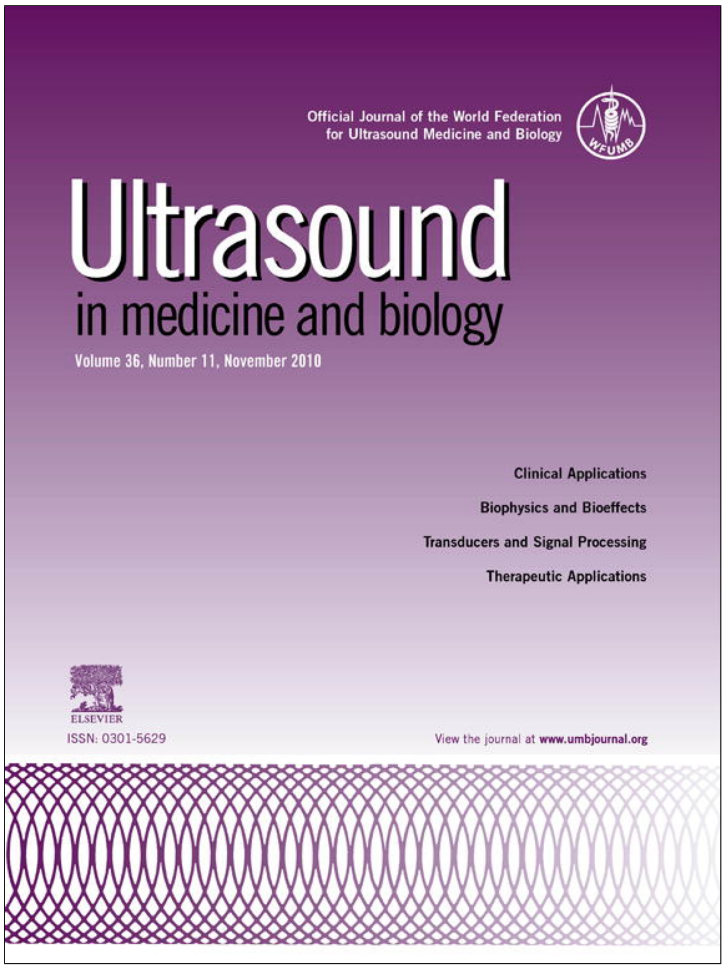

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● *Original Contribution*

DELIVERY OF TFPI-2 USING ULTRASOUND WITH A MICROBUBBLE AGENT (SONOVUE) INHIBITS INTIMAL HYPERPLASIA AFTER BALLOON INJURY IN A RABBIT CAROTID ARTERY MODEL

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Abstract—Here we report a new, simple and efficient method by using ultrasound and a microbubble agent (SonoVue) for delivering a gene to balloon-injured carotid arteries for restenosis prophylaxis. The tissue factor pathway inhibitor-2 (TFPI-2) has been shown to inhibit the postinjury intimae hyperplasia in atherosclerotic vessels. New Zealand white rabbits were divided into 4 groups with 14 in each, a treatment control for balloon injury, a gene vehicle control, a gene delivery of TFPI-2 without using ultrasound and a gene delivery of TFPI-2 using ultrasound. After four weeks, the injured artery neointimal proliferation was significantly lower in the TFPI-2 group with ultrasound than the control groups ($p < 0.01$) according to the measurement of the mean luminal diameters by B-mode ultrasonography. The ratio of intimal/media area and the stenosis rate in the gene delivery facilitated by ultrasound were significantly lower than those of the nonultrasound gene delivering method ($p < 0.01$). (E-mail: chenjuanlinda@gmail.com) **© 2010 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **TFPI-2, Intimal hyperplasia, Ultrasound, Carotid artery, SonoVue.**

INTRODUCTION

Postinjury intimal hyperplasia and vascular stenosis remain unresolved problems in the treatment of vascular disorders. Catheter-based interventions, including balloon angioplasty and stenting, initially restore blood flow in obstructed arteries in more than 95% of patients. Within six months, however, vasospasm, thrombosis and intimal hyperplasia cause clinically significant renarrowing of arteries in 15–40% of treated patients (Lefkovits and Topol 1997).

The pathogenesis of intimal hyperplasia after vascular injury is believed to involve diverse signaling cascades that ultimately converge on vascular smooth muscle cells (VSMC). These cascades, which are involved in blood clotting, include the tissue factor (TF)/factor VIIa complex, factor Xa, and thrombin (Ko et al. 1996; McNamara et al. 1993; Sato et al. 1997), and they exert chemotactic and mitogenic activity on VSMC and other cells.

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Tissue factor (TF), a member of the superfamily of cytokine receptors, plays a role in embryonic blood vessel formation (Carmeliet et al. 1996) and is the initiator of blood coagulation and thrombosis (Rapaport and Rao 1992). In animal models of vascular injury, however, the inhibitor of TF (TF pathway inhibitor, or TFPI) (Han et al. 1999; Jang et al. 1995; Oltrona et al. 1997) has been recognized as a key factor for counteracting the TF pathway of blood coagulation and thrombosis in the pathogenesis of postinjury intimal hyperplasia.

The effect of TFPI on the development of intimal hyperplasia may play a prominent role when vascular insult takes place in an atherosclerotic vessel. Indeed, previous studies have reported that overexpression of TFPI-2 by local viral-mediated gene transfer in normal rat carotid arteries can reduce neointimal proliferation after balloon angioplasty (Zoldhelyi et al. 2001). Although this method resulted in rapid cellular uptake and higher transfection efficiency, it is still far from feasible as a human therapy. The adenoviral vector seems to be very efficient when applied via intra-arterial incubation within the local lumen (Feldman et al. 1996), but there are some theoretical and practical disadvantages to this approach, including strong immunogenicity (Dzau et al. 1996). Despite the efficiency, the safety of

the gene transfer method has become a considerable issue because infusion of adenovirus has recently been shown to cause adverse side effects (Marshall 1999). Although angioplasty can successfully save patients with coronary artery disease, 30–40% of patients who undergo angioplasty develop restenosis (Bult 2000; Mehan and Meier 1993). Because the rate of restenosis is so high, there is a need to establish alternative and efficient gene transfer approaches for the prophylaxis of restenosis. When considering actual treatment for re-occlusion of the vessel, it is desirable to look for nonvirus-mediated TFPI-2 transfection. Here, we demonstrated the feasibility of TFPI-2 in the treatment of intimal hyperplasia after balloon injury in a rabbit carotid artery model using the ultrasound-SonoVue delivery method.

MATERIALS AND METHODS

Plasmid construction and preparation

The pEGFP-TFPI-2 plasmid was constructed in our laboratory. The 4.7-kb plasmid pEGFP-N1, an expression vector for the enhanced green fluorescent protein (EGFP) gene, was obtained from Invitrogen (Carlsbad, CA, USA). The 753-bp human TFPI-2 gene (from Proteintech Group, Inc., Chicago, IL, USA; GenBank: NM001032281) was inserted into compatible enzyme restriction sites (XhoI and BamHI) of pEGFP-N1 to generate pEGFP-TFPI-2. The forward primer was TGT GGG ATC CAT GAT TTA CAC AAT GAAG, and the reverse primer was TAT ACT CGA GCG ACA TAG GCA TGA AAT GCT ATC. The polymerase chain reaction product of the TFPI-2 gene was digested with the restriction enzymes XhoI and BamHI. The desired fragment was cloned into pEGFP-N1 to generate pEGFP-TFPI-2. The sequences of the constructed plasmids were confirmed by DNA sequencing. High-purity plasmid DNA was prepared by the following described experiments in large scale.

In vivo *gene transfer into balloon-injured arteries.* Healthy New Zealand white rabbits of both genders (2.5 kg, age range 24 to 39 months) were obtained from the Center of Medical Experimental Animals (Hubei Province, China). All rabbits were treated under protocols approved by the animal care committee of Huazhong University of Science and Technology. All rabbit experiments were approved by the Institute of Animal Care and Use Committee.

A total of 56 New Zealand white rabbits were used for TFPI-2 expression studies. Anesthesia was induced with xylazine and ketamine and maintained after endotracheal intubation with isoflurane in oxygen. A 5-F sheath was inserted into the right femoral artery, and heparin (150 U/kg) was administered. A balloon angioplasty catheter with a 20 × 2.5-mm balloon was introduced into the femoral sheath and was advanced over a guide wire to the right common carotid artery. The balloon was inflated five times to 8 atm (1 atm = 101.3 kPa) (30 s each inflation; 60-s intervals between inflations). After the last inflation, the balloon was withdrawn to the caudal end of the injured carotid segment. The cut-down sites and the skin wounds were repaired and the rabbits allowed to recover.

A mixture of 100 μg of pEGFP-TFPI-2 (1 μg/μL) and 200 μL of SonoVue (Bracco Diagnostics, Inc., Stony Brook, NY, USA) (45 μg/mL) was incubated for 20 min at room temperature and was injected into the ear vein through a 22-gauge intravenous cannula, followed by a flush of 2 mL of 0.9% normal saline solution. After injection, the right common carotid artery was quickly sonoporated for 3 min using ultrasound until the SonoVue microbubbles had largely disrupted from the circulatory system.

Dalteparin 60 U/kg (Fragmin, Pharmacia & Upjohn Inc., Bridgewater, NJ, USA) was given 1 h after surgery and was repeated once after 12 h. The rabbits were euthanized 3 d or 4 wk after surgery, and the carotid arteries were harvested.

The rabbits were divided into four groups: (i) a treatment control for balloon injury (0.9% NaCl, $n = 14$); (ii) a gene delivery of TFPI-2 without using ultrasound (SonoVue + TFPI-2, $n = 14$); (iii) a gene vehicle control (US + SonoVue + EGFP, $n = 14$); and (iv) a gene delivery of TFPI-2 using ultrasound (US + SonoVue + TFPI-2, $n = 14$). Of the 56 rabbits, 28 were used in the acute study (3 d after surgery), and 28 used in the 4 weeks study. Both genders of rabbits were evenly and randomly distributed in each group. In all rabbits that had undergone surgery, the right carotid artery served as the injured group specimens, and the contralateral side was used as self-control.

Ultrasound (US) equipment

For gene transfer system, US was generated using a 1.5-MHz therapeutic US (Accusonic, Metron Medical Australia Pty. Ltd., Carrum Downs, Australia) with a pulse repetition frequency of 100 Hz and a probe area of 0.8 mm^2. The nominal spatial peak-temporal average (SPTA) intensity varied from 0.2–3.0 W/cm^2. The US parameters in this experiment were set at an intensity of 1 W/cm^2 with a 20% duty cycle.

For measurement of luminal diameters of rabbit carotid arteries *in vivo*, rabbits in each group ($n = 7$) were anesthetized with trichloroacetaldehyde monohydrate (200 mg/kg, ip) four weeks post-surgery. GE Vivid7 Ultrasound Machine (GPS Medical, Inc., Indianapolis, IN, USA) was used to examine the luminal diameters of the rabbit carotid arteries. The settings of ultrasound scanner used in this study were performed as follows: imaging mode (B-mode), MI (1.2 as read on screen),

frequency (13 MHz), depth (3.5 to 4.0 cm), focus position (0.5 to 1) and focus number (1).

Transfer efficiency assessment

After surgery, the vessels were harvested at 3 d for fluorescence microscopy and Western blot analyses of TFPI-2 expression. Some carotid arteries were embedded in cryoprotective compound (dimethyl sulfoxide, Triangle Biomedical Sciences, Durham, NC, USA) and transferred to –80°C. Tissue sections in 10 μM were observed by fluorescence microscopy (Olympus Optical Company, Ltd., Tokyo, Japan).

The carotid arteries used for Western blotting were homogenized in 1 mL of lysis buffer containing 50 mM NaCl, 10 mM Tris, 1 mM EDTA, 1 mM PMSF, 0.5 mM Na3VO4.12H2O, 50 mM NaF and 1 mM benzamidine. The samples were centrifuged at 12,500 g for 15 min at 4°C. The protein concentration was determined using the BCA Protein Assay Reagent (Pierce Com., Rockford, IL, USA). Equal amounts of lysate protein (20 μg) for each sample were loaded on a 7.5% sodium dodecylsulphate (SDS)-polyacrylamide gel for electrophoresis and were blotted onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Nonspecific binding to the membranes was blocked with 3% BSA in tris buffered saline (TBS) for 1 h at room temperature. They were then incubated with a monoclonal antibody to human TFPI (Santa Cruz, CA, USA) overnight at 4°C. Western blots were visualized using enhanced chemiluminescence detection reagents (Sigma, St Louis, MO, USA) according to the manufacturer's instructions. Quantification of protein bands was performed via scanning with the Bio-Rad GelDocTM XR and Chemi DocTM XRS systems (Bio-Rad, Hercules, CA, USA), and the bands were analyzed using Quantity One 1-D Analysis Software Version 4.6.3. (Bio-Rad, Hercules, CA, USA).

Immunohistochemistry

Four weeks after injury, the rabbits were killed with a sodium pentobarbital overdose. The injured carotid artery and a segment of the contralateral uninjured carotid artery of each rabbit were collected into 4% paraformaldehyde, cut into 2-mm rings and embedded in paraffin. Five-micrometer tissue sections were fixed for 15 min in 4% formaldehyde in phosphate-buffered saline, incubated in 3% H_2O_2, blocked in 2% horse serum and exposed for 1 h to a monoclonal antibody to proliferating cell nuclear antigen (PCNA) (Santa Cruz, CA, USA). The primary antibody was diluted 1:200. The immunoreaction was visualized using horseradish peroxidase (HRP)-labeled immunoglobulin G as the secondary antibody (Zymed, South San Francisco, CA, USA) and diaminobenzidine tetrachloride (500 μg/mL) (Sigma) as the substrate.

Some sections harvested four weeks after the initiation of treatment were stained with hematoxylin and eosin (HE). Magnified images were captured using an Axiophot microscope (Zeiss, Austin, TX, USA) and a digital camera (Leaf Systems Lumina, Southboro, MA, USA). Images were processed with software from Optima Imaging Analysis Systems (Version 6.5, Media Cybernetics, Silver Spring, MD, USA).

Vasomotor studies

The vasomotor studies were conducted as described by Channon *et al.* (1998). Freshly harvested vessels free of fat and connective tissues were cut into helical strips, mounted in 30-mL organ baths containing Krebs-Henseleit buffer (mmol/L: NaCl 120, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4, $NaHCO_3$ and glucose 5.5 mmol/L, pH 7.4) and maintained at 37°C and oxygenated with 95% O_2/5% CO_2. Vessels were equilibrated for 60 min, with changes of bathing fluid every 15 min. Isometric tension studies were performed using a Grass model 7D polygraph (Grass Instrument Company, Quincy, MA, USA). Optimal resting tension was determined in baseline studies, and the response to vasoactive drugs was then determined. Cumulative dose-response curves to phenylnephrine (PE, 10^{-9} to 10^{-4} mol/L) were established. The vessels were then submaximally precontracted with PE (typically 3×10^{-6} mol/L), and endothelial function was evaluated by vascular relaxation to acetylcholine (Ach, 10^{-8} to 10^{-4} mol/L). Contractile responses were measured from the polygraph chart and expressed as a percentage of the maximal contraction or, for relaxations, as a percentage of the precontracted tension.

Statistical analysis

One-way analysis of variance followed by the *post hoc* test was used to determine significant differences in multiple comparisons among groups. A level of $p < 0.05$ was considered statistically significant.

RESULTS

Efficiency of TFPI-2 gene transfer by the ultrasound-SonoVue delivery system in a rabbit carotid artery model

Initially, we performed *in vivo* transfer of pEGFP-TFPI-2 and pEGFP constructs into injured rabbit carotid arteries using ultrasound with SonoVue. Infusion was performed immediately after balloon inflation. At 3 d after infusion, the fluorescence in the injured rabbit carotid arteries was detected by fluorescence microscopy. Vessels from the treatment control group revealed no specific fluorescence (Fig. 1a). A paucity of fluorescence was detected in the intima area of the vessel wall of the

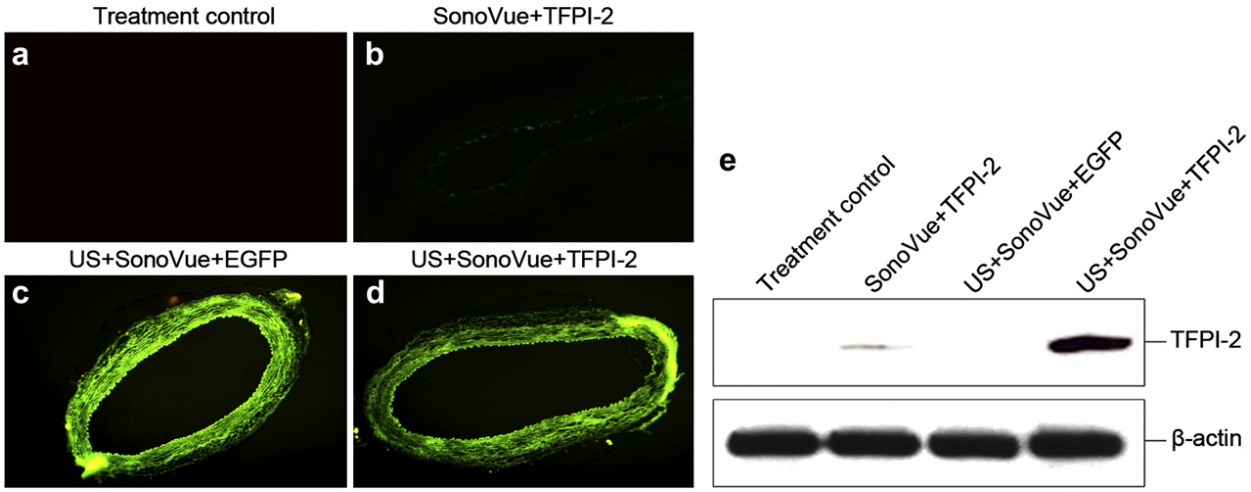

Fig. 1. Overexpression of TFPI-2 in rabbit carotid artery by using ultrasound with SonoVue. Representative images of EGFP-TFPI-2 expression in blood vessels are (a) treatment control, (b) SonoVue+TFPI-2 group, (c) US+SonoVue+EGFP group and (d) US+SonoVue+TFPI-2 group. Human TFPI-2 protein expression analyzed by Western blot is shown in (e). Fluorescence microscopic images are taken at 10× magnifications. These data are representative of three independent experiments.

TFPI-2 gene transfer without using US (Fig. 1b) and the strong fluorescence was detected in the deep area of the vessel wall of the TFPI-2 gene delivery with US (Fig. 1d). With the gene vehicle control, there was also some degree of plasmid DNA fluorescence detection (Fig. 1c). However, this vehicle DNA expression had no effect on the vessel neoplasma proliferation (Figs. 2 and 3). Western blots showed that the specific human TFPI-2 band was detected in the groups with TFPI-2 transfection at 3 d after gene transfer (Fig. 1e). Moreover,

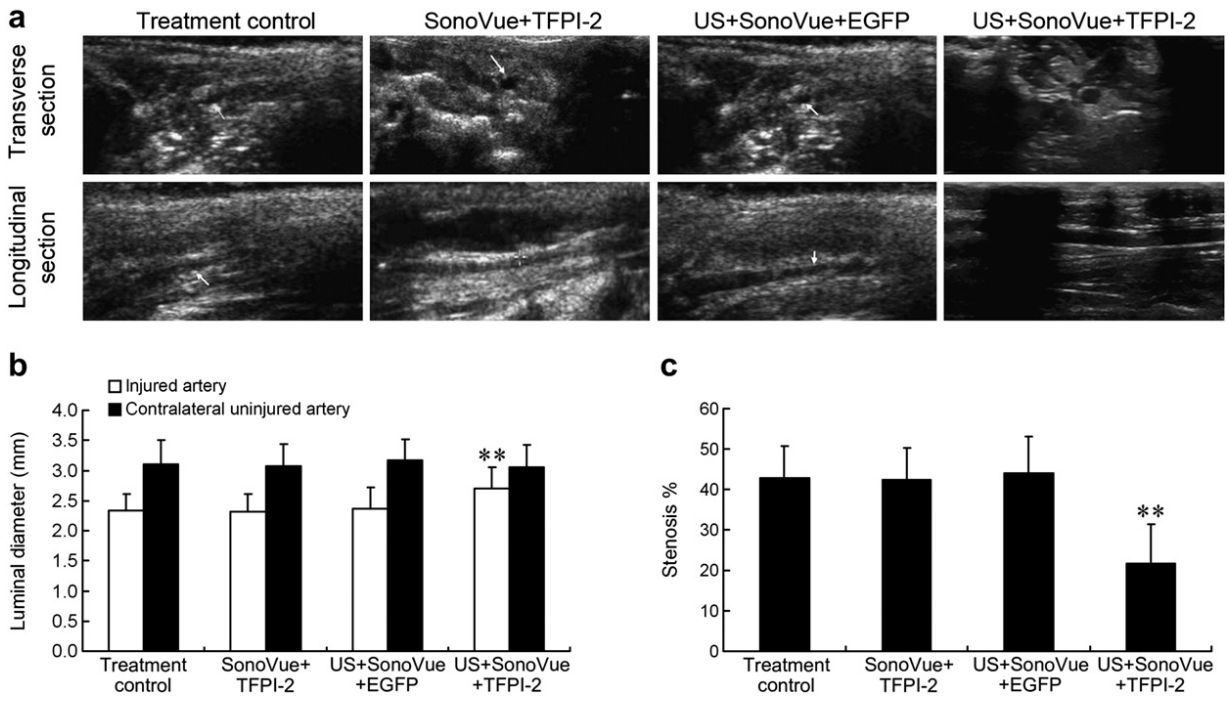

Fig. 2. Effect of TFPI-2 transfer on stenosis in rabbit balloon injury model at four weeks after gene delivery. (a) Representative B-mode ultrasound images of balloon-injured vessels with or without expression of TFPI-2. The measurements in (b) and (c) were B-mode images derived from *in vivo* ultrasound scans. The average luminal diameter in the injured arteries and contralateral uninjured arteries is shown in (b) and the degree of stenosis in the injured arteries is shown in (c). $^{**}p < 0.01$ compared with all other groups.

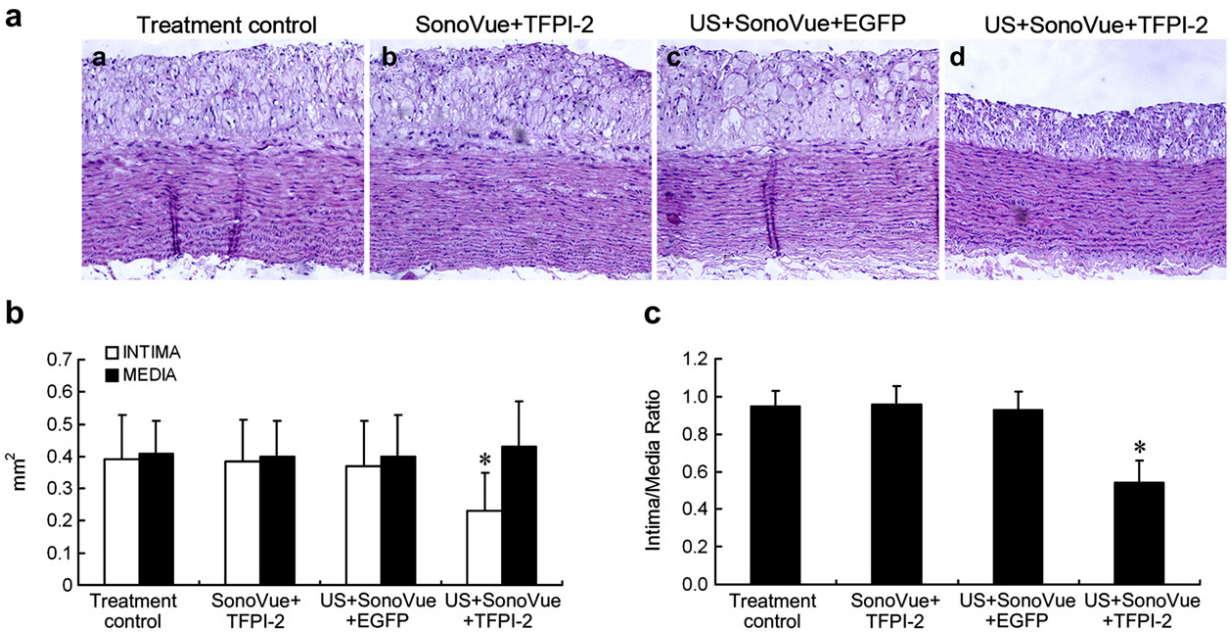

Fig. 3. Effect of TFPI-2 expression on intimal hyperplasia in rabbit balloon injury model at four weeks after its delivery. The top (a) is the representative images of Hematoxylin-eosin staining in rabbit balloon-injured carotid arteries: (a) treatment control, (b) SonoVue+TFPI-2 group, (c) US+SonoVue+EGFP group and (d) US+SonoVue+TFPI-2 group. The bottom (b) is the representative quantitative histomorphometry of intima/media with or without ultrasonic transfer of TFPI-2 (*left*) and individual intima/media ratios observed in injured arterial sections (*right*). $*p < 0.05$ *vs.* other groups.

the fluorescence of US+SonoVue+TFPI-2 group was still observed up to two weeks after gene infusion, whereas the weak fluorescence of the SonoVue+TFPI-2 group disappeared at 7 d after treatment (data not shown). These findings established the feasibility of using TFPI-2 with the ultrasound-SonoVue approach to treat vascular disease.

Effects of TFPI-2 gene transfer into rabbit balloon-injured carotid arteries

We examined the inhibitory effects of TFPI-2 transfection on intimal hyperplasia after balloon injury in a rabbit carotid artery model.

As shown in Figs. 2 and 3, TFPI-2 could significantly suppress intimal hyperplasia and the stenosis rate in the carotid arteries of the injured rabbits.

At 4 weeks after surgery, we measured the luminal diameters using high-frequency B- mode ultrasonography, and we calculated the intima/media areas ratio using histopathology measurements. The lumens of the arteries from the treatment control group, SonoVue+TFPI-2 group and US+SonoVue+EGFP group had more plaques than those of the US+SonoVue+TFPI-2 group, in which there was no plaque (Fig. 2a). The mean luminal diameters of the arteries of the US+SonoVue+TFPI-2 group, the SonoVue+TFPI-2 group, US+SonoVue+EGFP group and the treatment control group were 2.71 ± 0.35 mm, 2.39 ± 0.25 mm, 2.34 ± 0.19 mm and 2.32 ± 0.21 mm, respectively. There were statistically significant differences between the US+SonoVue+TFPI-2 group and other groups ($p < 0.01$) (Fig. 2b). The results indicated that a trend toward a larger lumen size was caused exclusively by a reduction in neointima formation. Stenosis (42.97 ± 9.8%, treatment control group; 21.62 ± 7.7%, US+SonoVue+TFPI-2 group) was of borderline statistical significance (Fig. 2c).

The mean intima/media ratio of the TFPI-2-transferred injured carotid arteries was reduced by 41% ($p < 0.05$) compared with that of treatment control group vessels, same as the reduction of the absolute intima area (0.39 ± 0.14 mm^2, treatment control group; 0.23 ± 0.12 mm^2, US+SonoVue+TFPI-2 group). There was no significant change in the medial area (Fig. 3).

Effect of TFPI-2 gene transfer on DNA synthesis in rabbit balloon-injured carotid arteries

At four weeks after transfer, we assessed DNA synthesis in rabbit balloon-injured carotid arteries by PCNA staining. Inhibition of neointimal formation by TFPI-2 gene transfer using ultrasound with SonoVue was associated with a significant decrease in DNA synthesis in neointimal cells, as assessed by PCNA-positive staining ($p < 0.01$, Fig. 4). Many PCNA-positive cells could be detected in the neointimal area

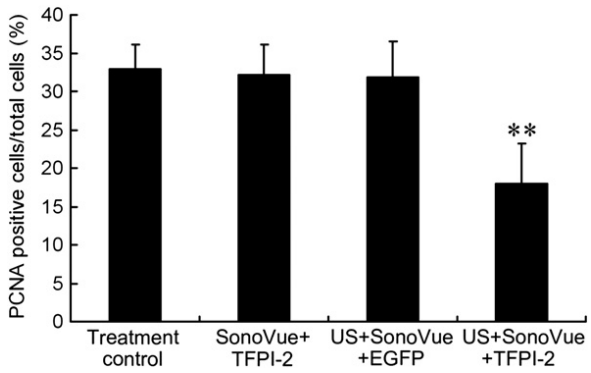

Fig. 4. The role of TFPI-2 expression in affecting the ratio of PCNA-positive cells to total cells in rabbit balloon injury model four weeks after the TFPI-2 gene was administered. $**p < 0.01$ *vs.* other groups.

of blood vessels transferred with TFPI-2 using ultrasound without SonoVue.

Effect of TFPI-2 gene transfer on vasomotor tension in rabbit balloon-injured carotid arteries

We next sought to determine whether TFPI-2 gene transfer could reverse the vasomotor impairment known to occur in balloon-injured carotid artery. Four weeks after operation, we measured the effect of TFPI-2 gene transfer on vasomotor tension. Interestingly, the arteries of the US+SonoVue+TFPI-2 group were seen to be relaxed to 47 ± 5% of precontracted tension ($p < 0.01$ *vs.* other groups arteries). The degree of contraction in response to PE and relaxation in response to sodium nitroprusside (SNP) was not affected by any of the groups (data not shown). The results showed that TFPI-2 gene transfer reversed the vasomotor deficit associated with intimal hyperplasia after balloon injury in rabbit carotid artery model.

DISCUSSION

Drug treatments developed to reduce intimal hyperplasia and restenosis include systemic administration of antithrombotic drugs. However, these therapies have not been consistently effective, and their dosing is limited given their associated hemorrhagic risk (Lefkovits and Topol 1997; Topol et al. 1994; Welsch et al. 1991). Gene therapy is one of the potential therapies for preventing restenosis after angioplasty (Bult 2000; Mehan and Meier 1993).

TFPI-2 was chosen as a model of gene therapy because transfection of TFPI-2 using a viral envelope system has been reported to inhibit neointimal formation (Zoldhelyi et al. 2001). Intriguingly, neointimal formation was significantly inhibited in blood vessels transferred with TFPI-2 using ultrasound with SonoVue. The present study demonstrated the feasibility of ultrasound-mediated TFPI-2 gene transfer into balloon-injured blood vessels.

Several recent studies (Gosk-Bierska and Adamiec 2005; Ivanciu et al. 2007) have reported that TFPI-2 has a major role in the inhibition of thrombosis and arterial hyperplasia. Previous studies (Nishida et al. 1999; Zoldhelyi et al. 1996) have shown that adenoviral gene transfer of TFPI exerts significant protection against the development of cyclic flow reductions, in accordance with reports that administration of recombinant TFPI markedly reduces the procoagulant properties of balloon-injured rabbit arteries (Speidel et al. 1995; St. Pierre et al. 1999; Zoldhelyi et al. 2001). We therefore speculate that the antithrombotic properties of local TFPI gene transfer may play an important role as a potential therapeutic approach to prevent intimal hyperplasia.

Most of the preclinical studies of the treatment of restenosis have used intraluminal injection of an adenoviral vector because of its high transfection efficiency. However, immune response to viruses can limit the application of gene therapy with a viral vector (Chen et al. 1999). Viruses are antigenic and can cause allergic reactions. A death occurred in one recent clinical trial of gene therapy using a viral vector; this may have been caused by an immune reaction related to the viral vector (Fleiner et al. 2004). In addition, there is a concern about mutagenesis with some of the viral vectors (Moreno et al. 2004).

In considering the treatment of restenosis, transfection of therapeutic genes into patients who will not develop restenosis seems to be inevitable, because although as many as 30–40% of patients develop restenosis after angioplasty, there are still a high percentage of patients who will not have this problem. Thus, the safety of the vector system should be carefully evaluated with regard to receiving the gene therapy. From this viewpoint, a viral vector may not be ideal. Therefore, it is critical to develop a novel safe synthetic vector that could be administered intravenously to provide targeted gene delivery to a localized tissue with high efficiency.

Ultrasound energy can be used to cavitate (rupture) ultrasound contrast agents and to deliver genes locally to a tissue (Moulton et al. 2003). Indeed, the transfer efficiency of plasmid DNA was increased by ultrasound in cultured cells (Huber and Pfisterer 2000; Manome et al. 2000). Our preliminary study revealed a marked increase in the transfer efficiency when using ultrasound in cultured human umbilical vein endothelial cells (HUVECs) (data not shown), consistent with previous reports (Huber and Pfisterer 2000; Manome et al. 2000). Therefore, in this study we used ultrasound-mediated gene transfer using an echo-contrast microbubble agent (SonoVue) because ultrasound induces cell membrane

porosity (Tachibana et al. 1999). This idea is based on the previous observation that the presence of an ultrasound contrast agent, such as albumin, causes microbubbles (Tachibana and Tachibana 1995).

SonoVue is a type of microbubble ultrasound contrast agent. It can increase image definition in ultrasonically diagnostic imaging and has been used for the delivery of drugs (Kaufmann et al. 2007). We found that TFPI-2 gene transfer was markedly improved by using ultrasound with SonoVue (Fig. 1). The increase in transfer efficiency might be a result of transient holes in the cell membrane produced by the spreading of bubbles.

Using the combined microbubbles and ultrasound cannot only create cavitation effect but can also locally enhance the capillary permeability. Experimental studies show that capillary permeability effects occur in experimental animals when using microbubbles and an ultrasound transducer. This process has been shown experimentally to increase transcapillary passage of macromolecules (Skyba et al. 1998). We also observed that the fluorescence was detected in the deep area of the vessel wall including both the media and intima (Fig. 1). Based on these results, we speculated that this subendothelial gene transfer that we obtained may be the result of a certain mechanism by which ultrasound cavitation probably opened micropores in the blood vessel walls and the microbubbles with TFPI-2 plasmid could easily pass through the artery intima and enter the media, where they were destroyed.

Given the high transfer efficiency of TFPI-2 using ultrasound with SonoVue, we examined the feasibility of gene therapy to inhibit neointimal formation after balloon injury in a rabbit model.

After local gene transfer of TFPI using ultrasound with SonoVue, we found that overexpression of TFPI-2 markedly inhibited intimal hyperplasia, as reflected by a 41% reduction in the intima/media ratio of carotid segments examined four weeks after balloon injury (Figs. 2 and 3). No impairment of systemic hemostasis or excess bleeding was observed in this or previous studies of adenoviral TFPI gene transfer *in vivo* (Nishida et al. 1999), indicating the potential of locally overexpressed TFPI to inhibit neointima formation without detectable hemorrhagic risk. We found that the trend toward increased lumen size in TFPI-2-transferred vessels was not associated with changes in the luminal diameters, which were virtually identical to those of controls (Figs. 2 and 3).

The efficacy of overexpressed TFPI in reducing neointima formation at sites of vascular injury suggests that TF and its downstream proteases, factor Xa and thrombin, may function *in vivo* much like cytokines—promoting intimal hyperplasia and vessel obstruction through signals activating migration and proliferation of VSMC (Ko et al. 1996; McNamara et al. 1993; Sato et al. 1997).

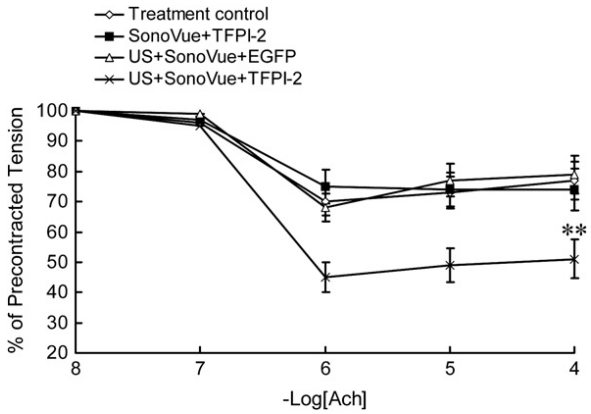

Fig. 5. The change in vasomotor function in rabbit balloon injury model at four weeks after animals received TFPI-2 gene. $**p < 0.01$ *vs.* other groups.

In this study, a significant decrease in DNA synthesis was found, as assessed by PCNA-positive staining, in neointimal cells after TFPI-2 gene transfer using ultrasound with SonoVue (Fig. 4). It was also found that TFPI-2 gene transfer reversed the vasomotor deficit associated with intimal hyperplasia after balloon injury in rabbit carotid artery model (Fig. 5).

These data clearly demonstrate the clinical utility of a therapeutic strategy based on ultrasound-SonoVue-mediated transfer. It is possible to achieve high transfer efficiency without a viral vector. Avoiding viral gene transfer, such as using adenoviruses, may increase the safety of gene therapy and extend its application to a wide variety of targeted diseases. Overall, the present study demonstrated a novel nonviral method to transfer TFPI-2 into blood vessels. A novel therapeutic strategy using TFPI-2 with SonoVue and ultrasound may be useful to inhibit restenosis in clinical practice.

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