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TGF-beta 1/Smad3 expression and its effects on carotid intimal hyperplasia

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1. ABSTRACT

We investigated expression of TGF-beta 1/Smad3 after balloon injury in rat carotid arteries as well as the effects of blocking the TGF-beta 1/smad3 signaling pathway on carotid intimal hyperplasia. Rats were randomly divided into control group (C group) and injury group (S group). The latter were randomly divided into intervention group (antisense Smad3 adenovirus vector transfection after balloon injury) and blank control group (blank adenovirus vector transfection after balloon injury). Expression of Smad3 mRNA 1 d, 3 d, 1 week, 2 weeks and 1 month after injury and intima/media thickness ratios 1 d, 2 weeks and 3 months after injury in the intervention group were significantly lower than those in the injury group. Ki-67 expression in the intervention group was inhibited as shown in immunohistochemistry studies. These results demonstrated that antisense Smad3 adenovirus vector transfection can block TGF-beta 1/Smad3 signal transduction and thus inhibit intimal hyperplasia.

2. INTRODUCTION

Fifty million people in the USA undergo percutaneous coronary intervention (PCI) each year (1). In general, after treated with medical or bare carrier respectively after 6 month, the incidences of restenosis were 8.9% and 29% s (2). Neointimal hyperplasia (NIH) is an important reason for in-stent restenosis (3). The vascular smooth muscle cell (VSMC) differentiates into a proliferating and secretory phenotype after inflammatory stimulation (4). The proliferation and migration of VSMCs is the foundation of NIH (5). Although there have been many detailed biological studies and hypotheses about NIH, the molecular mechanism of NIH formation after vascular injury is incompletely understood.

Transforming growth factor beta-1 (TGF-beta 1) is a cell growth factor with hormone-like activity. Several studies have show that TGF-beta 1 is associated with the formation of atherosclerotic plaques (6), restenosis after

PCI (7) and repair of blood-vessel walls after injury (8). *In vivo* and *in vitro* studies have shown that TGF-beta 1 expression was increased in locally damaged vessel walls. TGF-beta 1expression increased at 6 h, and was maintained until 14 days after balloon injury (9–10). TGF-beta 1 stimulates neointimal proliferation as well as the synthesis and remodeling of the extracellular matrix (ECM) to promote restenosis (11).

TGF-beta 1 can promote neointimal hyperplasia *in vivo* (12). Tranilast can effectively reduce neointimal thickening and restenosis by reducing TGF-beta 1 activity or by directly inhibiting the TGF-beta 1 (13–14). In VSMCs, TGF-beta 1 stimulates fibronectin synthesis through the Smad3 signaling pathway, thereby increasing neointimal deposition of the ECM (15), and participating in restenosis. Neointimal formation is closely related to the TGF-beta 1/Smad3 signaling pathway, but the mechanism of action remains unclear.

3. MATERIALS AND METHODS

3.1. Materials

Male Sprague-Dawley (SD) rats (age, 6 weeks; 350–450 g) were supplied by Shanghai Lake Hayes. Adenovirus venom and Smad3 adenovirus solution (16) was supplied by Shanghai Ji Kaiji Chemical Technology Company Limited (Shanghai, China).

3.2. Establishment of an animal model for each group and sample collection

Ninety rats (age, 6 weeks) underwent injury to the left common carotid artery using an Edward 2F arterial embolectomy catheter. This was done to establish a balloon-injury carotid artery model according to reported methods (17). Rats in the balloon injury group (S group; N = 30) underwent carotid artery balloon injury only. In the intervention group (N = 30), the thrombectomy catheter was withdrawn. An intravenous catheter was inserted. Adenovirus was injected through the external carotid artery as well as 0.5 mL antisense Smad3 adenovirus (6×1010 pfu/mL). In the blank control group (N = 30), 0.5 mL adenovirus venom (6×1010 pfu/mL) was injected. All rats in the three groups were killed after 1 day, 3 days, 1 week, 2 week and 1 month (N = 5).

3.3. Examination of TFG-β1 in serum

Serum concentrations of TGF-beta 1 in each group were determined by enzyme-linked immunosorbent assay (ELISA) according to manufacturer instructions. A standard curve was constructed using the standard proteins in the kit. The final serum concentration of TGF-beta 1 was determined according to the optical density (OD) obtained using the standard curve.

3.4. Pathological examination

Biopsy specimens were paraffin fixed and processed for hematoxylin and eosin (H&E) staining. The morphology of the vessel wall was assessed by light microscopy. The image analysis system (Image Pro Plus 4.5 software; Media Cybernetics, Silver Spring, MD, USA) was used to measure the cross-sectional area of the intima and media, as well as to calculate the ratio of the thickness of the intima and media.

3.5. Quantitative real-time polymerase chain reaction (PCR)

Total RNA (2 μg) was extracted from the artery using TRIZOL, and 2 mg RNA was synthesized to cDNA. The primers of Smad3 were: forward 5′-CATTACCATCCCCAGGTCAC-3′; reverse 5′-CGTAACTCATGGTGGCTGTG-3′. The primers of b-actin were forward 5′-CTGTCCCTGTATGCCTCTG-3′, reverse 5′-TGTCACGCACGATTTCC-3′. Amplification was conducted under the following conditions: 95°C for 5 min, 40 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 45 s. The actin gene was used as an endogenous control to normalize differences.

3.6. Immunohistochemistry

Thin sections were dewaxed and dehydrated. Endogenous peroxidase activity was blocked by goat serum (10%) (room temperature, 30 min). Serum was then removed. Anti-Smad3 antibody (1:200 dilution; Sigma–Aldrich, St Louis, MO, USA) or anti-Ki-67 antibody (1:200 dilution; Sigma–Aldrich) was added. The mixture was allowed to incubate for 1 h at 37°C. It was then washed with phosphate-buffered saline (PBS). Horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added and the mixture allowed to incubate for 30 min. It was then washed with PBS, detected with 3,3'-Diaminobenzidine (DAB) and stained with hematoxylin.

3.7. Statistical analysis

Results are mean ± SD. Statistical analyses were undertaken using SPSS version 11.0 (SPSS, Chicago, IL, USA). The statistical significance of differences was assessed using ANOVA for multiple comparisons. $p<0.05$ was considered significant.

4. RESULTS

4.1. TGF-beta 1/Smad3 expression after vascular injury

The concentration of TGF-beta 1 in the injury group and normal control group at each time point was significantly different ($P < 0.05$). TGF-beta 1 began to increase 1 day after injury and reached a peak at 3 days ($P < 0.05$); it was ~6-times that of the normal control. It remained at a relatively high level 1–2 weeks after injury and then gradually decreased (Figure 1A).

Real-time PCR analyses showed that Smad3 gene expression was significantly higher 1 day after vascular injury (it was ~20-times that of the normal group) and reached a peak 3 days after injury. The Smad3 gene was stably expressed at a high level for 1–2 weeks after injury. It continued to decline after injury for 1 month, and reached normal expression levels at 3 months ($P < 0.05$) (Figure 1B). Immunohistochemistry results were in accordance with the protein expression results of Smad3 (Figure 1C).

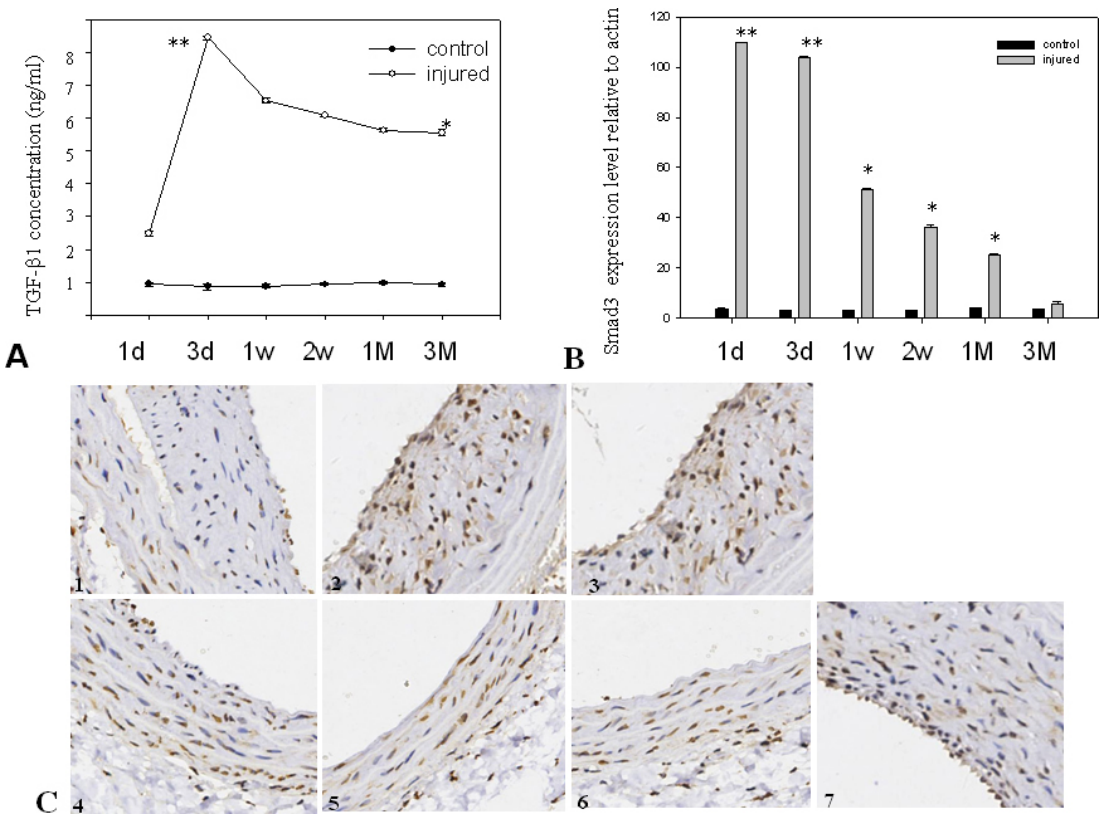

Figure 1. TGF-beta 1/Smad3 expression after vascular injury. A: TGF-beta 1 concentration was examined by ELISA; B; real-time-PCR was used to detect Smad3 mRNA expression; C: Smad3 expression was observed by immunohistochemistry. 1–7 represent the time points 1 day, 3 days, 1 week, 2 weeks, 1 month and 3 months after injury, respectively. *P < 0.05 vs. control, **P < 0.01 vs. control.

4.2. NIH after vascular injury

Intimal hyperplasia was examined using H&E staining, and was analyzed at different time points.

One day after injury, endothelial cells were denuded, the internal elastic plate was fractured, the middle of smooth muscle cells (SMCs) was elongated, the middle membrane was damaged, and the number of SMCs near the luminal side was increased (Figure 2A). Seven days and 14 days after injury, the continuity of the endothelial cell layer was gradually restored, cells migrated and proliferated to the inner surface of blood vessels, and the intima began to form. One month after injury, the neointima showed significant proliferation with formation of the ECM, but cell proliferation decreased, gradually narrowing the lumen. Three months after injury, the neointima was further thickened, intimal cells were relatively stable, and considerable accumulation of stromal cells, luminal stenosis, and an intima/media thickness (IMT) ratio of ≥1:1 was noted. After injury for 2 weeks, 1 month, and 3 months in each group, the IMT ratios were significantly different compared with normal controls (Figure 2B).

4.3. *In vivo* Smad3 expression in vessels after transduction of the antisense adenovirus of smad3

Compared with the injury group, expression of Smad3 mRNA in the intervention group was significantly inhibited 1 day and 3 days after injury. It reached a peak in the second week, but was lower than that seen in the injury group (P<0.05), and then gradually declined to a minimum. The immunohistochemistry results of Smad3 expression were in agreement with the mRNA results (Figure 3B).

4.4. Effect of the antisense adenovirus of Smad3 on neointimal hyperplasia *in vivo*

Compared with the control group, the difference in intima/media area ratio was significant (P<0.05) in the intervention group at 1 day, 2 weeks, and 3 months. Compared with the injury group, NIH was less in the antisense Smad3 group 1 day, 2 weeks, and 3 months after injury (Figure 4).

4. 5. Expression of Ki-67 in blood vessels

Compared with the control group, immunohistochemical results showed that expression of Ki-67 was lower in the intervention group 1 day, 2 weeks, and 3 months after injury: this difference was significant (P <0.05) (Figure 5).

5. DISCUSSION

According to Tsai *et al.* (17-19), when simulating injury due to balloon withdrawal, TGF-beta 1 can promote the proliferation of SMCs in rat carotid arteries through the samd3 pathway. This is consistent with our finding that the expression of Smad3 was higher in the intimal and medial

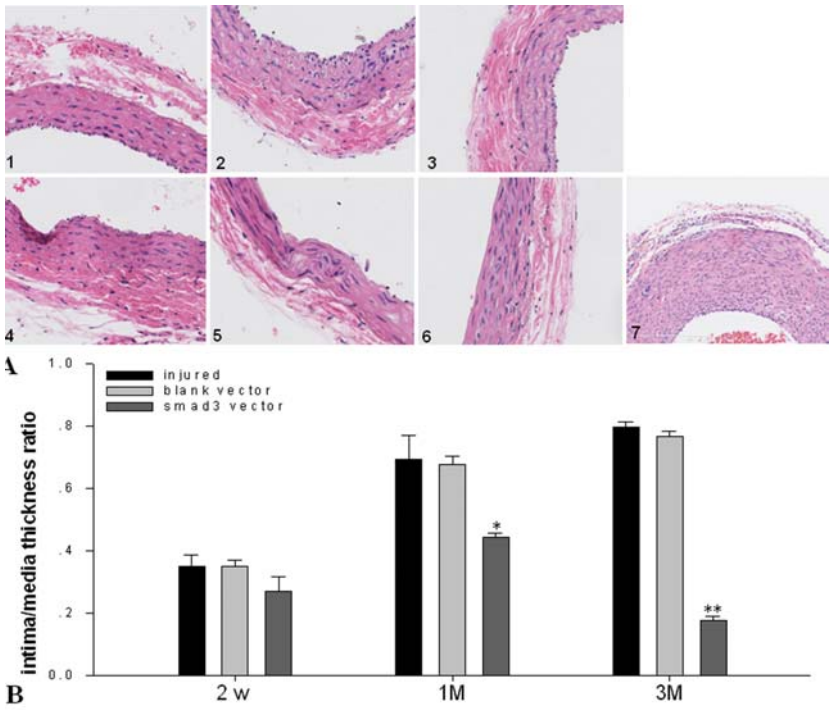

Figure 2. Detection of neointimal hyperplasia after vascular injury. A: neointimal hyperplasia was observed by H&E staining, 1-7 represent the time points 1 day, 3 days, 1 week, 2 weeks, 1 month and 3 months after injury, respectively, magnification, ×100; B: neointimal/media thickness was calculated; *P < 0.05 vs. control, **P < 0.01 vs. control.

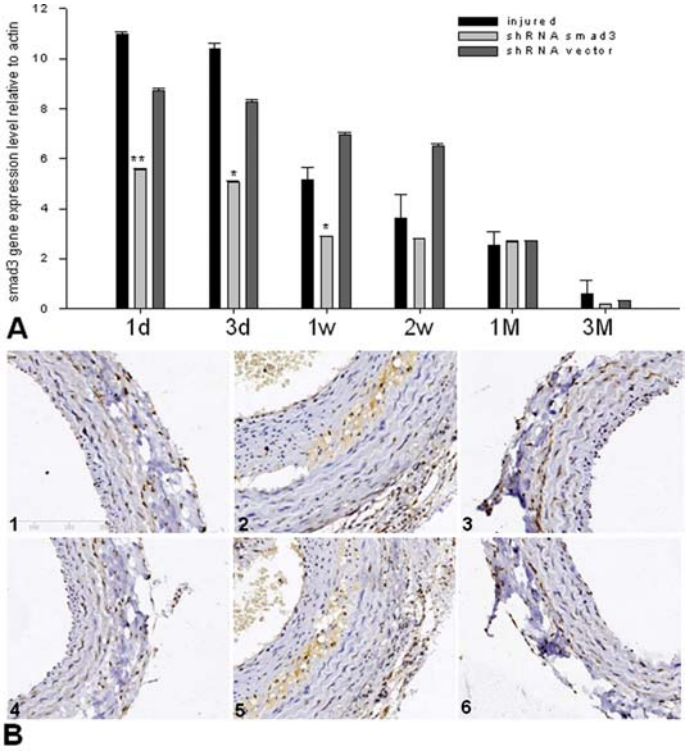

Figure 3. Effect of transduction of the antisense adenovirus of Smad3. A: Smad3 gene expression was examined by real-time-PCR; B: Smad3 expression was observed by immunohistochemistry, 1–3 represent the injured group, 4–6 represent transduction of the Smad3 gene group; both groups were 1 day, 2 weeks, and 3 months, respectively. *P < 0.05 vs. control, **P < 0.01 vs. control.

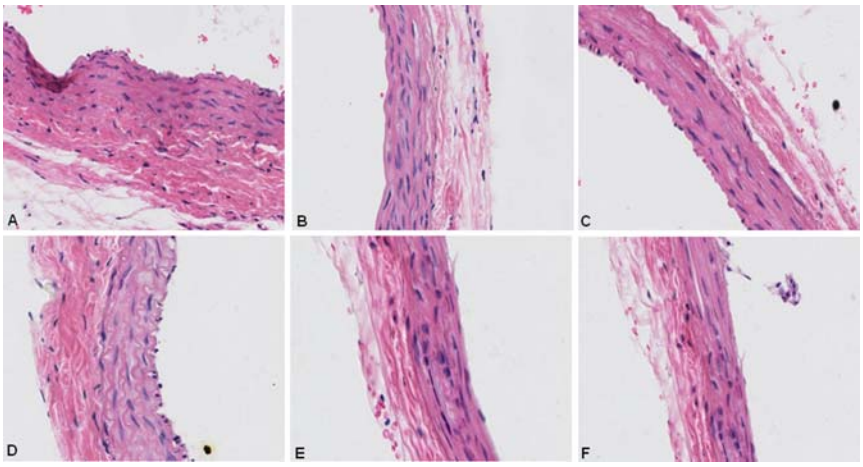

Figure 4. Effect of transduction of the antisense adenovirus of Smad3 on neointimal hyperplasia *in vivo* through H&E staining. A–C represent the injured group, D–F represent the transduction of the Smad3 gene group; both groups were 1 day, 2 weeks, and 3 months, respectively, magnification, ×100.

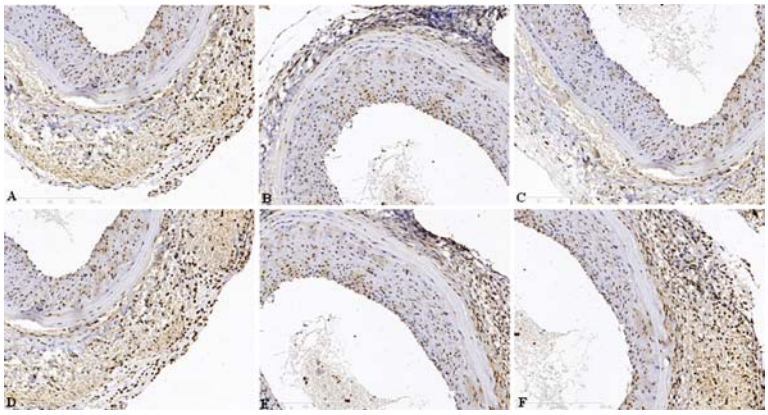

Figure 5. Effect of transduction of the antisense adenovirus of Smad3 on Ki-67 expression. A–C represent the injured group, D–F represent the transduction of the Smad3 gene group, both groups were 1 day, 2 weeks, and 3 months, respectively.

part of the injured carotid artery as identified by histochemistry and transfection assay. Furthermore, Smad3 was identified to be co-localized with proliferating cell nuclear antigen (PCNA). Taken together, these results demonstrated that Smad3 was upregulated in SMCs and promoted cell proliferation.

Serum concentrations of TGF-beta 1 in each group were determined 1 day, 3 days, 1 week, 2 weeks, 1 month and 3 months after injury. Serum concentrations of TGF-beta 1 were significantly increased in early acute injury. This may have been caused mainly by thrombosis formation, platelet-derived growth factors, and the inflammatory response. The increased TGF-beta 1 level in the early phase of balloon injury participated in repair of the vessel wall. After 2 weeks, the concentration of TGF-beta 1 decreased, but remained at a higher level compared with that of the control group. This may be related to TGF-beta 1, which promotes the proliferation of SMCs and deposition of collagen. This is the peak phase of local tissue repair and intimal narrowing after balloon injury. Several studies have showed that intimal hyperplasia can be inhibited at this stage by decreasing TGF-beta 1 levels (20-21).

Smad3 mRNA expression was significantly increased 1 day after vascular injury ($P < 0.05$), was maintained at a high level 2 weeks after injury ($P<0.05$), and gradually decreased to normal levels 3 months after injury: these results are consistent with previous studies (19). The TGF-beta 1/Smad3 pathway may be involved in vascular repair and intimal hyperplasia after balloon injury.

We constructed the antisense Smad3 adenovirus vector and transduced this vector into injured vascular tissue. The results showed that, compared with the injury group, Smad3 and the TGF-beta 1/Smad3 signaling pathway reduced the IMT ratios at different time points. Therefore, Smad3 mRNA can be inhibited by antisense adenovirus and the TGF-beta 1/Smad3 signaling pathway can be blocked, leading to the inhibition of intimal thickening and reduction of the IMT ratio. This could

provide a new methof of gene therapy to cure restenosis after percutaneous transluminal coronary angioplasty.

Ki-67 was measured to explore the effect of inhibition of the TGF-beta 1/Smad3 signaling pathway on cell proliferation. Ki-67 level was reduced by blocking the TGF-beta 1/Smad3 signaling pathway. This indicated that cell proliferation could be inhibited by blocking the TGF-beta 1/Smad3 signaling pathway.

In conclusion, upregulation of the TGF-beta 1/Smad3 pathway is related to neointimal formation after vascular injury. Intimal hyperplasia could be inhibited by blocking the TGF-beta 1/Smad3 signaling pathway, and reduced proliferation of SMCs may play an important part in this process. These results provided new methods for the prevention and treatment of NIH.

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Abbreviations: PCI: percutaneous coronary intervention, PCI, VSMC: vascular smooth muscle cell, CAD: coronary artery disease

Key Words: Balloon injury, Intimal hyperplasia, TGF-beta 1/Stand3 signaling pathway, Antisense Smad3, Adenovirus

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