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Efficient delivery of connective tissue growth factor shRNA using PAMAM nanoparticles

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ABSTRACT. The aim of this study was to detect the anti-fibrosis activity of connective tissue growth factor (CTGF) small hairpin RNA (shRNA) mediated by polyamidoamine dendrimer nanoparticles in rat myocardial cell lines and myocardium. CTGF shRNAs were constructed from inverted oligonucleotides and a polyamidoamine nanoparticle vector was used to transfer shRNA into H9c2 myocardial cells and spontaneously hypertensive rats. The expression of CTGF, transforming growth factor-β_1, and laminin were measured by semi-quantitative reverse transcription-polymerase chain reaction, Western blotting, and immunohistochemistry. pCTGF-shRNA significantly reduced CTGF upregulation induced by angiotensin II in H9c2 myocardial cells. The mRNA and protein expression of CTGF and laminin in pCTGF-shRNA-transferred spontaneously hypertensive rats decreased significantly compared to the control group and pHK-shRNA group ($P < 0.05$). The expression of transforming growth factor-β_1 showed no significant difference among the 3 groups ($P > 0.05$). pCTGF-shRNA mediated

by polyamidoamine can be used to successfully reduce myocardial CTGF and laminin expression, suggesting that this system can be used to improve myocardial fibrosis therapy.

Key words: Connective tissue growth factor; Fibrosis; Myocardial cells; Polyamidoamine dendrimers; RNAi; Spontaneously hypertensive rat

INTRODUCTION

Short hairpin RNA (shRNA) is widely used for targeted gene silencing through RNA interference (RNAi) (Lambeth and Smith, 2013). After its discovery in 1998, numerous therapeutic applications of RNAi in biomedical research have been reported, such as for treating human immunodeficiency virus, viral hepatitis, and several other diseases (Ambesajir et al., 2012). Although many vehicles for shRNA delivery have been examined, efficient shRNA delivery remains elusive (Morille et al., 2008). Polyamidoamine (PAMAM) dendrimers have been developed as non-viral nanoparticles. They are considered to be non-immunogenic and non-genotoxic and show low toxicity toward cells. PAMAMs are thought to be useful as transfection vehicles for plasmid DNA (Santos et al., 2010), particularly for siRNA (Ofek et al., 2010; Liu et al., 2011; Arima et al., 2012).

Connective tissue growth factor (CTGF), a cysteine-rich 36-38 kDa peptide, belongs to the CCN gene family. CTGF is involved in a wide range of cellular activities, including cell proliferation, migration, extracellular matrix protein production, and granulation tissue formation (Xiao et al., 2010). CTGF is regarded as the prime mediator of transforming growth factor (TGF)-β_1-induced fibrosis. Furthermore, CTGF plays a specific and key role in the fibrotic pathway as it shows high expression in hypertension-related cardiac fibrosis (Gravning et al., 2012; Zambrano et al., 2013). Thus, identification of a more effective method of reducing CTGF may enable the prevention of relief of fibrosis and heart remodeling.

In this study, we constructed PAMAM vectors expressing CTGF-shRNA and investigated their transfection efficiencies and biological impact in H9c2 myocardial cells and spontaneous hypertension rats (SHR).

MATERIAL AND METHODS

Construction of CTGF shRNA PAMAM vectors

Two target sequences and 1 negative sequence were designed according to the coding sequence of the rat CTGF gene (GenBank NM_022266): MCTGF1, GAGTCCTTCCAAAG CAGTT; MCTGF2, CGATGGCGAGATCATGAAA; HK, GACTTCATAAGGCGCATGC. BLAST analysis was performed to ensure that no homology existed with other genes. The shRNAs were ligated to the linearized pGenesil-1 vector (Genesil; Wuhan, China) to form plasmids pCTGF1-shRNA, pCTGF2-shRNA, and pHK-shRNA. Plasmid sequences were confirmed using *Sal* restriction endonuclease (TakaRa; Shiga, Japan) and the sequences were confirmed before extraction using a Tip100 column (Qiagen; Hilden, Germany).

G9 PAMAM was provided by Dr. Yanming Wang from the Key Laboratory of Bioactive Materials, Nankai University. According to the protocol (Yi et al., 2008) to form

a dendrimer-plasmid nanocomplex, 100 μL physiological saline containing 50 μg pCTGF-shRNA or pHK-shRNA plasmid was added to 100 μL G9 PAMAM dendrimer solutions (6.5 μg/μL). The mixture was incubated at room temperature for 10-20 min to form complexes, with gentle occasional vortexing. The mixture was then centrifuged at 80,000 *g* for 45 min and the pellet containing the heavier PAMAM G9/pCTGF2-shRNA or PAMAM G9/pHK-shRNA was collected. The preparation was conducted for every experiment to obtain a fresh sample.

In vitro experiment

The H9c2 myocardial cell line (ATCC; Manassas, VA, USA) was used in *in vitro* experiments. H9c2 myocardial cells were divided into 5 groups: normal control group receiving pure Dulbecco's modified Eagle medium (DMEM); angiotensin (AngII) stimulation group receiving AngII; the pHK-shRNA transfection group, pCTGF1-shRNA-transfected group, and the pCTGF2-shRNA-transfected group were pre-treated with AngII. These groups were incubated for 3 h at 37°C, 5% CO_2 with PAMAM G9/pHK-shRNA, PAMAM G9/pCTGF1-shRNA, or PAMAM G9/pCTGF2-shRNA, respectively. After discarding the transfer fluid, DMEM (ATCC) was added to the H9c2 myocardial cells. The cells were collected after 24 h of transfection. Reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting were performed to screen for the most efficient RNAi plasmid for *in vivo* study. The expression of CTGF and TGF-β_1 mRNA and protein expression levels were measured by Western blot and immunohistochemistry analyses.

In vivo experiment

The Animal Ethics Review Board in the Third Xiangya Hospital of Central South University approved the animal experiments. Twenty-four male SHR (SLRC, Shanghai, China) were randomly divided into 3 groups: control group (N = 8), pCTGF-shRNA transfected group (N = 8), and pHK-shRNA transfection group (N = 8). All rats from the 3 groups were intramyocardially injected with 200 μL saline, PAMAM G9/pCTGF-shRNA transfection complex, and PAMAM G9/pHK-shRNA in the left ventricle using 27-G needles. Tail arterial blood pressure and body weight of SHRs were observed each week. Twenty-eight days after injection, all SHRs were sacrificed by anesthetic overdose. Left ventricular free wall and septa were weighed to calculate left ventricular mass/body mass. The expression of CTGF, TGF-β_1, and laminin (LN) protein expression were measured using semi-quantitative RT-PCR and Western blotting.

Semi-quantitative RT-PCR

RT-PCR was used according to our established laboratory procedures (Lu et al., 2012). Total RNA (1 μg) was extracted to synthesize cDNA using a reverse transcription kit (Promega; Madison, WI, USA). Specific oligonucleotide primers were designed based on the published genomic sequences (CTGF/NM_022266, TGF-β_1/NM_021578, β-actin/NM_031144). The PCR conditions were 95°C for 5 min to activate the polymerase, and each cycle consisted of 30 s at 95°C, 40 s at 55°C, and 50 s at 72°C (total of 30 cycles), followed by extension at

72°C for 10 min. Control reactions were carried out in parallel using non-reverse transcribed RNA and mixtures in which target RNA had been omitted. Amplification products were resolved by 6% polyacrylamide gel electrophoresis, and their optical densities were determined using computer-processed images, with the absorbance value of the β-actin product used as a control. The following primers were used for PCR: β-actin-F: 5'-TGGGTCAGAAGGACTCC TATG-3', β-actin-R: 5'-CAGGCAGCTCATAGCTCTTCT-3' (593 base pairs, bp); CTGF-F: 5'-CTAAGACCTGTGGAATGGGC-3', CTGF-R: 5'-CTCAAAGATGTCATTGCCCCC-3' (383 bp); TGF-β_1-F: 5'-GCGACTCCTGCTGCTTT-3', TGF-β_1-R: 5'-CTGGCGAGCCTTAGT-3' (350 bp).

Western blot

According to our established laboratory procedures (Lu et al., 2012), the total protein in different groups was extracted, and 30 mg protein from each sample was resolved on 12% sodium dodecyl sulfate-polyacrylamide gradient gels and electrophoretically transferred to nitrocellulose membranes. The blots were probed with the following antibodies: anti-CTGF (Abcam; Cambridge, UK), anti-β-actin (Sigma; St. Louis, MO, USA), anti-TGF-β_1 (Abcam), anti-LN antibody (1:2000), and anti-IgG-horseradish peroxidase antibody (Abcam) (1:5000).

Immunohistochemistry

Myocardial tissue sections were fixed in 2% paraformaldehyde and permeabilized. Antibodies for rat anti-CTGF (1:200; Abcam), rat anti-LN (1:200; Abcam), and rat anti-TGF-β_1 (1:200; Abcam) were used for immune staining. Conventional hematoxylin and eosin staining was used to determine the degree of cardiac hypertrophy in SHRs.

Statistical analysis

Statistical analyses were performed using the SPSS software, v. 11.5 (SPSS, Inc.; Chicago, IL, USA). The experimental results are reported as means ± standard deviation. One-way analysis of variance or the rank sum test was used for intergroup comparisons. Statistical significance was set at $P < 0.05$.

RESULTS

CTGF expression *in vitro*

Following stimulation with AngII, CTGF mRNA and protein expression in H9c2 myocardial cells was significantly increased compared with the control group ($P < 0.01$); CTGF mRNA and protein expression in the pCTGF1-shRNA-transfected group and pCTGF2-shRNA-transfected group were significantly lower than those in the AngII and pHK-shRNA-transfected groups ($P < 0.01$). In particular, the pCTGF2-shRNA-transfected group decreased significantly ($P < 0.05$); CTGF expression showed no significant difference between the pHK-shRNA-transfected group and the AngII group ($P > 0.05$) (Figure 1).

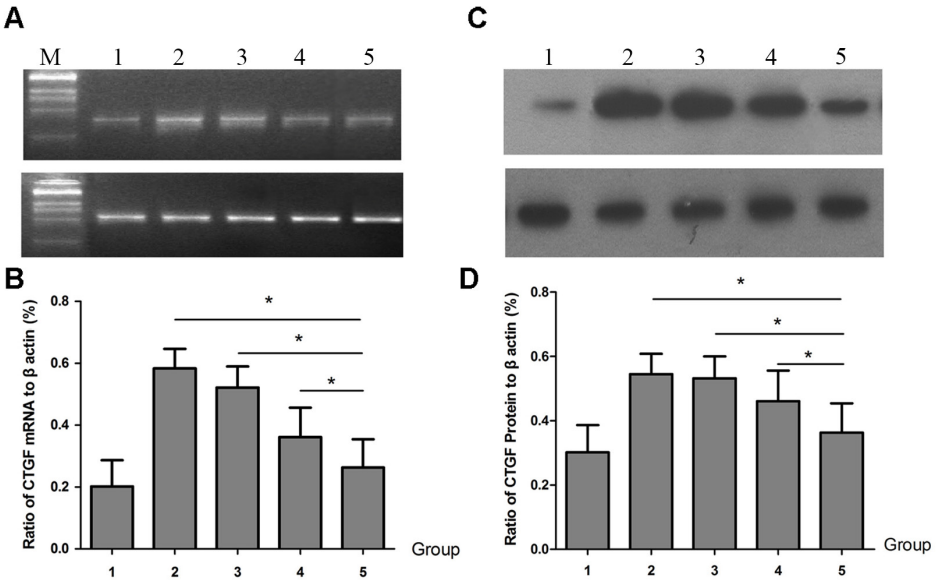

Figure 1. Connective tissue growth factor (CTGF) mRNA and protein expression in H9c2 myocardial cells. *Lane M* = DL2000 marker; *lane 1* = normal control group; *lane 2* = AngII group; *lane 3* = group transfected with pHK-shRNA; *lane 4* = group transfected with pCTGF1-shRNA; *lane 5* = group transfected with pCTGF2-shRNA. *P < 0.05 *vs* group transfected with pCTGF2-shRNA.

CTGF, TGF-β_1, and LN protein expression *in vivo*

Twenty-four SHRs were examined in this study. Western blot results of myocardial tissue showed that CTGF and LN protein expression in the pCTGF-shRNA-transfected group was significantly lower than that in the control group or pHK-shRNA-transfected group ($P < 0.05$), but there was no significant difference in TGF-β_1 expression among the 3 groups ($P > 0.05$) (Figure 2).

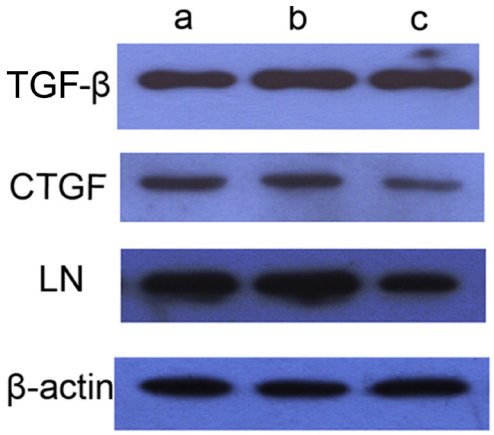

Figure 2. TGF-β_1, CTGF, and LN protein expression detected by Western blotting in spontaneous hypertension rat myocardium. *Lane a* = control group; *lane b* = group transfected with pHK-shRNA; *lane c* = group transfected with pCTGF-shRNA.

Immunohistochemistry

The left ventricular mass/body mass of the pCTGF-shRNA transfection group was lower than those in the pHK-shRNA transfected group and the control group (Figure 3A) and showed relatively mild fibrosis (Figure 3B). CTGF and LN expression was significantly decreased in the pCTGF-shRNA-transfected group, while the control group and pHK-shRNA-transfected group myocardial tissue samples showed abundant dense, brown CTGF expression in myocardial cells and myocardial interstitial tissues, and LN showed a brown-intensive trend in myocardial interstitial tissues. There was no significant difference in TGF-β_1 expression among the 3 groups (Figure 3C).

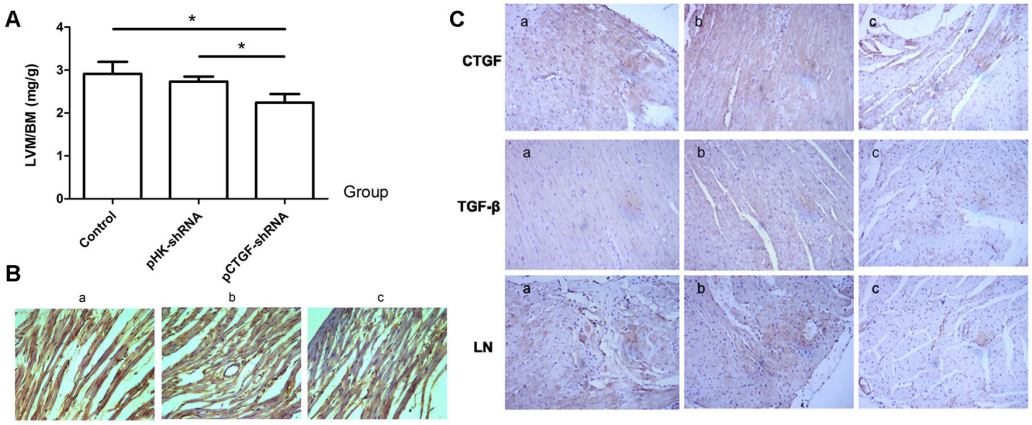

Figure 3. A. Difference in left ventricular mass/body mass in 3 spontaneous hypertension rat (SHR) groups *vs* pCTGF-shRNA group: *P < 0.05. **B.** Difference in left ventricular hypertrophy in 3 SHR groups (HE staining, 200X). **a.** Control group; **b.** group transfected with pHK-shRNA; **c.** group transfected with pCTGF-shRNA. **C.** Difference in CTGF, TGFβ_1 and LN in 3 SHR groups (hematoxylin, 200X). **a.** Control group; **b.** group transfected with pHK-shRNA; **c.** group transfected with pCTGF-shRNA. Brown indicates stained protein and blue indicates nucleus staining of myocardial cells.

DISCUSSION

PAMAM has been used for gene therapy in many diseases except for hypertension-related cardiac fibrosis. In this study, we constructed PAMAM vectors expressing CTGF-shRNA and investigated the transfection efficiencies and biological impacts in H9c2 myocardial cells and SHRs.

We constructed 2 pCTGF-shRNA plasmids that could mediate the synthesis of CTGF shRNA, and then transferred these plasmids into H9c2 and SHRs using PAMAM G9. The levels of CTGF mRNA and protein expression were low in myocardial cells, but significantly increased after 3-h incubation with AngII. This result was consistent with those of other studies (Rosin et al., 2013; Gao et al., 2014). However, the elevated level of CTGF and LN expression was inhibited in SHRs transfected with PAMAM-CTGF shRNA, indicating that our PAMAM-CTGF shRNA complex was functionally effective in SHRs.

TGF-β_1 plays an important role in the pathogenesis of cardiac remodeling and

fibrosis (Dobaczewski et al., 2011), but complete blockage is difficult because of its wide range of target cells and complex effects. We found that TGF-β_1 expression did not change with downregulation of CTGF and LN. However, the mechanism behind this is unclear. We speculate that when CTGF expression is inhibited by shRNA, TGF-β_1 synthesis might be decreased. However, compared to the increased TGF-β_1 expression by the active renin-angiotensin system in SHRs (Li et al., 2013), there was no slight downregulation in TGF-β_1, and therefore other biological effects of TGF-β_1 were unaffected.

Myocardial matrix accumulation is a characteristic of hypertension-related cardiac fibrosis. LN is one of the major components of the extracellular matrix. The results of our experiments showed that expression of LN protein remarkably increased with stimulation of high blood pressure, but decreased after injection with CTGF shRNA. This suggests that inhibition of CTGF expression can effectively prevent the production of extracellular matrix induced by hypertension in SHRs, and that CTGF is an essential mediator of extracellular matrix production induced by hypertension in SHRs.

Completion of the Human Genome Project deepened the understanding of human genetics. Consequently, the use of recombinant DNA in prevention and treatment of diseases has increased (Butler, 2010; Fishbein et al., 2010). However, gene therapy technology remains limited; a major challenge is identifying safe and effective vectors. PAMAM is a non-viral vector that performs significantly better than existing vectors with low gene transfer efficiency, cytotoxicity, or lack of cell-targeting capability. In particular, non-viral vectors are recognized for their ease of synthesis with controlled structure and size, minimal cytotoxicity, biodegradability, and high transfection efficiencies (Xu et al., 2010). Many researchers have used PAMAM as effective tools for gene therapy (Paul et al., 2012; Zhu et al., 2013). We used G9 PAMAM as the CTGF shRNA transfer vector because of its large capacity. Our study was limited because only the PAMAM carrier was used, and no other non-viral vector was directly compared. In future studies, we will compare the efficiency between PAMAM and other non-viral vectors in cardiac fibrosis gene therapy.

CONCLUSIONS

In summary, the PAMAM G9 vector was used to successfully mediate pCTGF-shRNA-transfected SHRs, and the nanocomplex reduced the expression of CTGF and LN in myocardial tissue, relieving myocardial fibrosis and indicating the feasibility of PAMAM-pCTGF-shRNA for improved myocardial fibrosis therapy applications.

Conflicts of interest

The authors declare no conflict of interest.

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