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ORIGINAL PAPER

Adenovirus-mediated gene transfer of tissue factor pathway inhibitor induces apoptosis in vascular smooth muscle cells

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Abstract
Objective To investigate the pro-apoptotic effect of tissue factor pathway inhibitor (TFPI) gene transfer mediated by adenovirus on vascular smooth muscle cells (VSMCs).
Methods Rat VSMCs were infected with recombinant adenovirus containing either the TFPI (Ad-TFPI) or LacZ (Ad-LacZ) gene or DMEM in vitro. TFPI expression was detected by ELISA. Apoptosis of VSMCs was determined by electron microscopy and flow cytometry. The expression of cytochrome c, procaspase-3, cleaved caspase-3, cleaved caspase-9 and inhibitor of apoptosis protein-1(IAP-1) were examined by western blot and RT-PCR.
Results TFPI protein was detected in the TFPI group after gene transfer and the peak expression was at the 3rd day. At the 3rd, 5th and 7th day after gene transfer, the apoptotic rates in the TFPI group were 11.95%, 71.96% and 37.83%, respectively, whereas those in the LacZ group were 1.34%, 1.83% and 6.37%, respectively. We observed cell contraction, slight mitochondrial swelling, nuclear pyknosis and apoptotic body formation in TFPI-treated VSMCs using electron microscopy. Cytochrome c, cleaved caspase-3 and cleaved caspase-9, which are all involved in mitochondrial pathway, were detected in the cytoplasm on the 3rd, 5th and 7th day after TFPI gene transfer. Procaspase-3 expression was significantly decreased over time in the TFPI group (each $P < 0.05$), which were not seen in the Ad-LacZ and DMEM groups. The expression of IAP-1 mRNA in the TFPI group was also decreased compared with the Ad-LacZ and DMEM groups (each $P < 0.05$) at the 3rd and 7th day after gene transfer.
Conclusion The results demonstrated that overexpression of TFPI gene might induce VSMC apoptosis in vitro through the mitochondrial pathway; meanwhile, IAP-1 expression is decreased. These findings indicated that TFPI might inhibit restenosis by inducing apoptosis in VSMCs.

Keywords Tissue factor pathway inhibitor · Vascular smooth muscle cell · Apoptosis · Restenosis · Caspase · Inhibitor of apoptosis protein-1

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Tissue factor pathway inhibitor (TFPI) is the major physiological inhibitor of tissue factor (TF) and plays a critical role in regulating TF-mediated blood coagulation. TFPI is a Kunitz-type protease inhibitor, which consists of three Kunitz-type domains. It inhibits TF activity by forming a quaternary complex through two steps. First, the Kunitz II domain binds to factor Xa, and then, the Kunitz I domain binds to the TF/FVIIa complex. Recent studies have demonstrated that TFPI is synthesized not only by endothelial cells but also by vascular smooth muscle cells (VSMCs) in the coronary arteries [1].

Restenosis after percutaneous transluminal coronary angioplasty (PTCA) or stents is still a limitation in interventional therapy and occurs in 15–30% of patients after angioplasty procedures or intracoronary stenting [2, 3]. The main causes of restenosis are thrombosis, neointimal hyperplasia and vascular remodeling [4]. Both our and other previous studies have demonstrated that TFPI gene transfer or recombinant TFPI (rTFPI) irrigation can significantly reduce restenosis by inhibiting thrombosis, preventing neointimal hyperplasia and reducing remodeling [5–8]. Insufficient VSMC apoptosis is one of the key

events in restenosis after angioplasty. Hamuro et al. [9] and Tasiou et al. [10] have demonstrated that TFPI can induce apoptosis in endothelial cells and malignant human gliomas. Lin et al. [11] have also found that the process of apoptosis triggered by rTFPI is, at least partially, actively conducted by rat mesangial cells possibly through the PI3-kinase-Akt signal pathway and not by binding to TF. However, there are no reports about the effect of TFPI on VSMC apoptosis.

In this study, we investigated the effect of TFPI on VSMC apoptosis. For the first time, we have demonstrated that the TFPI gene induced apoptosis in VSMCs. The mitochondrion is the target of many signal transduction molecules that promote cell apoptosis and is the integration element of the cell death pathway. Our results suggest that the mitochondrial pathway includes cytochrome c, caspase-9 and caspase-3 activation and may lead to VSMC apoptosis caused by TFPI gene transfer. We have further proved that inhibitor of apoptosis protein-1 (IAP-1) was decreased in the TFPI group when apoptosis appeared.

Materials and methods

Reagents

Anti-β-actin, anti-procaspase-3 and anti-caspase-9(cleaved) antibodies were purchased from Santa Cruz Biotechnology. Anti-caspase-3(cleaved) antibody was purchased from Cell Signaling Technologies. Anti-cytochrome c antibody was purchased from Neomarker Biotechnology. Adenoviruses containing human TFPI gene and LacZ gene (both were 5×10^8 pfu/ml) were obtained from Dr. Yin Xinhua.

Cell culture

Rat VSMCs were cultured by the explantation technique from rat aortic segments and used between passages three and six. VSMCs were maintained at 37°C, 5% CO_2 in DMEM containing 10% FBS (Hyclone).

Adenovirus infection

Vascular smooth muscle cells were grown in 6-well plates in DMEM containing 10% FBS. When the cells were nearly 70–80% confluent, the medium was changed to DMEM without FBS. The Ad-TFPI or Ad-LacZ was added to the medium, respectively, at a multiplicity of infection (MOI) of 100. Two hours later, the cells were washed three times with PBS, and the medium was changed to DMEM containing 10% FBS. As a control in all experiments, an identical group of cells was left uninfected but was incubated for 2 h in serum-free DMEM.

ELISA assay

At the 1st, 3rd, 5th and 7th days after gene transfer, cell culture mediums in each group were collected respectively each day, and quantitative determination of TFPI expression was performed using a specific ELISA kit for human TFPI protein (American Diagnostica Inc.) following the manufacturer's instructions.

Apoptosis analysis

Two methods were used to determine the presence of apoptosis. (a) For electron microscopy, as described previously [12], cells were seeded in 6-well plates, infected as indicated, fixed in 2.5% glutaraldehyde in Hank's modified salt solution, postfixed in 1% OsO_4 in 0.1 M cacodylate buffer, scraped off and dehydrated in a series of ethanol. Dehydration was completed in propylene oxide, and the specimens were embedded in Araldite. Ultrathin sections were produced on an ultramicrotome, mounted on copper grids and contrasted with lead citrate. Specimens were analyzed and documented by electron microscope. (b) An apoptosis detection kit was also utilized. The translocation of phosphatidylserine to the cell surface was detected by an Annexin V-FITC apoptosis detection kit (JINGMEI Biotech, China) at the 3rd, 5th and 7th days after gene transfer. The kit was used according to the manufacturer's protocol.

Western blot

Western blot analysis was performed by the method described previously [13]. At the 1st, 3rd, 5th and 7th days after gene transfer, the cells were washed with PBS and resuspended in cold lysis buffer with PMSF. The cell lysate was incubated on ice for 30 min and centrifuged at 12,000*g* for 15 min at 4°C. The protein content of the supernatant was determined by using a BCA-200 protein assay kit (Beyotime). Equal amounts of proteins (20–50 μg) were loaded into the gel and separated on 10%, 12% or 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The resolved proteins were transferred to PVDF membranes. After blocking with 5% nonfat milk in TBST for 1 h at 37°C, the blots were incubated overnight at 4°C with a primary antibody against β-actin (1:500 mouse monoclonal), cytochrome c (1:200 mouse monoclonal), cleaved caspase-9 (1:200 rabbit polyclonal), procaspase-3 (1:400 rabbit polyclonal) or cleaved caspase-3 (1:500 rabbit polyclonal) diluted in blocking buffer respectively. The membrane was washed with TBST and probed with horseradish peroxidase-conjugated secondary antibody for 1 h at 37°C. The membrane was washed three times in TBST and then treated with DAB according to the

manufacturer's protocol. Protein–antibody complexes conjugated with secondary antibody were visualized on the GIS Imaging System.

Reverse transcription-polymerase chain reaction (RT-PCR) of IAP-1

Approximately 5×10^6 cells were washed with PBS at the 3rd or 7th day after gene transfer in preparation for extracting total cellular RNA. The cells were then resuspended in 1 ml Trizol (Invitrogen), and the total RNA in each group was extracted according to the manufacturer's guidelines. RT was performed with 1 μg of isolated RNA and a reverse transcription kit (Promega). The RT reaction was carried out with random primers according to the manufacturer's protocol. The cDNA (1 μg) was amplified using gene-specific primers. Primers were designed to detect rat IAP-1 (forward: 5′-TGGCTACTTCAGTGG CTCCT-3′; reverse: 5′-GCAAAGCAGGCCACTCTATC-3′, 227 bp) and rat β-actin (forward: 5′-GGCTACAGCT TCAC CACCAC-3′; reverse: 5′-GCTTGCTGATCCACA TCTGC-3′, 499 bp) mRNA levels in each group at the 3rd and 7th days after gene transfer. PCR was performed using a standard protocol recommended by the manufacturer with primer concentrations optimized to 1 μM. Analysis was performed using the GIS detection system.

Statistical analysis

All results are expressed as the mean ± SEM. The variance analysis was used for statistical analysis, and a P value <0.05 was considered statistically significant.

Results

TFPI expression

At the 1st, 3rd, 5th, 7th days after gene transfer, TFPI protein was detected in the TFPI group (Table 1). The peak expression was at the 3rd day. This demonstrated that the exogenous TFPI gene was both transferred into VSMCs and successfully expressed.

TFPI induces apoptosis in rat VSMCs

Morphologically, the cell apoptosis induced by TFPI met all the classical features of apoptosis (Fig. 1). Early stages of apoptosis were characterized by cell contracting, cytoplasm condensing and mitochondria lightly swelling (TFPI group, the 3rd day). Later stages of apoptosis were characterized by nuclear pyknosis, mitochondria and endoplasmic reticulum distension, as well as the formation of apoptotic body (TFPI group, the 5th and 7th day). At the 7th day after Ad-TFPI infection, signs of apoptosis and secondary necrosis were seen in more than 70% of cells. However, no significant changes were observed in the LacZ or control groups.

Table 1 TFPI protein expression in each group

Group	1st Day	3rd Day	5th Day	7th Day
Ad-TFPI	14.32 ± 0.73	14.49 ± 0.87	12.19 ± 0.30	11.83 ± 1.15
Ad-LacZ	ND	ND	ND	ND
DMEM	ND	ND	ND	ND

ND indicates not detectable (mean ± SD, $n = 6$)

Also, TFPI expression led to the exposure of phosphatidylserine on the outside of the plasma membrane. FACS analyses of annexin V-FITC and PI-staining were performed. Cells in the lower quadrant on the right side are annexin V-FITC positive and PI negative, indicating that they are in the early stages of apoptosis (Fig. 2). The apoptotic rates in the TFPI group at the 3rd, 5th and 7th days after gene transfer are 11.95%, 71.96% and 37.83%, respectively, whereas the apoptotic rates in the LacZ group are 1.34%, 1.83% and 6.37%, respectively. At the 7th day, the early apoptotic rates in the TFPI group decreased, but later apoptotic rates increased compared with the 5th day.

These results imply that the inhibition of restenosis by TFPI may be due to its ability to induce VSMC apoptosis.

Molecular mechanisms of TFPI-induced apoptosis in VSMCs

Recent apoptosis studies have revealed that cytochrome c is a pro-apoptotic factor released from the inner mitochondrial membrane at early stages of apoptosis [14, 15]. Cytochrome c leakage from the mitochondria in the cytosolic fraction was detected when significant apoptosis occurred in the TFPI group, as shown in Fig. 3, but not in the LacZ group. These results support the hypothesis that cytochrome c is involved in TFPI-induced VSMC apoptosis.

In eukaryotic cells, the execution of apoptosis depends on the activation of a family of cysteine proteases, known as caspases [16]. Cell lysates from Ad-TFPI- or Ad-LacZ-transferred VSMCs were probed with specific antibodies. Decreases of procaspase-3 and increases of cleaved caspase-3 and caspase-9 were observed and indicate that caspase was activated in the TFPI group. Figure 3 showed that caspase-3 and caspase-9 were activated initially at the 3rd day after gene transfer. Figures 3 and 4 demonstrated that procaspase-3 degradation was observed, which is an indicator of caspase-3 activation, from the 3rd day in the TFPI group ($P < 0.05$). These results further suggested that the mitochondrial signaling pathway was involved in the TFPI-induced apoptotic process in VSMCs.

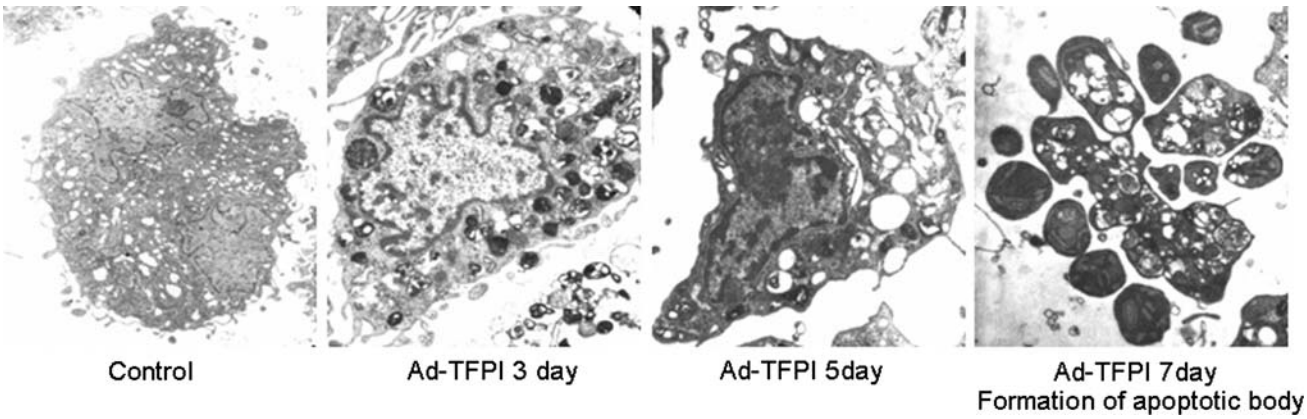


Fig. 1 Electron microscopic features of Ad-TFPI induced VSMC apoptosis. VSMCs were infected at 100 MOI with Ad-TFPI. The cells were fixed and analyzed by electron microscopy at different times after infection (original magnification, A × 4000, B, C, D × 8000)

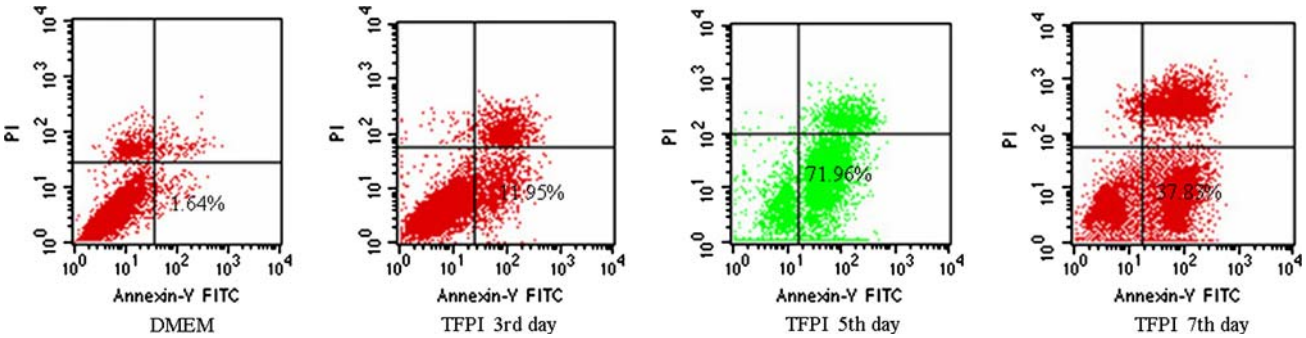
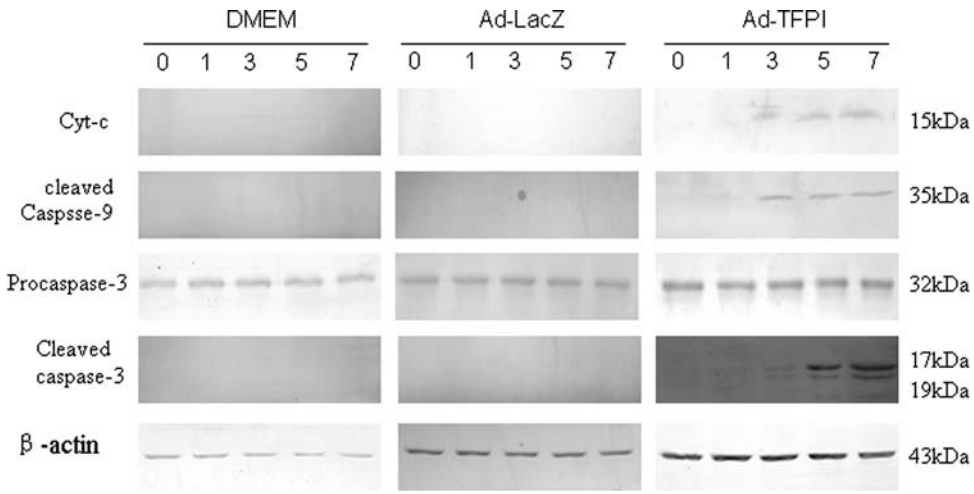


Fig. 2 Induction of apoptosis by TFPI in rat VSMCs at different times after gene transfer

Fig. 3 Western blot results of cyt-c, cleaved caspase-9, procaspase-3 and cleaved caspase-3 in the DMEM, Ad-LacZ and Ad-TFPI groups before and at the 1st, 3rd, 5th and 7th days after gene transfer



Role of IAP-1 during TFPI-induced apoptosis

The levels of rat IAP (RIAP)-1 mRNA were then analyzed by RT-PCR. Infecting VSMCs with Ad-TFPI at MOI 100 caused substantial down-regulation of RIAP-1 mRNA levels at the 3rd and 7th days after gene transfer. This was not observed in the LacZ or control groups (Fig. 5, $P < 0.05$).

Discussion

In this study, we demonstrated that TFPI gene transfer can induce VSMC apoptosis and that this effect is exerted through both activation of cytochrome *c*, caspase-9 and caspase-3 and inhibition of IAP-1 expression.

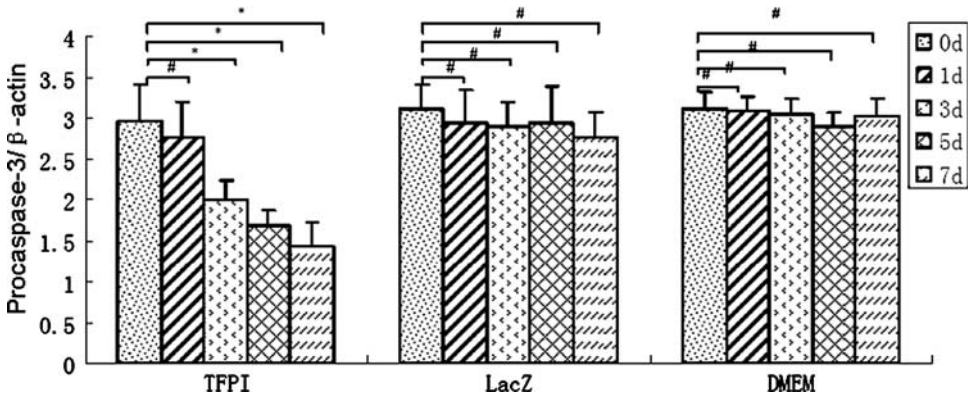


Fig. 4 Procaspase-3 expression in the TFPI, LacZ and DMEM groups before (0 d) and at different time points after gene transfer (1, 3, 5, 7 d). $^{\#}P > 0.05$, $^{*}P < 0.05$

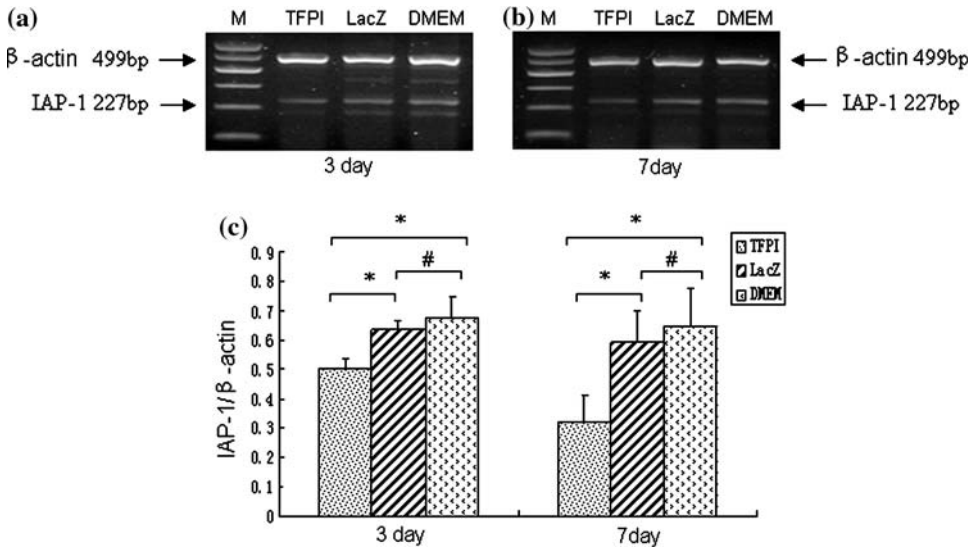


Fig. 5 IAP-1 mRNA expression in different groups at the 3rd and 7th days after gene transfer. (**a**) and (**b**) are RT-PCR results. (c) Shows the analysis of IAP-1 mRNA expression ($^{*}P < 0.05$, $^{\#}P > 0.05$)

Several studies have demonstrated that balloon injury of vessels induces VSMC apoptosis [17]. A rapid onset of apoptosis in medial VSMCs was observed within hours after balloon injury. Kamenz et al. [18] counted the numbers of cells in the intima and examined VSMC proliferation and apoptosis in injured carotid arteries at the 7th, 14th and 28th days after injury. They found that the cell number was highest at the 7th day. Intimal hyperplasia was most obvious on the 28th day. VSMC apoptotic rates were unchanged on the 7th, 14th and 28th days and were all lower than 1%. These results demonstrated that an insufficient increase in cell apoptosis, concomitant with intimal hyperplasia, might be the direct reason for the increasing intimal cell counts those are seen. Previous studies have demonstrated that increasing VSMC apoptosis can reduce neointimal hyperplasia in vivo [19]. Because TFPI can induce endothelial cell apoptosis, we hypothesize that it may prevent the occurrence of restenosis through the induction of VSMC apoptosis.

Malik et al. [20] established the model of balloon-injured porcine coronary arteries and found that medial VSMC apoptosis peaked at 18 h after injury and that decreased apoptosis was observed on the 3rd and 7th days but not on the 14th day. Previous studies have demonstrated that there was weak TFPI expression at the first day after gene transfer but that expression peaked at the 3rd day [21]. In this study we also found that the peak expression of TFPI protein was at the 3rd day by ELISA. As a result, we examined apoptosis at the 3rd, 5th and 7th days after gene transfer and observed, both by electron microscopy and flow cytometry, that TFPI could induce VSMC apoptosis. In detail, TFPI induces apoptosis by the 3rd day after gene transfer. The early apoptosis rate is about 11.95%. The apoptosis rate at the 5th and 7th days are 71.96% and 37.83%, respectively, but the later apoptosis rate is increased on the 7th day, and there are also some necrotic cells observed in the TFPI group by electron microscopy. This is consistent with lower levels of apoptosis on the vessel wall after injury mentioned above and, therefore, contributes to the insufficient increase in cell apoptosis in correspondence with intimal hyperplasia.

The cellular apoptotic process is controlled by both intrinsic and extrinsic mechanisms. Recent studies have revealed that cytochrome *c* is a pro-apoptotic factor.

Leakage of cytochrome c from the inner mitochondrial membrane has been proposed as a central event during the early steps of apoptosis [14, 15]. In particular, cytochrome *c* that is released from the mitochondria into the cytoplasm binds to Apaf-1 during apoptosis [22, 23]. The Apaf-1/cytochrome *c* complex promotes the autocatalytic activation of caspase-9 [24, 25], and activated caspase-9 can cleave and activate procaspase-3 directly, leading to a cascade of additional caspase activation and apoptosis. Studies with cells derived from caspase-9 knock-out mice indicate that caspase-9 is necessary for apoptosis induced by multiple stimuli those are known to trigger cytochrome *c* release from mitochondria [26, 27].

Similar to other cells, VSMCs can also undergo apoptosis in response to a broad array of stress via exogenous and endogenous pathways, such as mitochondria. In this study, we first hypothesized that TFPI gene transfer might induce VSMC apoptosis through the mitochondrial pathway. The increased cytochrome c level in cytoplasm, decreased expression of procaspase-3 and activation of the caspases-3 and -9 were detected in TFPI gene transferred VSMCs but not in the LacZ and DMEM groups. These results further suggested that the mitochondrial signaling pathway may be really involved in the TFPI-induced apoptotic process in VSMCs. George et al. [28] demonstrated that TFPI-2 increases both the activity of caspase-9 and caspase-3 and the expression of cleaved caspase-9 and caspase-3 in U-251 cells, which are similar to our results.

On the other hand, we assume that TFPI exerts its cell-killing activity via a pathway independent of mitochondria. In this aspect, TFPI might cause apoptosis through the IAP pathway. Among apoptotic regulators, considerable interest has been focused on the IAP-1 family, which was first identified as a family of negative programmed cell death regulators [29]. Recent studies demonstrated that several human IAPs (XIAP, c-IAP1 and c-IAP2) inhibit caspases directly [30, 31]. In the caspase activation pathway, IAPs bind directly to procaspase-9 and prevent its processing and activation induced by cytochrome *c*, both in intact cells and cell extracts, where caspase activation is induced by the addition of exogenous cytochrome *c* [32]. IAPs can also bind directly to and potently inhibit the next caspase in the cytochrome *c*/Apaf-1-induced cascade, caspase-3 [22]; therefore, Srinivasula et al. [33] presumed that IAP family proteins can also interfere with a reported amplification loop in which active caspase-3 cleaves and activates additional procaspase-9 molecules. In this study, we observed that IAP-1 mRNA levels in the TFPI group were significantly decreased, as compared to those in the LacZ and control groups at the 3rd and 7th days. Its decrease is consistent with caspase-9 and caspase-3 activation.

Results from the present study highlight the molecular mechanisms underlying TFPI-induced anti-restenosis activity. TFPI-induced cytochrome c leakage from the mitochondria resulted in caspase-9 and caspase-3 activation and eventually caused VSMC apoptosis. TFPI also decreased IAP-1 expression and, therefore, reduced the inhibitory effect of IAP-1 on caspase-3 activation. Because TFPI regulates an array of cellular processes including proliferation, migration and apoptosis [28, 34, 35], which are essential to neointimal hyperplasia and restenosis, adenovirus-mediated TFPI gene transfer may provide a useful tool for the local treatment of these and other vascular proliferative disorders.

Conclusion

The present results demonstrated that overexpression of TFPI gene might induce VSMC apoptosis in vitro through the mitochondrial pathway; meanwhile, IAP-1 expression is decreased. These findings indicated that TFPI might inhibit restenosis by inducing apoptosis in VSMCs.

References

1. Caplice N, Mueske C, Kleppe L, Broze G, Simari R (1997) Expression and regulation of tissue factor pathway inhibitor in arteries and vascular smooth muscle cells. Circulation 96(Suppl I): I–663 (Abstract)
2. Mehran R, Dangas G, Abizaid AS et al (1999) Angiographic patterns of in-stent restenosis: classification and implications for long-term outcome. Circulation 100:1872–1878
3. Williams DO, Holubkov R, Yeh W et al (2000) Percutaneous coronary intervention in the current era compared with 1985–1986: the National Heart, Lung, and Blood Institute Registries. Circulation 102:2945–2951
4. Lefkovits J, Topol EJ (1997) Pharmacological approaches for the prevention of restenosis after percutaneous coronary intervention. Prog Cardiovasc Dis 40:141–158
5. Koop C, Holzenbein T, Steiner S et al (2004) Inhibition of restenosis by tissue factor pathway inhibitor: in vivo and in vitro evidence for suppressed monocyte chemoattraction and reduced gelatinolytic activity. Blood 103:1653–1661
6. Singh R, Pan S, Mueske C et al (2001) Role for tissue factor pathway in murine model of vascular remodeling. Circ Res 89:71–76
7. Zoldhelyi P, Chen ZQ, Shelat H, McNatt JM, Willerson JT (2001) Local gene transfer of tissue factor pathway inhibitor regulates intimal hyperplasia in atherosclerotic arteries. Pro Natl Acad Sci 98:4078–4083
8. Yin XH, Yutani C, Ikeda Y et al (2002) Tissue factor pathway inhibitor gene delivery using HVJ-AVE liposomes markedly reduces restenosis in atherosclerotic arteries. Cardiovasc Res 56:454–463
9. Hamuro T, Kamikubo Y, Nakahara Y et al (1998) Human recombinant tissue factor pathway inhibitor induces apoptosis in cultured human endothelial cells. FEBS Lett 421:197–202
10. Tasiou A, Konduri SD, Yanamandra N et al (2001) A novel role of tissue factor pathway inhibitor-2 in apoptosis of malignant human gliomas. Int J Oncol 19: 591–597
11. Lin YF, Zhang N, Guo HS et al (2007) 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. Apoptosis 12:2163–2173
12. Ho YS, Wang YJ, Lin JK (1996) Induction of p53 and p21/WAF1/CIP1 expression by nitric oxide and their association with apoptosis in human cancer cells. Mol Carcinog 16:20–31
13. Ho YS, Lee HM, Mou TC, Wang YJ, Lin JK (1997) Suppression of nitric oxide-induced apoptosis by *N*-acetyl-L-cysteine through modulation of glutathione, bcl-2 and bax protein levels. Mol Carcinog 19:101–113
14. Mignotte B, Vayssiere JL (1998) Mitochondria and apoptosis. Eur J Biochem 252:1–15
15. Zou H, Li Y, Liu X, Wang X (1999) An APAF-1.cytochrome *c* multimeric complex is a functional apoptosome that activates procaspase-9. J Biol Chem 274:11549–11556
16. Alnemri ES, Livingston DJ, Nicholson DW et al (1996) Human ICE/CED-3 protease nomenclature (letter). Cell 87:171
17. Walsh K, Smith RC, Kim HS (2000) Vascular cell apoptosis in remodeling, restenosis, and plaque rupture. Circ Res 87:184–188
18. Kamenz J, Seibold W, Wohlfrom M et al (2000) Incidence of intimal proliferation and apoptosis following balloon angioplasty in an atherosclerotic rabbit model. Cardiovasc Res 45:766–776
19. Lim S, Jin CJ, Kim M et al (2006) PPARgamma gene transfer sustains apoptosis, inhibits vascular smooth muscle cell proliferation, and reduces neointima formation after balloon injury in rats. Arterioscler Thromb Vasc Biol 26:808–813
20. Malik N, Francis SE, Holt CM et al (1998) Apoptosis and cell proliferation after porcine coronary angioplasty. Circulation 98:1657–1665
21. Atsuchi N, Nishida T, Marutsuka K et al (2001) Combination of a brief irrigation with tissue factor pathway inhibitor (TFPI) and adenovirus-mediated local TFPI gene transfer additively reduces neointima formation in balloon-injured rabbit carotid arteries. Circulation 103:570–575
22. Li P, Nijhawan D, Budihardjo I et al (1997) Cytochrome c and dATP-dependent formation of Apaf-1/Caspase-9 complex initiates an apoptotic protease cascade. Cell 91:479–489
23. Zou H, Henzel WJ, Liu X, Lutschg A, Wang X (1997) Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome c-dependent activation of caspase-3. Cell 90:405–413
24. Hu Y, Benedict MA, Ding L, Nunez Q (1999) Role of cytochrome *c* and dATP/ATP hydrolysis in Apaf-1-mediated caspase-9 activation and apoptosis. EMBO J 18:3586–3595
25. Slee EA, Harte MT, Kluck RM et al (1999) Ordering the cytochrome *c*-initiated caspase cascade; hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner. J Cell Biol 144:281–292
26. Hakem R, Hakem A, Duncan GS et al (1998) Differential requirement for caspase 9 in apoptotic pathways in vivo. Cell 94:339–352
27. Kuida K, Haydar TF, Kuan CY et al (1998) Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. Cell 94:325–337
28. George J, Gondi CS, Dinh DH, Gujrati M, Rao JS (2007) Restoration of tissue factor pathway inhibitor-2 in a human glioblastoma cell line triggers caspase-mediated pathway and apoptosis. Clin Cancer Res 13:3507–3517
29. Crook NE, Clem RJ, Miller LK (1993) An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif. J Virol 67:2168–2174
30. Deveraux QL, Takahashi R, Salvesen GS, Reed JC (1997) X-linked IAP is a direct inhibitor of cell death proteases. Nature 388:300–304
31. Roy N, Deveraux QL, Takahashi R, Salvesen GS, Reed JC (1997) The c-IAP-1 and c-IaP-2 proteins are direct inhibitors of specific caspases. EMBO J 16:6914–6925
32. Deveraux QL, Roy N, Stennicke HR et al (1998) IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. EMBO J 17:2215–2223
33. Srinivasula SM, Ahmad M, Fernandes-Alnemri T, Alnemri ES (1998) Autoactivation of procaspase-9 by apaf-1-mediated oligomerization. Mol Cell 1:949–957
34. Sato Y, Kataoka H, Asada Y et al (1999) Overexpression of tissue factor pathway inhibitor in aortic smooth muscle cells inhibits migration induced by tissue factor/factor VIIa complex. Thromb Res 94:401–406
35. Kamikubo Y, Nakahara Y, Takemoto S et al (1997) Human recombinant tissue factor pathway inhibitor prevents the proliferation of cultured human neonatal aortic smooth muscle cells. FEBS Lett 407:116–120