Modulation of the Extracellular Matrix in Advanced Atherosclerosis

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5 Lesional Overexpression of Murine Tissue Factor Pathway Inhibitor-2 (TFPI-2) Paradoxically Decreases Intimal Collagen Content in ApoE Deficient Mice

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Abstract

Remodeling of the extracellular matrix is instrumental in atherogenesis and plaque stability. The serine protease inhibitor tissue factor pathway inhibitor-2 (TFPI-2) has been proven to be a potent MMP inhibitor and is expressed in human plaques. Thus far, the characterization and functionality of murine TFPI-2 has not been established. This study aims to generate and characterize murine TFPI-2 and to evaluate its role in atherogenesis.

Murine TFPI-2 was successfully cloned into an adenoviral vector. Transduced endothelial cells displayed impaired invasive capacity and the secreted protein prolonged plasma coagulation time (P=0.002). In addition, atherosclerotic plaques were elicited in apoE deficient mice (n=20) by collar placement on the carotid artery. Lesional gene transduction of mTFPI-2 did not affect lesion size of vessel remodeling. However, intimal collagen content had significantly decreased compared with controls (P=0.01). This was accompanied by a ~40% increase in necrotic area (P=0.05). In vitro analyses revealed that mTFPI-2 dramatically impairs SMC invasive activity and reduced SMC proliferation by approximately 50% (P=0.03). Moreover, collagen synthesis remained unaffected and MMP-9 gene expression was increased more than 2-fold (P=0.03) by mTFPI-2 overexpressing SMCs.

In conclusion, mTFPI-2 prolongs plasma coagulation time, impaired cellular invasion and reduced intimal collagen in atherosclerotic plaques, suggesting that mTFPI-2 has an adverse effect on plaque stability.

Introduction

Remodeling of the vascular extracellular matrix (ECM) is an important process in atherosclerosis affecting luminal patency, lesion progression and plaque stability.[1-4] It involves the activity of several proteolytic enzymes, such as matrix metalloproteinases and cathepsins, which are regulated at four different levels: 1) gene expression, 2) trafficking of intracellular proteases to the plasmalemma and subsequent secretion, 3) activation of the inactive zymogen and 4) inhibition of proteolytic activity by endogenous inhibitors.[3] While extracellular cathepsin activity is regulated by several cystatins[5], that of MMPs can be silenced by 1 of the 4 known tissue inhibitors of metalloproteinases (TIMP-1 to -4).[6]

Recently, a novel inhibitor of MMP activity has been identified. Originally detected in human placenta and designated placental protein 5 (PP5) in 1977 [7], tissue pathway inhibitor-2 (TFPI-2) showed significant structural homology to TFPI-1,[8] an important physiological anticoagulant. Apart from placenta it could also be detected in other tissues, including mesenchymal and endothelial cells.[9, 10] While both proteins posses 3 Kunitz-type domains and are able to inhibit a variety of coagulation related factors including factor Xa, factor XIa, kallikrein and plasmin, the anti-coagulating capacity of TFPI-2 is rather modest and hardly plays a significant role in vivo.[11, 12] Surprisingly, TFPI-2 was senn to display much higher potency to inhibit other proteolytic enzymes, i.e. MMP-1, -2, -3, -9 and –13.[13-15] In addition, TFPI-2 promotes VSMC mitogenic activity in vitro.[16]

Also, it was demonstrated that TFPI-2 is expressed in human plaques at levels that inversely correlated to that of lesional MMP.[15, 17] TFPI-2, predominantly co-localized with SMCs in the media and the fibrous cap, while macrophage enriched areas at the shoulder regions stained only weakly for this serine protease inhibitor, indicating a stabilizing role for TFPI-2 in advanced atherosclerotic plaques.[15] Complementary observations showed that TFPI-2 mRNA levels are strongly upregulated in invasive human arterial SMCs.[18]

Currently, no data is available on murine TFPI-2 expression and function in mouse models of atherosclerosis. Because overall TFPI-2 expression patterns differ substantially in humans and mice, showing high-level expression in murine hepatocytes, Hisaka and colleagues suggested that murine and human TFPI-2 may have different functions in vivo.[19] A notion that is supported by the relatively low sequence homology of human and mouse TFPI-2.[20, 21] In the present study we cloned mTFPI-2 into an adenoviral vector and characterized its functionality in vitro. Targeted mTFPI-2 overexpression in collar-induced carotid artery plaques did not affect lesion progression, but strongly reduced intimal collagen content, which could at least in part be attributable to a changed MMP-9/TIMP-1 expression ratio. Taken together, these data suggest that murine TFPI-2 surprisingly has a negative influence on plaque matrix turnover, pointing to a detrimental role of TFPI-2 in murine plaque stability.

Methods

Animals

Male apoE deficient mice on a C57Bl/6 background (n=20), 20 weeks of age, were obtained from our in house breeding stock. Mice were placed on a western type diet containing 0.25% cholesterol (Special Diets Services, Witham, Essex, UK). High fat diet and water were provided ad libitum. All animal work was approved by the regulatory authority of Leiden University and performed in compliance with the Dutch government guidelines.

Cloning of full length TFPI-2 gene into an adenoviral vector
Replication-deficient recombinant adenoviruses were generated essentially as has previously been described by Vogelstein et al.[22] Briefly, full length TFPI-2 was amplified by PCR from C57Bl/6 liver cDNA (Fwd: 5'-CGTACCGCGTACAGAGAACCACAGCA-3', Rev: 5'-TCTAGAGGAAGGCAGCTGGTATGAAT-5') and subsequently cloned into the Sma-I site of pGEX4T-2 (Pharmacia). After sequence verification, the Acc65-I and Xba-I digested (MBI Fermentas) insert was transferred into the shuttle vector pShuttleCMV. The resultant plasmid was linearized (PmeI) and co-transfected into E. coli BJ5183 cells with adenoviral backbone plasmid DNA (pAdEasy-1). The linearized recombinant plasmid then was transfected into E1A expressing 911 cells for adenoviral packaging. TFPI-2 recombinant adenovirusses were purified and titred. Stock titres were determined on 293 overlay plaque assays and expressed as plaque forming units/ml (pfu/ml).

Validation of Ad.TFPI-2 in vitro by coagulation assay
To validate TFPI-2 functionality in vitro, COS cells were transduced with Ad.TFPI-2 or with Ad.Empty at 50 pfu/cell for 3 h and incubated for an additional 24 hours. Media samples were subjected to an in vitro clotting assay in which clotting of citrated (0.38%) mouse plasma is induced with recombinant human tissue factor (Innovin, final dilution $4*10^5$ times) in presence of $CaCl_2$ (17 mM) and phospholipid vesicles (10 μM) in Hepes buffer (25 mM Hepes, 137 mM NaCl, 3.5 mM KCl, 10.5 mM $CaCl_2$, 0.1% BSA, pH 7.4). To assess the effect of TFPI-2 on coagulation, conditioned medium from Ad.TFPI-2 infected COS cells (50 m.o.i.) was collected 3 h after infection and added at indicated amounts. Conditioned medium from Ad.Empty infected COS cells was used as a control. Turbidity was monitored in time at 450 nm and 37°C in a FLUOstar OPTIMA microplate reader (BMG Labtech GmbH, Offenburg Germany) and expressed relative to the clotting time in absence of media samples.

Carotid collar placement and transgene expression
Carotid atherosclerotic lesions were induced by perivascular collar placement as previously described.[23] Briefly, mice were anesthetized with a subcutaneous injection of ketamin (60 mg/kg; Eurovet, Netherlands), fentanyl citrate and fluanisone (1.26 mg/kg and 2 mg/kg, respectively; Janssen Animal Health). A constrictive silastic manchette was placed perivascularly at both carotids facilitating atherosclerotic lesion formation proximal to the collar within 3 to 6 weeks.[23] High-fat diet started 14 days prior to collar placement. At week 3 after collar placement, when early lesions had already formed, plaques (n=39) were incubated intraluminally with an adenovirus carrying the murine TFPI-2 (Ad.TFPI2) or empty transgene (Ad.Empty) under control of the CMV promoter. Collars were left in situ to ensure continued plaque progression after gene transfer. Three weeks after transduction, lesions were analyzed histologically with regard to morphology and composition.

Tissue harvesting and preparation for histological analysis
Mice were sacrificed two weeks after infection. One day prior to sacrifice phenylephrin (8 μg/kg i.v.; Sigma Diagnostics, St. Louis, MO) was administered to all mice to assess plaque vulnerability to hemodynamic challenge. Before harvesting, the arterial bed was perfused with phosphate buffered saline (PBS) and formaldehyde.
Transverse, serial cryosections (5μm thick) were prepared from OCT-embedded carotid artery and routinely stained with hematoxylin (Sigma Diagnostics) and eosin (Merck Diagnostica) or Masson's trichrome (Accustain kit, Sigma). Collagen staining was performed by a 90 minute incubation of cryosections in 0.1% Sirius Red (Direct red 80, Sigma) in saturated picric acid and subsequent rinsing in 0.01 M HCl and distilled water. Perl's staining was applied to detect intralesional iron deposits. Corresponding sections were stained immunohistochemically with antibodies directed at mouse metallophilic macrophages (monoclonal mouse IgG_{2a}, clone MoMa2, dilution 1:50; Sigma) and α-SM-actin (monoclonal mouse IgG_{2a}, clone 1A4, dilution 1:500; Sigma). Macrophage, smooth muscle cell (SMC) and collagen positive areas were determined by computer-assisted color-gated measurement, and related to the total intimal surface area. To assess apoptotic cell death sections were subjected to TUNEL staining according to the protocols provided by the manufacturer (Roche).

Morphometry and morphology
In hematoxylin-eosin stained sections, the site of maximal plaque size was selected for morphometry. Images were digitized and analyzed as previously described.[23] The stage of lesion progression was assessed with classification criteria defined by Virmani et al.[24] Of six defined categories (i.e 1: fibrous lesion, 2: atheromatous lesion, 3: thin cap atheroma, 4: healed rupture, 5: plaque rupture or intraplaque hemorrhage and 6: plaque erosion) the first two classes are considered stable, whereas lesions in classes 3 to 6 are perceived as plaques with characteristics of vulnerability. Lesions were blindly allocated to the different classes by evaluating subsequent sections with a maximal interval of 100 μm. Thin cap atheroma is defined as lesions with a thin fibrous cap (≤3 cell-layers) accompanied by a large necrotic core (>40% of total plaque area). Plaque erosion was defined as thrombus formation without apparent plaque rupture.

Expression analysis
VSMCs were incubated with Ad.TFPI-2 or Ad.Empty at 300 pfu/cell for 18h and RNA was isolated using the TRIZOL method according to the manufacturer's instructions (Invitrogen, Netherlands). Purified RNA was DNase treated (DNase I, 10U/µg total RNA) and reverse transcribed (RevertAid M-MulV Reverse Transcriptase) according to the protocols provided by the manufacturer. Quantitative gene expression analysis was performed on an ABI PRISM 7700 machine (Applied Biosystems, Foster City, CA) by SYBR Green technology. Primers were designed for murine desmin, α-SM-actin, MGP, Osteopontin, Hsp47, Procollagen type III, MMP-3, MMP-9, MMP-13 and TIMP-1 using PrimerExpress 1.7 (Applied Biosystems) and validated for identical efficiencies (table 1). Target gene mRNA levels were expressed relative to the housekeeping gene (36b4) and calculated by subtracting the threshold cycle number (Ct) of the target gene from the Ct of 36b4 and raising two to the power of this difference.

Table1. Primer sequences (3' – 5')

Gene	Forward primer	Reverse primer
36B4	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG
α-SM-actin	TCCCTGGAGAAGAGCTACGAACT	GATGCCCGCTGACTCCAT
Desmin	GATGCAGCCACTCTAGCTCGTATT	CTCCTCTTCATGCACTTTCTTAAGG
MGP	GCATGTGTTGCTTGCTCCTTAC	TCATTACTTTCAACCCGCAGAA
Osteopontin	CAGGCATTCTCGGAGGAAC	GAGCTGGCCAGAATCAGTCACTTT
Procol III	TGCCCAACTGCGCTTCA	CCAGCCTGACAGGTTGGAAA
Hsp47	ACAAGATGCGAGATGAGTTGTAGAGT	TAGCACCCATGTGTCTCAGGAA
MMP-3	TTTAAAGGAAATCAGTTCTGGGCTATAC	CGTAAGTGTGGGACCCAGAC
MMP-9	CTGGCGTGTGAGTTTCCAAAAT	TGCACGGTTGAAGCAAAGAA
MMP-13	CAACCTATTCCTGGTTGCTGC	ATCAGAGCTTCAGCCTTGGC
TIMP-1	ACACCCCAGTCATGGAAAGC	CTTAGGCGGCCCGTGAT

Migration and invasion assay
TFPI-2 or mock virus transduced vSMCs, isolated from C57Bl/6 murine aortas, were incubated on standard medium for 16 h, harvested and seeded into a 24-wells plate placed at an angle of 70°. After cells had adhered the culture plate was placed at an angle of 35° and migration was monitored during the next 2 days. In addition, transfected vSMCs and H5V cells were cultured in a 2D Matrigel Matrix (Becton Dickinson Biosciences, San Jose, CA) containing laminin, collagen type IV and heparin sulphate. The formation of filopodia, the resulting cell-cell contacts between neighboring cells and the matrix invasive capacity were closely monitored during the following 2 days. Both the average distance of migrated cells as well as the amount of cellular nodules per field was quantified with Leica Qwin software.

Proliferation assay
VSMCs were transduced with 300 pfu/cell Ad.TFPI-2 or mock virus for 3 h. After 16 h incubation on standard medium, cells were starved in DMEM containing 0.5% fetal calf serum for 24 hours to synchronize the cell cycle. The cells were then incubated for 5 hours in standard medium containing 0.5 µCi [^{3}H]thymidin (Amersham). Thymidin incorporation was measured by LCS (Packard 1500 Tricarb).

Collagen synthesis assay
VSMCs were transduced with 300 pfu/cell Ad.TFPI-2 or mock virus for 3 h. After 16 h on standard medium cells were incubated with 37kBq [^{3}H]Proline (Amersham) for 24 h. Cells were washed in 20 mM TrisHCl, pH 7.6 and collected in 400 µl 20 mM TrisHCl, 0.36 mM $CaCl_2$, pH7.6. Collagen incorporated proline was released by collagenase treatment (Worthington collagenase, 4000 U/mL) of cell lysate for 5 h at 37°C and non-digested proteins were precipitated by adding 100 µl 50% TCA on ice for 30 minutes. After centrifugation, supernatant was collected and presence of [^{3}H]Proline was measured by LCS analysis (Packard 1500 Tricarb). IFN-γ (Leinco Technologies; 1000 U/mL), Brefeldin A (1µM) and serum free media served as negative controls.

Statistics
Morphometric analysis was compared using the Mann Whitney U test or the 2-tailed Student t-test depending on normality. Significance of differences in classification was assessed using the Yates' corrected Fisher test. Expression analysis (dCt-values) and other in vitro assays were analyzed by a 2-tailed Student t-test. Values are displayed as mean±SEM and P-values≤0.05 were considered significant.

Results

Validation of transgene functionality

To validate the functionality of the adenoviral construct, H5V or COS cells were transduced with either Ad.mTFPI-2 or mock virus (300 m.o.i.). Ad.mTFPI-2 upregulated mTFPI-2 mRNA levels by 4-fold in murine endothelial cells (Figure 1) compared to controls, showing that mTFPI-2 was successfully cloned into the adenoviral vector. In addition, media of mTFPI-2 overexpressing COS cells significantly and dose-dependently prolonged TF/PLP/Ca^{++} induced clotting time of murine plasma (P=0.002, Figure 2A), indicating that mTFPI-2 transduction not only increased mTFPI-2 gene expression, but also resulted in the secretion of a functional protein with a tissue factor inhibitory activity. To test the effect of mTFPI-2 on endothelial cell migration in a Matrigel matrix, H5V endothelial cells, transduced with Ad.mTFPI-2 or mock virus, showed a 60% reduction of filopodia formation and cell-cell contacts (P=0.004, Figure 3), possibly attributable to impaired degradation of the Matrigel matrix.

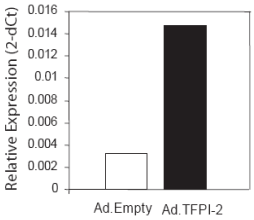

Figure 1. The H5V endothelial cell line was incubated with 300 pfu/cell Ad.Empty or Ad.mTFPI2 for 3 hours and harvested 24 h later. The expression of mTFPI-2 was raised almost 4-fold in Ad.mTFPI-2 transduced cells.

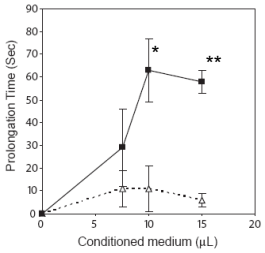

Figure 2. Coagulation of citrated plasma samples from C57Bl/6 mice was induced by adding recombinant tissue factor, $CaCl_2$, and phospholipid carrier vesicles. The addition of conditioned medium from Ad.TFPI-2 transduced COS cells (■) dose-dependently prolonged coagulation time compared with that from Ad.Empty transduced cells (△). Values are mean±SEM. * P=0.035; ** P=0.002.

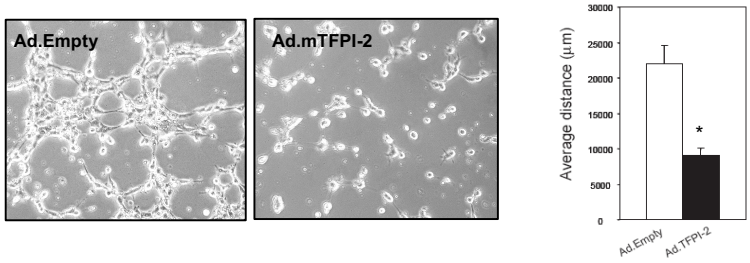

Figure 3. H5V endothelial cells were transduced with 300 pfu/cell Ad.mTFPI-2 or Ad.Empty and subsequently cultured in a Matrigel matrix. During the following 2 days mTFPI-2 gene transfer resulted in a dramatically impaired matrix invasion as can be appreciated by the reduction of filopodia formation and cell-cell contacts as well as by the absence of a cellular network. The average distance per field was reduced by approximately 60% with TFPI-2 overexpression (n=6, * P=0.004).

Lesion targeted mTFPI-2 transfer did not affect plaque size or vessel remodeling

In an attempt to elucidate the role of murine TFPI-2 in atherosclerosis, bi-clamped carotid artery segments containing collar-induced carotid lesions were incubated transluminally with either Ad.Empty or Ad.mTFPI-2 at an early stage of lesion progression (i.e. 3 weeks after collar placement). This localized overexpression did not affect bodyweight or serum lipid levels (data not shown) and mice remained in good health throughout the entire experiment.
Although TFPI-2 was expected to attenuate initial plaque progression by impairing SMC migration via MMP inhibition, no effect on lesion size could be detected 3 weeks after gene transfer (Figure 4A). Also, medial area (Figure 4B) and total vessel area (data not shown), reflecting vessel remodeling, were unaffected by lesion targeted mTFPI-2 overexpression.

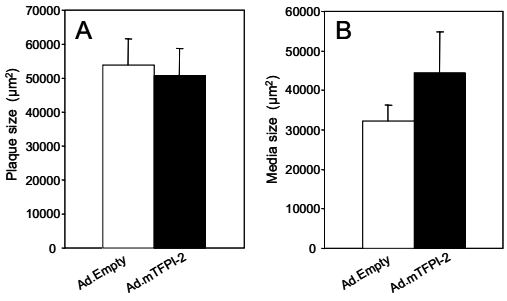

Figure 4. Atherogenesis was induced by carotid collar placement. Lesional mTFPI-2 gene transfer during early lesion development (3 weeks) did not affect plaque progression (A) or vessel remodeling as indicated by media size (B). Values are mean±SEM.

mTFPI-2 overexpression markedly reduced intimal collagen content and increased relative necrotic core area

Because TFPI-2 is thought to reduce SMC migration, lesional ASMA positive cells were stained and quantified relative to the intimal area. This did not reveal any change in intimal ASMA positive SMC content (Figure 5A), although it must be noted that not all intimal SMCs express this cytoskeletal protein.
Surprisingly, intimal collagen was reduced by as much as 65% from 17±4% in control vessels to only 6±1% in mTFPI-2 treated arteries (P=0.01, Figure 5B). This could not be explained by altered ASMA positive SMC numbers and even is in disagreement with the supposedly inhibitory action of mTFPI-2 on matrix degrading proteases. Alternatively, a shift in VSMC phenotype may be attributable to an enhanced inflammatory activity causing a dysbalance in matrix homeostasis. Therefore, the amount of infiltrated macrophages was evaluated, but did not reveal any difference in intimal macrophage accumulation (Figure 5C). Necrotic core content however was found to be increased from 33±11% in controls to 56±5% in Ad.TFPI-2 treated vessels (P<0.05; Fig.5D). In keeping, the relative amount of TUNEL positive cells, reflecting the apoptotic rate, tended to increase 2-fold by overexpression of TFPI-2 from 1.4% to 2.8%, but this did not reach statistical significance (P=0.08; Fig.5E).

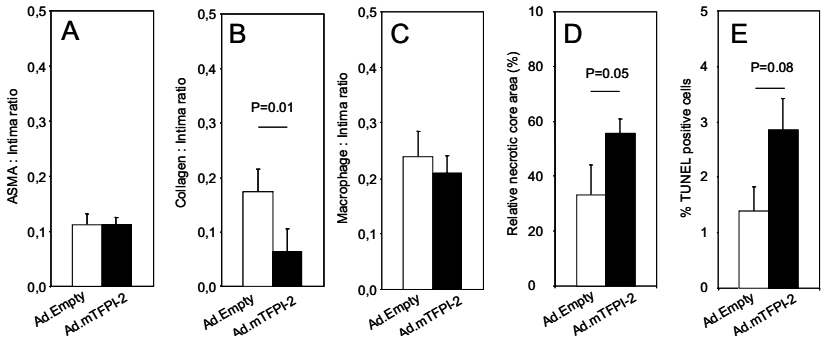

Figure 5. Lesional overexpression of mTFPI-2 resulted in a significantly reduced intimal collagen content (A). The relative amount of α-SM-actin (ASMA) positive SMCs (B) and macrophages (C) had not been changed by mTFPI-2 gene transfer. Necrotic core area relative to intimal area, however, was significantly enlarged upon mTFPI-2 overexpression (D). Also, the apoptotic rate as indicated by TUNEL positivity tended to increase (E). Values are mean±SEM.

In vitro effects of mTFPI-2 on SMC behavior

As proliferating SMCs in atherosclerotic lesions are not detectable by α-SM-actin staining and phenotypic modulation could explain the observed reduction in intimal collagen content, the effect on VSMC proliferation after mTFPI-2 overexpression was assessed by thymidine incorporation. In contrast to observations with human TFPI-2, showing a mitogenic response of VSMCs, we detected a reduced proliferation rate in cultured murine VSMCs overexpressing mTFPI-2 (Figure 6).
In a 2D Matrigel Matrix, essentially consisting of laminin, collagen type IV and heparin sulphate, mTFPI-2 gene transfer led to a dramatic decrease in matrix degradation. While Ad.Empty transduced SMCs formed extensive networks of cells, only a few mTFPI-2 transduced cells were capable of showing such invasive behavior. Both total length of these networks (Figure 7A-B) and the number of noduli (not shown) had significantly decreased by more than 80% (P=0.0003). By contrast, SMC migration on gelatin coated culture dishes was not impaired by mTFPI-2 overexpression (data not shown), suggesting that matrix invasion of SMCs had diminished by reduced pericellular protease activity rather than by an intrinsically impaired motility.

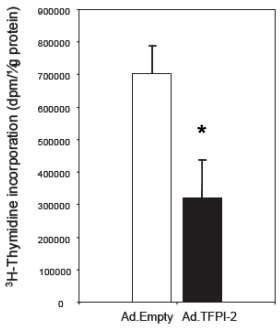

Figure 6. VSMCs, transduced with either Ad.mTFPI-2 or mock virus, were equilibrated overnight on serum free media. Proliferation commenced by adding fetal calf serum and was monitored by ^{3}H-thymidine incorporation. mTFPI-2 overexpression reduced SMC proliferation by approximately 50%. Values are mean±SEM. * P=0.03

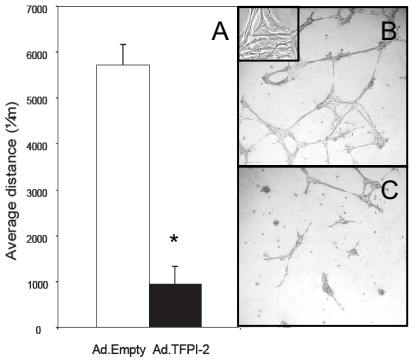

Figure 7. VSMCs were transduced with 300 pfu/cell Ad.Empty (A) or Ad.mTFPI-2 (B) and subsequently cultured in a Matrigel matrix. In accordance with the effects observed in H5V endothelial cells, mTFPI-2 gene transfer almost completely inhibited SMC invasion, whereas controls formed an extensive cellular network throughout the media. * P=0.0003

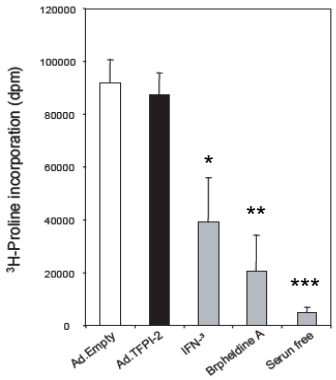

Figure 8. Collagen synthesis was assessed by ^{3}H-proline incorporation and did not differ between TFPI-2 overexpressing cells and controls. IFN-γ significantly reduced collagen synthesis by approximately 55%. Similarly brefeldine A (1 µg/mL) and serum free media inhibited proline uptake by 70% and 90% respectively. Values are mean±SEM. * P=0.03; ** P=0.005; *** P=0.0003

To verify if TFPI-2 might have reduced plaque collagen content by impairing its synthesis directly, a proline incorporation assay was performed. Transduction of VSMCs with Ad.mTFPI-2 did not affect collagen synthesis, whereas IFN-γ and serum starvation as well as brefeldine A treatment all significantly reduced collagen synthesis (Figure 8). This might suggest that lesional mTFPI-2 overexpression did not alter the VSMC matrix synthesizing capacity in vivo.
In line with these findings, gene expression analysis of mock and mTFPI-2 transduced SMCs could not reveal any phenotypic changes as α-SM-actin, desmin, MGP or osteopontin expression, all markers of SMC phenotype, remained unchanged (data not shown). However, MMP-9 mRNA levels were significantly upregulated 2 days after infection (P=0.03, Figure 9). Conversely, TIMP-1 expression tended to be downregulated by mTFPI-2 transduction (P=0.06). Taken together, the MMP-9/TIMP-1 ratio, a crude measure for proteolytic activity[25, 26], was increased 7-fold by mTFPI-2 overexpression in murine VSMCs (P=0.03, not shown). Paradoxically, overexpression of the MMP inhibiting TFPI-2 may thus lead to enhanced proteolytic activity.

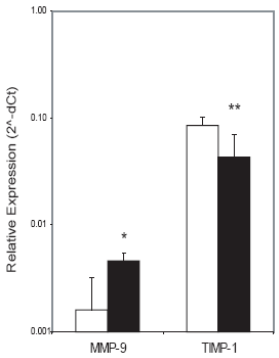

Figure 9. VSMCs were transduced with 300 pfu/cell Ad.Empty (A) or Ad.mTFPI-2 (B) and harvested for RT-PCR analysis one day later. mTFPI-2 overexpressing SMCs displayed an enhanced expression of MMP-9, while TIMP-1 mRNA levels tended to diminish. The MMP-9:TIMP-1 ratio, indicating proteolytic activity, increased 7-fold (P=0.03).
Values are mean±SEM. * P=0.03; ** P=0.06

Discussion

The matrix-bound serine protease inhibitor TFPI-2 has been shown to be a potent inhibitor of MMP activity.[13, 15] In fact, its affinity for MMPs was shown to be considerably higher than that for plasmin, factor VIIa, factor Xa or other factors important for coagulation, suggesting that its physiological function not so much resembles the structural homologue TFPI-1, but rather may be more related to matrix homeostasis. Furthermore, murine TFPI-2 displays only 50% homology with its human counterpart [21] and because its distribution pattern, displaying high expression in the liver, greatly differs with that of humans, it is suggested that this protein contributes different physiological functions in both organisms.[19] This study is the first to characterize murine TFPI-2 and to evaluate its role in atherogenesis.

Murine TFPI-2 was successfully cloned into an adenoviral vector and in vitro validation showed a significant upregulation of TFPI-2 in H5V endothelial cells. Unfortunately, mTFPI-2 antibodies are currently not available, complicating the confirmation of TFPI-2 expression at a protein level. However, murine plasma clotting time was significantly and dose-dependently prolonged by conditioned medium from Ad.mTFPI-2 transduced COS cells. Moreover, endothelial cell migration through a Matrigel matrix was dramatically impaired by TFPI-2 overexpression. These observations are highly suggestive of a functional protein and are in line with earlier observations with human and bovine TFPI-2. [27-29]

Notwithstanding these analogies, mTFPI-2 overexpression in the arterial wall rendered some surprising results. While mice overexpressing the MMP inhibitor TIMP-1 show reduced plaque size and an increased intimal collagen content,[30] lesional overexpression of mTFPI-2 did not affect plaque size, nor any other vessel dimension and paradoxically resulted in a profoundly decreased intimal collagen. Both α-SM-actin and macrophage staining did not reveal any differences between groups, indicating that increased intimal macrophage content or a reduced number of α-SM-actin positive SMCs cannot explain the reduction in intimal collagen content.

However, proliferating SMCs only weakly express α-SM-actin[31] hence masking a potential effect of mTFPI-2 on intimal SMC content. Indeed, in vitro, mTFPI-2 inhibited SMC proliferation. This is in contrast to human TFPI-2 which has been demonstrated to be a mitogen for VSMCs.[16] Furthermore, the migration of both ECs and SMCs on a Matrigel matrix was almost completely abolished by mTFPI-2

gene transfer. Because SMC migration on a gelatin coated culture dish was not affected by mTFPI-2, we conclude that mTFPI-2 impairs the degradation of the pericellular matrix rather than affected intrinsic SMC motility. These findings could indicate that mTFPI-2 attenuates medial SMC invasion towards the arterial intima.

Programmed cell death may also be involved in the reduced intimal collagen. The amount of TUNEL positive cells tended to rise in mTFPI-2 overexpressing plaques, but this did not reach statistical significance. However, the relative necrotic core area proved to be substantially larger in these lesions. While SMC death negatively affects collagen production, macrophage cell death may lead to enhanced inflammation and subsequent collagen breakdown by increased expression of matrix degrading proteases. The rate of collagen synthesis itself was not affected by mTFPI-2, suggesting that SMC phenotype may not necessarily be influenced by this protease inhibitor. Indeed, expression levels of α-SM-actin, desmin, MGP and osteopontin remained unaffected. However, it must be noted that SMC phenotypic regulation is extremely complex and involves the expression of numerous markers that greatly overlap in various phenotypic states.[32-35] Hence, the absence of an effect on the afore-mentioned markers in vitro cannot fully exclude phenotypic modulation on other levels in vivo.

Gene expression analysis further revealed that MMP-9 expression had increased more than 2-fold, while TIMP-1 expression tended to decrease. Considering the significant increase of the MMP-9/TIMP-1 ratio, a measure for proteolytic activity within the plaque, it may be surprising that TFPI-2 impaired matrix invasion in vitro. This could be explained by the high affinity of TFPI-2 for proteoglycans[36] in the direct vicinity of the cells that produce this inhibitor and thus attenuates proteolytic activity only within the pericellular space, while secreted MMP-9 could diffuse further into the matrix to exert its proteolytic actions. Therefore, it may be speculated that TFPI-2 impairs SMC proliferation and invasion by inhibiting pericellular matrix degradation, while promoting accelerated proteolysis deeper into the extracellular matrix.

In summary, adenoviral transfer of mTFPI-2 resulted in the secretion of a functional protein sharing many characteristics with human TFPI-2, such as impairing plasma clotting and cell migration. However, in contrast to its human analogue, murine TFPI-2 attenuated SMC proliferation and enhanced the expression of MMP-9, suggesting that murine TFPI-2 displays an alternative physiological functionality. Additional studies are presently being conducted to further unravel the biological actions of murine TFPI-2. Overexpression of this serine protease inhibitor in the carotid arterial wall during early plaque formation did not affect lesion progression, but resulted in significantly decreased intimal collagen content and larger necrotic cores. In vitro analysis of mTFPI-2 functionality revealed that overexpression of this protein was accompanied by a decreased proliferation and invasion of SMCs, while it did not alter collagen synthesis. Together with the mTFPI-2 induced expression of MMP-9 by VSMCs, these observations could at least partially explain the reduced intimal collagen content in vivo. In conclusion, mTFPI-2 gene transduction impaired invasive capacity of both endothelial and smooth muscle cells in vitro. In vivo, lesional mTFPI-2 overexpression resulted in a strong reduction of intimal collagen accompanied by larger necrotic cores, suggesting that mTFPI-2 has an adverse effect on plaque stability.

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