Curcumin suppresses transforming growth factor-β1-induced cardiac fibroblast differentiation via inhibition of Smad-2 and p38 MAPK signaling pathways

HUZI LIU1*, AIJUN LIU2*, CHUNLI SHI3 and BAO LI4

1Department of Cardiac Surgery, Shanxi Cardiovascular Hospital, Taiyuan, Shanxi 030024; 2Pediatric Heart Center, Beijing Anzhen Hospital, Capital Medical University, Beijing 100029; 3Outpatient Department and 4Department of Cardiology, Shanxi Cardiovascular Hospital, Taiyuan, Shanxi 030024, P.R. China

Received November 16, 2014; Accepted December 10, 2015

DOI: 10.3892/etm.2016.2969

Abstract. The differentiation of cardiac fibroblasts (CFs) into myofibroblasts and the subsequent deposition of the extracellular matrix is associated with myocardial fibrosis following various types of myocardial injury. In the present study, the effect of curcumin, which is a pharmacologically-safe natural compound from the Curcuma longa herb, on transforming growth factor (TGF)-β1-induced CFs was investigated, and the underlying molecular mechanisms were examined. The expression levels of α-smooth muscle actin (SMA) stress fibers were investigated using western blotting and immunofluorescence in cultured neonatal rat CFs. Protein and mRNA expression levels of α-SMA and collagen type I (ColI) were determined by western blotting and reverse transcription-quantitative polymerase chain reaction. In addition, the activation of Smad2 and p38 was examined using western blotting. Curcumin, SB431542 (a TGF-βR-Smad2 inhibitor) and SB203580 (a p38 inhibitor) were used to inhibit the stimulation by TGF-β1. The results demonstrated that the TGF-β1-induced expression of α-SMA and ColI was suppressed by curcumin at the mRNA and protein levels, while SB431542 and SB203580 induced similar effects. Furthermore, phosphorylated Smad-2 and p38 were upregulated in TGF-β1-induced CFs, and these effects were substantially inhibited by curcumin administration. In conclusion, the results of the present study demonstrated that treatment with curcumin effectively suppresses TGF-β1-induced CF differentiation via Smad-2 and p38 signaling pathways. Thus, curcumin may be a potential therapeutic agent for the treatment of cardiac fibrosis.

Introduction

Cardiac fibroblast differentiation, excessive biosynthesis and destruction of the interstitial extracellular matrix (ECM) in the ventricles of the heart are key features of cardiac fibrosis, which is a consequence of cardiac remodeling initiated by pathological events associated with various cardiovascular disorders (1). Induced by transforming growth factor (TGF)-β1 and other factors, cardiac fibroblasts (CFs) differentiate into α-smooth muscle actin (SMA) fiber-rich cardiac myofibroblasts that facilitate contractility and increase ECM modulation ability (1). Although these changes are important for wound repair and are beneficial for the maintenance of cardiac function, continuous myocardial fibrosis may result in abnormal myocardial stiffness and, ultimately, ventricular dysfunction (1).

Curcumin, 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, is a natural polyphenolic compound from the Curcuma longa herb, possessing multiple biological and medicinal activities (2). The pharmacological safety of curcumin has previously been demonstrated in various animal models, and curcumin is nontoxic even at high doses (3). Previous studies have demonstrated that curcumin has anti-oxidative, anti-inflammatory, anti-proliferative, pro-apoptotic and anti-carcinogenic properties (4-8). Furthermore, previous investigations into the effects of curcumin in heart disease have indicated that curcumin has a regulatory role in the cardiac remodeling process; curcumin has been demonstrated to ameliorate and reverse cardiac fibrosis, cardiac hypertrophy and heart failure in animal models (9-13). Therefore, curcumin may represent a novel therapeutic strategy for the treatment of cardiac remodeling.

TGF-β1 is a key mediator of the differentiation of fibroblasts to myofibroblasts (14), and this TGF-β1-induced effect has been demonstrated to be associated with the Smad2 and p38 mitogen-activated protein kinase (MAPK) signaling pathways (15-17). Although it has previously been demonstrated...
that curcumin has an inhibitory effect on ECM secretion in cultured CFs (10), the effect of curcumin on the differentiation of CFs and the underlying mechanisms are yet to be fully elucidated. In the present study, using cultured CFs from neonatal rats, it was demonstrated that curcumin has an inhibitory effect on TGF-β1-induced cardiac fibroblast differentiation. Furthermore, the role of Smad2 and p38 MAPK signaling in the activation of CFs and the anti-fibrotic mechanism of curcumin in the modulation of the TGF-β1 induced effects was addressed.

Materials and methods

Reagents. TGF-β1 was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Curcumin and rabbit monoclonal anti-α-SMA antibody (SP171) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rabbit polyclonal anti-collagen type I (Coll; ab34710) and -vimentin antibody (zm-0260), -glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (TA-08), and anti-vimentin antibody (α-SMA) was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Curcumin and rabbit monoclonal anti-α-SMA antibody were purchased from Abcam (Cambridge, MA, USA), whereas mouse monoclonal anti-vimentin antibody (zm-0260), -glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Abcam; ab9484), and horseradish peroxidase (HRP)-conjugated goat anti-rabbit and goat anti-mouse immunoglobulin (Ig)G secondary antibodies (ZB-2301 and ZB-2305) were purchased from ZhongsanJinqiao Biotechnology Co., Ltd. (Beijing, China). Phosphorylated and non-phosphorylated monoclonal rabbit anti-rat Smad2 (#3108 and #5339) and p38 (#9215 and #2371) primary antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). SB431542, a TGF-β-Smad2/3 inhibitor was purchased from Cayman Chemical Company (Ann Arbor, MI, USA) and SB20358, a p38 inhibitor, was purchased from Merck Millipore (Tarrytown, NY, USA). SB20358, a p38 inhibitor, was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). SB431542, a TGF-β1 inhibitor (SB431542) or 10 µM p38 MAPK inhibitor (SB203580) for 30 min, prior to treatment with recombiant 10 mg/ml TGF-β1 for 24 h. In order to investigate the activation of Smad2 and p38, CFs were treated with 10 ng/ml TGF-β1 for 1 h. Cells were subsequently harvested and stored at -80°C prior to the determination of protein and mRNA expression levels.

Immunofluorescent staining. CFs were cultured on coverslips in 6-well plates (2.5x10^4 cells/well). Growth was arrested and CFs were treated as described earlier. Following a 24-h treatment, CFs were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 (Applygen Technologies, Inc., Beijing, China). Non-specific binding was blocked via incubation with 10% normal goat serum (Applygen Technologies, Inc.). Subsequently, α-SMA was detected using a Cy5-conjugated goat anti-rabbit α-SMA polyclonal antibody (Abcam; ab6564) and stained cells were visualized using the BX51 Fluorescence Microscope (Olympus Corporation, Tokyo, Japan).

Western blotting. CFs were harvested in lysis buffer (Applygen Technologies, Inc.) containing 20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM Na_3VO_4 (pH 7.5), 1 mM phenylmethanesulfonyl fluoride, 1 mM benzamidine, 10 µg/ml leupeptin and 10 µg/ml aprotonin. Protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA), according to the manufacturer's protocol. Following boiling for 1 min to denature, 20 µg protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Membranes were blocked with 5% fat-free milk in Tris-buffered saline with Tween-20 (TBST; 10 mM Tris HCl, 150 mM NaCl and 0.1% Tween-20) for 1 h at room temperature, and incubated overnight with primary antibodies (1:2,500) at 4°C. Following each incubation, membranes were washed three times for 10 min with TBST and subsequently incubated with HRP-conjugated secondary antibody (1:5,000) for 1 h at room temperature. Using chemiluminescence (Applygen Technologies, Inc.), the membranes were scanned and quantified using Quantity-One software, version 4.2 (Bio-Rad Laboratories, Inc.), and the results were presented as the optical density of phosphorylated-protein/total protein or of the target protein/GAPDH.

Cell culture and treatment. The present study was conducted with the approval of the Ethics Committee of Experimental Animal Center of Shanxi Cardiovascular Hospital (Taiyuan, China), according to the regulations outlined by the National Institutes of Health Guidelines on the Use of Laboratory Animals. CFs were isolated from neonatal (1-3-days-old) Sprague-Dawley rats via trypsin digestion methods, as previously described (18). CFs were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, and were passaged using trypsin (1:3). Second passage CFs were used in the present study and were serum-starved for 24 h at 80% confluence in order to induce quiescence. Immunocytoc hemical analysis demonstrated that the purity of the CFs used was >95%, according to positive staining for vimentin and negative staining for von Willebrand factor.

In order to elucidate the potential effects of curcumin on the various signaling pathways in TGF-β1-induced CFs, CFs were pretreated with 20 µmol/l curcumin, 10 µM TGF-β1-Smad2 inhibitor (SB431542) or 10 µM p38 MAPK inhibitor (SB203580) for 30 min, prior to treatment with recombiant...
Target genes were amplified using SYBR Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). The PCR cycling conditions were as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 35 sec and 60°C for 1 min, with a final extension step of 72°C for 5 min. Relative mRNA levels were calculated using the $2^{-\Delta\Delta Cq}$ method (19), where $\Delta Cq$ was the difference between GAPDH and target gene critical threshold cycle (Cq) values.

**Statistical analyses.** Statistical analyses in this study were conducted using the SPSS software, version 16.0 (SPSS, Inc., Chicago, IL, USA). The results are presented as the mean ± standard error of the mean. Between-groups differences were assessed using one-way factorial analysis of variance. P<0.05 was considered to indicate a statistically significant difference. Each assay in the present study was performed in triplicate.
Results

Treatment with curcumin impairs TGF-β1-induced cardiac fibroblast differentiation. To investigate the effects of curcumin on the differentiation of CFs into myofibroblasts, western blotting was used to detect the protein expression levels of α-SMA and ColI (Fig. 1A). The results demonstrated that curcumin significantly suppressed the TGF-β1-induced protein expression of α-SMA and ColI in CFs (P<0.05), in a dose-dependent manner. These anti-differentiation effects were further validated by immunofluorescence staining of α-SMA (Fig. 1B). As compared with untreated CFs, cells induced by 10 ng/ml TGF-β1 exhibited bright fluorescence staining signals for α-SMA and prominent stress fibers; by contrast, treatment with 20 µM curcumin significantly attenuated α-SMA fluorescence signals and morphological characteristics of CFs induced by 10 ng/ml TGF-β1 (P<0.05). Furthermore, RT-qPCR was performed to analyze the mRNA expression levels of α-SMA and ColI. The results demonstrated that α-SMA and ColI mRNA expression levels in TGF-β1-induced CFs were also significantly and dose-dependently decreased following treatment with curcumin (P<0.05; Fig. 2).

TGF-β1-activated Smad2 and p38 signaling pathways in CFs. To investigate the downstream signaling pathways in TGF-β1-stimulated CFs, the levels of activated (phosphorylated) Smad2 and p38 were measured at various time points between 15 and 60 min after TGF-β1 administration (Fig. 3). As determined by western blotting, phosphorylated Smad2 and p38 (p-Smad2 and p-p38) levels were significantly increased following stimulation with TGF-β1 (P<0.05). Total Smad2 and p38 protein expression levels were also examined and found to be unaffected by TGF-β1 administration. These results indicate that treatment with TGF-β1 activates the Smad2 and p38 signaling pathways in CFs.
Smad2 inhibitor, SB431542, and p38 inhibitor, SB203580, suppress TGF-β1-induced α-SMA and Coll expression levels. To assess whether Smad2 and p38 were associated with the TGF-β1-induced differentiation of CFs, the Smad2 and p38 inhibitors, SB431542 and SB203580, respectively, were used to block these signaling pathways (Fig. 4). When administered alone, SB431542 and SB203580 exhibited no significant effect on cardiac fibroblast differentiation, as detected by consistent mRNA α-SMA and Coll expression levels (P>0.05). However, pretreatment with SB431542 or SB203580 prior to TGF-β1 administration significantly suppressed the TGF-β1-induced mRNA expression levels of α-SMA and Coll (P<0.05).

Curcumin inhibits TGF-β1-induced activation of the Smad2 signaling pathway. In CFs, TGF-β1 administration induced an early and significant increase in p-Smad2 expression levels over the baseline value in 30 min, as analyzed using western blotting (P<0.05; Fig. 5). Pre-treatment with curcumin induced a significant decrease in the degree of Smad2 phosphorylation and, as hypothesized, SB431542 (P<0.05), which is a well established TGF-βR-Smad2 inhibitor, effectively eliminated the activation of Smad2. Furthermore, pre-treatment with the p38 inhibitor, SB203580, also significantly attenuated the TGF-β1-induced Smad2 phosphorylation (P<0.05).

Curcumin inhibits TGF-β1-induced activation of the p38 pathway. The effects of curcumin on the TGF-β1-induced activation of the p38 signaling pathway were also examined. Western blot analysis demonstrated that, in TGF-β1-stimulated CFs, curcumin pre-treatment significantly decreased p-p38 expression levels, and SB203580 effectively eliminated p38 activation (P<0.05). Furthermore, SB431542 administration also significantly inhibited the phosphorylation of p38, thus preventing its activation (P<0.05; Fig. 6).

Discussion

In the present study, curcumin was demonstrated to dose-dependently inhibit the TGF-β1-induced cardiac fibroblast differentiation. Furthermore, the results indicated that these effects may be mediated by the Smad2 and p38 signaling pathways. Therefore, the present results suggested...
that curcumin may be a potential therapeutic agent for the treatment of myocardial fibrosis, which has been associated with the pathogenesis of heart failure (1).

The transformation of CFs to cardiac myofibroblasts is a key event in cardiac fibrosis. Cardiac myofibroblasts are absent from normal myocardium, however, they are the predominant source of excessive extracellular collagen in cardiac fibrosis (20,21). In addition to aggravating cardiac dysfunction, previous studies have demonstrated that the persistence of cardiac myofibroblasts also contributes to malignant arrhythmia (22-24). Therefore, factors associated with the formation of cardiac myofibroblasts are of considerable clinical interest.

High expression levels of α-SMA are a hallmark of the formation of cardiac myofibroblasts (24,25). Collagen, particularly fibrillar ColI, is the predominant component of the ECM and is excessively synthesized by cardiac myofibroblasts (26,27). The results of the present study demonstrated that curcumin administration effectively suppressed TGF-β1-induced cardiac fibroblast differentiation, as determined by the decreased expression of α-SMA and ColI, at the protein and mRNA levels. These findings are consistent with previous studies which have demonstrated that curcumin is capable of inducing anti-fibrotic effects in cultured CFs (10,28).

TGF-β1, which is the most well-characterized cytokine, induces the differentiation of CFs to cardiac myofibroblasts (29,30). Activation of the Smad cascade has an essential role in the differentiation of myofibroblasts (15,16,31) and is regarded as the classical mediator of TGF-β1. Following TGF-β1 stimulation, the TGF-β1RI serine-threonine kinase phosphorylates the receptor-Smads (R-Smads), and Smad2 subsequently forms a complex with Smad3 that, in turn, associates with a Co-Smad (Smad4) and translocates into the nucleus, where it acts as a transcription factor (32). In addition to Smad-dependent pathways, previous studies have suggested that Smad independent pathways, such as p38 and Kukreja RC: Curcumin prevents cardiac remodeling following myocardial infarction. Br J Pharmacol 167: 1550-1562, 2012.


