Preventive Effects of Salvianolic Acid B on Transforming Growth Factor-β1-Induced Epithelial-to-Mesenchymal Transition of Human Kidney Cells

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Epithelial-mesenchymal transition (EMT) is an important mechanism in kidney fibrosis. While Salvianolic acid-B (Sal B) has been well appreciated to show a protective effect of tissue fibrosis, the objective of this study was to investigate the influence of Sal B on the transdifferentiation of renal tubular epithelial cells. Human kidney proximal tubular cell line (HK-2) was used as the proximal tubular cell model and EMT was induced with 5 ng/ml of human transforming growth factor (TGF-β1). The effects of the Sal B on cell morphology were observed by phase contrast microscopy, and the possible mechanisms were studied by immunocytochemistry and real-time reverse transcription-polymerase chain reaction. Our results revealed that Sal B could inhibit TGF-β1-induced myofibroblast phenotype and restored the epithelial morphology in a dose-dependent manner. It was partially through modulating α-smooth muscle actin (SMA) increase and E-cadherin reduction. These observations strongly suggest that Sal B is a potent inhibitor of TGF-β1-induced EMT and might be a promising agent for treating tubulointerstitial fibrosis.

Key words Salvianolic acid-B; epithelial-to-mesenchymal transition; kidney fibrosis

Tubulointerstitial fibrosis is the common pathway in progressive renal disease leading to functional deterioration and eventual loss of renal function, irrespective of the diverse initial causes.1,2 Tubulointerstitial fibrosis is involved in the accumulation of extracellular matrix components and loss of tubular architecture, while proximal tubular epithelial cells (PTEC) are increasingly being recognized for playing a central role in renal tubulointerstitial fibrosis.3 Recent studies have demonstrated that a critical step in the pathogenesis of tubulointerstitial fibrosis is epithelial-mesenchymal transition (EMT), whereby renal tubular epithelial cells change phenotypically and functionally into myofibroblasts. The factor most capable of inducing and completing EMT is transforming growth factor-β1 (TGF-β1), which is characterized by the loss expression of E-cadherin and the increasing expression of α-smooth muscle actin (α-SMA).4,5 Therefore, EMT in the kidney should be of significant interest as a therapeutic target, and in this regard, it is quite important to prevent tubular epithelial cell to EMT in tubulointerstitial fibrosis.

Radix Salviae Miltiorrhizae, one of popular traditional Chinese medicine, has been widely used around the world for numerous diseases, including cerebrovascular diseases and coronary artery diseases.7,8 Salvianolic acid-B (Sal B) is the most abundant and bioactive component from Radix Salviae Miltiorrhizae,9 which has multiple pharmacological activities such as antioxidative effect,10,11 anti-fibrogenic effect,12 myocardial salvage effect and cardioprotective effect.13-16 Extensive pharmacological studies have shown that Sal B could effectively reverse liver fibrosis.17-21 A recent clinical trial also indicated that Sal B could markedly reduce liver fibrosis in patients with chronic hepatitis B,22 which is capable of reducing α-SMA expression, collagen synthesis and deposition in TGF-β1-stimulated hepatic stellate cells.23

Radix Salviae Miltiorrhizae has also widely been used for the treatment of renal diseases in traditional Chinese medicine. Although the clinical efficacy of Radix Salviae Miltiorrhizae has been established,12,24-26 the active compounds and mechanism against renal diseases were still unclear. Interestingly, our study demonstrated that Sal B can evidently inhibit renal interstitial fibrosis in vivo, which might be related with the down regulation of TGF-β1 expression.27 However, the role of Sal B in renal fibrosis is less understood, in contrast to the detailed knowledge about the antifibrotic function in the liver. In the present study, we investigated the protective effects of Sal B on the transdifferentiation of human kidney PTEC, to elucidate the mechanism of Sal B effect on renal fibrosis.

MATERIALS AND METHODS

Materials Sal B (purity 99%) was extracted and identified by the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), and diluted into 0.1—100 μmol/l with phosphate buffer solution (PBS). Recombinant human TGF-β1 was purchased from Peprotech, U.S.A. Antibodies against α-SMA and E-cadherin were from Sigma, U.S.A. and Cell Signaling. Dulbecco’s Modified Eagle’s Medium/F12 (DMEM/F12), trypsin and N-(2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (HEPES) were from Invitrogen, U.S.A. and fetal calf serum (FCS) was from Sijiqing, China.

Cell Culture and Treatment The immortalized human kidney proximal tubular cell line (HK-2, ATCC) was cultured in DMEM/F12 containing 2.50 g/l HEPES, 1.80 g/l sodium bicarbonate, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FCS at 37 °C in 5% CO2. After digested with 0.25% trypsin, 1×105 cells were grown in 50 ml plastic culture bottles. For experiments, HK-2 cells were cultured overnight in DMEM/F12 5% FCS, then media were changed to fresh DMEM/F12 5% FCS containing TGF-β1 plus Sal B with different concentration (0.1 μmol/l, 1 μmol/l, 10 μmol/l, 100 μmol/l), with both positive (5%FCS + TGF-β1), negative (5% FCS) controls. Cultures were continued for a further 72 h.

MTT Assay The prolified fraction of Sal B was assessed
using MTT (3-[4,5-dimethyl-2-thiazoly]-2,5-diphenyl-2-tetrazoliumbromide) assay. For cell viability assays $1 \times 10^3$ cells per well were seeded onto 96-well plates with 200 $\mu$l of DMEM/F12. The cells were cultured for 24 h before treatment with Sal B or TGF-$\beta$1. After a further 72 h, a MTT solution (5 $mg/ml$) was added to the wells containing cells (final concentration 0.5 $mg/ml$). MTT was dissolved in Hank’s balanced salt solution (HBSS). The cells were incubated for 4 h at 37 °C in 5% CO$_2$. Thereafter, the medium was removed and added to blue formazan crystals dissolved in 200 $\mu$l dimethylulfoxide (DMSO); the absorbance was measured at 570 nm using a microplate reader. Data were expressed as relative viability calculated as follows: relative viability ($\%) = (\text{absorbance}_{\text{Treatment}}/\text{absorbance}_{\text{Control}}) \times 100$. The arithmetic mean OD of 8 wells for each group was calculated.

**Morphological Assessment of HK-2 Cell** The cells were grown in 6-well plates, followed growth factor and Sal B treatment, and photographed using a JVC KY-FSSBE digital camera coupled to a Nikon TMS microscope. Length/breadth ratios and mean cell areas were used to quantify morphological changes. For each drug concentration, 80 cells were measured.$^{58,28}$

**Immunocytochemistry** HK-2 Cells were fixed on coverslips using ice-cold 4% formalin for 30 min followed by washing three times in PBS. Cells were then permeabilized with 0.25% Triton X-100 for 10 min followed by three washings in PBS. Following fixation and permeabilization, the cells were incubated with 10% BSA in PBS at 4 °C overnight. The cells were then washed in the same manner and incubated for 1 h at room temperature with (1) a mouse monoclonal antibody against human $\alpha$-SMA (1:100; Abcam) and (2) a rabbit monoclonal antibody against human E-cadherin (1:200, Cell Signaling) diluted with 3% BSA in PBS at 4 °C overnight. The cells were then washed in the same manner and incubated for 1 h at room temperature with (1) FITC-conjugated anti-mouse immunoglobulin (Ig) G antibody (1:100, Sigma) and (2) PE-conjugated anti-rabbit IgG antibody (1:200, Sigma) diluted with 3% BSA in PBS. Finally, after rinsing the cells three times with PBS, coverslips were mounted on the slides using an antifade regent (Fluoro-Gaurd; Bio-Rad). Slides were washed three times in PBS. Coverslips were mounted on the slides using 3% BSA in PBS at 4 °C overnight. The cells were then washed in the same manner and incubated for 1 h at room temperature with (1) a mouse monoclonal antibody against human $\alpha$-SMA (1:100; Abcam) and (2) a rabbit monoclonal antibody against human E-cadherin (1:200, Cell Signaling) diluted with 3% BSA in PBS at 4 °C overnight. The cells were then washed in the same manner and incubated for 1 h at room temperature with (1) FITC-conjugated anti-mouse immunoglobulin (Ig) G antibody (1:100, Sigma) and (2) PE-conjugated anti-rabbit IgG antibody (1:200, Sigma) diluted with 3% BSA in PBS. Finally, after rinsing the cells three times with PBS, coverslips were mounted on the slides using an antifade regent (Fluoro-Gaurd; Bio-Rad). Slides were blinded and three random fields were digitized using a Nikon microscope attached to a digital camera. Each experiment was repeated 3 times.

**Semiquantitative Real-Time Analysis** Total RNA was extracted from the HK-2 cells using TRIzol reagent according to the manufacturer’s instructions. Reverse transcription was performed using the Superscript III RT kit (Invitrogen) according to the manufacturer’s protocols. mRNA levels of $\alpha$-SMA and E-cadherin were analyzed, while house keeping gene-“glyceraldehyde-3-phosphate dehydrogenase (GAPDH)” was used as the internal standard. The oligonucleotide primers used were: for $\alpha$-SAM, sense 5’-CCAGCTATGT-GTGAAGAGAGG-3′ and anti-sense 5’-GTGATTCCTT-TCTGATTCGCT-3′; for E-cadherin, sense 5’-TGCTGCA-AGGTCCCTCTTGG-3′ and anti-sense 5’-AGTCCCAGGG-CGTAAGCAACAG-3′; while, for GAPDH, sense 5’-CTCA-GACACCATGGGGAAGTGA-3′ and anti-sense ATCTTG-AGCTGTGTTCATA-3′. Real-time polymerase chain reaction (PCR) amplification was performed using the SYBR Green master mix (Applied Biosystems) and the Prism 7300 Real-time PCR Detection System (Applied Biosystems). Cycling conditions were 95 °C for 10 min followed by 40 repeats of 95 °C for 15 s and 60 °C for 1 min. Relative amounts of mRNA were normalized by GAPDH and calculated using the delta–delta method from threshold cycle numbers.$^{23}$ Standardization of real-time PCR gene expression data were representative of four independent experiments.

**Statistical Analysis** All data are expressed as mean ± S.D. Multiple groups of values were compared using one-way analysis of variance (ANOVA) and by Fisher’s LSD test ($p＜0.05$). Data were analyzed by SPSS 10.0 programs for Windows. A value of $p＜0.05$ was considered significant.

**RESULTS**

**TGF-$\beta$1-Induced EMT of HK-2 Cells** As TGF-$\beta$1 is a well-characterized inducer of EMT in renal tubular epithelial cells, we first analyzed the changes in HK-2 cells treated by TGF-$\beta$1 alone. HK-2 cells exhibit typical cobblestone morphology of epithelial cells when grown in culture (Fig. 1A). They profoundly showed morphologic changes after TGF-$\beta$1 treatment for 72 h, with cells becoming elongated in shape, disassociating from neighboring cells and losing their cobblestone monolayer pattern (Fig. 1B). Mean cell area for PTEC controls at 72 h (1942.3±85.9 $\mu$m$^2$) was significantly greater than for TGF-$\beta$1-induced myofibroblast controls (1580.4±126.2 $\mu$m$^2$, $p＜0.001$; Table 1).

The $\alpha$-SMA and E-cadherin expression of HK-2 cells after TGF-$\beta$1 treatment for 72 h (Figs. 2, 3). After HK-2 cells were incubated in medium containing 5 ng/ml of TGF-$\beta$1 alone for 72 h, both protein and mRNA levels of $\alpha$-SMA were up-regulated, while reduction expression of E-cadherin was detected. The changes in mRNA for $\alpha$-SMA or E-cadherin reached the statistical significance ($p＜0.001$; respec-

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**Fig. 1.** Preventive Effect of Sal B on HK-2 Cells Morphology

The cells were grown in 24-well plates for 72 h and then treated with 5%FCS (A), 5%FCS + TGF-$\beta$1 (5 ng/ml, B) and TGF-$\beta$1 (5 ng/ml) plus Sal B (0.1 $\mu$mol/l, 1 $\mu$mol/l, 10 $\mu$mol/l, 100 $\mu$mol/l, from C to F) for another 72 h. Photographs were taken using a Nikon TMS microscope.
It was confirmed that HK-2 cells were successfully transdifferentiated into myofibroblasts.

**Influence of Sal B on HK-2 Cells** After being cultured for 24 h, HK-2 cells were exposed to Sal B or/and TGF-β1 for a further 72 h in DMEM/F12 5% FCS then cell survival was assessed by MTT assay. As shown in Table 2, the relative viability of cells was increased by Sal B with 100 μmol/l, while the other concentrations did not show a significant inhibitory effect. It suggested the effect of Sal B at the concentration used in this study is not mediated by the cytotoxic responses. The appropriate concentration of Sal B was safe to prevent the EMT of HK-2 cells.

**Preventive Effects of Sal B on TGF-β1-Induced EMT** of HK-2 Cells The preventive effect of Sal B in TGF-β1-induced EMT was analyzed in present study. Simultaneous incubation of Sal B with TGF-β1 protected the change to the myofibroblast phenotype and largely restored the epithelial morphology of the HK-2 cells (Fig. 1). Moreover, the de-
crease in cell area and increase in the length/breadth ratio normally induced by TGF-β1 was prevented by Sal B (Table 1). In line with this finding, Sal B treatment showed obvious dose-dependent roles. Sal B at high concentration appeared to return the morphology to that of PTEC (Fig. 1F), with a significant increase in cell areas and reduction in the length/breadth ratio (p<0.001, respectively) compared with cells maintained in TGF-β1 supplemented media alone. But, the lowest concentration did not successfully prevent EMT-related changes in morphology.

All concentration significantly prevented the change in α-SMA and E-cadherin (Fig. 2). Sal B showed a dose-dependent prevention of α-SMA increase and E-cadherin reduction, with the high concentration significantly inhibiting the lesions compared to the TGF-β1 controls (p<0.001, respectively; Fig. 3). The α-SMA induction was completely abolished by 100 μmol/l Sal B, but also showing significantly lower levels in E-cadherin compared to FCS controls (p<0.05). These results were confirmed by immunofluorescence showing abundant expression of E-cadherin and reduced expression of α-SMA following Sal B treatment, when compared with the TGF-β1 controls (Fig. 2). These results indicate that Sal B not only prevents the de novo expression of the myofibroblast marker α-SMA in HK-2 cells but also prohibits the loss of the epithelial marker E-cadherin.

DISCUSSION

Emerging evidence suggests that EMT is a major event in the pathogenesis of tubulointerstitial fibrosis. In response to TGF-β1, tubular epithelial cells transdifferentiate to myofibroblasts. The phenotypic conversion involves loss of epithelial polarity and E-cadherin, disruption of tubular basement membrane, acquisition of spindle-like morphology, and production of matrix proteins. Acquisition of spindle-like morphology, loss of epithelial E-cadherin, and de novo synthesis of α-SMA are specific markers of EMT.

HK-2 cells are one of the best-characterized renal epithelial cells and have been used by a number of studies to investigate various aspects of EMT. In this study, exposure of HK-2 cells to TGF-β1 for 3 d induced a complete conversion of the epithelial cells to myofibroblasts as evidenced by acquisition of spindle-like morphology, loss of E-cadherin, and activation of α-SMA. The time frame required for this transition was similar to previous reports.

Sal B is the most abundant and bioactive component from Radix Salviae Miltiorrhiza, which has been well considered to have multiple pharmacological activities such as antioxidative effect, antifibrogenic effect, myocardial salvage effect. Recently many reports revealed that Sal B had potential protective effects in renal disease. But, there is insufficient information on its protective properties in nephropathy cellular model.

This study is the first to investigate the effects of Sal B on EMT in human PTEC. Our results demonstrated that Sal B exhibited a remarkable inhibitory effect on TGF-β1-induced EMT. With the high concentration of Sal B, this inhibition was complete as evidenced by a full restoration of epithelial morphology, mediated by an increase of E-cadherin expression and a complete abolishment of α-SMA expression, simultaneously. These observations strongly suggest that Sal B could be essential in maintaining the structural and functional integrity of normal tubular epithelium. Regarding to TGF-β1 is a key modulator of organ fibrosis after tissue injury and EMT is a critical step in the pathogenesis of tubulointerstitial fibrosis, our data shed new light on the mechanism by which Sal B elicits its renoprotective action in the pathogenesis of tubulointerstitial fibrosis.

Furthermore, Sal B showed a dose-dependent preventive effects of TGF-β1-induced EMT, although it at all concentrations exhibited the inhibitory effect. At low concentrations, Sal B only had partial effects, while at high concentration, Sal B could completely prevent the TGF-β1-induced EMT. These results suggest that only the high concentration of Sal B exhibited a remarkable renoprotective action by targeting EMT in the pathogenesis of tubulointerstitial fibrosis. The result was instructive in the clinical application of Sal B.

Sal B has been reported to be rapidly and completely taken up by hepatocytes in vivo, where most of the Sal B was methylated. Liu et al. reported that Sal B showed no obvious side effects even if in antifibrotic treatment of liver fibrosis in patients with chronic hepatitis B. Our results showed that Sal B at all concentrations had nontoxicity in HK-2 cells and the relative viability of HK-2 cells was even increased by Sal B with high concentration. This may explain that Sal B is highly security in the clinical application. It was also corroborated with better performance and fewer side effects of Radix Salviae Miltiorrhiza confirmed during the long-time clinical usement.

Although in vitro models do not reliably reflect the clinical setting and ongoing clinical trials are required, this study provided evidence that Sal B exhibits a remarkable ability to prevent this epithelial to myofibroblast transition. It demonstrates the significant implications for developing clinically relevant therapeutic strategies for renal tubulointerstitial fibrosis.

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