Biochemical and Biophysical Research Communications xxx (2015) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Astragaloside IV suppresses transforming growth factor-β1 induced fibrosis of cultured mouse renal fibroblasts via inhibition of the MAPK and NF-κB signaling pathways

Xiajing Che, Qin Wang, Yuanyuan Xie, Weijia Xu, Xinghua Shao, Shan Mou**, Zhaohui Ni*

Department of Nephrology, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China

ARTICLE INFO

Article history:
Received 14 July 2015
Accepted 23 July 2015
Available online xxx

Keywords:
Astragaloside IV
Renal fibroblasts
Proliferation
Transdifferentiation
ECM production
Transforming growth factor-β (TGF-β)

ABSTRACT

Renal fibrosis, a progressive process characterized by the accumulation of extracellular matrix (ECM) leading to organ dysfunction, is a characteristic of chronic kidney diseases. Among fibrogenic factors known to regulate the renal fibrotic process, transforming growth factor-β (TGF-β) plays a central role. In the present study, we examined the effect of Astragaloside IV (AS-IV), a component of the traditional Chinese medicinal plant *Astragalus membranaceus*, on the processes associated with renal fibrosis in cultured mouse renal fibroblasts treated with TGF-β1. RT-PCR, western blotting, immunofluorescence staining and collagen assays showed that AS-IV suppressed TGF-β1 induced fibroblast proliferation, transdifferentiation, and ECM production in a dose-dependent manner. Examination of the underlying mechanisms showed that the effect of AS-IV on the inhibition of fibroblast differentiation and ECM formation were mediated by its modulation of the activity of the MAPK and NF-κB signaling pathways. Taken together, our results indicate that AS-IV alleviates renal interstitial fibrosis via a mechanism involving the MAPK and NF-κB signaling pathways and demonstrate the therapeutic potential of AS-IV for the treatment of chronic kidney diseases.

1. Introduction

Fibrosis is the excessive accumulation of extracellular matrix (ECM) caused by chronic inflammation, resulting in the disruption of normal tissue architecture and organ dysfunction [1]. Renal interstitial fibrosis, which is a feature of chronic kidney disease, involves the proliferation of renal fibroblasts and deposition of ECM, a dynamic structure composed of proteoglycans and fibrous proteins such as collagen, fibronectin, and laminin [2,3]. The cytokine transforming growth factor-β (TGF-β) functions as an important mediator of fibrogenesis and plays a critical role in the pathogenesis of kidney diseases [3]. The TGF-β superfamily is implicated in a wide range of cellular functions including the regulation of tissue homeostasis and repair, immune and inflammatory responses, extracellular matrix deposition, and cell differentiation and growth [1]. TGF-β signaling involves a canonical or Smad dependent pathway, which involves the Smad family of transcriptional activators, and non-canonical signaling, by which TGF-β activates members of the mitogen-activated protein kinase (MAPK) family including extracellular signal-regulated kinase (ERK), p38 MAPK and c-Jun-N-terminal kinase (JNK) [4]. Three TGF-β isoforms exist in mammals (TGF-β1–3), and despite being expressed in a tissue-specific manner, they signal through the same surface receptors and have similar targets [5]. TGF-β1 is ubiquitously found in mammalian tissues and is primarily responsible for the development of tissue fibrosis. TGF-β1 induces the activation of fibroblasts to undergo a phenotypic transition to myofibroblasts, which are the effectors of the fibrotic state and are characterized by their association with contractile proteins such as α-smooth muscle actin (α-SMA) and non-muscle myosin [6]. In addition to its role in myofibroblast transdifferentiation, TGF-β promotes matrix preservation and deposition by inducing the expression and increasing the stability of type I collagen, suppressing the activity of matrix metalloproteases, and inducing the synthesis of protease inhibitors such as plasminogen activator inhibitor-1 (PAI-1) and tissue inhibitors of metalloproteinases (TIMPs) [7,8]. TGF-β also plays an

* Corresponding author. Department of Nephrology, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, 160 Pujian Road, Shanghai 200127, China.
** Corresponding author. Department of Nephrology, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, 160 Pujian Road, Shanghai 200127, China.
E-mail addresses: shan_mou@126.com (S. Mou), doctor_nzh@126.com (Z. Ni).

http://dx.doi.org/10.1016/j.bbrc.2015.07.116
0006-291X/

important role in endothelial–mesenchymal transition, which is characterized by the downregulation of epithelial markers such as E-cadherin and upregulation of mesenchymal markers such as vimentin and α-SMA [9].

Astragaloside IV (AS-IV), the main active component of the traditional Chinese medicinal plant *Astragalus membranaceus*, has anti-inflammatory, anti-hypertensive, anti-diabetes, and myocardial protective properties [10]. AS-IV was previously shown have an antifibrotic effect mediated by the MAPK pathway in human kidney tubular epithelial cells and by TGF-β/Smad signaling in TGF-β stimulated rat renal fibroblasts *in vitro* and in a mouse model of unilateral ureteral obstruction *in vivo* [11,12].

In the present study, we investigated the effect of AS-IV on TGF-β1 induced cell proliferation, myofibroblast differentiation and ECM production in cultured mouse renal fibroblasts to assess its potential value as an antifibrotic agent for the treatment of kidney diseases.

2. Materials and methods

2.1. Cell preparation and culture

Primary renalfibroblasts were obtained from Balb/c mice (5–6w) using the method reported by Lonnemann et al. [13]. Briefly, kidneys were dissected, cut off the cortex with a surgical blade, and renal fibroblasts were isolated in 2.5 mg/ml trypsin and 1 mg/ml collagenase II (Sigma, St. Louis, MO) in phosphate buffered saline (PBS) for 20 min at 37 °C. Cells were cultured in DMEM supplemented with 15% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in a 5% CO_2 incubator at 37 °C. Before each experiment, primary cultured renal fibroblasts were incubated in 1% FCS-DMEM for 24 h tominimize serum-induced effects. TGF-β1 (Sigma) was dissolved in PBS containing 0.1% bovine serumalbumin (BSA).

2.2. Immunofluorescence staining

For immunofluorescence detection, cultured fibroblasts were washed three times in PBS, fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, and exposed to the indicated primary antibodies (Santa Cruz, CA, USA) overnight at 4 °C. After exposure to secondary antibodies and counterstaining with DAPI, cells were visualized under a fluorescence microscope (Olympus Corp, Tokyo, Japan).

2.3. Cell proliferation assay (MTT assay)

Cell viability was evaluated by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells (1×10^3 cells/well) were seeded into 96-well plates in 100 μl culture medium for 72 h before the addition of 100 μl of MTT reagent (Promega, USA) (0.5 g/l) to each well and incubation for 90 min at 37 °C. Precipitates were dissolved in DMSO and absorbance was read at 560 nm with a reference at 690 nm using a microplate reader.

2.4. Real-time polymerase chain reaction (PCR) analysis

Total RNA was extracted from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and the cDNA was synthesized using oligo (dT) primers with the transcriptor first-strand cDNA synthesis kit. Selected gene differences were confirmed by real-time PCR using SYBR-Green and the results were normalized against GAPDH gene expression. The sequences of all primers used in this study are presented below:

α-smooth muscle actin (sense sequence 5′-GGT GCT GTC TCT CTA GCC TCT GGA-3′ and anti-sense sequence 5′-CCC ATC AGG CAA CTC GAT ACT CTT C-3′); collagen type I (sense sequence 5′-CAT CAC CTA CCA CTG CAA GAA C-3′ and anti-sense sequence 5′-ACG TCG AAG CCG AAT TCC-3′); fibronectin (sense sequence 5′-GGA TGC TCC TGC TGT CAC-3′ and anti-sense sequence 5′-CTG TTT GAT CTG GAC CTG CAG-3′); GAPDH (sense sequence 5′-GTG GAT ATT GTT GCC ATC AAT GAC C-3′ and anti-sense sequence 5′-GCC CCA GCC TTC TTC ATG GTG GT-3′).

2.5. Western blot analysis

Total protein was extracted from cultured fibroblasts using conventional methods and 50 μg of each sample were separated by SDS-PAGE, transferred onto nitrocellulose membranes, blocked with 5% skim milk in PBS, and exposed to the indicated primary antibodies overnight at 4 °C. After exposure to the adequate peroxidase-conjugated secondary antibodies, signals were visualized with FluorChem E (Cell Biosciences, Santa Clara, CA, USA). Specific protein expression levels were normalized to β-actin for total cell lysates and cytosolic proteins on the same nitrocellulose membrane.

2.6. Collagen measurement

The amount of total soluble collagen in the culture supernatant of renal fibroblasts treated as indicated was measured using the Sircol assay (Biocolor, Belfast, UK). One milliliter of Sirius red dye was added to 400 mL of supernatant, incubated with rotation for 30 min at room temperature, centrifuged at 12,000 rpm for 10 min, and the precipitate containing the collagen-dye complex was solubilized in 0.5 M sodium hydroxide. The dye concentration was quantified by spectrophotometry at 540 nm.

2.7. Statistical analysis

For all results, data were obtained from at least 3 independent experiments. The statistical significance of differences between control and experimental data was analyzed by unpaired t tests or one-way analysis of variance, followed by Tukey's test (GraphPad Prism, version 5, Graph Pad Software, San Diego, CA, USA). Significance was established at the 95% confidence level. A *P* value of less than 0.05 was considered statistically significant.

3. Results

3.1. Characterization of primary cultured neonatal rat renal fibroblasts

Cultured rat renal fibroblasts were visualized under a light microscope, which showed their characteristic spindle-shaped morphology. Immunofluorescence staining showed positive expression of vimentin and negative cytokeratin staining, indicating the purity of the isolated primary renal fibroblasts.

3.2. Effect of AS-IV on renal fibroblast proliferation

The cell viability of fibroblasts stimulated with TGF-β1 and treated with different concentrations of AS-IV for 48 h was determined using the MTT assay. The results showed that AS-IV suppressed the TGF-β1induced increase in cell viability in a dose dependent manner (Fig. 1A). Western blot assessment of the expression of proliferating cell nuclear antigen (PCNA) as a marker of cell proliferation showed that PCNA was upregulated in fibroblasts in response to TGF-β1, whereas its expression was downregulated by AS-IV in a dose-dependent manner (Fig. 1B).

Please cite this article in press as: X. Che, et al., Astragaloside IV suppresses transforming growth factor-β1 induced fibrosis of cultured mouse renal fibroblasts via inhibition of the MAPK and NF-κB signaling pathways, Biochemical and Biophysical Research Communications (2015), http://dx.doi.org/10.1016/j.bbrc.2015.07.116