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

Running title: Chen et al.; hTFPI on VSMC inhibits MIF and atherosclerosis

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Abstract

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

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

Conclusions—Our data indicate that TF plays a hitherto unreported role in the generation of MIF by SMC in atherosclerosis-prone ApoE<sup>-/-</sup> 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.

Key words: anticoagulant, atherosclerosis, inflammation, muscle, smooth
Introduction

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 (VLDL) and low-density lipoprotein (LDL) which activates endothelial cells (ECs) and smooth muscle cells (SMCs) \(^1,2\) to upregulate leukocyte adhesion molecules and secrete chemokines such as CCL2 and macrophage migration inhibitory factor (MIF) \(^3\), in turn promoting infiltration of leukocytes and lymphocytes into the subendothelial space, an event which begins the process of atheroma formation.

Coagulation proteases play a role in atherosclerosis; they catalyse fibrin formation as well as signalling though protease activated receptors (PAR). Increased activity of tissue factor (TF), an 47-Kd cell membrane-bound glycoprotein that initiates the serine protease cascade, is seen in the neointima and underlying media of atherosclerotic plaques \(^4,6\) and TF is expressed by EC \(^7\), monocytes/macrophages \(^8\) and SMC \(^9\). 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 \(^10-13\) using a strain of transgenic mice expressing a membrane-tethered human Tissue Factor Pathway Inhibitor (hTFPI) fusion protein on \(\alpha\)-smooth muscle actin (SMA)\(^+\) cells (\(\alpha\)-TFPI-Tg mice). Here, we crossed \(\alpha\)-TFPI-Tg mice with Apolipoprotein E–deficient (ApoE\(^-\)) mice to generate a new strain (ApX4) to explore how inhibition of TF on SMC influences the development of atherosclerosis.
Methods

An expanded methods section is available in Supplemental Materials.

Generation, breeding and diets of mice

α-TFPI-Tg \(^{10}\) were crossed with ApoE\(^{-/-}\) mice (The Jackson Laboratory, Bar Harbor, Maine 04609, USA). 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 twenty weeks after a normal Chow diet (NCD). Some animals were fed a high-fat diet (HFD) for six or twelve weeks (35% fat, 1.25% cholesterol, and 0.5% cholic acid; Special Diet Services, Essex UK) \(^{14}\), starting at six weeks age. Mice were housed in a temperature-controlled Specific Pathogen-Free environment at 22–24 °C and all procedures were approved by the UK Home Office.

Aortic transplantation

A sleeve anastomosis technique was used for aortic transplantation \(^{12}\). Simply, a 5mm of the segment of infrarenal donor (MIF\(^{-/-}\) 15, C57BL/6 (Harlan Olac, Bicester, UK), α-TFPI-Tg or PAR-1,-2 deficient mice (Jackson, Bar Harbour, ME)) aorta, flushed with 300 µl of saline containing 50U of heparin, was transplanted into ApoE\(^{-/-}\) recipients. Blood flow was confirmed by direct inspection after the clamps removed.

Northern blot analysis

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

**Serum and plasma assays**

Human and mouse TFPI were detected using specific ELISA kits (Quantikine, R&D Systems, UK and Antibodies, Atlanta GA USA respectively). Plasma hTFPI activity was estimated using a chromogenic assay (Actichrome, American Diagnostica Inc, Stamford, CT, USA). Total cholesterol (TC), high-density lipoprotein (HDL), LDL and Triacylglycerol were determined with commercial kits (Cell Biolabs, Cambridge UK; and Abcam, Cambridge, UK) according to the manufacturer’s protocols. Plasma TNF-α, IFN-γ and MIF were measured by ELISA according to the manufacturer’s instructions (R&D systems, Abingdon, UK), as were thrombin anti-thrombin (TAT) complexes (Dickinson, San Jose, CA) and D-dimers (Diagnostica Stago, FR, USA).

**Morphometric analysis**

Hearts were embedded in paraffin and sectioned through the aortic root before staining with elastin/van Gieson using 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 which was determined using Image-Pro Plus TM software version 4.0 (Media Cybernetics, Silver Spring, USA). At least three 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 and embedded in OCT compound (VWR International, Dorset, UK), sectioned at 5μm thickness and fixed in methanol at –20°C. Frozen sections were immersed in 1% bovine serum albumin–phosphate-buffered saline (BSA-PBS) for 30 minutes and then incubated overnight at 4°C with the antibodies defined in supplemental material. Stained sections were mounted in Vectashield with DAPI (Vector Laboratories Inc, CA USA). 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 10x/0.40CS and 20x/0.70IMM (Leica, Planapo, Wetzlar, Germany). Images were processed using associated Leica-TCS-NT software. All immunohistochemistry was performed at 22°C. The staining area was expressed as ratio of the total lesion area, calculated using 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 six random sections from each sample.

Culture of primary SMCs

Isolation and culture of SMCs has been described previously. Briefly, mouse aortae with adventitia removed were washed with DMEM (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 mM) (all from Sigma, Dorset, UK) at 37°C for 45 min. After passage through a cell strainer and centrifugation, the resulting SMCs were cultured in DMEM supplemented with 10% fetal calf serum (FCS), 100U/mL penicillin, and 100μg/mL streptomycin until subconfluent at 37°C in a humidified 5% CO2 incubator.

SMC-MIF release assay in vitro and FIX, FX & thrombin generation assays

SMCs (1×10^6 cells/well in a 24-well plate) were serum-starved for 24 hours before addition of
either thrombin 50nM, FXa (0 - 30nM) (Enzyme Research Laboratories, Swansea, United Kingdom), increasing concentrations of PAR-1 and -2 agonists (Peptides International, Kentucky, USA), p38 MAP Kinase inhibitor SB203580 (10μM), p42/p44 inhibitor PD98059 (50μM) or NF-κB inhibitor SN502 (20μM) (Merck Millipore, Hertfordshire, UK) for variable amounts of time. MIF was determined in supernatants.

In separate assays, washed SMCs (10^6 cells/well of a 96-well plate) were suspended in DMEM containing either 5nM FXa or 10nM FVIIa at 4°C. After 15 minutes, pre-prepared 10nM FVa and 0.5 μM prothrombin in HBS buffer (Life Technologies, NY, USA) were added for thrombin generation; FIX, FX, FXI or FXII at variable concentrations were added to assess FIXa, FXa, FXIa or FXIIa generation (Enzyme Research Laboratories). After defined times, aliquots of the reaction mixture were transferred into Tris-EDTA buffer with the chromogenic substrate Biophen CS-51(09) (Hyphen BioMed. Neuville sur Oise, France) for assessment of FIXa, or S-2238, S-2222, S-2366 or S-2302 for thrombin, FXa, FXIa or FXIIa generation respectively (Chromagenix, Milan, Italy). Absorbance at 405 nm was converted to concentrations using purified standards control assays. To assess MIF production under these conditions, the assay was terminated after 20 minutes by washing the cells five times with PBS, before re-culturing in DMEM containing 2% FCS for 24 hours to obtain the supernatant used for MIF assessment.

**RNA isolation and qRT-PCR analysis**

Total RNA from SMCs was isolated using TRIzol reagent according to the manufacturer's instructions (RNasy Plus Mini Kit, Qiagen, Hilden, Germany). First strand cDNA was synthesized from 1 μg total RNA using SuperScript III first-strand synthesis system (Invitrogen, Paisley, UK) as recommended by the manufacturer. qRT-PCR was performed using SYBR
Green Mix (Thermo Scientific) in an qRT-PCR system (AB7500, Applied Biosystems, Foster City, CA, USA). Details of MIF and β-actin primers, thermal cycling conditions and quantification of MIF RNA are in supplemental material. Fold increase in MIF was determined using the $2^{-\Delta\Delta CT}$ (Livak) method.

**Statistical Analysis**

Statistical analysis was performed using GraphPad Prism software. The Mann Whitney test was used for comparison of two groups and Kruskal-Wallis test for 3 or more groups. All data are presented as Mean ± SEM except table one, in which data is presented as Mean ± SD; a p value < 0.05 was considered significant. The pairwise p-values are two-sided and due to the nature of the study, there are no adjustments for multiple comparisons.

**Results**

**Baseline Characteristics of ApX4 mice**

Transgene frequencies, assessed by PCR (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 (see table 1). 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&D), medium and small arteries, arterioles and larger venules of all major tissues (suppl figure1) and in vitro-cultured aortic SMC from ApX4 but not ApoE<sup>−/−</sup> mice (figure 1E). In mice fed a NCD, there were no significant differences in circulating mouse or human TFPI nor in human TFPI activity when comparing ApoE<sup>−/−</sup> and ApX4 mice (Table 1). After a HFD, levels of all three tended to increase in ApX4 or (ApX4 x ApoE<sup>−/−</sup>) hTFPI+ littermates, though differences remained statistically insignificant (Table 1). There were no significant differences in the levels
of circulating lipids in ApX4 compared to ApoE−/− mice or in hTFPI-positive or negative
littermates fed either type of diet (Table 1). Expression of the hTFPI fusion protein had no
significant impact on circulating TAT complexes or D-dimers (Table 1), 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 x ApoE−/−) hTFPI+ littermates developed significantly attenuated
atherosclerosis after being fed a HFD for 6 or 12 weeks, compared to ApoE−/− or negative
littermate controls (figure 2). Obvious atheromatous lesions were also visible in ApoE−/− mice
fed a NCD for 20 weeks but these were almost undetectable in ApX4 mice (suppl figure 2).
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 to TFPI- mice (figure 3 and suppl figure 2). Analysis of circulating plasma TNFα and
IFNγ showed that atheroma development in ApX4 mice (fed either type of diet) occurred in the
context of significantly reduced systemic inflammation (figure 4A&B).

Specific inhibition of MIF expression by SMA+ cells in ApX4 mice
Plasma levels of MIF in 20 week ApX4 mice fed a NCD were approximately 25% those seen in
age-matched ApoE−/− mice (figure 4C). After a HFD, plasma MIF concentration was still
significantly reduced compared to negative littermates or ApoE−/− mice (figure 4C).
By confocal analysis, cells expressing MIF occupied a significant proportion of typical
atheromatous plaques in ApoE−/− mice (figure 4D&E). In the α-SMA-rich lesions seen in mice
fed a NCD, MIF was predominantly expressed by α-SMA+ cells (figure 4E). In contrast, there
was little MIF staining within the small plaque areas seen in ApX4 mice, even though α-SMA+
cells occupied the same proportional area (figure 4D&F). After a HFD, all SMA+ cells in ApoE−
mice co-expressed MIF and these accounted for approximately 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 ApX4 mice was significantly reduced, consistent with reduced infiltration of leukocytes in these lesions (figure 4F). Most importantly, although the proportional area occupied by SMA+ cells in ApX4 mice fed a HFD for 12 weeks was similar to that seen in ApoE−/− (15 ± 0.5% vs. 27 ± 1.8%; p<0.05), none of these cells co-expressed 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 ApX4 mice.

Additionally, in ApX4 mice fed a NCD, there was no MIF expression by SMC within non-atheromatous areas of the vessel wall, compared to in ApoE−/− mice, in which all the media stained strongly for MIF (figure 4D). These data indicate that MIF expression by SMC was independent of plaque development. Similar findings in medial SMC were seen in all the mice fed a HFD (suppl figure 3). However, in these mice, it was obvious that CD31+ intimal cells distant from atheroma were MIF+ in both hTFPI+ and hTFPI− mice indicating that EC expression of MIF was induced under these conditions and this was not prevented by expression of the hTFPI on medial SMC.

Vessel wall MIF is required for 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 a HFD was unaltered by the transplanted section (Figure 5). Aortic transplants from mice completely deficient in MIF were resistant to atheroma development, compared to MIF+ littermates, which developed exaggerated atheroma (Figure 5
A, C and H). Transplants from α-TFPI-Tg mice were also resistant, indicating that expression of hTFPI fusion protein on SMC had the same functional impact as complete MIF deficiency. Aortic sections from PAR-1 and -2 deficient mice were also protected compared to controls, suggesting that the effect of hTFPI might be to limit signaling through PAR on SMC. Atheroma resistance was associated with absent MIF production by SMC in the transplanted segments (suppl figure 4A) but not with alterations in plasma level of MIF (suppl Figure 4B) indicating the importance of vessel wall rather than systemic MIF for development of atheroma in this model.

**MIF secretion by SMC is TF- and PAR-dependent.**

To explore how hTFPI influenced MIF expression, SMC from both ApoE−/− and ApX4 mice were first shown to be TF-expressing (suppl figure 5A-D). In vitro, the generation of FIIa, FXa and thrombin were completely dependent on TF and FVIIa (suppl figure 5E-I) and SMC were unable to convert FXI or FXII into their activated forms (suppl figure 5J), indicating no involvement of the intrinsic pathway in generation of FXa or thrombin in vitro by SMC.

SMC 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 antagonists to either PAR-1 or PAR-2 (figure 6B) whereas thrombin was inhibited by a PAR-1 but not PAR-2 antagonist (figure 6E). When FXa (figure 6C) or thrombin (figure 6F&G) were 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 & FVa or FVIIa, FX & FVa were provided (figures 6F&G) and ApX4 SMC were resistant to the effects of exogenously added FXa (figure 6A) and FXa made in-situ (figure 6C). PAR-1 and
-2-dependent MIF secretion was transcriptionally-induced and involved signaling through p42/p44 ERK and NFKB pathways (suppl figure 6). PAR-1 induced MIF was also partly dependent on p38 kinase (suppl figure 6). All this data indicates that MIF production by SMC 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. SMC from these mice started to express MIF, like those from ApoE⁻/⁻ mice (figure 7A). This also significantly increased circulating MIF concentrations, compared to animals injected with an isotype control, to levels similar to those seen in age and sex-matched ApoE⁻/⁻ mice (figure 6H), suggesting that, since hTFPI was expressed only on SMA+ cells, plasma MIF levels were determined by MIF released from SMC. Additionally, these effects were abolished by co-administration of active site-inhibited FVIIa (but not FXIIa), and mimicked by administration of a PAR-1 but not PAR-2 agonist (figure 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 all 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 SMC. Analysis of the arterial walls in these animals revealed changes in expression of MIF within SMC 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 co-expressed TF and associated PAR-1 signalling by FXa and thrombin, inhibition of which by the hTFPI fusion protein in ApX4 mice is associated with
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 18. However, there are two potential mechanisms operating in these mice, the first involving loss of the direct effects of TFPI on circulating lipids 19, 20 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 due to a direct effect of soluble TFPI on VLDL metabolism 20.

The importance of the second mechanism is illustrated when ApoE<sup>-/-</sup> mice are made deficient in HCII, a natural thrombin inhibitor, as they develop severe atheroma 21. Additionally, the direct thrombin inhibitor melagatran reduces lesion progression and maintains plaque stability in ApoE<sup>-/-</sup> mice 22.

In this paper, utilizing a new strain of transgenic mice expressing a membrane-tethered hTFPI fusion protein on SMA<sup>+</sup> cells, we provide an explanation of the molecular and cellular basis of this second mechanism and highlight the importance of regulation of TF activity in the evolution of atherosclerosis in ApoE<sup>-/-</sup> mice. The hTFPI fusion protein used in ApX4 represents a hybrid of the two main isoforms of TFPI (α and β. Physiological TFPIα and β mouse and human) are generated by alternative splicing and differ in their C-terminal structure and cellular localization 23. TFPIα is a secreted protein with three tandem Kunitz type domains (K1-3) 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, and this
prevented inflammatory cell recruitment into the vessel wall. Resistance to atheroma formation
was seen in mice fed either a NCD or a HFD, but was more significant in mice fed Chow, in
which MIF expression which was almost exclusively restricted to α-SMA+ cells. After a high
cholesterol diet, MIF was also obviously expressed by CD31+ ECs and by infiltrating leukocytes
within atheromatous plaques, which might explain why plasma MIF levels were higher in ApX4
mice after a HFD. Importantly, MIF was expressed by SMC 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 25. These results therefore indicate
a crucial role for TF in the generation of MIF by SMC 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, T cells and binding, amongst
other ligands, CXC chemokine receptors 26. MIF expression within atheromatous plaques has
been closely associated with progression and instability in human disease 27. Moreover,
deficiency of MIF is known to significantly impair atheroma development in LDL-R deficient
mice \(^{28}\) and inhibition of MIF activity (using an anti-MIF antibody) prevents atherosclerosis in ApoE\(^{-/-}\) mice \(^{29}\).

In this context, it is MIF expression by cells in the vessel wall that is important, by initiating leukocyte recruitment \(^{25}\). In vitro experiments have suggested that MIF expression is induced in SMC by hypoxia \(^{30}\) and oxidised LDL \(^{31}\). Coagulation factors including FXa and thrombin are also known to induce MIF secretion by ECs in vitro, through activation of PAR-2 and PAR-1 respectively \(^{32}\), and have been shown to mediate secretion of other cytokines from SMC \(^{33}\). Moreover, thrombin has been shown to induce MIF expression in diverse cell types \(^{34,35}\).

Our data confirms that FXa and thrombin induce MIF expression in SMC, and that SMC expressing the tethered hTFPI fusion protein are resistant to both these factors (when thrombin has to be generated from prothrombin in situ by prothrombinase). The signalling pathways we have defined are similar to those described for coagulation protease-induced MIF expression in EC \(^{32,36}\), thrombin signalling \(^{37}\) and MIF secretion in SMC \(^{38}\).

All these data are consistent with previous work from our group, in which we demonstrated that TF and thrombin are necessary for in situ generation of local CXC chemokine gradients to initiate leukocyte recruitment after transplantation \(^{39}\) and suggest that coagulation factors may perform a critical role of initiating leukocyte migration into or across the vessel wall.

In summary, our data indicates that the proatherogenic stimuli that promote MIF expression by SMC 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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**Conflict of Interest Disclosures:** None.

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Table 1. Characteristics of ApoE-deficient mice with or without hTFPI fusion protein transgene (n=10 per group, Mean ± SD).

<table>
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<tr>
<th></th>
<th>Fed HFD aged 6-12 weeks</th>
<th>Fed HFD aged 6-18 weeks</th>
<th>Fed Chow diet aged 0-20 weeks</th>
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<td><strong>Body Weight (g)</strong></td>
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<td>Before HFD</td>
<td>21.8±2.15</td>
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<td>After HFD</td>
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<td>28.2±2.09</td>
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<td>After chow diet</td>
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<td>Mouse TFPI (ng/ml)</td>
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<td>Human TFPI activity (U/ml)*</td>
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<td>Plasma cholesterol (mmol/L)</td>
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<td>Plasma triglycerides (mmol/L)</td>
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<td>LDL (mmol/L)</td>
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Figure Legends:

**Figure 1.** Baseline characterisation of ApX4 mice. A: PCR analysis of genomic DNA using hTFPI specific primers. B: Northern blot analysis of mRNA purified from aortas using a Dig-labelled hTFPI cDNA probe. GAPDH cDNA was used as an internal control. Representative of other tissues as described in text. C&D: Immunohistology of consecutive sections through mouse aorta. Sections stained with DAPI (4,6 diamidino-2-phenylindole) nuclear stain (blue), anti-human TFPI (green) and (red) anti-CD31 (C) or anti-α-SMA (D). Yellow indicates co-localisation. E: Immunocytofluorescence of isolated medial SMC from ApoE−/− and ApX4 mice. Slides stained with DAPI (blue), anti-human TFPI (green) and anti-α-SMA (red). Yellow indicates co-localisation.

**Figure 2.** hTFPI fusion protein on α-SMA+ cells inhibits atheroma development after HFD. Atheromatous lesions were assessed in the aorta (A&B) and aortic root (C&D) after a high fat diet (HFD) for 6 or 12 weeks. Quantitative assessments (A&C) show the area occupied by atheroma as a proportion of the total area (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 aortic root.

**Figure 3.** Inflammatory cell recruitment into atheromatous lesions after HFD (6 weeks). Immunohistology of aortic sinus from mice fed a HFD for 6 weeks. A: 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. B: Quantitative analysis of infiltration into aortic root atheromatous lesions. N=10 male mice per group.

**Figure 4.** Cytokine production accompanying atheroma development. Analysis of plasma at indicated times for TNFα (A), IFNγ (B) and MIF (C). Samples from 10 mice per group were pooled and analysed in triplicate by ELISA. Error bars = SEM. D: Immunohistological analysis of consecutive sections from aortic sinus from mice fed a NCD for 20 weeks. Representative three colour images of sections stained with DAPI (blue), anti-MIF (green) and anti-α-SMA (red). L=lumen. Yellow = co-localisation. E&F: Quantitative analysis of the area (in μm²×10⁻³±SEM) of atheroma in sections from aortic sinuses (n= 10 mice per group, 3 sinuses per mouse) (blue columns) compared to the area staining positively with anti-MIF (red columns), anti-SMA (purple columns) and the area where staining for both was co-localised (green columns).

**Figure 5.** Aortic transplant model to illustrate importance of local vessel wall MIF production. A-F: Representative Oil Red O-stained en face preparations of ApoE⁻/⁻ aortas containing a transplanted segment (indicated) from donors as indicated. Atheromatous lesions were assessed in the recipient aorta and in the graft after mice were fed a HFD for 6 weeks post-transplantation. G&H: Quantitative assessments (A&C) show the area occupied by atheroma as a proportion of the total area examined (n=6 males each group). G: Recipient aortas. H: Transplanted aortic segment.

**Figure 6.** MIF expression by SMC is TF- and FVIIa-dependent and inhibited by hTFPI. A-G: In vitro ELISA analysis of MIF secretion into the supernatants by 1 x 10⁶ stimulated SMC. A:
ApoE<sup>-/-</sup> (circles) or ApX4 (squares) SMC exposed to increasing concentrations of FXa. B: ApoE<sup>-/-</sup> SMC exposed to increasing concentrations of FXa without inhibitors (circles) or with addition of 10nM PAR-1 (squares) or PAR-2 antagonist (triangles). C: MIF made after incubation with 80nM FX for the indicated amount of time; ApoE<sup>-/-</sup> SMC incubated with FX alone (circles), FX and 10nM FVIIa (squares) or FX, FVIIa and 80μg/ml of inhibitory anti-TF antibody (triangles). Diamonds shows MIF made by ApX4 SMC incubated with 80nM FX and 10nM FVIIa. D: ApoE<sup>-/-</sup> (circles) or ApX4 (squares) SMC exposed to increasing concentrations of thrombin. E: ApoE<sup>-/-</sup> SMC exposed to increasing concentrations of thrombin without inhibitors (circles) or with addition of 10nM PAR-1 (squares) or PAR-2 antagonist (triangles). F: MIF secretion after incubation with prothrombin. SMC incubated with FVa (10nM), prothrombin (0.5μM) and anionic phospholipids either with or without FXa (5nM), as indicated. In colorimetric assays, thrombin was only generated in the presence of FXa by SMC from ApoE<sup>-/-</sup> mice. G: MIF made after incubation with 0.5μM prothrombin for the indicated amount of time; ApoE<sup>-/-</sup> SMC incubated with 80nM FX and 10nM FVa (circles), FX, FVa and 10nM FVIIa (squares) or FX, FVa, FVIIa and 80μg/ml of inhibitory anti-TF antibody (triangles). Diamonds shows MIF made by ApX4 SMC incubated with 80nM FX, 10nM FVa and 10nM FVIIa. In all experiments, baseline MIF levels (no coagulation factors added) have been subtracted form the values displayed. Values obtained from mean of 3 values. Error bars (SEM) included on all datapoints. H&I: Analysis of plasma MIF in different groups of young mice fed NCD. Samples from 3 mice per group were pooled and analysed in triplicate by ELISA. In all graphs, error bars = SEM. H: Comparison of ApoE<sup>-/-</sup> mice injected with PBS with ApX4 mice injected with anti-TFPI antibody (with or without either active site inhibited FVIIa (FVIIai) or FXIIa (FXIIai)), control istotype, or PAR agonists as indicated. Compared to isotype control, *p<0.05. Compared to anti-
TFPI, †p=0.05, §p=NS. I: ApoE⁻/⁻ mice injected with PBS, PAR-1 or -2 antagonists, active site inhibited FXa (FXai), FVIIai or FXIIai or inhibitory anti-TF antibody as indicated. Compared to PBS control, **p<0.005, †p=NS. Compared to PAR-1 antagonist, §p=NS.

**Figure 7.** Immunohistological analysis of consecutive sections from aortic sinus from 6-week old mice fed a NCD. A&B: Representative three colour images of sections stained with DAPI (blue), anti-MIF (green) and anti-α-SMA (red). Mice were injected intravenously daily for 3 days with substances indicated at the doses stated in methods. In A, apart from where indicated, all sections from ApX4 mice. L=lumen. Yellow = co-localisation.
Figure 1

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Figure 2

A

![Graph showing lesion area (% of total aorta) for different groups: ApoE-/- Littermates TFPI+ TFPI- ApoE-/- ApX4 for HFD 6W and HFD 12W.](image)

B

![Images showing aorta sections for different groups: ApoE-/- Littermates TFPI+ TFPI- ApoE-/- ApX4 for HFD 6W and HFD 12W.](image)

C

![Graph showing lesion area (% of aortic sinus) for different groups: ApoE-/- Littermates TFPI+ TFPI- ApoE-/- ApX4 for HFD 6W and HFD 12W.](image)

D

![Images showing histological sections for different groups: Littermate TFPI- vs Ap4, 250μm.](image)
**Figure 3**

(ApoE\(^{-/-}\) x ApX4) littermates

(A) Immunofluorescence images showing the area of staining within atheroma (% of lesional area) for CD68, CD11b, and CD3. The images illustrate the differences between ApoE\(^{-/-}\) and (ApoE\(^{-/-}\) x ApX4) littermates for TFPI+ and TFPI- conditions.

1. **CD68**
   - ApoE\(^{-/-}\): L
   - TFPI+: L
   - TFPI-: L
   - P = 0.11

2. **CD11b**
   - ApoE\(^{-/-}\): L
   - TFPI+: L
   - TFPI-: L
   - P = 0.08

3. **CD3**
   - ApoE\(^{-/-}\): L
   - TFPI+: L
   - TFPI-: L
   - P = 0.42

**B**

Graphical representation of the area of staining within atheroma (% of lesional area) for CD68, CD11b, and CD3.

1. **CD68**
   - ApoE\(^{-/-}\): 75
   - (ApoE\(^{-/-}\) x ApX4) littermates: 50
   - TFPI+:
   - TFPI-:
   - P = 0.08

2. **CD11b**
   - ApoE\(^{-/-}\): 75
   - (ApoE\(^{-/-}\) x ApX4) littermates: 50
   - TFPI+:
   - TFPI-:
   - P = 0.01

3. **CD3**
   - ApoE\(^{-/-}\): 15
   - (ApoE\(^{-/-}\) x ApX4) littermates: 10
   - TFPI+:
   - TFPI-:
   - P = 0.01
Figure 4

A

B

C

D

E

F

Figure 4

ApoE-/- ApoE-/- ApX4 ApoE-/ ApX4

Chow 20 weeks

HFD 6 weeks

HFD 12 weeks

ApoE-/- ApoE-/- ApX4 ApoE-/- ApX4

TFPI+ TFPI-

TFPI-

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Figure 5

A: MIF+ littermate donor

B: MIF -/- donor

C: C57BL/6 donor

D: α-TFPI-Tg donor

E: PAR-1 -/- donor

F: PAR-2 -/- donor

G

Lesion area (% of recipient aorta)

H

Lesion area (% of transplanted segment)

Donors

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

MIF concentration in supernatant (ng/ml)

[FXa] (nM)

Time (mins)

[Thrombin] (nM)

Plasma [MIF] (ng/ml)

ApoE<sup>−/−</sup> Apo<sup>x4</sup> ApoE<sup>−/−</sup>

PBS
Istotype control
Anti-TFPI
PAR-1 antag
PAR-2 antag
FVIIai
anti-TF
FXIIai
FXai

H

I

P<sup>=0.001</sup>
Figure 7

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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
Daxin Chen, Min Xia, Claudia Hayford, Vikki Semik, El-Li Tham, Stuart Hurst, Ying Chen, Henry H. Tam, Jun Pan, Yucheng Wang, Xiaojin Tan, Huiyao Lan, Huahao Shen, Vijay V. Kakkar, Qingbo Xu, John H. McVey and Anthony Dorling

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http://circ.ahajournals.org/content/suppl/2016/12/29/CIRCULATIONAHA.114.013423.DC2

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SUPPLEMENTAL MATERIAL

Methods

Generation, breeding and diets of mice

α-TFPI-Tg and ApoE\(^{-/-}\) strains are on a C57BL/6J background. For some experiments, hTFPI+ or hTFPI-negative littermates were generated by crossing ApX4 with ApoE\(^{-/-}\) 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 Diagnostic 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'-TGCCCAGAACCACAAGAAGAGTAA-3' (Forward) and 5'-TCGCTACCACCGAATAAACAAAACAAGA-3' (Reverse) were used for qRT-PCR, and β-actin primers 5'-CTGTGGCAGCTCCACGAAACTA-3' (Forward) and 5'-AGTACTTGGCGCTCAGGAGGA-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 \(2^{-\Delta\Delta C_T}\) (Livak) method.
Supplemental Figure 1

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

ApoE⁻/⁻

CD31
αSMA

hTFPI
Combined

ApX4

CD31
αSMA
Supplemental Figure 2

A. Scatter plot showing lesion area (% of total aorta) with ApoE/− and ApX4. The plot includes statistical significance (P<0.001).

B. Staining images for ApoE/− and ApX4 showing lesion areas.

C. Scatter plot showing lesion area (% of aortic sinus) with ApoE/− and ApX4. The plot includes statistical significance (P<0.001).

D. Staining images for ApoE/− and ApX4 showing lesion areas with scale bar 250 µm.

E. Immunofluorescence images showing CD68, CD11b, and CD3 staining for ApoE/− and ApX4.

F. Bar graphs showing area of staining within atheroma (% of lesional area) for CD68, CD11b, and CD3. The graphs include statistical significance (P<0.001) for each comparison.
Supplemental Figure 4

A

B

Plasma [MIF] (ng/ml)

0 10 20 30 40 50 60 70

MIF+ littermate MIF-/- C57BL/6 α-TFPI-Tg PAR-1-/- PAR-2-/-
Supplemental Figure 6

Fold increase in MIF mRNA

Time of incubation with PAR agonists (hours)

E

PAR-1 agonist
PAR-2 agonist
PD98509
SB203580
SN50

[\text{MIF}]^\text{(ng/ml)}

p<0.001

p=0.001

p=0.01

p=0.2
SUPPLEMENTAL FIGURE LEGENDS

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

Immunohistology analysis of ApoE\(^{-/-}\) 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-\(\alpha\)-SMA as indicated. Yellow = co-localisation.

Supplemental Figure 2 - Human TFPI fusion protein on \(\alpha\)-SMA\(^{+}\) 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 1x10\(^6\) 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 관여한다. 그러나 이들이 영향을 미치는 세포 분자 수준의 기전은 알려진 바 없다.

방법 및 결과

결론
TF는 SMCs에서의 MIF 생산을 유도한다. 이는 ApoE-/-마우스에서 죽상경화를 우발하는데, 이를 차단하면 염증세포의 침윤이 억제되어 죽상경화의 발생이 저해된다. 이 연구는 병태생리적 이해도를 높여주며, 죽상경화 예방을 위한 새로운 치료 전략을 제시해 주고 있다.