Peptide corresponding to the C terminus of tissue factor pathway inhibitor inhibits mesangial cell proliferation and activation in vivo

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1. Introduction

Mesangial cells (MsCs) are one of the resident cell types in the glomerulus and are important with respect to its function and structure. The activation and proliferation of MsCs occur in several types of glomerulonephritis, particularly proliferative glomerulonephritis, producing a series of protein factors and matrix components that impair the normal structure and function of the glomerulus. To inhibit proliferation or induction of apoptosis is considered to be one mechanism that can be used to treat these diseases. In previous studies, we found that the tissue factor pathway inhibitor (TFPI) induces the apoptosis of cultured rat MsCs. Here, we expressed a series of TFPI fragments as fusion proteins to maltose binding protein (MBP-TFPI162–188, MBP-TFPI187–241, MBP-TFPI240–276, MBP-TFPI162–241, MBP-TFPI187–241, and MBP-TFPI187–276) and applied them to cultured rat mesangial cells. The C terminus of TFPI, a peptide corresponding to residues 240–276 of TFPI, was confirmed to induce apoptosis of MsCs in vitro. To observe the effect of this peptide on MsCs in vivo, we performed intramuscular gene transfer treatment on a rat model of proliferative glomerulonephritis with a plasmid containing the gene for the C terminus of TFPI. This revealed that the C terminus of TFPI exhibited suppressive effects on the activation and proliferation of MsCs and, thereby, improved renal function. Our data indicate that the C terminus of TFPI could be used in the treatment of proliferative glomerulonephritis.

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treated serum-starved MsCs with these fragments. Overall, we found that the C terminus of TFPI is essential for inducing apoptosis.

The anti-Thy1 nephritis rat model is a well established model for researching mesangial proliferative glomerulonephritis characterized by MsC proliferation and matrix expansion [19,1]. To test the effect of the C terminus of TFPI on MsCs in vivo, we constructed a plasmid expressing a gene for the C terminus of TFPI that was fused to the human serum albumin signal peptide (HSA-SP), and a control plasmid expressing only the HSA-SP gene. The plasmids were transformed to muscle tissue via an electroporation-control plasmid expressing only the HSA-SP gene. The plasmids were fused to the human serum albumin signal peptide (HSA-SP), and a control plasmid expressing only the HSA-SP gene. The plasmids were transformed to muscle tissue via an electroporation-control plasmid expressing only the HSA-SP gene. The plasmids were transformed to muscle tissue via an electroporation-control plasmid expressing only the HSA-SP gene.

Using this technique, we demonstrated that the C terminus of TFPI inhibits the proliferation and activation of MsCs rather than apoptosis in vivo. The renal function was also protected by the expression of the C terminus of TFPI. These findings suggest the clinical usage of a peptide corresponding to the C terminus of TFPI for the treatment of proliferative glomerulonephritis (PGN).

2. Materials and methods

2.1. Expression and purification of the TFPI fragment

To identify the required structure that induces apoptosis in cultured rat MsCs, we expressed a series of TFPI fragments (Fig. 1A) with the pMAL-TM Protein Fusion and Purification System from NEB. Briefly, a gene for each fragment was amplified by PCR and inserted into the pMAL-c2E vector (NEB) with EcoRI and HindIII endonuclease sites. The expression vector was transformed into competent E. coli TB1 cells to prepare the expression strain for each fragment of TFPI. The expression strain was then cultured in rich medium (1% tryptone, 0.5% yeast extract, 0.2% glucose, 0.5% NaCl and 100 µg/ml ampicillin) at 37 ºC in a shaking incubator (250 rpm). When the OD600 of the culture had increased to ~0.8, isopropylthiogalactoside (IPTG) was added to the medium to a final concentration of 0.5 mM to induce protein expression, and the temperature was modified to 28 ºC. Five hours later, the bacteria were collected by centrifugation, washed, and resuspended in buffer 1 (20 mM Tris–HCl pH 7.4, 200 mM NaCl, 1 mM EDTA) and frozen at −80 ºC for several hours. The suspension was then thawed at room temperature, sonicated, and centrifuged to remove any inclusion bodies or other cell debris. The supernatant was retained for the purification steps.

Affinity and ion-exchange chromatography methods were used for protein purification. First, the supernatant was flowed through an amylose resin column buffered with buffer 1. Then, the column was washed with buffer 1 to record a baseline, and eluted with elution buffer (buffer 1 + 10 mM maltose). The target fractions were collected and dialyzed against buffer 2 (50 mM Tris–HCl pH 8.0, 1 mM EDTA) for 12 h at 4 ºC. The desalted fraction was then loaded on a Q-Sepharose Fast Flow (Amersham) column pre-balanced with buffer 2. The column was washed with buffer 2 to record a baseline and the proteins were divided by elution with an increasing salinity gradient. The purified proteins were dialyzed against phosphate buffer (PB, 20 mM, pH 7.4) and freeze dried.

2.2. Cell culture and apoptosis analysis

The rat MsCs used in this experiment had undergone 5–10 passages and were maintained in DMEM medium (GIBCO) supplemented with 10% newborn calf serum (GIBCO), 100 units/ml penicillin and 100 µg/ml streptomycin, at 37 ºC in a 5% CO2 incubator.

For the Hoechst 33258 assay, the cells were seeded on sterile glass cover slips in six-well plates and grown in medium containing 10% newborn calf serum to about 80% confluence, and were made quiescent by serum starvation for 24 h. Batches of quiescent cells were treated with individual fusion proteins at a final concentation of 2 µM. Twenty-four hours later, the cells were fixed, washed twice with phosphate-buffered saline (PBS), stained with Hoechst 33258 staining solution (Beyotime) for 5 min at room temperature, and observed under a fluorescence microscope using a 4’6-diamidino-2-phenylindole (DAPI) filter. Apoptosis was determined according to the appearance of fragmented or condensed nuclei.

For flow cytometry, cells were seeded in 10 cm dishes in complete DMEM medium. At approximately 80% confluency, the cells were washed and serum-starved for 24 h, and then exposed to the appropriate concentration of experimental proteins (2 µM of each protein for the comparison of apoptosis between MBP-TFPI187–276 and MBP-TFPI240–276, or stepwise concentrations of 0.5, 1 and 2 µM for the quantitative analysis of the effect of MBP-TFPI240–276). After 24 h, adherent cells and floating cells were...
harvested. The collected cells were washed with PBS and fixed in citric acid. The apoptotic ratio was analyzed by flow cytometry with propidium iodide (PI) staining of the nuclei and was performed at the Institute of Cell Biology (Shanghai, China).

2.3. Electroporation-enhanced intramuscular gene transfer

To evaluate the in vivo effect of the C terminus of TFPI on MsCs, its gene was fused to the HSA-SP gene with an overlap PCR method and was inserted in the vector VRnew with the EcoRV and BamHI endonuclease sites. The product, referred to as VRnew-SP(HSA)-TFPI240–276 enables the secretory expression of the C terminus of TFPI in muscle tissue. The control vector (VRnew-SP(HSA)) contains only the HSA gene. The validated plasmids were amplified and dissolved in normal saline (NS) to 1 μg/μl.

Rats were anesthetized with pentobarbital sodium in NS (3%, 4 mg/100 g bodyweight) and hair was removed from both hind legs with depilatory paste. The rats were then injected in both tibialis anterior muscles with 100 μg of either the VRnew-SP(HSA) or the VRnew-SP(HSA)-TFPI240–276 plasmids, or with an equal volume of vehicle. The needle was fitted with a plastic collar to limit muscle penetration to approximately 2 mm. After DNA injection, the local skin was covered with conducting gel. Muscle electroporation was performed using electrodes fitted to a pair of calipers. Eight pulses of 200 V/cm and pulse length of 20 ms were delivered at 1 s intervals.

2.4. Animal models

Anti-Thy1 serum (ATS) was prepared as described by Chen et al. [4]. Male Sprague–Dawley rats weighing 90–110 g were obtained from the Shanghai Laboratory Animal Center of the Shanghai Institutes for Biological Science. The body weight and breeding condition were consistent for all rats. After being housed for 3 days, 24 animals were randomly allocated into four groups (n = 6). As shown in Fig. 4A, the animals in group A received an injection of normal serum on days 0 and 7, while animals in groups B–D were injected with ATS to induce the anti-Thy1 nephritis model. Electroporation-enhanced intramuscular gene transfer was performed on days 1 and 8; groups A and B received NS, group C received VRnew-SP(HSA) and group D received VRnew-SP(HSA)-TFPI240–276. Serum and 24 h urine samples were collected on days 0, 6 and 15. Animals were sacrificed at the end of day 15. For each animal, the left kidney was excised and fixed in 10% formalin for the pathology analysis. Urine creatinine and serum creatinine levels were detected using an ADVIA 1650 automatic biochemistry analyzer (Bayer Company). Creatinine clearance rate (Ccr) was calculated based on urine volume, urine creatinine and serum creatinine. Animal studies were conducted using a protocol approved by the committee for the care and use of laboratory animals of Fudan University.

2.5. Glomerulus histopathology

The kidney was fixed in 10% formalin, dehydrated and then embedded in paraffin. Three-micrometer-thick sections were stained with hematoxylin–eosin (HE) or periodic acid Schiff (PAS) reagents. In each HE-stained section, 100 glomeruli were selected for analysis. In PAS-stained sections, deposition of ECM was confirmed by quantitative analysis with Image-Pro Plus 6.0 software.

2.6. Immunohistochemistry

Sections were baked at 56 °C for 8 h, dewaxed and hydrated. Antigens were retrieved by microwave-treatment in 0.01 M citrate buffer (pH 6.0) for 20 min. After the sections had cooled to room temperature, they were soaked in 3% hydrogen peroxidase for 30 min to quench endogenous peroxidase activity. The sections were washed with Tris-buffered saline (TBS; 0.05 M, pH 7.6) three times for 2 min per wash. Then, the primary antibody (mAb anti-α-SMA smooth muscle actin [SMA] or proliferating cell nuclear antigen [PCNA], Boster) was added and the sections were placed in a humidified cabinet and incubated at 37 °C for 1 h and then at 4 °C overnight. After washing with TBS three times, the sections were incubated with mouse/rabbit HRP-IgG (Gentech Company) at 37 °C for 45 min and washed again with TBS. Positive staining was visualized as a reaction to 3,3′-diaminobenzidine (DAB) for 40 s. Subsequently, the sections were stained with hematoxylin for 20 s and soaked in hot water (55 °C) for 3 s. The sections were then dehydrated and covered with a glass cover slip.

2.7. Measurements and statistical analyses

The histopathology assessments were performed under a light microscope by a researcher blind to the treatment groups. The quantification of ECM in the mesangium and α-SMA staining was determined with Image-Pro Plus 6.0 software from nine fields of view at 200×. Statistical analyses were performed using SPSS 11.0 software. The changes in Ccr were analyzed by a mixed linear regression analysis method. Histopathology grading was performed using a rank-sum test and the differences in numbers of total cells, PAS staining, PCNA-positive cells and α-SMA staining were validated by LSD t-tests.

3. Results

3.1. Expression and purification of TFPI fragments

All of the fusion proteins and a free maltose binding protein were expressed after the expression induction with IPTG (Fig. 1B). The molecular weight of each protein was confirmed by SDS-PAGE analysis. The purity of each protein exceeded 95% (Fig. 1C).

3.2. The C terminus of TFPI induces MsC apoptosis in vitro

In the Hoechst 33258 assay detection, cells treated with MBP-TFPI162–276, MBP-TFPI187–276 and MBP-TFPI240–276 exhibited nuclear condensation and fragmentation (Fig. 2D, G and I) whereas the MBP-, MBP-TFPI162–188-, MBP-TFPI187–241- and MBP-TFPI162–241-treated cells showed none of these morphological changes (Fig. 2E, F and H). Therefore, the TFPI162–276, TFPI187–276 and TFPI240–276 fragments, but not the TFPI162–188, TFPI187–241 or TFPI162–241 fragments, induced apoptosis in cultured MsCs. This suggests that the TFPI240–276 region of the C terminus of TFPI is required for apoptosis induction.

However, to exclude a synergistic effect of the KD3 to C terminus of TFPI, we compared the apoptosis ratio between MBP-TFPI187–276- and MBP-TFPI240–276-treated MsCs using flow cytometry. We found no statistical difference in the apoptosis ratio between the MBP-TFPI187–276- and MBP-TFPI240–276-treated MsCs (Fig. 3A). This means that the C terminus of TFPI is adequate for apoptosis induction.

We also examined the effect of the C terminus on MsCs using flow cytometry, which revealed that MBP-TFPI240–276 induces apoptosis in a dose-dependent manner (Fig. 3B).
3.3. Transgenic therapy with the C terminus of TFPI preserved renal function

Renal function is reflected by the value of Ccr. As shown in Fig. 4B, Ccr showed a natural increase as the body weight of each rat increased by about 50 g each week. At the end of the study, the average bodyweight of each group was about 220 g; however, there were marked differences in Ccr between each group. The group treated with normal serum had the largest increment in Ccr ($\Delta$Ccr = 1.8 ml/min) from days 0 to 15. By contrast, the increment in Ccr in the NS- and VRnew-SP (HSA)-treated groups was much smaller ($\Delta$Ccr = 0.75 and 0.88 ml/min). The increment in Ccr in the VRnew-SP (HSA)-TFPI240–276-treated group was partly restored ($\Delta$Ccr = 1.28 ml/min), which indicates that transgenic therapy with the C terminus of TFPI improved renal function.

3.4. The C terminus of TFPI ameliorates glomerulus pathologies

In the histological study, the glomeruli of rats in groups B and C showed marked changes, including mesangial hypercellularity and ECM deposition on the hematoxylin–eosin-stained sections. By contrast, the glomeruli from group A were largely free of these changes. Group D, which was treated with the C terminus of TFPI, showed a significant decrease in the histological changes compared with group C (upper panel in Fig. 4C). These changes in the glomeruli were evaluated semiquantitatively by grading and cell counting. Group B had a high proportion of glomeruli in grades 3 and 4 (44.3%), while in group A was 6.4%; group C was lower than group B (31%); group D was markedly decreased to 19.7%. Furthermore, the proportion of grade 1 glomeruli was markedly increased in group D compared with group C (46.2%/24.5%) (Fig. 4D). The total number of glomerular cells in group D was less than that in groups B and C (69.3 ± 1.15 vs. 79 ± 1.95 and 75.4 ± 1.27; group A: 61.3 ± 1.05) (Fig. 4E). PAS staining revealed that ECM deposition was decreased in group D compared with groups B and C (0.51 vs. 0.57 and 0.56) (Fig. 4C(2) and F).

Fig. 2. Effects of fusion proteins on apoptosis of MsCs. Light blue spots indicate apoptotic cells. Each fusion protein was added to a final concentration of 2 μM: (A) control (PB); (B) MBP; (C) rTFPI; (D) MBP-TFPI162–276; (E) MBP-TFPI162–188; (F) MBP-TFPI187–241; (G) MBP-TFPI240–276; (H) MBP-TFPI240–241; (I) MBP-TFPI187–276 (Hoechst 33258 staining, 400×).

Fig. 3. The C terminus of TFPI induces apoptosis of cultured rat MsCs. (A) Apoptosis ratio of MsCs treated with 2 μM of MBP-TFPI187–276, MBP-TFPI187–241 or MBP-TFPI240–276 for 24 h; **P < 0.01 (compared with the MBP group). (B) MBP-TFPI240–276 induces apoptosis in a dose-dependent manner; **P < 0.01, *P < 0.05. Data are expressed as means ± SEM in parts A and B.
Fig. 4. The C terminus of TFPI ameliorates renal function and pathology in the anti-Thy1 nephritis model. (A) Induction of anti-Thy1 nephritis. Rats were intravenously injected with 500 μl/100 g bodyweight of anti-Thy1 serum (ATS) (groups B–D) to induce the anti-Thy1 nephritis model. The control group (group A) was injected with normal rabbit serum. (B) The C terminus of TFPI maintained the progressive increase in creatinine clearance rate (Ccr). Urine and blood were sampled on days 0, 6 and 15. (C) Kidney glomerulus. Upper panel (1), HE staining (400x); lower panel (2), PAS staining (400x). Both panels, a: group A; b: group B; c: group C; d: group D. (D) The distribution of glomeruli pathology grade was decreased in group D than in group C ($\chi^2 = 48.475, p = 0.0001$). (E) The total number of cells in the glomeruli from VRnew-SP (HSA)-TFPI240–276-treated rats is less than that in the therapy control group. (F) ECM deposition in glomeruli mesangium was less in group D than in groups B and C. **$P < 0.01$, *$P < 0.05$. Data are expressed as means ± SEM in (E) and (F).
3.5. The C terminus of TFPI attenuates MsC proliferation and activation

In response to injury, MsCs change from the smooth muscle phenotype to the embryonic myofibroblastic phenotype, and express the cytoskeletal protein $\alpha$-SMA, while PCNA is a biomarker for cell proliferation. We detected the in situ expression of $\alpha$-SMA and PCNA via immunohistochemistry (Fig. 5A) to examine the proliferation and activation of MsCs. We found that the mean number of PCNA-positive cells in the glomeruli of rats in group D was less than that in groups B and C (11.2 vs. 13.2 and 12.3) (Fig. 5B). Similarly, the mean optical density of $\alpha$-SMA staining in the glomeruli was lower in group D than in groups B and C (0.51 vs. 0.61 and 0.57) (Fig. 5C). This suggests that the C terminus of TFPI inhibited the proliferation and activation of MsCs in vivo.

4. Discussion

The function of the region of TFPI after the second Kunitz-type domain appears to be complex. This region is less associated with its anticoagulation effect but plays active roles in non-coagulant bioactivity, by forming complexes with heparin, low-density lipoprotein (LDL), lipopolysaccharide (LPS), low-density lipoprotein receptor-related protein (LRP) or glycosaminoglycans (GAGs), which induces the cellular binding, internalization of TFPI (or its complex) or anti-inflammatory effect [2]. By creating truncated TFPI proteins, our group, and others, have found that this region exerts an antiproliferative or apoptotic effect on MsCs, human umbilical vein endothelial cells (HUVECs) and vascular smooth muscle cells (vSMCs) [17,9].

In the present study, we divided this region into several fragments, which were fused with MBP, and expressed and purified in a prokaryotic system. These proteins are soluble and active, which enabled us to directly and comprehensively analyze the effect of this region on MsCs. We found that the essential structure that induces apoptosis in MsCs lies in the C terminus of TFPI starting from residue 240. We have characterized the basic sequence of this region and chemically synthesized the corresponding polypeptide (LIKTKRRKQRVKIA). However, this polypeptide was unable to induce apoptosis in MsCs (data not shown). This is consistent with the findings reported by Hembrough et al., who found that a peptide corresponding to residues 254–276 of TFPI inhibited the proliferation of HUVECs by binding to VLDL, while a sequence that was shorter than that synthesized by us was able to maintain the VLDL-binding capacity but was unable to inhibit the growth of HUVECs [10,27]. Because we have also detected the expression of VLDL in

![Fig. 5. The C terminus of TFPI attenuates mesangial cell proliferation and activation. The upper panel in (A) shows PCNA staining, the lower panel shows $\alpha$-SMA staining (immunohistochemistry, 200×). The number of PCNA-positive cells (B) and the $\alpha$-SMA staining intensity (expressed as the mean optical density in C) were decreased in the VRnew-SP (HSA)-TFPI240-276-treated group. **P < 0.01, *P < 0.05. Data are expressed as means ± SEM in (B) and (C).]
MsCs (data not shown), we hypothesized that TFPI induces apoptosis in MsCs by binding to VLDL, which is associated with the inhibition of the PI3-kinase/Akt pathway [20]. It seems that a complete C terminal peptide is essential for this process; however, characterization of the molecular mechanism requires further research.

We are also interested in whether this peptide can induce MSC apoptosis in vivo and whether it could be used in a clinical setting. To achieve these objectives, we used intramuscular gene transfer treatment and found a baseline effect in the comparison between the NS- and VRnew-Sp (HSA)-treated groups (Figs. 4D and E, 5B and C). However, our data confirmed that the C terminus of TFPI has an antiproliferative effect on MsCs. The number of PCNA-positive cells and the total number of cells in the glomeruli were decreased in the VRnew-Sp (HSA)-TFPI540–276-treated group compared with the control groups (NS or VRnew-Sp (HSA)-treated). During proliferation, MsCs are activated and transformed to an embryonic myofibroblastic phenotype, as demonstrated by the increased expression of α-SMA [6,15]. Using immunohistochemistry, we found that the expression of α-SMA was decreased in the treated group compared with the control groups, which indicates that the C terminus of TFPI inhibited the activation of MsCs in glomerulonephritis. Notably, this phenomenon is reflected from that of the in vitro experiments for we have not detected apoptosis of MsCs in renal sections (data not shown), which might be due to lower expression efficiency and/or the complexity of the internal environment.

MsC proliferation results in hypercellularity in the glomerulus and, thus, enhanced production of active factors and abnormal matrix components such as collagen I and III to cause matrix expansion in the mesangium [14,24]. In our study, these histopathological changes were attenuated in the VRnew-Sp (HSA)-TFPI540–276-treated group, which might be due to the inhibition of proliferation and activation of MsCs by the C terminus of TFPI. Moreover, renal protection was provided by treatment with the C terminus of TFPI. The Ccr on day 15 was markedly higher in the VRnew-Sp (HSA)-TFPI540–276-treated group than that in the control groups.

Clinical studies have revealed that TF and its inhibitor, TFPI, are expressed in glomeruli during glomerulonephritis, which implies that a balance between coagulation and anti-coagulation is important in the development of glomerulonephritis [5,21,22]. Endothelial cells, mesangial cells and macrophages produce TF under stimulating conditions. TF increases the activation of FX by FVIIa, and the resulting product causes fibrin deposition and proliferation of resident cells [12,23]. Via inhibition of TF and FXα, TFPI was shown to reduce fibrin deposition, proteinuria and renal impairment in a rabbit crescentic glomerulonephritis model [7]. However, an in vivo effect of TFPI on mesangial cells has not been described until now. We suggest that TFPI exerts an antiproliferative effect on resident cells in the glomeruli either directly via its C terminus or indirectly through the inhibition of FXα, which benefits the turnover of glomerulonephritis.

Because the induction of apoptosis of resident cells is important in the treatment of PGN and because proliferation of MsCs and/or endothelial cells is a frequent phenomenon in these diseases [8,29], we believe that administration of the C terminus of TFPI, which is more readily available and has less effect on coagulation than full-length TFPI, could be used in the treatment of PGN.

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