Curcumin Ameliorates Renal Fibrosis by Inhibiting Local Fibroblast Proliferation and Extracellular Matrix Deposition

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Abstract. Renal fibrosis is mainly characterized by activation and proliferation of interstitial fibroblasts and by excessive synthesis and accumulation of extracellular matrix (ECM) components, including fibronectin (FN) and collagen. This study investigated the effects of curcumin on proliferation of renal interstitial fibroblasts and their underlying mechanisms in vivo and in vitro. ECM components were visualized by Sirius red and immunohistochemistry staining and quantified by western blot analysis in mice with unilateral ureteral obstruction (UUO). Duplex staining for proliferating cell nuclear antigen and α-smooth muscle actin (α-SMA), as well as MTT and flow cytometry assays, were performed to measure fibroblast proliferation. Protein expression of phosphorylated Smad2/3 (p-Smad2/3) and peroxisome proliferator-activated receptor-γ (PPAR-γ) were assessed by western blotting. Curcumin treatment decreased the accumulation of type I collagen and FN in the kidney of animals with UUO. Activation of rat renal interstitial fibroblasts (NRK-49F) was induced by TGF-β1. Curcumin treatment inhibited fibroblast proliferation and the cell cycle was arrested in the G1 phase. Curcumin treatment upregulated the expression of PPAR-γ and downregulated the expression of p-Smad2/3. These results suggest that curcumin treatment ameliorates renal fibrosis by reducing fibroblast proliferation and ECM accumulation mediated by PPAR-γ and Smad-dependent TGF-β1 signaling.

Keywords: curcumin, renal fibrosis, fibroblast proliferation, transforming growth factor-β1, extracellular matrix

Introduction

Chronic kidney disease (CKD) is a growing public health problem, and new pharmacological agents are urgently needed to treat this disease. Renal fibrosis, a common pathological alteration in patients with CKD, is characterized by tubular atrophy/dilation and excessive deposition of extracellular matrix (ECM) (1). In an injured kidney, fibroblasts transform into myofibroblast-like cells with activation phenotypes characterized by the expression of α-smooth muscles actin (α-SMA) and increase in cellular proliferation and ECM production (2–4). Therefore, interstitial fibroblasts are target cells for the treatment of renal fibrosis. Fibroblast proliferation may serve an important function in the resolution of renal fibrosis by eliminating the major sources of both collagen and matrix.

Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is a polyphenolic compound isolated from the rhizomes of Curcuma longa (turmeric). This compound exhibits a wide range of antifibrotic properties, including anti-epithelial–mesenchymal transition (EMT), anti-apoptosis, anti-oxidation, and anti-inflammation properties. Peroxisome proliferator-activated receptors (PPARs) play an important role in the general transcriptional control of numerous cellular processes, including lipid metabolism, glucose homeostasis, cell cycle progression, cell differentiation, inflammation, and extracellular matrix remodeling (5). We previously demonstrated that curcumin dramatically induced gene expression of PPAR-γ in activated hepatic stellate cells (HSC) and rat cardiac fibroblasts, which facilitated its trans-activation activity, leading to the inhibition of HSC and fibroblast proliferation, and the suppression of ECM production (6, 7). However, whether curcumin can inhibit renal fibroblast proliferation has yet to be
tested. It is well known that all signaling pathways activated by chronic renal injury converge on upregulation of TGF-β1 signaling. TGF-β1 induces renal fibrosis by activating interstitial fibroblasts to produce massive amounts of matrix components (8). TGF-β1–mediated activation of Smad signaling is critical in renal fibrosis (9). In this study, we investigated the effects of curcumin on renal fibroblast proliferation and ECM production in vivo and in vitro. Potential mechanisms explaining these effects are discussed.

**Materials and Methods**

**Reagents**

The following reagents were used: HRP and DAB quanto detection system (Lab Vision corporation, CA, USA); anti-mouse α-smooth muscles actin (α-SMA), PPAR-γ, and collagen I antibodies (Boster Company, Wuhan, China); CY3-goat anti-mouse Immunoglobulin G and FITC-goat anti-rabbit (Google Company, Wuhan, China); antibodies to PCNA, FN, p-Smad2/3, and cyclin D (Sigma, St. Louis, MO, USA); curcumin (Sangon, Shanghai, China); TGF-β1 (R&D Systems, Minneapolis, MN, USA); and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO, USA).

**Animal model of renal fibrosis**

Male C57 mice (7 – 10-week-old) weighing 18 – 24 g were obtained from Wuhan University (China). Unilateral ureteral obstruction (UUO) was performed as described previously, and the left kidney and ureter were exposed through a flank incision. The left ureter was ligated with 4-0 silk. Finally, the wound was closed in layers. Control animals underwent identical surgical procedures, but the left ureter was simply manipulated. Renal fibrosis was induced in mice with UUO. The mice were divided into the sham operation group, UUO model group, first curcumin group, and second curcumin group. Mice from the first and second curcumin groups were administered with curcumin by gastro gavage at daily doses of 50 and 100 mg/kg (curcumin dissolved in carboxymethyl cellulose sodium), beginning the first day after surgery, and continued until groups of mice were killed 14 days later.

**Cell culture and treatments**

All experiments were performed using rat normal renal interstitial fibroblast cells (NRK-49F) and rat normal renal tubular epithelial cells (NRK-52E) cultured in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum. The cells were then treated with TGF-β1 (10 ng/ml) or curcumin (10, 20, and 30 μM) to evaluate the suppressive effects of curcumin on TGF-β1–induced activation of fibroblasts and tubular epithelial cells.

**Sirius red staining**

To estimate the degrees of fibrosis, sirius red staining was performed. The slides were deparaffinized and rehydrated in distilled water. The sections were first treated in 37°C in Sirius red solution for 30 min. Finally, the slides were dehydrated and cleared through graded ethyl alcohol.

**Immunohistochemistry staining**

Slides were deparaffinized in xylene and hydrated in graded ethanol. Tissue sections were treated with 3% hydrogen peroxide (H2O2) solution for 15 min at room temperature, followed by washing with phosphate-buffered saline (PBS) 3 times. The antigens were retrieved by boiling the sections in tris-ethylenediamine tetra-acetic acid buffer for 15 min in a microwave oven under high power. After cooling, the slides were washed 3 times in PBS. Subsequently, sections were incubated with primary antibodies at 37°C for 1 h. The sections were washed, further incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit/mouse polyclonal antibody for 10 min at 37°C, and developed with 3,3-diaminobenzidine (DAB) for color reaction. The color reaction was stopped by washing in distilled water twice and sections were then counterstained with hematoxylin.

**Immunofluorescence staining**

For duplex immunofluorescence staining, the tissue sections were deparaffinized in xylene and hydrated in graded ethanol. The antigens were retrieved by boiling the sections in tris-ethylenediamine tetra-acetic acid buffer for 15 min in a microwave oven (high power). Tissue sections were treated with 0.5% bovine serum albumin (BSA) solution for 15 min at room temperature, followed by washing with PBS 3 times. Sections were then incubated first in solutions containing both mouse anti-α-SMA and rabbit anti-PCNA antibodies for 1 h and then with CY3-goat anti-mouse and FITC-goat anti-rabbit Immunoglobulin G secondary antibodies for 1 h. For cell immunofluorescence staining, cells cultured on coverslips were washed with cold PBS twice and then fixed with cold methanol:acetone (1:1) for 15 min at 20°C. Following 3 washes with PBS containing 0.5% BSA, the cells were blocked with 0.1% Triton X-100 and 2% BSA in PBS buffer for 30 min at room temperature and then incubated with specific primary antibodies and secondary antibodies as described above. Cell nuclei were double-stained with DAPI.
Fig. 1. Renal paraffin sections from different groups were stained with Sirius red. Every field in each section was analyzed at 200 × magnification. A) Sham group: No fibrotic septa and other lesions were observed; UUO model group: a large number of collagen fibers were observed; 50 and 100 mg/kg–treated group: number of collagen fibers decreased. B) Image-Pro Plus analysis of positive area in renal sections of different groups. *P < 0.05 vs. the sham group, #P < 0.05 vs. the UUO group, △P < 0.05 vs. the 50 mg/kg curcumin group.

Fig. 2. Renal paraffin sections were immunostained with fibronectin and collagen I antibody, and image analysis was performed at 200 × magnification. Positive expression of fibronectin and collagen I was observed only in interstitial cells. A) Sham group: No fibrotic septa and other lesions were observed, UUO model group: a large number of collagen fibers were observed, 50 and 100 mg/kg–treated group: numbers of fibronectin and collagen I decreased. B) Western blot analysis of fibronectin and collagen I expression in the sham, UUO, and UUO with curcumin treatment (50 and 100 mg/kg body weight) groups. C) Quantification of fibronectin and collagen I expression was performed by densitometric analysis. *P < 0.05 vs. the sham group, #P < 0.05 vs. the UUO group.

Fig. 3. Renal paraffin sections were dually stained with α-SMA and PCNA to indicate cellular proliferation of fibroblasts. Image analysis was performed at 200 × magnification. The red granules in interstitial cells mark positive expression of α-SMA, and the bluish green granules indicate positive expression of PCNA (green) in the nucleus (blue). Notably, many double-positive (PCNA + SMA) cells are seen in the UUO and treatment groups. A) Sham group: no proliferative cell was observed; UUO model group: a large number of positive cells were observed; 50 and 100 mg/kg–treated group: Number of positive cells decreased. B) Quantification of double-positive (PCNA + SMA) cells was performed using Image-Pro Plus. *P < 0.05 vs. the sham group, *P < 0.05 vs. the UUO group, †P < 0.05 vs. the 50 mg/kg curcumin group.
Western blot analysis

Kidney and cell culture samples were sonicated and resuspended in 0.4 ml RIPA lysis buffer. Protein concentration estimations were performed with a detergent-compatible protein assay kit and 100 μg total protein was loaded in each well and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels. Gels were electroblotted onto a polyvinylidene difluoride membrane. Blots were incubated first with primary antibodies overnight at 4°C and with peroxidase-conjugated secondary antibodies for 60 min at room temperature. Bound antibodies were detected using an ECL advanced system. Band intensities were analyzed using the Quantity One software.

MTT and FCM

Stock MTT solution (20 μl) was added into each well and then incubated for 4 h in an incubator. The supernatant from each well was drawn out and then added with 150 μl of dimethyl sulfoxide (DMSO). The plate was vigorously shaken for 15 min to dissolve the resulting blue crystals. Absorbance was measured at 492 nm using an enzyme-linked immunosorbent assay. Inhibitory rate was calculated as \((1 - \frac{A_1c}{AO}) \times 100\), where AO and A1 represent the total absorbance of the control and experimental groups, respectively. FCM with propidium iodide labeling was performed to detect cell cycle stage. The proportions of cells in G1, G2/M, and S phases were then quantified.

Statistical analysis

All experiments were repeated three times. Statistical analyses were performed by conducting one-way analysis of variance (ANOVA) and Tukey’s post hoc test. Data are shown as the mean ± S.D. \(P < 0.05\) was considered to indicate a statistically significant difference.

Results

Curcumin decreased the accumulation of extracellular matrix

Sirius red staining illustrated that curcumin treatment reduced collagen deposition in UUO-induced renal fibrosis (Fig. 1A). Image analysis showed that the percentage of positive area was lower in the curcumin-
treated groups than in the UUO model group (Fig. 1B). Immunohistochemistry and/or western blotting showed that curcumin treatment reduced the accumulation of type I collagen and fibronectin (Fig. 2: A – C).

Curcumin inhibited fibroblast proliferation in vivo and in vitro

Fibroblast proliferation, as identified by two-color immunostaining for α-SMA and PCNA, was prominent in the UUO kidney and in fibroblasts activated by TGF-β1. This was substantially reduced by curcumin treatment (Figs. 3 and 6). Curcumin treatment inhibited cell proliferation in dose- (Fig. 5A) and time-dependent (Fig. 5B) manner. FCM showed that curcumin affected the cell cycle of fibroblasts and retarded fibroblasts in the G1 phase (Fig. 7: A and B). The progression of G1/S was affected by the G1 phase–related regulatory protein cyclin D1. As shown in Fig. 7C, the TGF-β1 group

![Fig. 6. NRK-49F cells were dually stained with α-SMA and PCNA to determine cellular proliferation of fibroblasts. Image analysis was performed at 200 × magnification. The red granules in interstitial cells mark positive expression of α-SMA, while the bluish green granules mark positive expression of PCNA (green) in the nuclei (blue). Many double-positive (PCNA + SMA) cells are seen in the TGF-β1 and treatment groups (arrow). A) TGF-β1 group: a large number of positive cells were observed; Curcumin treatment group: number of positive cells decreased. B) *P < 0.05 vs. TGF-β1 group alone, #P < 0.05 vs. curcumin low dosage treatment group.](image)

![Fig. 7. Effects of curcumin on cell cycle redistribution of NRK-49F cells measured by flow cytometry. Passaged NRK-49F cells (in serum-free medium) were exposed to curcumin (10 – 30 μM) and incubated for 48 h. A) After fixation and labeling with propidium iodide (PI), cells labeled with PI were detected by flow cytometry. B) Cell cycle arrest occurred in the G1 phase, resulting in increase in the number of cells in this phase. Flow cytometry analysis showed that curcumin affected the cell cycle of fibroblasts by retarding fibroblasts in the G1 phase. C) G1 was affected by the G1 phase–related regulatory protein cyclin D1. The control group showed significantly higher cyclin D1 expression than the other experimental groups. D) Curcumin inhibited protein expression of cyclin D1 (*P < 0.05).](image)
showed a significantly higher level of cyclin D1 than the other experimental groups. As shown in Fig. 7D, curcumin dramatically restrained the expression of cyclin D1 proteins ($P < 0.05$). Of note, curcumin treatment did not inhibit the proliferation of tubular epithelial cells (Fig. 5A).

Curcumin treatment increased PPAR-$\gamma$ expression level in mice with UUO

Our data showed that total PPAR-$\gamma$ expression was increased after UUO, and curcumin treatment for 24 h increased PPAR-$\gamma$ expression level (Fig. 4: A and B). The inhibitory effects of curcumin on fibroblast proliferation were attenuated by PPAR-$\gamma$.

Curcumin inhibits fibroblast proliferation via smad2/3 in vivo and in vitro

To investigate whether curcumin is involved in fibroblast proliferation through Smad pathways, we examined levels of the p-Smad2/3 protein in TGF-$\beta$1–treated cells pre-incubated with or without curcumin and in mice with UUO. Western blot analysis showed that curcumin treatment significantly decreased p-Smad2/3 expression in mice with UUO (Fig. 4C) and in NRK-49F cells (Fig. 8).

Discussion

The current study demonstrated that mice with UUO exhibited significant fibrotic changes that were associated with fibroblast proliferation. Curcumin treatment significantly decreased fibrosis. Furthermore, curcumin at different concentrations inhibited NRK-49F stimulated by TGF-$\beta$1. Previous studies reported that curcumin exerts its antifibrotic action by a) reducing EMT (10); b) inducing apoptosis in myofibroblasts (11); c) modulating inflammatory responses (12); d) blocking NF-$\kappa$B signaling, ERK signaling, and the Rac1/MLK3/JNK pathway (13 – 15); e) attenuating oxidative stress (16); and f) regulating ion channels and transporters (17). However, we found out that suppression of fibroblast proliferation via activation of PPAR-$\gamma$ might be another antifibrotic mechanism by which curcumin functions. Renal fibrosis is generally preceded by massive activation and expansion of interstitial fibroblasts and myofibroblasts because these cells are the principal matrix-producing effector cells. The sizes of activated fibroblasts and myofibroblast population in fibrotic kidneys are believed to be a determining factor in the progression and prognosis of a wide variety of chronic kidney diseases (18). It is recognized that local fibroblast proliferation is an important mechanism of myofibroblast accumulation (19). However, there is controversy regarding the origin of renal myofibroblasts. The traditional concept holds that myofibroblasts primarily derive from local stromal cells in the kidney such as resident fibroblasts and pericytes. Recent studies using state-of-the-art fate mapping techniques have cast serious doubt on the significance of EMT as generator of myofibroblasts in renal fibrosis. It is noteworthy that this was not found in our study to result in generalized inhibition of cell proliferation, since tubular epithelial cell proliferation, which accounts for the majority of proliferating cells in the UUO kidney, was not significantly affected by curcumin treatment in vitro. However, we confirmed that curcumin may inhibit local fibroblast proliferation to decrease the sources of myofibroblasts. Proliferation of myofibroblasts of other origin may need to be confirmed in additional studies.

PPAR-$\gamma$ is present and active in multiple renal cell types. Studies have shown that PPAR-$\gamma$ agonists can ameliorate renal fibrotic lesions in diabetic nephropathy and non-diabetic chronic kidney diseases (20, 21). In this study, we demonstrated that curcumin induces the expression of PPAR-$\gamma$ in a UUO model. The mechanisms by which curcumin induces PPAR-$\gamma$ expression remain poorly understood.

TGF-$\beta$1 is the prime stimulator of fibroblast activation, as shown here and in previous studies. TGF-$\beta$1 can also stimulate fibroblast proliferation and we demonstrated that curcumin can inhibit this in vitro proliferative response in a dose- and time-dependent manner as well. The concentration range of curcumin (10 – 30 $\mu$M) used in previous studies was similar to that used in the present study (22). TGF-$\beta$1 mediates its biological effects...
through downstream signaling effectors that belong to the Smads protein family (23). Earlier studies revealed that TGF-β1 specifically initiates phosphorylation of Smad2/3 (24). Thus, disruption of p-Smad2/3 may potentially lead to interception of TGF-β1 signaling that results in blockage of fibroblast proliferation and myofibroblastic activation. In this study, TGF-β1 significantly increased p-Smad 2/3 expression in NRK-49F cells, while curcumin almost reversed these effects.

In summary, curcumin can inhibit renal fibrosis, as evidenced by reduced fibroblast proliferation and ECM accumulation via PPAR-γ and Smad2/3. Our findings raise the possibility that curcumin can be developed as a therapeutic agent to ameliorate renal fibrosis.

References