Original Article

Knockdown of ANGPTL2 reduces transforming growth factor-β1-induced fibrogenesis in cardiac fibroblasts

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Abstract: Angiopoietin-like 2 (ANGPTL2) is a member of ANGPTL family and plays important roles in various inflammatory diseases, tumorigenesis and fibrosis. However, the role of ANGPTL2 in cardiac fibrosis remains unclear. Thus, the objective of this study was to investigate the role of ANGPTL2 in cardiac fibrosis. Cardiac fibroblasts (CFs) were treated with TGF-β1 for different times. The expression of ANGPTL2 was detected by qRT-qPCR and western blot. In addition, CFs were transfected with si-ANGPTL2 or scramble and then stimulated with 10 ng/ml TGF-β1. Cell proliferation was determined by the colony formation assay. The expression of α-SMA, collagen I, p-Smad3 and Smad3 was analyzed by western blot. Our results demonstrated for the first time that ANGPTL2 mRNA and protein levels were significantly up-regulated in transforming growth factor beta 1 (TGF-β1)-stimulated CFs. In addition, knockdown of ANGPTL2 inhibited cell proliferation and ECM expression in TGF-β1-stimulated CFs. Further mechanism study indicated that knockdown of ANGPTL2 significantly inhibited the TGF-β1-induced p-Smad3 in CFs. In conclusion, this study showed that knockdown of ANGPTL2 inhibited cell proliferation and ECM expression in TGF-β1-stimulated CFs through interfering with TGF-β1/Smad3 signaling pathway. Thus, ANGPTL2 may be a potential therapeutic target for cardiac fibrosis.

Keywords: Cardiac fibrosis, ANGPTL2, TGF-β1, ECM

Introduction

Cardiac fibrosis is an important pathological feature of cardiac remodeling associated with various heart diseases, including hypertension, myocardial infarction, and heart failure. It is characterized by excessive proliferation of cardiac fibroblasts (CFs) and abundant deposition of extracellular matrix (ECM) [1]. Although significant therapeutic progress has been made in the past years [2-4], the mechanism underlying cardiac fibrosis remains elusive. CFs, the most prevalent cell type in the heart, play a central role in the development of cardiac fibrosis [5]. In response to transforming growth factor beta 1 (TGF-β1), these cells can transform to myofibroblast and contribute to cardiac fibrosis [6, 7]. Thus, suppressing myofibroblast differentiation may be an effective strategy to alleviate cardiac fibrosis.

Angiopoietin-like (ANGPTL) proteins are structurally similar to the angiopoietin family proteins which contain both N-terminal coiled-coil domain and C-terminal fibrinogen like domain [8]. ANGPTL2 is a member of ANGPTL family and plays important roles in various inflammatory diseases and tumorigenesis [9-11]. It has been reported that knockdown of ANGPTL2 expression decreased the proliferative, migration and invasive capacity in human esophageal cancer cells [12]. In addition, ANGPTL2 was found to be involved in fibrosis. A study by Morinaga et al. reported that the expression of ANGPTL2 was significantly up-regulated in renal tubule epithelial cells during progression of renal fibrosis, and ANGPTL2 deficiency obvious reduced renal fibrosis in a mouse unilateral ureteral obstruction model [13]. However, the role of ANGPTL2 in cardiac fibrosis remains unclear. Thus, the objective of this study was to investigate the role of ANGPTL2 in cardiac fibrosis.

Materials and methods

Cell culture

This study was performed with approval from the Animal Ethics Committee of Cixi People’s
The role of ANGPTL2 in cardiac fibrosis

Hospital (China). Cardiac fibroblasts (CFs) were harvested from SD neonate rats and routinely cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO, USA), 100 U/ml penicillin and 100 U/ml streptomycin (Sigma) at 37°C in a humidified incubator.

RNA interference and cell transfection

Small interfering RNA targeting ANGPTL2 (siANGPTL2) and non-targeting scrambled siRNA (scramble) were designed and synthesized by GenePharma (Shanghai, China). CFs were cultured to 80% confluence and transfected with (si-ANGPTL2) or scramble using Lipofectamine™RNAiMAX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The efficiency of transfection was verified by RT-PCR and western blot.

Colony formation assay

The transfected CFs (1×10⁴ cells/well) were seeded into six-well plates. Two weeks later, colonies were fixed with 4% paraformaldehyde (Sigma) and stained with crystal violet (Sigma) for 30 min. The number of colonies with diameter more than 1.5 mm was counted.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from CFs using Trizol reagent (Takara Biotechnology, Dalian, China) and reverse-transcribed to complementary DNA (cDNA) using the Transcriptor First Strand cDNA Synthesis Kit (Invitrogen). The specific primers for ANGPTL2 were forward, 5'-GAAGCATGAAGGCCTGCTC-3' and reverse, 5'-CAGCAGTCAAACACCAGTA-3'; and β-actin forward, 5'-GAGGCACCTTCCAGCTCTC-3' and reverse, 5'-GGATGTCCACGTCACTCTC-3'. The PCR products were subjected to 2% agarose gel electrophoresis. The relative change in gene expression was calculated using the 2-ΔCt method and standardized to the expression of β-actin.

Western blot

Total proteins were extracted using RIPA lysis buffer (Beyotime, Jiangsu, China), and the protein concentration was determined by BCA protein assay kit (Pierce, Rockford, USA). Equal amounts of proteins (30 µg per lane) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% gel, transferred to a PVDF membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% non-fat milk at room temperature for 1 h, and incubated with primary antibodies (anti-ANGPTL2, anti-α-SMA, anti-collagenI, anti-Smad3, anti-p-Smad3 and anti-GAPDH) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) over night at 4°C. After being extensively washed with PBS containing 0.1% Triton X-100, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) for 30 min at room temperature. Finally, immune complexes were visualized using the enhanced chemiluminescence (ECL) detection system (Invitrogen, Carlsbad, CA, USA). Target protein levels were nor-
The role of ANGPTL2 in cardiac fibrosis

Immunofluorescence staining

The transfected CFs were fixed with 4% paraformaldehyde (Sigma) for 15 min, permeabilized in 0.2% Triton X-100 in PBS, blocked with 10% goat serum, and then stained with anti-α-SMA antibody for overnight, and then incubated with the secondary antibody, goat anti-Mouse IgG Alexa Fluor 488. Finally, the cells were mounted onto glass slides with Slow Fade Gold anti-fade reagent with DAPI. The number of positive cells was counted under a quantitative digital image analysis system (Image ProPlus).

Statistical analysis

The results were analyzed using SPSS 13.0 software (Chicago, IL, USA). Data are expressed as mean ± standard deviation (SD). Comparisons between two groups and among multiple groups were conducted by Student t-test and one-way analysis of variance (ANOVA), respectively. A P value of < 0.05 was considered statistically significant.

Results

ANGPTL2 was upregulated in TGF-β1-induced CFs

We examined the expression of ANGPTL2 in TGF-β1-stimulated CFs using qRT-PCR and western blot. As shown in Figure 1A and 1B, the expression of ANGPTL2 at both mRNA and protein levels was significantly up-regulated in CFs induced by TGF-β1, exhibiting a time-dependent manner, as compared with the untreated group.

Knockdown of ANGPTL2 inhibited cell proliferation induced by TGF-β1 in CFs

In order to examine the effect of ANGPTL2 on CF proliferation, CFs were transfected with si-ANGPTL2 or scramble, respectively. After transfection, the expression of ANGPTL2 at both mRNA and protein levels was markedly decreased in CFs (Figure 2A and 2B). Then, we performed the colony formation assay to detect the effect of ANGPTL2 on cell proliferation in CFs induced by TGF-β1. As indicated in Figure 2C, TGF-β1 treatment markedly induced the colony formation of CFs, compared with the control group. However, knockdown of ANGPTL2
The role of ANGPTL2 in cardiac fibrosis

Knockdown of ANGPTL2 efficiently suppressed TGF-β1-induced CFs proliferation.

Knockdown of ANGPTL2 attenuated cardiac myofibroblast differentiation and decreased the expression of α-SMA and pro-fibrotic molecules induced by TGF-β1 in CFs.

We examined the effect of ANGPTL2 knockdown on cardiac myofibroblast differentiation. As shown in Figure 3A, knockdown of ANGPTL2 significantly prevented cardiac myofibroblast differentiation. Cardiac fibrosis is characterized by the excessive deposition of ECM components, such as α-SMA and collagen I, thus, we investigated the effects of ANGPTL2 on α-SMA and collagen I expression in TGF-β1-stimulated CFs. Western blot analysis indicated that TGF-β1 treatment significantly induced the protein expression of α-SMA and collagen I in CFs. However, knockdown of ANGPTL2 strikingly inhibited the TGF-β1-induced α-SMA and collagen I expression in CFs (Figure 3B).

Knockdown of ANGPTL2 attenuated TGF-β1-induced Smad3 phosphorylation in CFs

The TGF-β1/Smad3 signaling pathway plays a critical role in the development and progression of cardiac fibrosis. Therefore, we examined the effect of ANGPTL2 on the activation of TGF-β1/Smad3 pathway in CFs. As shown in Figure 4, TGF-β1 treatment caused a marked increase in phosphorylated Smad3 (p-Smad3) in CFs. However, knockdown of ANGPTL2 significantly inhibited the TGF-β1-induced p-Smad3.

Figure 3. Knockdown of ANGPTL2 attenuated cardiac myofibroblast differentiation and decreased the expression of α-SMA and pro-fibrotic molecules induced by TGF-β1 in CFs. CFs were transfected with si-ANGPTL2 or scramble and then stimulated with 10 ng/ml TGF-β1 for 24 h. A. CFs are examined with immunofluorescent staining for α-SMA. Blue dot represents the nuclear of CFs, red represents α-SMA protein. B. The protein expression of α-SMA and collagen I were analyzed by western blot. C. Quantification analysis was performed using Gel-Pro Analyzer version 4.0 software. All data are shown as means ± SD and experiments were repeated three times. *P < 0.05 vs control group, #P < 0.05 vs TGF-β1 + scramble group.
Discussion

In this study, we demonstrated that ANGPTL2 mRNA and protein levels were significantly up-regulated in TGF-β1-stimulated CFs. In addition, knockdown of ANGPTL2 inhibited cell proliferation and ECM expression in TGF-β1-stimulated CFs. Further mechanism study indicated that knockdown of ANGPTL2 significantly inhibited the TGF-β1-induced p-Smad3 in CFs.

TGF-β1 is a powerful fibrogenic that promotes the deposition of ECM and facilitates cardiac fibrosis [14]. Thus, in the current study, we used TGF-β1 to establish a fibrosis model in CFs, then investigated the role of ANGPTL2 in cardiac fibrosis. Our data showed that TGF-β1 treatment induced significant expression of ECM in CFs. In addition, we observed that the expression of ANGPTL2 at both mRNA and protein levels was significantly up-regulated in CFs induced by TGF-β1. These results suggest that ANGPTL2 is involved in regulating cardiac fibrosis.

Several studies demonstrated that excessive CFs proliferation induced cardiac fibrosis [15, 16]. Moreover, ANGPTL2 has been shown to regulate the proliferation and migration of several cell lines. Toiyama et al. confirmed that knockdown of ANGPTL2 significantly suppressed the proliferation, migration, and invasion of colorectal cancer cells [17]. Consistent with the previous studies, in this study, we found that TGF-β1 treatment markedly induced the proliferation of CFs. However, knockdown of ANGPTL2 efficiently suppressed TGF-β1-induced CFs proliferation. These results suggest that knockdown of ANGPTL2 ameliorated cardiac fibrosis through suppressing CFs proliferation.

Increasing evidences have reported that ECM crucially contributes to the pathogenic process of cardiac fibrosis [18-20]. In addition, several reports showed that TGF-β1 treatment significantly induced the protein expression of α-SMA and collagen I in cultured CFs. However, knockdown of ANGPTL2 strikingly inhibited the TGF-β1-induced α-SMA and collagen I expression in CFs. These results imply that knockdown of ANGPTL2 can effectively ameliorate cardiac fibrosis, which is at least partly related to the reduction of ECM expression in TGF-β1-stimulated CFs.

TGF-β1 signals through trans-membrane receptors, which activate Smad2/3 phosphorylation: in turn, the Smad complex translocates into the nucleus where they induce the expression of pro-fibrotic target genes, including α-SMA and collagen I [22]. It has been reported that overexpression of TGF-β in transgenic mice leads to cardiac fibrosis, whereas blockade of TGF-β with neutralizing antibodies obviously attenuates cardiac fibrosis [23]. Therefore, blocking the TGF-β1/Smads transduction pathway may be a good approach to the prevention and treatment of cardiac fibrosis [24-26]. In the current study, we observed that TGF-β1 treatment caused a marked increase in p-Smad3 in CFs. However, knockdown of ANGPTL2 significantly inhibited the TGF-β1-induced p-Smad3. These data suggest that knockdown of ANGPTL2 inhibited cell proliferation and ECM expression in TGF-β1-stimulated CFs through interfering with TGF-β1/Smad3 signaling pathway.

In conclusion, this study demonstrated for the first time that knockdown of ANGPTL2 inhibited...
cell proliferation and ECM expression in TGF-β1-stimulated CFs through interfering with TGF-β1/Smad3 signaling pathway. Thus, ANGPTL2 may be a potential therapeutic target for cardiac fibrosis.

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Disclosure of conflict of interest

None.

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References

The role of ANGPTL2 in cardiac fibrosis


