Tranilast attenuates TGF-β1-induced epithelial-mesenchymal transition in the NRK-52E cells

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Abstract: We previously reported that tranilast can halt the pathogenesis of chronic cyclosporine nephrotoxicity in rats via the transforming growth factor-β (TGF-β) /Smad pathway, an important signaling system involved in epithelial-mesenchymal transition (EMT), but the exact underlying cellular mechanisms are not yet clear. Thus, by selecting [0] TGF–β1–induced normal rat kidney proximal tubular epithelial cells (NRK-52E) as a model, we demonstrated potential modifying effect of tranilast on EMT-induced by TGF–β1 in vitro. NRK-52E cells were incubated with the blank vehicle (Dulbecco's modified Eagle's medium and F-12 (DMEM/F12) added with 10% fetal bovine serum (FBS)), 10 ng/ml TGF-β1 alone or together with 100, 200 or 400μM tranilast for 48 h after incubation in medium containing 1% FBS for 24 h. Cell morphological changes were observed to confirm occurrence of EMT. Protein expressions of two typical markers of EMT, E-cadherin and α-smooth muscle actin (α-SMA), were assessed by western blotting and flow cytometry, respectively. Our results showed that TGF-β1 induced spindle-like morphological transition, the loss of E-cadherin protein and upregulation of expression of α-SMA. However, the TGF-β1-produced changes in cellular morphology, E-cadherin and α-SMA were inversed by tranlilast in concentration-dependent manner. Our findings indicate that tranilast can directly inhibit EMT. Thus, it may be implied that regulation of EMT be the target to prevent renal tubulointerstitial fibrosis.

Keywords: α-SMA, E-cadherin, EMT, Tranilast, TGF-β1

INTRODUCTION

The progression of chronic nephrological disorders eventually results in the end-stage renal diseases (ESRD). The patients suffered with ESRD may need life-long dialysis, and some time, they need renal transplantation for life support. The pathogenesis of ESRD is characterized by renal tubulointerstitial fibrosis (RIF), in which the tubulointerstitial myofibroblasts play a key role (Simonson, 2007). Major tubulointerstitial myofibroblasts are produced from tubular epithelial cells, then these cells can undergo an transition from epithelial to mesenchymal (Liu, 2004; Strutz and Zeisberg, 2006). It is well-known that EMT is regulated by a number of growth factors in various ways. Among all many factors identified, transforming growth factor-β1 (TGF-β1) is the most effective one in induction of EMT. Several studies have demonstrated that typical phenotypic alterations similar to EMT in cultured tubular epithelial cells treated with TGF-β1 (Hay, 1990; Hay, 2005; Thiery and Sleeman, 2006). There also some studies showed that in the absence of EMT, tubulointerstitial fibrosis was inhibited. Thus, these studies provide strong evidence for a specific role of EMT in the development of tubulointerstitial fibrosis.

Tranilast, with chemical structure name as N- (3, 4-dimethoxycinnamoyl) anthranilic acid, is a chemical drug clinically used for treating allergic diseases including bronchial asthma, allergic rhinitis, hypertrophic scars and atopic dermatitis (Suzawa *et al.*, 1992), and scleroderma (Taniguchi *et al.*, 1994). Some of these diseases are associated with excessive fibrosis. In addition, tranilast also could attenuate tubulointerstitial fibrosis caused by unilateral ureteral obstruction (UUO) in rats (Kaneyama *et al.*, 2010; Kelly *et al.*, 2004) and advanced experimental diabetic nephropathy (Mifsud *et al.*, 2003). Our recent study showed that tranilast has significant inhibiting effects on renal fibrosis in rats with chronic cyclosporine nephrotoxicity by regulation of the TGF-β/Smad pathway in vivo (Tao *et al.*, 2011). However, the exact underlying mechanisms remain unclear. Isaji *et al.* found that tranilast inhibits the TGF-β-independent morphological changes of fibroblasts to myofibroblast-like cells and their proliferation in culture (Isaji *et al.*, 1994). In addition, Xu *et al.* demonstrated that tranilast can inhibit TGF-β-induced fibrogenesis in mesangial cells and fibroblasts isolated from kidneys (Xu *et al.*, 2007). Although several studies demonstrate that tranilast inhibits the production of TGF-β, the biochemical and cellular mechanisms need to be clarified. The present study investigated the in vitro effect of tranilast on TGF-β1-induced EMT in the NRK-52E cells, which is a normal proximal tubular epithelial cell line produced from rat kidney. Our study may shed light on the potential underlying mechanisms of tranilast in anti-fibrosis.

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MATERIALS AND METHODS

Reagents

The NRK-52E cells was purchased from Maisha Bio (Shanghai, China). Dulbecco's modified Eagle's medium and F-12 (DMEM/F12) was offered by Invitrogen (Grand Island, NY, USA). 1×TBS/Casein Blocker was purchased from Bio-Rad (Hercules, CA, USA). Recombinant human TGF-β1 was provided by Sigma (MO, USA). Polyclonal mouse antibodies against E-cadherin and α-smooth muscle actin(α-SMA) were purchased from Abcam (Hong Kong, China). A polyclonal goat antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from GenScript (NJ, USA). The cell permeabilization kit was purchased from ANDER GRUB Bio Research GmbH (Branch Vienna, Austria). FITC-labeled goat anti-rabbit IgG was purchased from BD (New Jersey, USA).

Cell culture

NRK-52E cells were maintained in DMEM/F12 containing 10% fetal bovine serum (FBS), 100, 000 U/L penicillin and 100 mg/L streptomycin at 37°C in a 100% humidified atmosphere with 5% CO_2. Cells were seeded in 24-well plates at low density and allowed to grow to approximately 80% confluence and then incubated in medium containing 1% FBS for 24 h. Then, cells were exposed or treated TGF-β1with at the concentration of 10 ng/ml without or with various concentrations of tranilast, and cells were treated with serum-free medium to be the blank control (Xu *et al.*, 2007). The effective concentration of tranilast was screened by a dose-response curve using 100, 200 or 400 μM tranilast (Qi *et al.*, 2006), which were extrapolated from our recent in vivo study (Tao *et al.*, 2011).

Morphological changes

Cells were grown in 6-well plates, followed by treatment with TGF-β1 alone or together with tranilast, and then observed under a microscope and photographed.

Western blotting

In order to harvest cells, the cells were washed with ice cold PBS, lysed with cell lysis buffer and then stored at -20°C. Lysates were separated by SDS-PAGE using 4–12% acrylamide gels. Following electrophoresis, separated proteins were transferred onto PVDF membranes. Nonspecific binding sites were blocked with 1×TBS/Casein Blocker overnight at 4°C. Then, membranes were incubated with corresponding primary antibodies for 1 h at room temperature, followed by washing three times and then incubation with horseradish peroxidase-conjugated secondary antibodies (1:10 dilution) for 1 h. Blots were visualized by enhanced chemiluminescence, and band densities were determined using Quantity One software (Bio-Rad, Hercules, CA, USA) in the Gel Doc™ XR system (Bio-Rad). For quantification, band densities were normalized to those of GAPDH, which severed as internal reference.

Flow cytometric analysis

The treated NRK-52E cells were fixed with 4% formaldehyde for half an hour and then permeabilized for intracellular α-SMA staining, according to the manufacturer's operational protocol. However, due to that E-cadherin is distributed at the cell surface; it was measured using unpermeabilized cells. Subsequently, cells were incubated with 10 μg antibodies (anti-E-cadherin or anti-α-SMA)/10^6 cells. FITC-conjugated goat anti-rabbit IgG, the secondary antibody was used to marker cells. For negative controls, primary antibodies were omitted. The duration of antibody labeling lasted for 30 min at room temperature, then cells were washed three times with 1 ml PBS containing 1% FBS (Adhim *et al.*, 2011; Geetha *et al.*, 2009). The cells were resuspended and then analyzed using a BD FACSCalibur.

STATISTICAL ANALYSIS

Results were presented as the means ± standard deviation (SD). Statistical comparisons between groups were performed by one-way analysis of variance, followed by the Student-Newman-Keuls test as a post-hoc test, using SPSS 13.0 software (Chicago, IL, USA). $P<0.05$ was considered statistically significant.

RESULTS

Morphological observation

After NRK-52E cells were treated with TGF-β1 at the concentration of 10 ng/ml for 48 h, gross morphological changes may be observed. The TGF-β1-treated cells became elongated in shape and separated from neighbor-ring cells. However, when NRK-52E cells were co-treated with 200 or 400μM tranilast with TGF-β1, morphological changes were largely restored to typical epithelial morphological appearance, though 100μM tranilast did not prevent EMT-related changes (figs not shown).

Effect of tranilast on the expression of E-cadherin

As shown in figs. 1A and 1B, untreated cells expressed a relatively high level of the epithelial cell protein E-cadherin, and treatment with 10 ng/ml TGF-β1 for 48 h resulted in the loss of E-cadherin expression. The magnitude of decreasing was nearly three times ($P<0.01$). However, compared with TGF-β1 alone, expression of E-cadherin in cells treated with both TGF-β1 and tranilast was concentration-dependently increased. When the concentration of tranilast reached to 400μM, expression of E-cadherin was increased 2.16 times ($P<0.05$).

To further identify the effect of tranilast on the expression of E-cadherin after TGF-β1 treated, flow cytometric analysis was employed to determine the significant changes (fig. 2A). Compared with control group, the portion of E-cadherin (+) cells in TGF-β1-treated group decreased from 1.09% to 0.11% ($P<0.05$). However, co-

treatment with 100, 200 and 400 μM tranilast attenuated the percentage of E-cadherin (+) cells to 0.39, 0.53, 0.6, respectively (P<0.05).

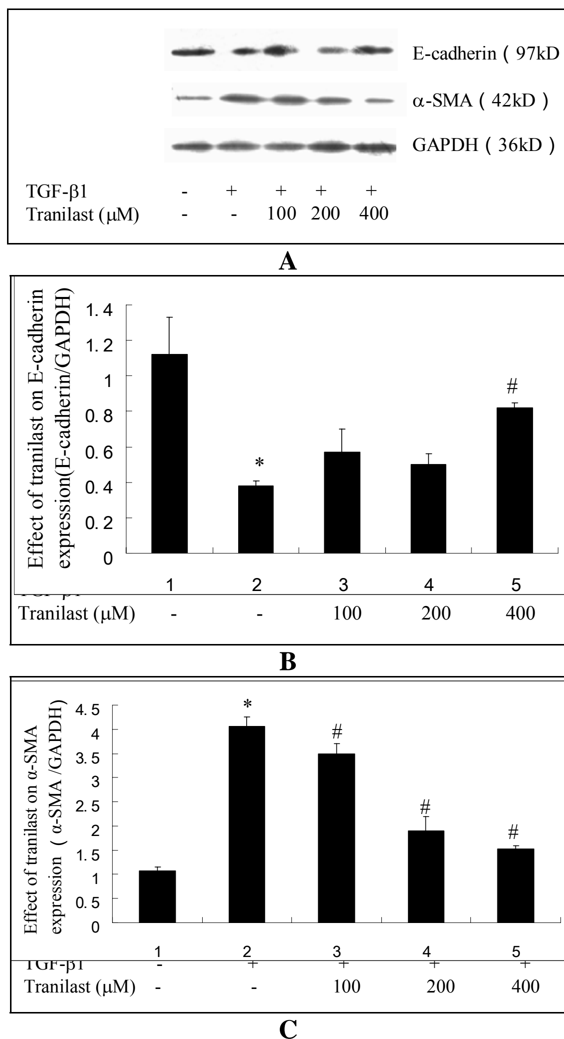

Fig. 1: Alterations of α-SMA and E-cadherin expression in NRK-52E cells by tranilast treatment, as indicated by western blot. (A.) Western blot of E-cadherin and α-SMA expression in NRK-52E cells. (B.) Effect of tranilast on E-cadherin expression (n=4). (C.) Effect of tranilast on α-SMA expression (n =4). Results are the means ± SD. *P< 0.01 vs control; $^{\#}P$ < 0.01 vs TGF-β1

Effect of tranilast on the expression of α-SMA

The relative expression of the mesenchymal marker α-SMA increased significantly in cells incubated with TGF-β1 (10 ng/ml) as compared with the controls (P<0.05; figs 1A and 1C). Tranilast at 100, 200 and 400 μM suppressed the increase of protein levels of α-SMA by 13.8%, 53.2% and 65.8%, respectively (P<0.05) (fig. 1C).

Similar results were obtained by flow cytometric analysis (fig. 2B). TGF-β1 (10 ng/ml) addition after 48 h resulted in a 6-fold increase in the portion of α-SMA (+) cells. Co-treatment with 100, 200 and 400 μM tranilast, there was significant decrease, compared with the TGF-β1-treated group (from 11.36±0.13% to 6.52±0.17%, 2.34±0.10% and 2.07±0.56%, P<0.05, respectively).

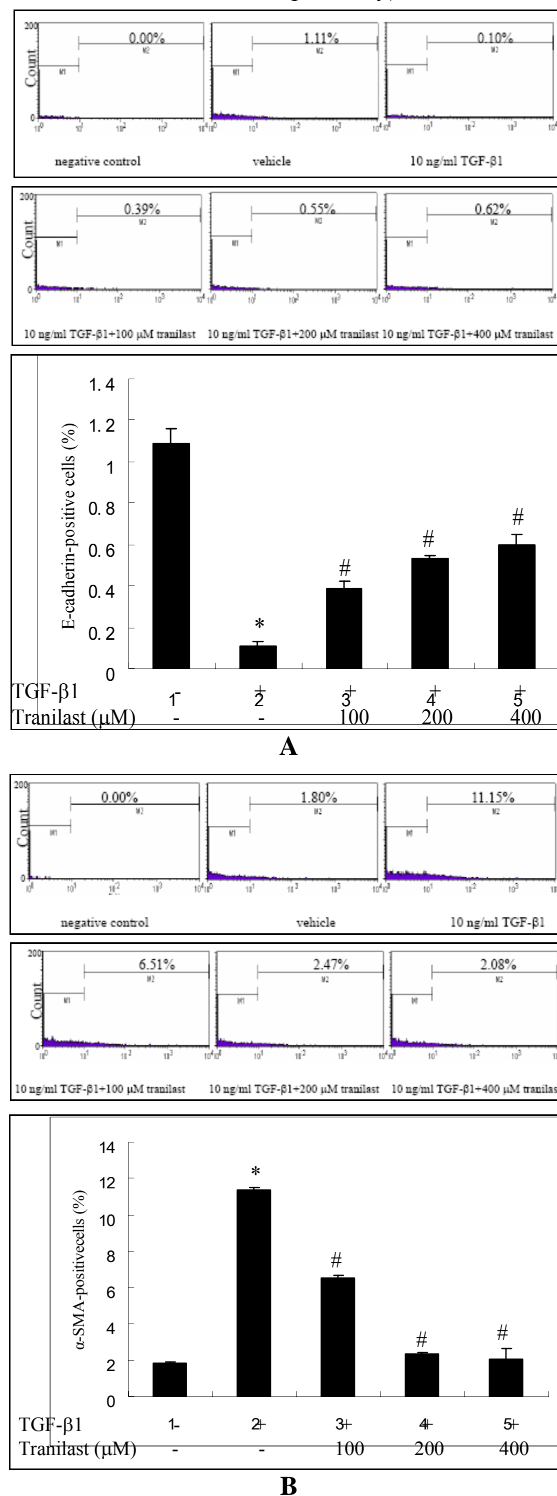

Fig. 2: Flow cytometric detection of E-cadherin and α-SMA. (A.) Effect of tranilast on E-cadherin expression (n =5). (B.) Effect of tranilast on α-SMA expression (n=5). Data are the means ±SD. *P <0.01 vs control; $^{\#}$ P<0.01 vs TGF-β1

DISCUSSION

Tranilast, a well-recognized anti-allergic agent, has been shown to suppress collagen synthesis in human proximal tubule cells, cortical fibroblasts (Qi *et al.,* 2006) and rat alveolar epithelial cells (Xu *et al.,* 2007). In addition, tranilast treatment could also attenuate tubulointerstitial fibrosis in animals with chronic renal diseases (Kaneyama *et al*., 2010; Kelly *et al*., 2004; Mifsud *et al*., 2003).

Both clinical and animal studies of RIF indicate that EMT is critical in progressive RIF. The most compelling evidence has shown that tubular EMT contributes up to 36% of fibroblasts (Kalluri and Neilson, 2003) in response to inflammatory cytokines, in which TGF-β1 is predominant (García-Sánchez *et al*., 2010; van Meeteren and Ten Dijke, 2012). Furthermore, TGF-β1 treatment in mesenchymal cells from the kidney for 24-48 h resulted in EMT-like morphological changes and induces significant matrix accumulation (Xu *et al*., 2007). More interestingly, studies have strongly indicated the important roles of E-cadherin and α-SMA in the development of normal epithelium (Horster *et al*., 1999; Junwei and Youhua, 2001; Strutz *et al*., 2002) as well as in the initiation of EMT in renal fibrogenesis (Tang *et al*., 2011; Vitalone *et al*., 2008; Vongwiwatana *et al*., 2005). Many studies have shown that the key features of EMT include early loss of cell-cell contacts due to down-regulation of E-cadherin, acquisition of spindle-like morphology, and finally de novo synthesis of α-SMA, the hallmark of the myofibroblast phenotype (Masszi *et al*., 2004; Zeisberg *et al*., 2001; Zeisberg and Kalluri, 2004). These features are perfectly consistent with our finding that, in the presence of TGF-β1 for 48 h, proximal tubular epithelial cells undergo EMT, as evidenced by downregulated expression of E-cadherin protein, enhancement of α-SMA protein expression and occurrence of a spindle-like morphology.

Kaneyama and co-workers have shown that tranilast attenuates kidney fibrosis in UUO rats by modifying EMT-like changes, tubular basement membrane damage and macrophage infiltration. All above modifications were associated with reduced TGF-β (Kaneyama *et al*., 2010). This observation supports our previous studies in which tranilast modulates renal fibrogenesis in rats with chronic cyclosporine nephrotoxicity, and its mechanism is closely associated with regulation of the TGF-β/Smad pathway (Tao *et al*., 2011) and inhibition of macrophage infiltration (Tao *et al*., 2009). Therefore, our results provide further evidence indicating that tranilast may suppress EMT induced by TGF-β1, which may result in the generation of fibrocytes and reduced accumulation of collagens and fibronectin. Several studies show that tranilast can inhibit EMT and TGF-β1 production in whole animals. However, because both of these two phenomena are associated with attenuation of fibrosis, the predominant event cannot be distinguished. We first demonstrated that tranilast can suppress EMT induced by TGF-β in a concentration-dependent manner. Thus, the role of EMT and TGF-β inhibition in the anti-fibroic effect of tranilast requires further study.

In conclusion, based on morphological observation and demonstrating the loss of E-cadherin and enhancement of α-SMA associated with EMT, we found that tranilast prevents EMT triggered by TGF-β in NRK-52E cells and then suppressed the initiation and progression of renal fibrosis. The underlying mechanism of EMT inhibition by tranilast warrants further studies.

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