CLINICAL RESEARCH

The effect of tissue factor pathway inhibitor on the expression of monocyte chemotactic protein-3 and IκB-α stimulated by tumour necrosis factor-α in cultured vascular smooth muscle cells

Effet de l’inhibition de la voie du facteur tissulaire sur l’expression de la protéine-3 et de IκB-α chimiotactique stimulé par le TNF-α dans des cultures de cellules vasculaires musculaires lisses

Yong Zhao a,1, Yu Fu b,1, Jing Hu c, Yue Liu b, Xinhua Yin b,∗

a Department of General Surgery, The First Affiliated Hospital of Harbin Medical University, Harbin, China
b Department of Cardiology, The First Affiliated Hospital of Harbin Medical University, Harbin, China
c Department of Oncology, The Third Affiliated Hospital of Harbin Medical University, Harbin, China

Received 8 May 2012; received in revised form 18 August 2012; accepted 4 September 2012
Available online 21 December 2012

KEYWORDS
Gene therapy; IκB-α;

Summary
Background. – In recent years, the importance of inflammation in restenosis has been recognized gradually. When vascular injury occurs, NF-κB, which controls transcription of many inflammatory genes in restenosis (such as monocyte chemotactic protein-3 [MCP-3]), is

Abbreviations: Ad-LacZ, human recombinant adenovirus expressing bacterial β-galactosidase; Ad-TFPI, human recombinant adenovirus expressing TFPI; CASMC, coronary artery smooth muscle cell; DMEM, Dulbecco’s modified Eagle’s medium; ELISA, enzyme-linked immunosorbent assay; FBS, foetal bovine serum; MCP-3, monocyte chemotactic protein-3; mRNA, messenger ribonucleic acid; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PTCA, percutaneous transluminal coronary angioplasty; RNA, ribonucleic acid; rTFPI, recombinant tissue factor pathway inhibitor; TBST, tris buffered saline with Tween; TF, tissue factor; TFPI, tissue factor pathway inhibitor; TNF, tumour necrosis factor; VSMC, vascular smooth muscle cell.

∗ Corresponding author. Department of Cardiology, The First Affiliated Hospital of Harbin Medical University, No 23, Youzheng Road, Nangang Dis., Heilongjiang Province, 150001 Harbin China. Fax: +86 451 8555 5063.
E-mail address: yinxinhua0910@yahoo.com.cn (X. Yin).
1 Yu Fu contributed equally with Yong Zhao and is the co-first author for this paper.

1875-2136/$ – see front matter © 2012 Elsevier Masson SAS. All rights reserved.
http://dx.doi.org/10.1016/j.acvd.2012.09.003
Introduction

Restenosis after percutaneous transluminal coronary angioplasty (PTCA) and stent placement is closely related to thrombosis, neointimal hyperplasia, inflammation and vascular remodelling [1]. Coagulation and thrombosis are the initializing phases of restenosis; some active substances generated in this process can lead to the formation of neointima by stimulating vascular smooth muscle cell (VSMCs) migration and proliferation, and also by activating an inflammatory response. In recent years, recognition of the importance of inflammation in restenosis has increased gradually. From animal models and clinical experiments, researchers have found that endothelial injury, platelet and leukocyte interactions, cell chemotactic effects and inflammatory mediators are all key factors in the inflammatory response after PTCA and stenting. A method that prevents this inflammatory response may provide a new therapeutic strategy for inhibiting restenosis in clinical situations.

It is well known that the expressions of several genes involved in the inflammatory response and restenosis is regulated at the transcriptional level by NF-κB. In

activated by IkB degradation, leaving the NF-κB dimer-free to translocate to the nucleus to activate specific target genes.

Aims. — To investigate the effect of tissue factor pathway inhibitor (TFPI) on MCP-3 expression and IkB-α degradation stimulated by tumour necrosis factor (TNF-α) in vascular smooth muscle cells (VSMCs), thus further elucidating the mechanism of the inhibitory effect of TFPI on restenosis.

Methods. — Dulbecco’s modified Eagle’s medium or human recombinant adenoviruses expressing TFPI or bacterial β-galactosidase (LacZ) were used to infect rat aortic VSMCs in vitro. Enzyme-linked immunosorbent assays were used to detect exogenous TFPI expression and reverse transcription-polymerase chain reactions were used to detect MCP-3 expression after TNF-α stimulation in transfected cells. Western blotting and immunofluorescence microscopy were used to examine IkB-α expression.

Results. — TFPI protein was detected in the TFPI group after gene transfer. The cells were stimulated with TNF-α for 6 hours on the third day after gene transfer. MCP-3 messenger ribonucleic acid expression was lower in the TFPI group than in the LacZ group (P < 0.05) and IkB-α degradation was lower in the TFPI group than in the LacZ group in the cytoplasm (P < 0.05).

Conclusion. — TFPI inhibited MCP-3 expression induced by TNF-α; this effect may be propagated through the NF-κB pathway. TFPI gene transfer may be a new therapeutic strategy for inhibiting restenosis in clinical situations.

© 2012 Elsevier Masson SAS. All rights reserved.
balloon-injured arteries, NF-κB was found to be activated. The classical activation of the NF-κB pathway can be initiated by a wide range of extracellular stimuli. These agents can activate the cells and mediate phosphorylation of IκB, resulting in its degradation and leaving the NF-κB dimer-free to translocate to the nucleus to regulate many target genes, such as chemokines (e.g., monocyte chemotactic protein-1 [MCP-3]). Recently, MCP-3, which is a member of the CC chemokine group that includes MCP-1 and macrophage inflammatory proteins, was found to be involved in the pathological process of atherosclerosis and restenosis after vascular injury [2–4].

Tissue factor (TF) is the only initiator of coagulation in the classic extrinsic pathway [5]. Tissue factor pathway inhibitor (TFPI) is an anticoagulant protein found in serum. TFPI inhibits factor Xa and the TF/VIIa complex through the formation of TFPI/FXa/FVIIa/TF, regulates TF activity and exerts anticoagulant and antithrombotic effects. Work by our group and others has demonstrated that TFPI gene transfer or recombinant TFPI (rTFPI) irradiation can significantly reduce restenosis by inhibiting thrombosis and neointimal hyperplasia in balloon-injured arteries [6–9]. Many in vitro experiments have indicated that TFPI can induce VSMC apoptosis, inhibit VSMC migration and thereby reduce the formation of neointima [10,11]. At the present time, little is known about the effect of TFPI on inflammation. In this study, we transferred the TFPI gene, mediated by an adenovirus, into VSMCs cultured in vitro. The VSMCs were then stimulated with tumour necrosis factor (TNF)-α and we examined the effect of TFPI on the expression of MCP-3 to further investigate the anti-inflammatory effect of TFPI. To define the anti-inflammatory mechanism of TFPI, we also investigated the effect of TFPI on the degradation of IκB-α because it is known that expression of MCP-3 is regulated by the NF-κB pathway.

Methods

Reagents

The recombinant rat TNF-α was purchased from PeproTech Technologies, Rocky Hill, NJ, USA. The enzyme-linked immunosorbent assay (ELISA) kit for human TFPI protein was purchased from American Diagnostica, Inc., Stamford, CT, USA. The anti-β-actin and anti-IκB-α antibodies were purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA. The reverse transcription-polymerase chain reaction kit was purchased from Promega, Madison, WI, USA. The adenoviruses containing the human TFPI gene (Ad-TFPI) and the LacZ gene (Ad-LacZ) (both 5 × 10⁸ pfu/mL) were obtained from Dr. Yin Xinhua.

Cell culture

Rat VSMCs were isolated from the media of the thoracic aorta of male Wistar rats (170–200 g) and were cultured in Dulbecco’s modified Eagle’s medium (DMEM; HyClone, Cramlington, UK) supplemented with 10% foetal bovine serum (FBS). VSMCs were allowed to grow out from the tissue, which was consequently removed. After confluence was reached, cells were harvested by trypsinase. Cells between passages 3 and 6 were used in the experiments.

Adenovirus infection

The VSMCs were grown in six-well plates using DMEM containing 10% FBS. When 70 to 80% confluence was reached, the cells were serum-deprived for 24 hours. Ad-TFPI or Ad-LacZ was added to the medium at a multiplicity of infection of 100. Two hours later, the cells were washed three times with phosphate buffered saline (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 hours in serum-free DMEM.

Enzyme-linked immunosorbent assay

Cell culture media from each group (n = 5) were collected, respectively, at the first, third, fifth and seventh days after gene transfer. Quantitative determination of TFPI expression was performed using a specific ELISA kit for human TFPI protein (American Diagnostica, Inc.), following the manufacturer’s instructions.

Stimulation immediately after gene transfer

Immediately after gene transfer, some VSMCs were stimulated with TNF-α (10 ng/mL) for 6 hours and the medium was then changed to DMEM containing 10% FBS. The VSMCs that were not stimulated with TNF-α were considered to be the control group. On the third day, approximately 5 × 10⁶ cells were washed with PBS for detection.

Stimulation 3 days after gene transfer

On the third day after gene transfer, some VSMCs were stimulated with TNF-α (10 ng/mL) for 6 hours and were then washed with PBS for detection. The VSMCs that were not stimulated with TNF-α were considered the control group.

Expression of MCP-3 messenger ribonucleic acid (mRNA)

On the third day after gene transfer, approximately 5 × 10⁶ cells in each group (both those stimulated immediately and 3 days after gene transfer) were washed with PBS in preparation for extraction of the total cellular ribonucleic acid (RNA) (each group, n = 6). The cells were then suspended in 1 mL Trizol (Invitrogen, Carlsbad, CA, USA), and the total RNA in each group was extracted according to the manufacturer’s guidelines [10]. Reverse transcription was performed with 1 μg of isolated RNA using a reverse transcription kit (Promega). The reverse transcription reaction was carried out with random primers according to the manufacturer’s protocol. The complementary DNA (1 μg) was amplified using gene-specific primers. The following primers were designed to detect rat MCP-3 mRNA levels in each group (forward: 5′-CATGGAAGTCTGCTGAG-3′; reverse: 5′-TGAAACTTCTAGTCTGCTAG-3′; 494 base pairs) and rat β-actin (forward: 5′-GGCTACAGTCCTCCACAC-3′; reverse: 5′-GCTTTCGATGACACAC-3′; 499 base pairs). The PCR amplification cycles for the MCP-3 mRNA were performed
as follows: 94°C for 3 minutes; 94°C for 30 seconds, 54°C for 30 seconds, 72°C for 1 minute for 30 cycles; 72°C for 5 minutes. The PCR product was electrophoresed using a 1.5% agarose gel. The relative band intensities were measured using the Global Imaging Systems, Inc., Tampa, FL, USA (GIS) detection system. The expression levels of MCP-3 mRNA were expressed as the relative ratio to the amount of β-actin mRNA in each group.

Western blotting

On the third day after gene transfer, VSMCs were stimulated using TNF-α (10 ng/mL) for 6 hours (each group, n = 6). The cells were then washed with PBS and resuspended in cold lysis buffer with phenylmethanesulfonyl fluoride. The cell lysate was incubated on ice for 30 minutes and centrifuged at 12000 g for 15 minutes at 4°C. The protein content of the supernatant was determined by using a bicinchoninic acid (BCA)-200 protein assay kit (Beyotime, Haimen, Jiangsu, China) [10]. Equal amounts of the proteins (50 μg) were loaded into the gel and separated by 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis. The resolved proteins were transferred to polyvinylidene fluoride membranes. After blocking with 5% non-fat milk in tris buffered saline with Tween (TBST) for 1 hour at 37°C, the blots were incubated overnight at 4°C with a primary antibody against either actin (1:500 mouse monoclonal) or IκB-α (1:200 rabbit polyclonal) diluted in blocking buffer. The membrane was washed with TBST and probed with a horseradish peroxidase-conjugated secondary antibody for 1 hour at 37°C. The membrane was washed three times in TBST and treated with 3,3′-diaminobenzidine according to the manufacturer’s protocol. The protein-antibody complexes conjugated with the secondary antibody were visualized using the Global Imaging Systems, Inc. (GIS imaging system).

Immunofluorescence microscopy

VSMCs were grown in 24-well plates in DMEM containing 10% FBS with a slide placed in each well. Either Ad-TFPI or Ad-LacZ was transfected into the VSMCs as described above. At the third day after gene transfer, the VSMCs were stimulated with TNF-α (10 ng/mL) for 6 hours (each group, n = 7). The cells were then washed three times with PBS, fixed with paraformaldehyde for 30 minutes and washed three more times with PBS. After incubating with 0.1% Triton X-100 for 30 minutes and washing, the cells on the slides were incubated overnight at 4°C with a primary antibody against IκB-α (1:50 rabbit polyclonal) in a humidified chamber and were then rinsed three times in PBS, incubated with goat anti-rabbit IgG-fluorescein isothiocyanate-conjugated antibody diluted 1:50 in PBS for 30 minutes and rinsed three more times with PBS. Cell immunofluorescence was observed under microscopy.

Statistical analysis

All of the experiments were repeated at least five times, with similar patterns of results. All results are expressed as mean ± standard deviation. Analysis of variance was used for the statistical analysis and a value of P < 0.05 was considered statistically significant.

Results

Tissue factor pathway inhibitor expression

TFPI protein was detected in the TFPI group on the first, third, fifth and seventh days after gene transfer (Table 1). Peak expression occurred on the third day. This result demonstrated that the exogenous TFPI gene was transferred into the VSMCs and successfully expressed.

MCP-3 mRNA expression in VSMCs in response to TNF-α stimulation immediately after gene transfer

On the third day after gene transfer, the levels of MCP-3 mRNA were measured. The VSMCs that were not stimulated with TNF-α (10 ng/mL) expressed only a very low level of MCP-3 mRNA. In the presence of TNF-α immediately after gene transfer, the expression of MCP-3 mRNA was markedly increased. However, the level of MCP-3 mRNA in the TFPI group on the third day after gene transfer did not decrease compared with the levels in the Ad-LacZ and DMEM groups (p > 0.05, Fig. 1). These results indicated that TFPI could not inhibit the expression of MCP-3 stimulated immediately after gene transfer. We speculated that there was not enough effective TFPI protein expressed at this moment.

MCP-3 mRNA expression in VSMCs in response to TNF-α stimulation 3 days after gene transfer

The results showed that the expression of MCP-3 mRNA was markedly increased after stimulation on the third day after gene transfer and that the level of MCP-3 mRNA in the TFPI group was lower than the levels in the Ad-LacZ and DMEM groups (P < 0.05; Fig. 2). These results indicated that TFPI could inhibit the expression of MCP-3 that was stimulated by TNF-α on the third day after gene transfer.

Effect of TFPI on the expression of IκB-α using Western blotting in response to TNF-α stimulation 3 days after gene transfer

The data indicated that the levels of IκB-α in the cytoplasms of cells in each stimulated group were markedly decreased after the stimulation compared with those in the normal control group (P < 0.05), but that the expression of the IκB-α protein in the TFPI group was greater than that in the LacZ group (P < 0.05; Fig. 3). These results indicated that TFPI might be capable of inhibiting the degradation of IκB-α.

Effect of TFPI on the expression of IκB-α using immunofluorescence microscopy in response to TNF-α stimulation 3 days after gene transfer

The results showed that there was a moderate-intensity green fluorescence in the cytoplasm of the VSMCs with no TNF-α stimulation and that no green fluorescence was found in the nucleus. After TNF-α stimulation, the green
fluorescence in the cytoplasm of cells in each group was weakened compared with in the normal control group, which had no stimulation, but the fluorescence intensity in the TFPI group was slightly stronger than that in the LacZ group (Fig. 4). This result was consistent with the results from the Western blotting.

**Discussion**

PTCA and stenting have been the most common methods used to treat coronary artery disease since the world’s first angioplasty was successfully performed in 1977. Restenosis is still a limitation of interventional therapy after angioplasty procedures or intracoronary stenting. The mechanisms of restenosis are very complex. To date, many animal restenosis models have been established to explain the mechanisms of restenosis [12,13]. Increasing evidence has indicated that inflammation plays an important role in the development of restenosis.

TFPI is an endogenous anticoagulant protein and is the only substance known to regulate the TF-dependent coagulation pathway. TFPI, which consists of three Kunitz domains, inhibits TF activity by forming a quaternary complex through two steps. First, the Kunitz II domain binds to factor Xa and the Kunitz I domain then binds to the TF/FVIIa complex. Many studies have shown that TFPI reduces neointimal hyperplasia in local injured arteries by inhibiting VSMC proliferation and migration [14,15] and thereby has the potential for wide application in preventing thrombosis and restenosis. However, the effect of TFPI on inflammation has not yet been investigated in detail; only two reports currently exist regarding this topic. Nakamura et al. [16] demonstrated that the number of macrophages in the intima and media was significantly decreased by irrigating rTFPI into the local balloon-injured arteries. Kopp et al. [17] transferred Ad-TFPI into local injured rabbit femoral arteries and found that MCP-1 expression in the neointimal hyperplasia was markedly decreased in the TFPI-treated arteries, but the mechanism was not clear.

VSMCs play a key role in the intimal hyperplasia caused by inflammation. After endothelial denudation, VSMCs can be induced to generate many inflammatory factors (including chemokines), which then act on the macrophages and VSMCs again [18]. The chemokines that play an important role in leukocytic chemoattraction and activation belong to a group of proinflammatory factors. In the formation of neointima, some chemokines, such as MCP-1, are upregulated.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Tissue factor pathway inhibitor protein expression in each group.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>1st day</td>
</tr>
<tr>
<td>Ad-TFPI</td>
<td>13.62 ± 0.81</td>
</tr>
<tr>
<td>Ad-LacZ</td>
<td>ND</td>
</tr>
<tr>
<td>DMEM</td>
<td>ND</td>
</tr>
</tbody>
</table>

Data are mean ± standard deviation; n = 5. Ad-LacZ: human recombinant adenovirus expressing bacterial β-galactosidase; Ad-TFPI: human recombinant adenovirus expressing tissue factor pathway inhibitor; DMEM: Dulbecco’s modified Eagle’s medium; ND: not detectable.

Figure 1. Monocyte chemotactic protein-3 (MCP-3) messenger ribonucleic acid (mRNA) expression on the third day after gene transfer in different groups stimulated with tumour necrosis factor (TNF)-α immediately after gene transfer (n = 6). A. Representative autoradiographs of the MCP-3 polymerase chain reaction (PCR) products. B. Semiquantitative data regarding MCP-3 mRNA expression in vascular smooth muscle cells (VSMCs). Ad-LacZ: human recombinant adenovirus expressing bacterial β-galactosidase; Ad-TFPI: human recombinant adenovirus expressing tissue factor pathway inhibitor; DMEM: Dulbecco’s modified Eagle’s medium. *P < 0.05.
TFPI on monocyte chemotactic protein-3 and IκB-α expression

Figure 2. Monocyte chemotactic protein-3 (MCP-3) messenger ribonucleic acid (mRNA) expression on the third day after gene transfer in different groups stimulated with tumour necrosis factor (TNF)-α 3 days after gene transfer (n = 6). A. Representative autoradiographs of the MCP-3 polymerase chain reaction (PCR) products. B. Semiquantitative data regarding MCP-3 mRNA expression in vascular smooth muscle cells (VSMCs). Ad-LacZ: human recombinant adenovirus expressing bacterial β-galactosidase; Ad-TFPI: human recombinant adenovirus expressing tissue factor pathway inhibitor; DMEM: Dulbecco’s modified Eagle’s medium. *P < 0.05.

Figure 3. Western blotting was used to determine the expression of the IκB-α protein in the cytoplasm of each group after stimulation with tumour necrosis factor (TNF)-α at the third day after gene transfer (n = 6). A. Western blotting result of IκB-α protein expression. B. Semiquantitative data regarding IκB-α protein expression. Ad-LacZ: human recombinant adenovirus expressing bacterial β-galactosidase; Ad-TFPI: human recombinant adenovirus expressing tissue factor pathway inhibitor; DMEM: Dulbecco’s modified Eagle’s medium. *P < 0.05.

and activate and induce VSMC proliferation, migration and extracellular matrix secretion, which ultimately leads to macrophage accumulation and neointimal hyperplasia. MCP-3 is known to have biological functions in common with MCP-1 [19]. Wang et al. [4] found that stimulating cultured VSMCs with inflammatory factors could induce MCP-3 mRNA expression. In vivo, a significant induction of MCP-3 mRNA (a 41-fold increase compared with the control) was observed in the carotid artery 6 hours after balloon angioplasty. The expression tendency of MCP-3 was in accordance with that of MCP-1. Maddaluno et al. [20] also found that MCP-3 was produced by human coronary artery smooth muscle cells (CASMCs) and directly induced CASMC proliferation in vitro, which suggests a potential role for MCP-3 in vascular pathology. These results indicate that these two CC chemokines play a valuable role in restenosis after vascular injury.
In this study, we transferred the TFPI gene into VSMCs cultured in vitro and stimulated cells with TNF-α to observe the effect of TFPI on the expression of inflammatory factors. The results showed that after stimulation with TNF-α for 6 hours at the third day after gene transfer, the MCP-3 mRNA levels in the TFPI, LacZ and DMEM groups were all increased compared with those in the normal control group (each \( P < 0.05 \)); however, the MCP-3 mRNA level was significantly lower in the TFPI group than in the LacZ and DMEM groups (\( P < 0.05 \)). These results indicated that TFPI might inhibit the expression of MCP-3 mRNA stimulated by TNF-α. Thus, we speculated that TFPI might prevent macrophage accumulation and VSMC migration and proliferation by inhibiting the proinflammatory factor MCP-3 and therefore inhibit neointimal proliferation.

NF-κB is an important transcription factor that participates in the inflammatory reaction. Maruyama et al. [21] found that thrombin activates the thrombin receptor on the surface of VSMCs; the signal from thrombin/thrombin receptors then activates NF-κB and results in inflammation. Sharma et al. [22] showed that deletion of the cytoplasmic domain of TF could reduce NF-κB activation induced by endotoxin. Therefore, we speculated that TFPI might reduce the production of thrombin by inhibiting TF, block the activation of the NF-κB pathway and exert an anti-inflammatory effect. Using Western blotting and immunofluorescence techniques in this research, we investigated whether TFPI could inhibit the expression of inflammatory factors by blocking the NF-κB pathway. The data indicated that TFPI could inhibit the degradation of IκB-α in the cytoplasm. Therefore, it is preliminarily presumed that TFPI might exert an anti-inflammatory effect by blocking the activation of the NF-κB pathway; this should be demonstrated in detail in future studies.

In this study, we also stimulated VSMCs with TNF-α for 6 hours immediately after gene transfer and changed the medium to DMEM containing 10% FBS. On the third day after gene transfer, we found that TFPI could not inhibit MCP-3 mRNA expression using this stimulation method. We hypothesized that not enough TFPI protein was generated within 6 hours after gene transfer to inhibit inflammatory factors induced by TNF-α. A large quantity of stimulating and inflammatory factors is produced in a short period after balloon injury; however, during this short period of time, the TFPI gene transferred into the artery wall could not be translated into an active protein in time to exert its function. Therefore, as we previously described [23], the use of TFPI gene transfer and rTFPI irrigation at the same time in the injured arteries has been proved to be a better method.

The present findings showed that TFPI could inhibit the expression of proinflammatory factor MCP-3 and thus prevent VSMC proliferation and reduce neointimal thickening. At the same time, we found that TFPI could inhibit the degradation of IκB-α induced by TNF-α in the cytoplasm, and we
presumed that TFPI might exert an anti-inflammatory effect by blocking the activation of the NF-κB pathway. Because TFPI can regulate numerous pivotal molecular mechanisms involved in neointimal hyperplasia and restenosis, such as cell proliferation, migration, apoptosis and inflammation [14,24,25], adenovirus-mediated TFPI gene transfer may provide an effective method for the local treatment of restenosis and other vascular proliferative diseases. As TFPI has an anti-inflammatory effect, it may also be useful in the treatment of inflammatory diseases in the future.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

Acknowledgements

This work was supported by the Heilongjiang Science and Technology Tackling Fund (No G080C402-02), Scientific Research Fund of Heilongjiang Provincial Education Department (No 12511304), Natural Science Foundation of China (No 81200143) and Heilongjiang Traditional Chinese Medicine Scientific Research Project (ZHY12-W035).

References