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ORIGINAL PAPER

Recombinant tissue factor pathway inhibitor induces apoptosis in cultured rat mesangial cells via its Kunitz-3 domain and C-terminal through inhibiting PI3-kinase/Akt pathway

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Abstract Tissue factor pathway inhibitor (TFPI) is an endogenous inhibitor of tissue factor (TF) induced coagulation. In addition to its anticoagulation activity, TFPI has other functions such as antiproliferation and inducing apoptosis. In the present study, we investigated whether or not TFPI induced apoptosis in cultured rat mesangial cells (MsCs) and the possible signal pathway that involved in the apoptotic process. We demonstrated that recombinant TFPI (rTFPI) induced apoptosis in cultured MsCs via its Kunitz-3 domain and C-terminal in a dose- and time-dependent manner by Hoechst 33258 assay, flow cytometry, nucleosomal laddering of DNA, caspase 3 assay. Because the serine/threonine protein kinase Akt has attracted attention as a mediator of survival (anti-apoptotic) signal in MsCs, we investigated the expression of phosphospecific-Akt and its downstream signal phospho-IκB-α and some other signal molecules like Fas and bcl-2. The results indicated that the process of apoptosis triggered by rTFPI is, at least in part, actively conducted by rat MsCs possibly through PI3-Kinase-Akt signal pathway not by binding to tissue factor. Our findings suggest that rTFPI has the potential usefulness in inducing apoptosis of MsCs under inflammatory conditions.

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Introduction

Some kinds of proliferative glomerulonephritis show abnormally cell proliferation and/or extracellular matrix deposition and then process to glomerulosclerosis in their late stage. In many glomerular sclerosing diseases, mesangial cell proliferation is a major important pathological feature in progressive glomerular injuries [1]. Glomerular mesangial cells (MsCs) play significant roles in glomerular inflammation, regulating blood flow, secreting chemotactic cytokines and depositing extracellular matrix. Apoptosis is the major cell clearance mechanism for removal of large numbers of excess mesangial cells in experimental mesangial proliferation [2]. Development of proliferative glomerulonephritis in rat models involves apoptosis of MsCs at different stages of development of the diseases [2, 3]. After the inflammatory stimulating, apoptosis may be limited increases in MsC number, to mediate removal of surplus MsCs and to resume the structure and function of glomerular [2]. In the present study, we analyzed a death factor of MsC which might play a positive role in improving proliferative glomerulonephritis.

Tissue factor pathway inhibitor (TFPI) is a Kunize-type serine protease inhibitor which exhibits a strong and specific inhibitory activity against the tissue factor (TF)-mediated initiation of the blood coagulation cascade. TFPI is mainly synthesized and secreted by the endothelial cells, which inhibits factor Xa directly and the FVIIa/TF catalytic complex in a FXa-dependent fashion [4]. The function of TFPI as the physiologic inhibitor of TF-initiated coagulation has promoted interests in applying recombinant TFPI

(rTFPI) as a substitution therapy in some inflammations and cancers. Some studies show that TFPI has antiangiogenic and antitumor activities via its VLDL receptor binding fragment [5]. In addition, growing evidence indicates that TFPI may play a role in cell anti-proliferation and apoptosis. Kamikubo et al. [6] reported that human recombinant TFPI (hrTFPI) prevented the proliferation of cultured human neonatal aortic smooth muscle cells. Then Hamuro et al. [7] proved that hrTFPI induced apoptosis in culture human endothelial cells and Hembrough et al. [8] demonstrated that the antiproliferative activity of TFPI was mediated by VLDL receptor.

In addition to endothelial cells, TFPI is also produced by glomerular MsCs. Yamabe H et al. [9] found that cultured human MsCs have the ability to produce TFPI which inhibits fibrin formation. Some animal tests and clinical trials also show that TFPI is strongly expressed in human crescentic glomerulonephritis and has the potential to be of therapeutic benefit in the management of fibrin dependent human glomerulonephritis [10, 11]. But whether TFPI has any functions on MsC is still poorly understood. The aim of the present study was to investigate the effect of rTFPI on the apoptosis of rat MsCs in culture. We found that rTFPI inhibited the growth of MsCs and that its action was dependent on the induction of cell death, showing the typical characteristics of apoptosis but not necrosis. These results suggest that TFPI plays an important role in protecting the function of kidney from glomerulosclerosis via the induction of apoptosis in MsCs.

Phosphoinositide 3-kinase (PI3K) phosphorylates the 3′-OH position of the inositol ring of inositol phospholipids [12, 13]. PI3K plays a central role in a diverse range of cellular responses including cell growth, survival, and malignant transformation [14, 15]. Akt (also called protein kinase B) is a downstream signal of PI3K and it is a 60 KD serine/threonine kinase and is activated in response to stimulation of tyrosine kinase receptors such as platelet-derived growth factor (PDGF), insulin-like growth factor, and nerve growth factor [16–18]. Akt is also a critical mediator of survival signals to protect cells from apoptosis [19, 20]. Activation of a PI3K/Akt pathway in response to cytokines leads to phosphorylation and activation of the nuclear factor NFκB p65/RelA subunit, which regulates expression of anti-apoptotic genes [21, 22]. In the present study we investigated the expression of Akt and phospho-IκB-α and demonstrated that rTFPI induced MsCs apoptosis possibly through PI3K/Akt pathway.

Materials and methods

Animals

Male SD rats weighing 160–200 g were used in this study. Rats were housed under specific pathogen-free conditions. All animal experiments were performed in accordance with institutional guidelines, and the Review Board of Fudan University granted ethical permission for this study.

Induction of Thy1 glomerulonephritis

Experimental mesangial proliferative glomerulonephritis (Thy1 GN) was induced by a single intravenous injection of anti-rat Thy-1.1 monoclonal antibody (1 mg/kg) (Cedarlane Laboratories, Ontario, Canada) as described elsewhere [23] once a week for 4 weeks. These rats were sacrificed and nephrectomized 0, 14, 21 and 28 days after injection of the anti-Thy1.1 antibody. Four age-matched rats were injected with vehicle only and were sacrificed as controls.

Immunohistochemistry

Kidney sections of Thy1 GN were processed for immunohistochemistry following standard procedures. To study TFPI in glomerular, formaldehyde-fixed and paraffin-embedded tissue blocks were used. Kidney sections were rehydrated and treated with 1.5% hydrogen peroxide in methanol for 30 min. To expose the antigen, sections were then treated with 0.1% Trypsin/$CaCl_2$ in TBS for 30 min. In order to eliminate nonspecific staining, sections were incubated with the appropriate preimmune serum for 20 min at 37°C and then incubated with the anti-TFPI antibody (1:50 dilution) for 60 min at 37°C and then incubated with the appropriate biotinylated horse anti-mouse secondary antibodies followed by incubation with the avidin-biotin peroxidase complex. Peroxidase conjugates were subsequently localized using diaminobenzidine tetrahydrochloride. For TFPI, the brown area on an immunoperoxidase-stained section was selected for its color range.

Cell culture

Rat mesangial cells were maintained in DMEM medium (GIBCO) supplemented with 10% newborn calf serum (NCS from GIBCO), 100 units/ml penicillin, 100 μg/ml streptomycin, at 37°C in a 5% CO_2 incubator. Cells used in experiments were from 5 to10 passages. After an initial incubation in medium plus 10% NCS until approximately 80% confluence, the cells were made quiescent by serum deprivation for 24 h, then treated with or without PDGF, rTFPI, $rTFPI_{1\text{-}161}$ (lacking the Kunitz-3 domain and the C-terminus), or anti-Fas neutralizing antibody, ZB4, (Upstate) for the indicated time and concentration.

Hoechst 33258 assay

Cells were seeded on sterile cover glasses placed in the 6-well plates. When they grew to approximately 80% confluence, cells were washed and serum deprived in the presence and absence of experimental compounds. At the end of the incubation period, cells were fixed, washed twice with PBS and stained with Hoechst 33258 staining solution (Beyotime) for 5 min at room temperature and observed under fluorescence microscopy using a 4′,6-diamidino-2-phenylindole (DAPI) filter. Fragmented or condensed nuclei were scored as apoptotic.

Induction and determination of apoptosis by flow cytometry

The cells were seeded in 10 cm dishes in complete DMEM medium. At 80% confluence, cells were washed and serum deprived in the presence and absence of experimental compounds. At the end of the incubation period, the adhered cells were scraped from the bottom of the dish and pooled with the floating cells and centrifuged at 2,000 rpm for 5 min. The cells were washed with phosphate-buffered saline (PBS) before fixation in citric acid. After the fixation, the cells were sent to analyze for apoptotic ratio by flow cytometry by propidium iodide (PI) staining of nuclei which is based on the principle that after DNA fragmentation permeabilized cells exhibit a reduced chromatin stainability and accessibility to fluorochromes in the Institute of Cell Biology (Shanghai, China).

Detection of DNA fragmentation

The adherent and floating cells were collected. Cells were incubated in lysis buffer [10 mmol/l Tris, 1 mmol/l EDTA, 100 mmol/l NaCl, 5 g/l SDS, 1 μg/μl RNase A, pH 8.0] at 37°C for 30 min. At the end of incubation, proteinase K was added to a final concentration of 0.1 mg/ml and the incubation was continued at 55°C for 4 h. DNA was extracted with phenol/chloroform and precipitated with ethanol. DNA pellets were dissolved in TE buffer and analyzed on a 1.5% agarose gel.

Western blot analysis

Cells were seeded in 10 cm dishes in complete medium. At 80% confluence cells were washed and serum deprived in the presence and absence of experimental test compound. At the end of the indicated time interval, the dish was placed on ice, and floating cells were collected. The adhered cells were scraped from the bottom of the dish and pooled with the floating cells and centrifuged at 4,000 rpm for 5 min at 4°C to obtain the cell pellet. The cells were lysed by adding cell extraction buffer and the protease inhibitor cocktail (diluted 1:25) (Roche) on ice for 40 min. The lysates were centrifuged at 12,000 rpm for 5 min at 4°C. The supernatant was collected and total protein concentration was determined by BCA™ protein assay kit (Pierce). Approximately 60 μg protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gel. After electroblotting, the PVDF membranes were blocked in blocking buffer (Tris-buffered saline [TBS] containing 0.1% [vol/vol] Tween-20 and 5% [wt/vol] nonfat dry milk) for 2 h and washed in TBS with 0.1% (vol/vol) Tween-20 (TBS/T). The primary antibodies (diluted 1:400) and a second antibody consisting of horseradish peroxidase (HRP)—conjugated goat anti-rabbit IgG (diluted 1:2,000) were used for the detection of active caspase-3 (BioVision) and Fas (Santa Cruz Biotechnology). The primary antibodies (diluted 1:500) and a second antibody consisting of horseradish peroxidase (HRP)—conjugated goat anti-mouse IgG (diluted 1:2,000) were used for the detection of Bcl-2 (Santa Cruz Biotechnology). The primary antibodies (diluted 1:1000) and a second antibody consisting of horseradish peroxidase (HRP)—conjugated goat anti-rabbit/mouse IgG (diluted 1:2,000) were used for the detection of phosphospecific-Akt, total-Akt, phospho-IκB-α (Ser32/36) (Cell Signaling). The bands were visualized by the ECL detection system with 5–10 min exposure. Controls for protein loading were identified by β-actin (Sigma) as the internal standard.

Immunofluorescence

The cells were seeded on sterile cover glasses placed in the 6-well plates. At 50% confluence, they were fixed for 10 min in acetone at 4°C and air dried. The cells were then incubated for 1 h with anti-TFPI monoclonal antibody or anti-TF monoclonal antibody in 37°C. After washed in PBS for 3 times, the cells were incubated for 45 min with FITC-anti-mouse IgG. To investigate the interaction between rTFPI and TF, at 50% confluence cells were washed and serum deprived in the presence and absence of experimental test compound for 24 h. Confluent cells on sterile cover glasses then were washed with PBS, fixed and double-stained with anti-TFPI polyclonal antibody which has no cross-reaction with rat TFPI (American diagnostica Inc) and TRITC-conjugated goat anti-rabbit antibodies and with anti-TF monoclonal antibody and FITC-tagged horse anti-mouse secondary antibodies. rTFPI and TF were visualized with a confocal laser microscopy system (Leica, Wetzlar, Germany).

Results

TFPI expression changes in Thy1 GN

We examined TFPI expression in glomerulonephritis in vivo. We used an acute model of mesangial proliferative glomerulonephritis known as Thy1 GN. In Thy1 GN, the proliferation of MsCs began at day 3, proliferated obviously at day 7 and continue to increase by injected anti-rat Thy-1.1 monoclonal antibody once a week for 4 weeks. TFPI began to express obviously in the glomeruli at day 14, markly increased at day 21 and peaked at day 28 (Fig. 1). These findings indicated that TFPI expressed in the late stage of Thy1 GN and increased with the process of glomerulonephritis.

Mesangial cells express TFPI and TF in its cytoplasm or cytomembrane

TFPI is mainly synthesized and secreted by the endothelial cells. In addition to endothelial cells, many other vascular wall cells can synthesize TFPI, e.g., smooth muscle cells, monocytes, fibroblasts, and cardiomyocytes [24–28]. In order to make sure whether MsC in glomeruli synthesizes TFPI, we investigated the expression of TFPI in cultured rat mesangial cells by immunofluorescence. We found that TFPI was synthesized by MsCs and it was located in the cytoplasm (Fig. 2A, B). We also detected TF which can bind to Kunitz 1 domain of TFPI in MsCs by immunofluorescence and found that TF was expressed in MsCs and located in both cytomembrane and cytoplasm (Fig. 2C, D).

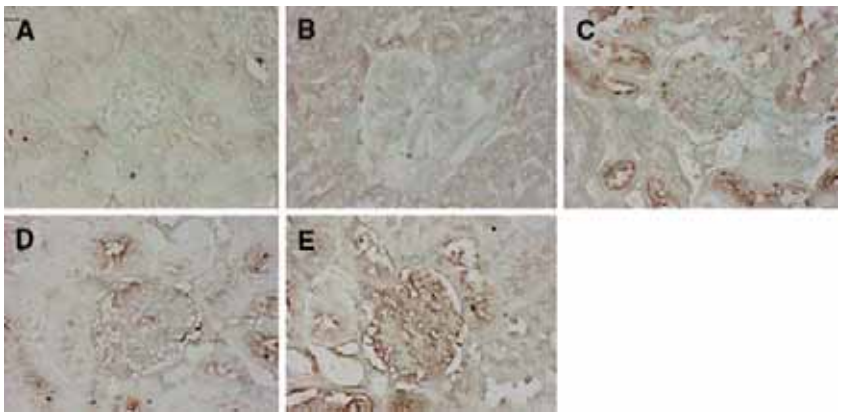

Fig. 1 Time course of expression of TFPI in Thy1 GN glomeruli. Kidney sections from Thy1 GN rats on day 0(**A**), 7(**B**), 14(**C**), 21(**D**) and 28(**E**) were subjected to immunohistochemical staining with antibody against TFPI. TFPI began to express obviously in the glomeruli at day 14, markly increased at day 21 and peaked at day 28

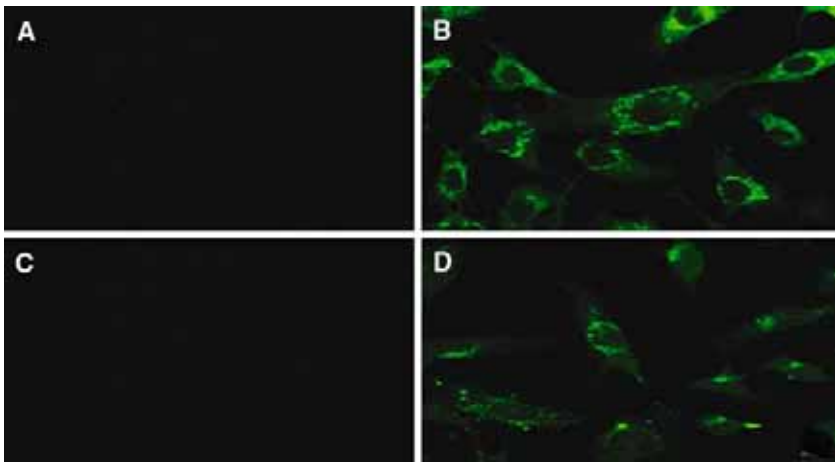

Fig. 2 Cultured rat MsCs express TFPI and TF. The cells were seeded on sterile cover glasses placed in the 6-well plates. At 50% confluence, they were fixed for 10 min in acetone at 4°C and air dried. The cells were then incubated with PBS (**A** and **C**) or anti-TFPI monoclonal antibody (**B**) or anti-TF monoclonal antibody (**D**) in 37°C for 1 h. After washed in PBS for 3 times, the cells were incubated for 45 min with FITC-anti-mouse IgG. Then they were photographed using a confocal microscope (original magnification ×630)

rTFPI promotes nuclear condensation in MsCs via its K3 domain and C-terminal

As apoptosis of kidney cells increased with the process of glomerulonephritis (data not shown), we want to know whether rTFPI has any effect on MsC apoptosis and its mechanism. The cells were therefore examined for possible apoptotic morphology changes and incubated with or without rTFPI in DMEM medium supplemented with 0.5% NCS, then were stained with Hoechst 33258. Fluorescence microscope revealed that compared with controls, condensed bright apoptotic nuclei were readily observed amidst the cells treated with rTFPI (Fig. 3A, B). To investigate the effect of rTFPI on the apoptosis of MsC in the process of MsC proliferation, we added PDGF, which is known to be a potent mediator in the proliferation of MsC [29], to DMEM medium. As a result, we found the same changes as we got in the earlier study (Fig. 3C, D). It suggests that rTFPI has an effect on MsC apoptosis when MsC is proliferating just like proliferative glomerulonephritis. When treated with $rTFPI_{1\text{-}161}$, obviously no nuclear condensation in MsCs was found (Fig. 3E, F).

rTFPI induces MsCs apoptosis in a dose and time dependent manner via its K3 domain and C-terminal

The cell morphology observation in Fig. 3 suggested that exposure of rTFPI to serum-starved MsCs produces an apoptotic effect. To obtain a quantitative characterization of the effect, we examined the cells for apoptotic changes by measuring DNA degradation using flow cytometry. Compared with the control, culturing of MsCs in rTFPI conditions for 24 h markedly increased the fraction of apoptotic cells (Fig. 4A). We also found the same result in the PDGF-treated cells which were incubated with rTFPI (Fig. 4B). These two results both show that addition of rTFPI to the serum-free medium induces cells to apoptosis in a dose-dependent manner (0.5–2 μM rTFPI). We also investigated the effect of rTFPI on the cells in different time. As a result, we found that rTFPI induced apoptosis in MsCs in a time-dependent manner (0–48 h) (Fig. 4C). As well as morphology examination, MsCs were also incubated with $rTFPI_{1\text{-}161}$ in DMEM medium supplemented with 0.5% NCS and were found that $rTFPI_{1\text{-}161}$ had no effect on MsCs apoptosis (Fig. 4A). All these results are consistent with those we found in Hoechst 33258 assay.

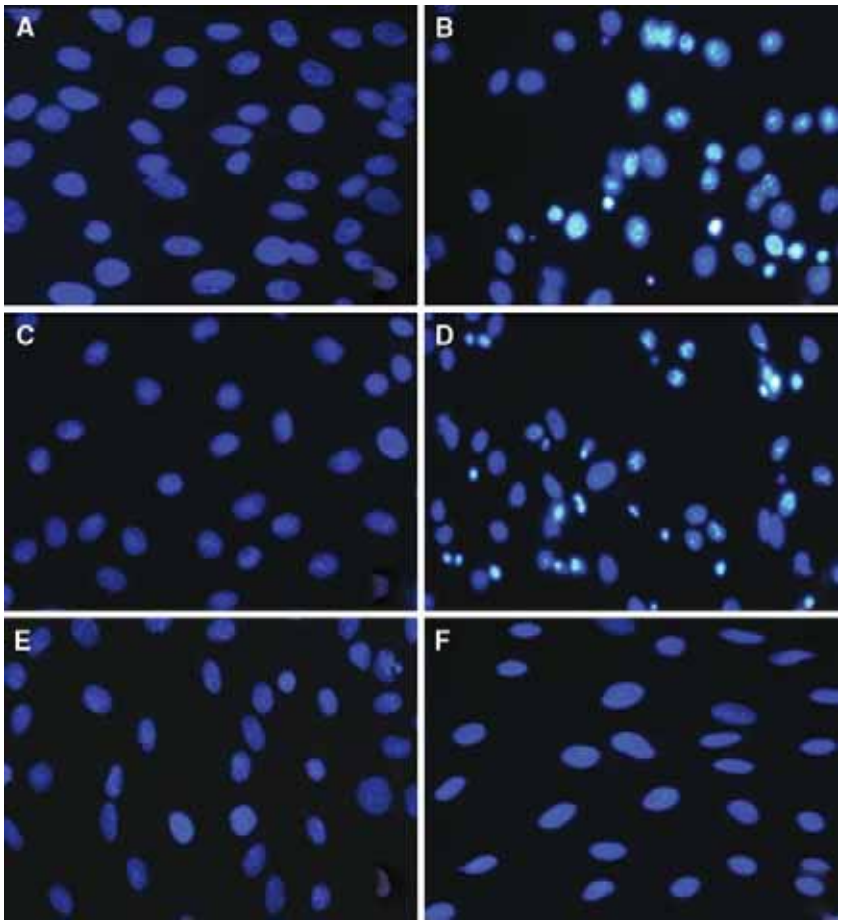

Fig. 3 Morphological effect of rTFPI and $rTFPI_{1\text{-}161}$ on the apoptosis in MsCs. Monolayers of MsCs were incubated with (**B**) or without (**A**) 2 μM rTFPI for 24 h. Monolayers of MsCs were pre-incubated with (**D**) or without (**C**) 2 μM rTFPI for 12 h and then added with PDGF for another 12 h. Monolayers of MsCs were incubated with (**F**) or without (**E**) 2 μM $rTFPI_{1\text{-}161}$ for 24 h. At the end of the incubation, the cells were washed, fixed, and stained with Hoechst 33258. All fields were representative of multiple fields observed in three independent experiments (original magnification ×400)

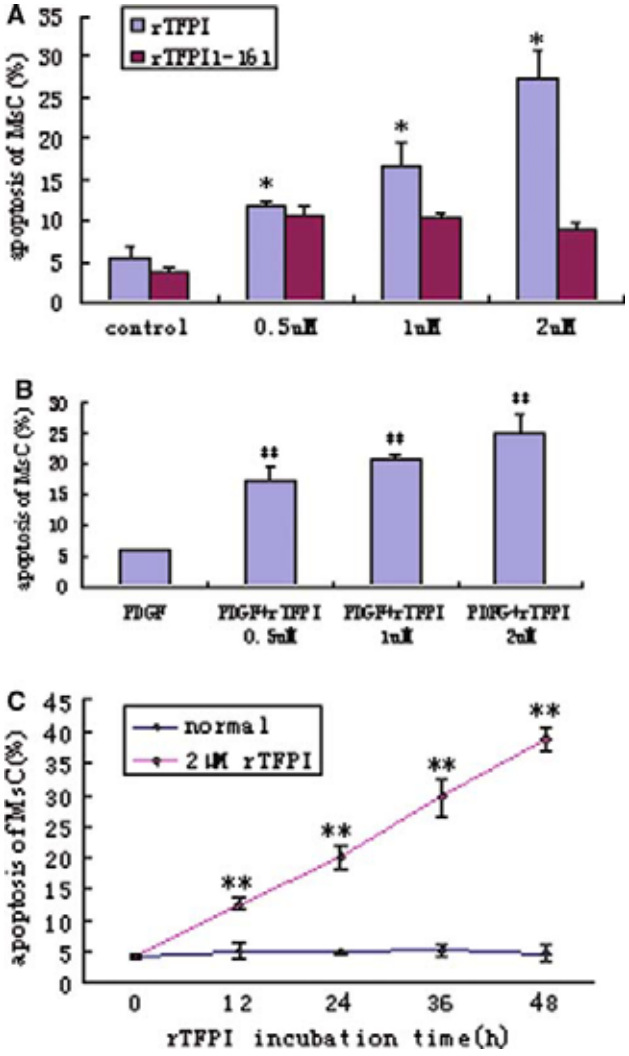

Fig. 4 Quantitation of MsCs apoptosis induced by rTFPI and $rTFPI_{1-161}$. (**A**) The cells were incubated with or without rTFPI or $rTFPI_{1-161}$ in different dose (0.5, 1, 2 μM) for 24 h. (**B**) The cells were pre-incubated with or without rTFPI in different dose (0.5, 1, 2 μM) for 12 h and then added with PDGF for another 12 h. (**C**) The cells were incubated with 2 μM rTFPI for 0, 12, 24, 36, or 48 h. The cells were collected, fixed, and sent to analyze for apoptotic ratio by flow cytometry. Each bar or point indicates the mean of cell apoptosis ± S.D. ($n = 4$). *$P < 0.05$ or **$P < 0.01$ versus control or 0 h

Effect of rTFPI and $rTFPI_{1-161}$ on promotion of DNA degradation

To establish the effect of rTFPI on MsCs further, we used gel electrophoretic analysis of DNA fragmentation to determine whether rTFPI triggered apoptosis in MsCs. The biochemical analyse demonstrated that the typical characteristic of apoptosis is in the cells incubation with 2 μM rTFPI for 48 h. As shown in Fig. 5, the electrophoretic analysis showed that the characteristic pattern of fragmentation of DNA in multiple of 180–200 bp was found after MsCs were treated with rTFPI. This result

Fig. 5 Estimation of apoptotic MsCs by using a gel electrophoretic analysis of DNA fragmentation. The cells were incubated with or without 2 μM rTFPI or $rTFPI_{1-161}$ for 48 h. Extracted DNA from adherent and floating cells was subjected to electrotesis on 1.5% agarose gels. Lane 1, DNA from cells incubated without 2 μM rTFPI; Lane 2, DNA from cells incubated with 2 μM rTFPI; Lane3, DNA from cells incubated without 2 μM $rTFPI_{1-161}$; Lane 4, DNA from cells incubated with 2 μM $rTFPI_{1-161}$

suggests that MsCs death induced by rTFPI was apoptosis. We also examined $rTFPI_{1-161}$ but did not find the characteristic pattern of fragmentation of DNA (Fig. 5).

rTFPI promotes caspase 3 activation

Caspases, a family of cysteine protease, are the central components of apoptotic response. Caspases are a conserved family of enzymes that irreversibly commit a cell to die. They are synthesized as latent zymogens organized in cascade system that upon activation stimulate apoptosis. If the program of apoptosis is stimulated, caspases will be activated. To obtain clues to the mechanism by which rTFPI induces apoptosis, we investigated the activation of the central apoptosis effector, caspase 3, by Western blot analysis using an antibody that recognizes the activated enzyme. rTFPI induced a dose-dependent appearance of a 18–20 KDa caspase 3 band in MsCs (Fig. 6), which represents the active caspase 3.

rTFPI inducing apoptosis of MsCs is not via binding to TF

TFPI is an endogenous inhibitor of TF and has the ability to inhibit TF/VIIa by binding of the first Kunitz domain of

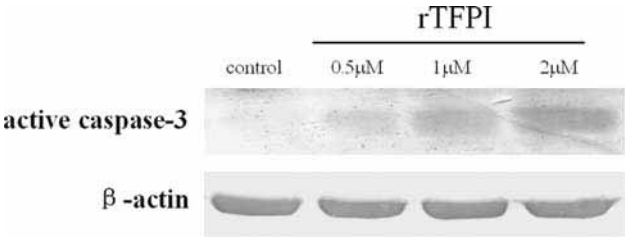

Fig. 6 Activation of caspase 3 in MsCs after rTFPI incubation. Monolayers of MsCs were incubated with rTFPI in different dose (0.5, 1, 2 μM) for 48 h. At the end of incubation, the cells were collected and lysed. Equal amounts of protein (60 μg) were subjected to SDS-PAGE followed by Western blot analysis with anti-caspase 3 antibody. The blots were stripped and reprobed with a *β*-actin-specific antibody as a measure of equal loading

TFPI to TF/VIIa [4], and TF is a transmembrane protein [30] which is also expressed in MsCs. In order to investigate whether rTFPI induces apoptosis of MsCs by binding to TF, MsCs were treated with rTFPI and their binding was detected by immunofluorescence. As Fig. 7 shows, the red fluorescence which represented rTFPI did not merge in the green fluorescence represented TF in MsC cytomembrane. This result suggests that rTFPI inducing apoptosis is not via binding to TF and there is another receptor interacting with rTFPI and tranducting the death signal.

rTFPI inducing apoptosis of MsCs is via Fas but not Bcl-2

Western blot analysis revealed that after MsCs incubated with rTFPI, expression of Fas was increased in a dose-dependent manner compared with control but Bcl-2 protein expression showed no change by the addition of 0.5–2 μM rTFPI (Fig. 8a). Thus, rTFPI inducing MsCs apoptosis maybe triggered the death receptor pathway but the regulation of Bcl-2 expression may not be involved in the potentiation of apoptosis by rTFPI. To verify further that Fas is involved in rTFPI-induced apoptosis, we used anti-Fas neutralizing antibody, ZB4. As a result, rTFPI-induced apoptosis was partly inhibited by blocking Fas (Fig. 8b).

PI3K/Akt pathway possibly involved in apoptosis induced by rTFPI in cultured MsCs

PI3k/Akt pathway is one of main signal pathways involved in MsCs proliferation. To investigate the function role of the PI3K/Akt pathway in the process of apoptosis induced by rTFPI, we examined the expression of phosphospecific-Akt and total Akt after treating MsCs with different dose of rTFPI. As a result, we found that expression of phospho-Akt was blocked after incubation of rTFPI in a dose-dependent manner (Fig. 9). We next examined the

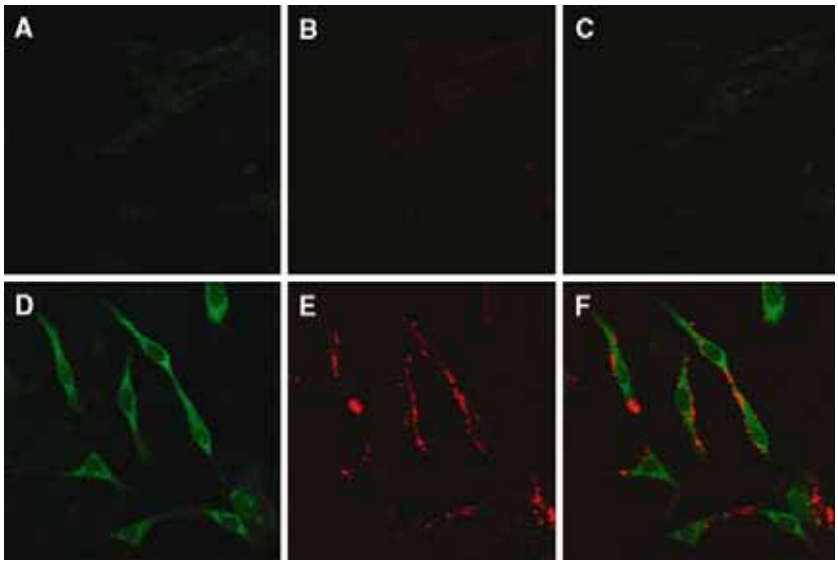

Fig. 7 rTFPI inducing apoptosis of MsCs is not via binding to TF. The cells were seeded on sterile cover glasses placed in the 6-well plates and incubated with or without 2 μM rTFPI for 48 h. At the end of incubation, they were fixed for 10 min in acetone at 4°C and air dried. The cells were then incubated with PBS (**A** and **B**) or anti-TFPI polyclonal antibody (**E**) and anti-TF monoclonal antibody (**D**) in 37°C for 1 h. After washed in PBS for 3 times, the cells were incubated for 45 min with TRITC-anti-rabbit IgG and then incubated for another 45 min with FITC-anti-mouse IgG. Then they were photographed using a confocal microscope (original magnification ×630). (**C**) A merged image of A and B. (**F**) A merged image of **D** and **E**

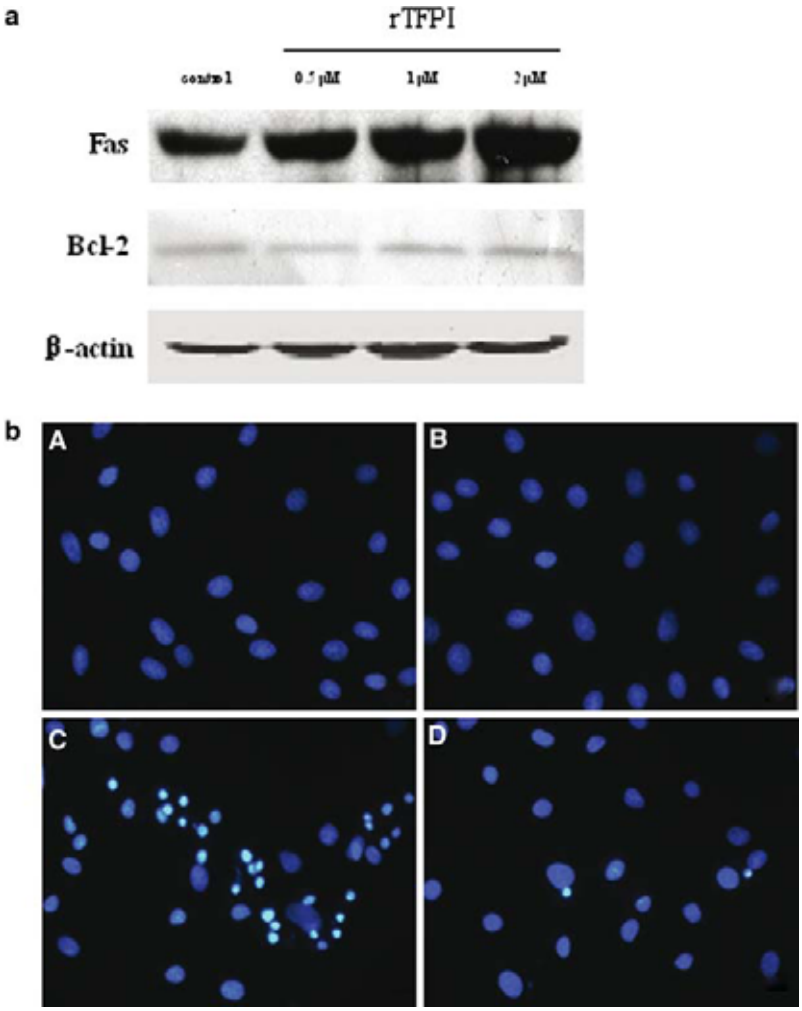

Fig. 8 Expression of Fas and Bcl-2 in MsCs by rTFPI incubation and Fas is involved in rTFPI-induced apoptosis. (**a**) Monolayers of MsCs were incubated with rTFPI in different dose (0.5, 1, 2 μM). At the end of incubation, the cells were collected and lysed. Equal amounts of protein (60 μg) were subjected to SDS-PAGE followed by Western blot analysis with anti-Fas polyclonal antibody or anti-Bcl-2 monoclonal antibody. The blots were stripped and reprobed with a *β*-actin-specific antibody as a measure of equal loading. (**b**) (A) normal MsCs; (B) Monolayers of MsCs were incubated with 1 μg/ml ZB4 for 24 h. (C) Monolayers of MsCs were incubated with 2 μM rTFPI for 24 h. (D) Monolayers of MsCs were pre-incubated with 1 μg/ml ZB4 for 2 h and then added with 2 μM rTFPI for another 24 h. At the end of the incubation, the cells were washed, fixed, and stained with Hoechst 33258. All fields were representative of multiple fields observed in three independent experiments (original magnification ×400)

downstream signal of Akt. Noting that NFκB is held in the cytoplasm as an inactive complex with inhibitor IκB and remains in an inactivate state without IκB phosphorylation, we evaluated the phosphorylation of IκB with phospho-IκB-specific antibody. As shown in Fig. 9, the phosphorylation of IκB was inhibited by rTFPI also in a dose-dependent manner. These results suggested that PI3K-Akt-NFκB pathway possibly involved in apoptosis induced by rTFPI in cultured MsCs and was blocked in the process of apoptosis (Fig. 10).

Discussion

In the present study, we demonstrated that rTFPI had a significant effect on inducing apoptosis in cultured rat MsCs despite MsCs proliferation just like glomerulonephritis. Apoptosis or programmed cell death is a form of cell death wherein the cells participate in their own demise, which is morphologically and biochemically different from necrosis, and occurs under physiologic and pathophysiologic conditions [7]. Apoptosis of MsC has an important significance in proliferative glomerulonephritis. Glomerular MsCs are thought to play a key role in promoting glomerular scarring as they elaborate extracellular matrix, secrete proinflammatory cytokines and regulate glomerular blood flow [31–33]. However, for many years, MsCs had been suggested that they might be particularly resistant to apoptosis for cultured MsCs can elaborate a number of growth factors with demonstrated or potential autocrine effects [34]. Furthermore, MsCs may apparently remain in a quiescent and viable state for many days in vitro in the presence of minimal added growth factors [31, 34]. But now we have discovered that apoptosis could be induced in MsCs.

Baker et al. and Shimizu et al. [2, 35] demonstrated that MsC apoptosis is a cell clearance mechanism counterbalancing MsC division in self-limited anti-Thy 1.1 glomerulonephritis, thereby contributing to the resolution of glomerular hypercellularity caused by experimentally induced MsC proliferation in rats. In the present study, we investigated TFPI expression in rat Thy1 GN and detected

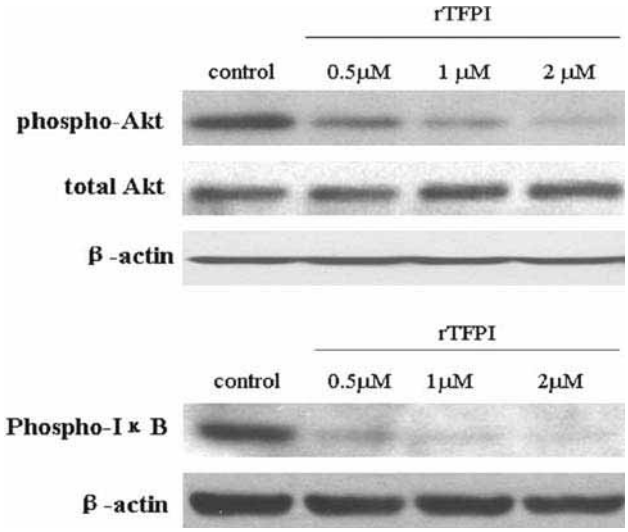

Fig. 9 Expression of phosphor-Akt, total Akt and phospho-IκB in MsCs after rTFPI incubation. Monolayers of MsCs were incubated with rTFPI in different dose (0.5, 1, 2 μM). At the end of incubation, the cells were collected and lysed. Equal amounts of protein (60 μg) were subjected to SDS-PAGE followed by Western blot analysis with anti-phosphor-Akt polyclonal antibody, anti-Akt polyclonal antibody or anti-phospho-IκB-α (Ser32/36) monoclonal antibody. The blots were stripped and reprobed with a β-actin-specific antibody as a measure of equal loading

the effect of rTFPI on the cultured rat MsCs by various methods involving morphological and biochemical analyses. Our experiments showed that TFPI expressed higher in the late stage of Thy-1 GN in vivo and rTFPI could inhibit the growth of cultured rat MsCs and its action contributed to the apoptotic cell death.

TFPI is the major physiologic inhibitor of the extrinsic coagulation pathway. A major pool of TFPI is the form associated with the surface of endothelial cells, which is speculated to play an important role in the regulation of the functions of vascular wall cells [4]. Recent findings indicate that TFPI has another function, i.e., the modulation of cell proliferation. Kamikubo et al. [6] found that rTFPI exhibited inhibitory activity toward the growth of cultured human neonatal aortic smooth muscle cells in addition to its anticoagulation activity. In their later study, they demonstrated that rTFPI completely inhibited the growth of cultured human umbilical vein endothelial cells and its action was dependent on the induction of cell death, showing the typical characteristics of apoptosis but not necrosis [7]. Some other researches also confirmed that TFPI had a significant effect on cell proliferation [5, 36, 37]. Yamabe H et al. [9] found that glomeruli MsC was one kind of the cells which synthesized TFPI. Our result was consistent with it and determined that TFPI was expressed in the cytoplasm of MsCs. In human crescent glomerulonephritis, TFPI was strongly expressed in the later stages of crescent formation and this late induction of TFPI may inhibit TF activity and favor reduced fibrin deposition in the chronic stages of crescent formation [10]. As the same with crescent glomerulonephritis, we found that TFPI was expressed in the later stages of Thy1 glomerulonephritis and continued to increase with the process of inflammation. But the endogenous TFPI is not enough to prevent the process of glomerulonephritis. Our result that rTFPI induces apoptosis in cultured rat MsCs suggests that exogenous rTFPI may help to improve the course of proliferative glomerulonephritis.

The mature TFPI consists of a negatively charged amino-terminal end, three tandemly repeated Kunitz type inhibitory domains, and a positively charged carboxy-terminal end [4]. As we know, TFPI is the endogenous inhibitor of TF [4] and in our present study we have proved that TF is expressed in MsCs. In another way, TF is a transmembrane protein and mediates some signal

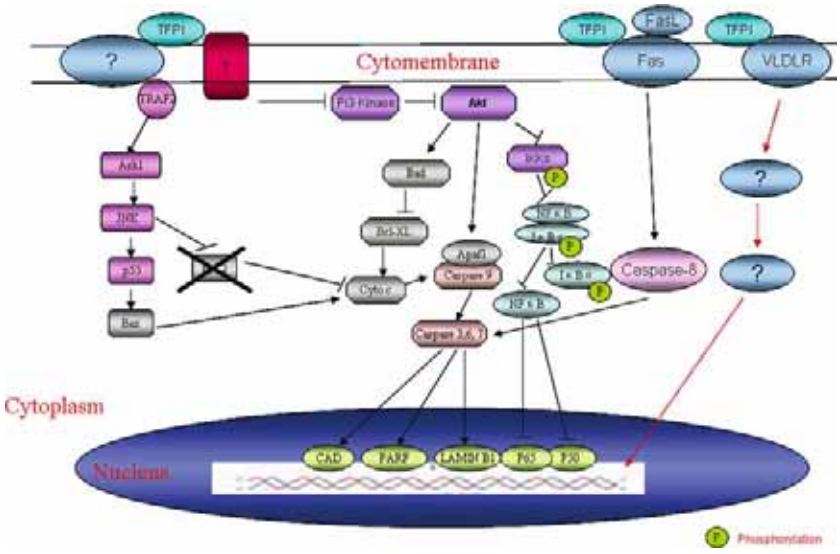

Fig. 10 The possible signal pathways of rTFPI inducing apoptosis in cultured rat MsCs. rTFPI inducing apoptosis in MsCs was not via inhibiting Bcl-2 expression. In the process of apoptosis, Fas and active caspase 3 were increased and those indicated that rTFPI inducing MsCs apoptosis may be through death receptor pathway and activate caspases. In this process phospho-Akt and phospho-IκB expressions were inhibited and these suggested that PI3K-Akt-NFκB pathway possibly involved in apoptosis induced by rTFPI in MsCs. It is possible that rTFPI induced apoptosis in MsCs by binding to VLDLR

transductions [38, 39]. At first we suspected that maybe rTFPI induced apoptosis in MsCs via binding to TF in cytomembrane of MsCs. But our result of confocal suggested that rTFPI was not binding to TF after added to DMEM medium supplemented with 0.5% NCS. Hembrough et al. [36] investigated that a truncated form of TFPI, containing only the first two Kunitz-type proteinase inhibitor domains, has very little antiproliferative activity. This result suggested that the K3 domain and carboxyl-terminal region of TFPI were responsible for its antiproliferative activity. In their following study, they demonstrated that the VLDL receptor binding fragment of TFPI in its C-terminal had apoptotic activity [5]. Optimal cell binding of TFPI requires its K3 domain and C-terminal [40]. After comparing the results of rTFPI and $rTFPI_{1\text{-}161}$, we consider that it is the K3 and C-terminal of TFPI inducing apoptosis of MsCs. We also found MsCs could express VLDL receptor by RT-PCR (data not shown). In the further study we will investigate whether rTFPI interacts with VLDL receptor in MsCs apoptosis.

Apoptosis can be triggered in vivo by various death insults such as hypoxia and growth factor deprivation or by activation of transmembrance death receptors of the FAS/TRAIL/TNF receptor 1 family [41–43]. The Bcl-2 protein is located in the outer mitochondrial membrane and plays a critical role in cell survival [44]. Bcl-2 opposes apoptotic cell death by inhibiting mitochondrial cytochrome-*c* release, which is a key step in the formation of apoptosomes [45]. Both pathways cause activation of caspase, cysteine aspartyl proteases, which are involved in a cascade of cleavage events that result in apoptosis. Caspases are synthesized as latent zymogens that are activated by proteolytic cleavage [41]. In our present study, we detected that Fas and caspase 3 were activated after adding rTFPI to MsCs. These results indicated that activating the death receptor probably is one of the pathways for rTFPI-induced MsCs apoptosis. But Bcl-2 expressed in MsCs incubated with rTFPI had no changes, which suggested that Bcl-2 did not involve in the signal pathway which induces apoptosis in MsCs by rTFPI stimulation.

In this study, we demonstrated that rTFPI suppressed phospho-Akt and phospho-IκB-α protein in a dose-dependent manner. The PI3-kinase-Akt pathway acts as a survival (anti-apoptotic) signal and plays a key role in the regulation of apoptotic change in MsCs [29]. Akt is a dowmstream mediator of PI3K and the growth factor receptor stimulation of Akt has been shown to be dependent on PI3K activity [29, 46]. Akt can exert its anti-apoptotic effects in several different ways. Shimamura et al. [29] identified that NFκB as a downstream mediator of the PI3K/Akt pathway in MsCs. Activation of cytoplasmic NFκB requires the degradation of an inhibitor protein, IκB, which traps NFκB in the cytoplasm. In response to signals, IκB is phosphorylated on two Ser residues in its NH_2-terminal regulatory domain, Ser32 and Ser36, and degraded [47]. Our data suggest that PI3K/Akt pathway possibly involved in apoptosis in MsCs and suppressed by rTFPI. In the future study we will further identify the effect of this pathway in rTFPI-induced apoptosis in MsCs.

Conclusion

In summary, the present data provide evidences for the first time that extrinsic rTFPI induces apoptosis in cultured rat MsCs via its K3 domain and C-terminal. The apoptotic effect is possibly through Fas and PI3K/Akt pathway but is not related to Bcl-2. Therefore, rTFPI may provide a new method to treat proliferative glomerulonephritis. Further studies are needed to elucidate the mechanism of rTFPI induced apoptosis in detail.

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