApX4 mice illustrate MIF expression by α-SMA-neg, CD31+ intimal cells in non-atheromatous areas of the vessel wall.

Supplemental Figure 4: MIF expression after aortic transplantation.
A: Representative three colour immunohistological images of consecutive sections of the recovered transplanted aortas from different donors, stained with DAPI (blue), anti-MIF (green) and (red) anti-α-SMA. L=lumen. Yellow = co-localisation.

B: Analysis of plasma MIF in ApoE−/− aortic transplant recipients, transplanted with aortic segment from different donors as indicated. Samples from 6 mice per group were pooled and analysed in triplicate by ELISA. In all graphs, error bars = SEM. Comparing all values, p=NS

Supplemental Figure 5: Generation of activated coagulation factors on SMC is TF-dependent.
A-D: Representative three colour immunofluorescence images of SMC (A&B) or consecutive sections from the aortic sinus of 6 week old mice fed a Chow diet (C&D) from ApoE−/− (A, C)) and ApX4 (B, D) mice. Sections stained as indicated with SMA (red), TF (green) and DAPI (blue). In last column, images have been overlayed; yellow = co-localisation.
E&F: Generation of FXa. E: Increasing concentrations of FX added to ApoE\(^{-/-}\) (squares, circles) or ApX4 (triangles) SMC for 20 minutes either with 10nM FVIIa (squares, triangles) or without FVIIa (circles). F: ApoE\(^{-/-}\) SMC pre-incubated with increasing concentrations of anti-TF antibody either with 10nM FVIIa (squares) or without FVIIa (circles), before addition of 80nM FX and incubation for a further 20 minutes.

G&H: Generation of thrombin. G: Increasing concentrations of prothrombin added to ApoE\(^{-/-}\) (squares, circles) or ApX4 (triangles) SMC for 20 minutes with 80nM FX/10nM FVa and 10nM FVIIa (squares, triangles) or without FVIIa (circles). H: ApoE\(^{-/-}\) SMC pre-incubated with increasing concentrations of anti-TF antibody and 0.5\(\mu\)M prothrombin with 10nM FVIIa (squares) or without FVIIa (circles), before addition of 80nM FX and 10nM FVa and incubation for a further 20 minutes.

I: Generation of FIXa: Increasing concentrations of FIX added to ApoE\(^{-/-}\) SMC for 20 minutes with 10nM FVIIa (squares), without FVIIa (circles) or with 10nM FVIIa and 160\(\mu\)g/ml anti-TF antibody (triangles).

J: Generation of intrinsic pathway activators: Increasing concentrations of FXI (circles), FXII (squares) added to ApoE\(^{-/-}\) SMC for 20 minutes. To assess FXa generation (triangles), wells also included 10nM FIX, 10nM FXI and 10nM FXII.

E-J: All experiments performed with 1\(\times\)10\(^6\) SMC in triplicate wells, with background values subtracted. Data points include ± SEM error bars.

**Supplemental Figure 6: PAR-1 and -2 signalling in SMC.**
A-E: SMCs from wild type mice incubated with PAR 1 (A&B) or PAR-2 (C&D) agonist at 37°C. Cells were harvested at indicated times for up to 48 hours to determine fold increase in relative MIF mRNA expression by qRT-PCR (compared to β-actin control) or to determine MIF protein concentrations in supernatants. Values are the means ± SE from three independent experiments.

A-D: relative MIF mRNA expression. The effect of increasing concentrations of agonists is shown in A (PAR-1) and C (PAR-2). Cells incubated with 1 µM (diamonds), 5 µM (squares), 10 µM (triangles) or 100 µM (circles) of respective agonist.

The effect of intracellular signalling pathway inhibitors is shown in B (PAR-1) and D (PAR-2). Agonists used at 10 µM. Cells were incubated with either no inhibitors (triangles) or 50 µM mitogen-activated protein kinase kinase (MEK) inhibitor PD98059 (squares), 10 µM p38-MAPK inhibitor SB203580 (diamonds) or 20 µM NF-κB inhibitor SN50 (circles) for 1 hour prior to addition of agonists.

E: MIF secretion by SMC induced by 24 hour incubation with 10 µM PAR-1 or-2 agonists. Cells were incubated with either no inhibitors or 50 µM PD98059, 10 µM SB203580 or 20 µM SN50 as indicated for 1 hour prior to addition of agonists.

In B, D and E, the signalling inhibitors had no impact on baseline expression levels of MIF compared to controls (data not shown).
평활근세포의 Tissue Factor가 염증세포의 침윤을 유도하여 죽상경화를 촉진시킨다

한 기 혼 교수 서울아산병원 심장내과

초록

배경

Tissue factor(TF)와 coagulation protease들은 죽상경화의 진행에 관여하다. 그러나 이들이 영향을 미치는 세포 분자 수준의 기전은 알려진 바 없다.

방법 및 결과


결론

TF는 SMCs에서 MIF 생산을 유도한다. 이는 ApoE⁻⁻ 마우스에서 죽상경화를 유발하는데, 이를 치료하면 염증세포의 침윤과 억제되어 죽상경화의 발생이 저해된다. 이 연구는 병태생리적 이해도를 높여주며, 죽상경화 예방을 위한 새로운 치료 전략을 제시해 주고 있다.