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Original Article

Apigenin inhibits TGF-β1-induced proliferation and migration of airway smooth muscle cells

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Abstract: It is well known that the proliferation and migration of ASM cells (ASMCs) plays an important role in the pathogenesis of airway remodeling in asthma. Previous studies reported that apigenin can inhibit airway remodeling in a mouse asthma model. However, its effects on the proliferation and migration of ASMCs in asthma remain unknown. Therefore, the aim of our present study was to investigate the effects of apigenin on ASMC proliferation and migration, and explore the possible molecular mechanism. We found that apigenin inhibited transforming growth factor-β1 (TGF-β1)-induced ASMC proliferation. The cell cycle was blocked at G1/S-interphase by apigenin. It also suppressed TGF-β1-induced ASMCs migration. Furthermore, apigenin inhibited TGF-β1-induced Smad 2 and Smad 3 phosphorylation in ASMCs. Taken together, these results suggested that apigenin inhibited the proliferation and migration of TGF-β1-stimulated ASMCs by inhibiting Smad signaling pathway. These data might provide useful information for treating asthma and show that apigenin has potential for attenuating airway remodeling.

Keywords: Asthma, apigenin, airway smooth muscle cells, transforming growth factor-β1

Introduction

Airway remodeling due to repeated airway wall damage and repair plays an important role in the pathophysiology of severe asthma. Airway remodeling in asthma is characterized by increased airway smooth muscle (ASM) mass, accompanied by cell migration [1]. It is well known that the proliferation and migration of ASM cells (ASMCs) plays an important role in airway remodeling. Ample evidence suggests that ASMC migration toward the airway epithelium in response to inflammatory mediators such as transforming growth factor-β1 (TGF-β1) contributes to the airway remodeling [2, 3]. Currently, some treatments have been used to therapy asthma airway remodeling, such as such as β2-agonists, theophylline, anticholinergics, corticosteroids, H1-anti-histamineandanti-leukotrienes [4, 5]. However, none of them can effectively retard the process of airway remodeling. This is largely attributed to a lack of complete understanding of the mechanisms for asthma. Therefore, further understanding of the molecular mechanisms of asthma progression and the development of new therapeutic tools based on these mechanisms are required.

Apigenin is a member of the flavone subclass of flavonoids and is widely distributed among fruits and vegetables. The health benefits of apigenin are a consequence of a number of biological activities ascribed to it, including antimicrobial [6], antitumor [7], and anti-inflammatory effects [8]. Previous studies demonstrated that apigenin inhibited ovalbumin (OVA)-induced an increase in the number of eosinophils in bronchoalveolar lavage (BAL) fluid, an increase in inflammatory cell infiltration into the lung around blood vessels and airways, airway luminal narrowing, and the development of airway hyper-responsiveness [9]; apigenin also attenuated TGF-β-induced fibroblast-to-myofibroblast transition (FMT) in human lung fibroblast, a key event in asthma progression [10]. However, its effects on the proliferation and migration of ASMCs in asthma remain unknown. Therefore, the aim of our present study was to investigate the effects of apigenin on ASMC proliferation and migration, and explore the possible molecular mechanism.

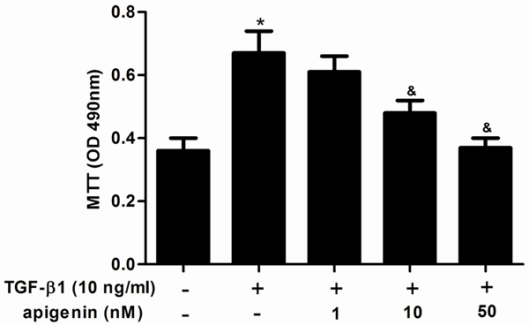

Figure 1. Apigenin inhibits cell proliferation in TGF-β1-stimulated ASMCs. ASMCs were cultured with various concentrations of apigenin (1, 10 and 50 nM) 30 min before TGF-β1 (10 ng/ml) stimulation for 24 h. Cell proliferation was determined by MTT assay. Experiments performed in triplicate, data shown are the mean results ± SD, $^{*}P < 0.05$ compared with control group, $^{\&}P < 0.05$ compared with TGF-β1 group.

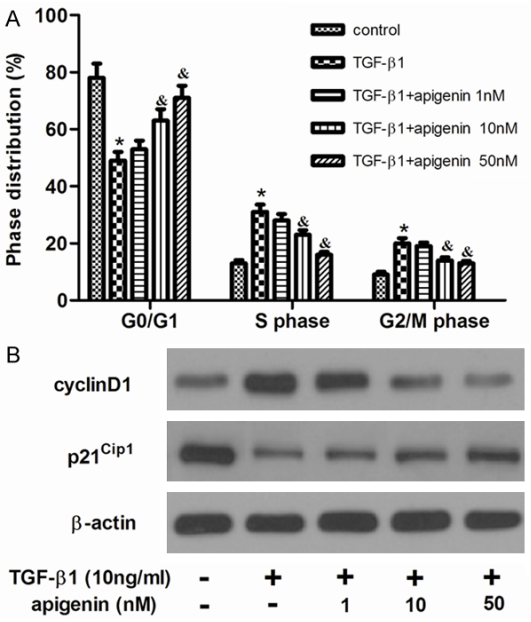

Figure 2. Apigenin inhibited the cell cycle progression in TGF-β1-stimulated ASMCs. ASMCs were cultured with various concentrations of apigenin (1, 10 and 50 nM) 30 min before TGF-β1 (10 ng/ml) stimulation for 24 h. A. Cell cycle distribution was determined by flow cytometric analysis. B. The levels of cyclinD1 and $p21^{Cip1}$ were detected by western blot analysis. Experiments performed in triplicate, data shown are the mean results ± SD, $^{*}P < 0.05$ compared with control group, $^{\&}P < 0.05$ compared with TGF-β1 group.

Materials and methods

Reagents

Apigenin (≥99% pure) was purchased from Sigma (Saint Louis, MO, USA). Anti-cyclinD1, anti-$p21^{Cip1}$, anti-p-Smad 2, anti-p-Smad 3 and ani-Smad 2/3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals and reagents were purchased from Sigma (Saint Louis, MO, USA).

Cell culture

Human ASMCs were isolated and grown from bronchi of resected unused lung tissue obtained from transplant donors. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplementedwith 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 U/ml streptomycin (all from Sigma, St. Louis, MO, USA). ASMC in passages 3 to 8 were used.

Evaluation of cell proliferation and cell cycle progression

The proliferation of cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, human ASMCs were plated at a density of 1.0×10^4/well in a 24-well tissue culture plate and then pre-incubated with apigenin (1, 10 and 50 nM) and vehicle DMSO for 30 min before stimulation with TGF-β1 (10 ng/ml) for 24 h. Then, 10 μL of 5 mg/mL MTT (Sigma, St. Louis, MO, USA) was added to each well. After incubation for 4 hat 37°C in the dark, the culture medium in each well was replaced with 150 mL of DMSO and the plates were shaken to dissolve the dark blue crystals. Absorbance was measured at 490 nm using an enzyme linked immunosorbent assay plate reader (Olympus, Tokyo, Japan).

ASMCs were stimulated with TGF-β1 (10 ng/ml) in the presence or absence of apigenin for 24 h in order to evaluate cell cycle progression. The harvested cell pellet was added to 3 mL of cold 70% ethanol and maintained at -20°C for 30 min. The cell pellet was resuspended with 1% Triton X-100, 0.1 mg/mL RNase A and 4 μg/mL propidium iodide after centrifuging. The flow cytometry (FC 500, Beckman Coulter, Inc., Fullerton, CA) was used to elucidate cell cycle progression.

Nucleosome ELISA assay for detection of apoptosis

ASMC apoptosis was carried out by using Nucleosome ELISA kit as described previously [11]. ASMCs were harvested. Nucleosome ELISAs were performed according to the manu-

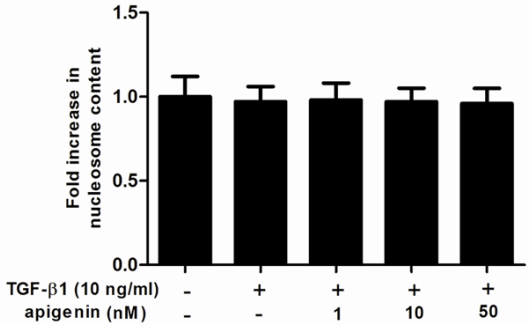

Figure 3. Effect of apigenin on cell apoptosis in TGF-β1-stimulated ASMCs. A. ASMCs were cultured with various concentrations of apigenin (1, 10 and 50 nM) 30 min before TGF-β1 (10 ng/ml) stimulation for 24 h. Cell apoptosis was carried out by using Nucleosome ELISA kit. Experiments performed in triplicate, data shown are the mean results ± SD.

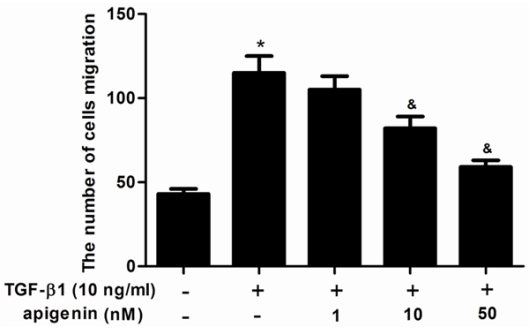

Figure 4. Apigenin inhibits cell migration in TGF-β1-stimulated ASMCs. ASMCs were cultured with various concentrations of apigenin (1, 10 and 50 nM) 30 min before TGF-β1 (10 ng/ml) stimulation for 24 h. Cell migration was measured by Transwell analysis. Experiments performed in triplicate, data shown are the mean results ± SD, $^{*}P < 0.05$ compared with control group, $^{\&}P < 0.05$ compared with TGF-β1 group.

facturer's instructions (Oncogene Research Products, Cambridge, MA, USA).

Transwell migration assay

Migration assays were performed using Transwell® cell culture chambers (Abcam PLC, Cambridge, UK). The lower compartment was filled with 0.5 ml of DMEM containing 1% FBS with TGF-β1 (10 ng/ml) alone or together with apigenin (1, 10 and 50 nM). ASMCs (1×10^5) were resuspended in 0.1 ml of DMEM and placed in the upper part of the Transwell plate. Subsequent to incubation in a humidified atmosphere of 5% CO_2 at 37°C for 24 h, the non-migrated cells were removed from the upper chamber using a cotton swab. Migrated cells were fixed using methanol for 10 min at 4°C and stained with 0.5% crystal violet solution for 30 min. The number of cells per five high power fields was counted using a microscope (Olympus, Tokyo, Japan).

Western blot

The protein in cell lysis was separated by 12% sodium dodecylsulfate polyacrylamide gel electrophoresis followed by transference to Immobilon-P Transfer Membranes (Millipore). After blocking in TBS buffer (50 mmol/L NaCl, 10 mmol/L Tris, pH 7.4) containing 5% nonfat milk, the blots were incubated with primary antibodies (anti-cyclinD1, anti-$p21^{Cip1}$, anti-p-Smad 2, anti-p-Smad 3 and ani-Smad 2/3) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. Membranes were then washed and incubated with horseradish peroxidase-conjugated secondary antibodies. Following washing, the sites of antibody binding were visualized via chemiluminescence (Boehringer Mannheim GmbH, Mannheim, Germany) according to the manufacturer's instructions.

Statistical analysis

Results from at least three independent experiments were expressed as mean ± SD. Statistical comparisons were performed using one way ANOVA and Dunnett's test. All analyses were performed using SPSS 13.0 software (SPSS, Chicago, IL, USA).

Results

Effect of apigenin on TGF-β1-induced ASMCs proliferation

First, we investigated the effect of apigenin on ASMC proliferation using MTT assay. As shown in **Figure 1**, TGF-β1 significantly increased the proliferation of AMSCs, compared with the control group. Whereas, apigenin treatment inhibited TGF-β1-induced ASMCs proliferation in a dose-dependent manner.

Effect of apigenin on cell cycle progression

The effects of apigenin on the cell cycle progression were elucidated. Growth-arrested cells were stimulated with TGF-β1in the presence or absence of apigenin for 24 h, and cell cycle profiles were obtained by flow cytometric analysis. As shown in **Figure 2A**, TGF-β1 markedly decreased the percentage of ASMCsat G0/G1

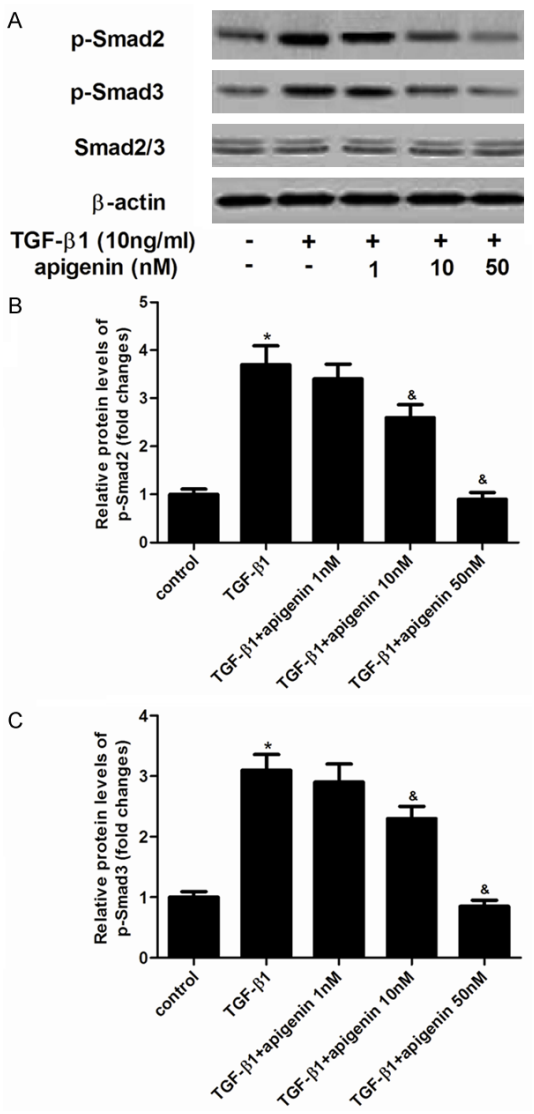

Figure 5. Effect of apigenin on Smad expression in TGF-β1-induced ASMCs. A. ASMCs were pretreated with/without various concentration of apigenin for 30 min, and stimulated with 10 ng/ml TGF-β1 for 30 min. The levels of phosphorylated Smad 2, phosphorylated Smad 3 and Smad 2/3 were detected by western blot analysis. B and C. quantitative analysis of p-Smad 2 and p-Smad 3 protein levels by Image-Pro Plus 6.0 software and normalized to β-actin. Experiments performed in triplicate, data shown are the mean results ± SD, $^{*}P < 0.05$ compared with control group, $^{\&}P < 0.05$ compared with TGF-β1 group.

phase and correspondingly increased their percentage at the SandG2/M phase. Whereas, apigenin treatment significantly reversed this event. Consistently, apigenin reduced the protein expression of the G1-S transition promoter cyclinD1, but enhanced the G1 gatekeeper $p21^{Cip1}$ (**Figure 2B**).

Effect of apigenin on TGF-β1-induced ASMCs apoptosis

Then we performed Nucleosome ELISA assay to measure the effect of apigenin on ASMC apoptosis. As shown in **Figure 3**, the result revealed that neither TGF-β1 nor apigenin can affect the apoptosis rate of ASMCs, compared with the control group. These results suggest that the anti-proliferative effect of apigenin was not mediated by ASMC apoptosis.

Effect of apigenin on TGF-β1-induced ASMCs migration

Increased migration of ASMCs was thought to participate in airway remodeling in asthma, so, we investigated the effect of apigenin on ASMC migration with the use of a Transwell chamber assay. As shown in **Figure 4**, TGF-β1 significantly increased ASMCs migration, compared with the control group. Whereas, apigenin significantly suppressed TGF-β1-induced ASMCs migration, compared with the TGF-β1 group.

Effect of apigenin on Smad expression

The TGF-β1-mediated signaling pathway depends on the phosphorylation of Smad 2/3. To further understand the molecular mechanisms by which apigenin inhibited TGF-β1-induced ASMCs proliferation and migration, the protein levels of Smad 2/3 were analyzed. As shown in **Figure 5**, TGF-β1 treatment stimulated phosphorylation of Smad 2 and Smad 3, however, apigenin prevented TGF-β1-induced phosphorylation of Smad 2 and Smad 3 in ASMCs.

Discussion

Here, we studied for the first time the effects of apigenin on ASMC proliferation and migration in TGF-β1-stimulated ASMCs. We found that apigenin inhibited TGF-β1-induced ASMC proliferation. The cell cycle was blocked at G1/S-interphase by apigenin. It also suppressed TGF-β1-induced ASMCs migration. Furthermore, apigenin inhibited TGF-β1-induced Smad 2 and Smad 3 phosphorylation in ASMCs.

Increasing evidence has demonstrated that apigenin inhibits cancer cell proliferation *in vitro* [12-14]. For example, Shi and coworkers demonstrated that apigenin inhibited human bladder cancer T-24 cell proliferation in a dose-dependent manner [15]. Similarly, recent studies have reported that apigenin significantly

inhibited both FBS- and platelet derived growth factor-BB (PDGF-BB)-induced proliferation of VSMCs in a concentration-dependent manner [16, 17]. ASMCs proliferation is a key process underlying the formation of airway remodeling in asthma [18]. Therefore, suppressing of ASMCs proliferation represents a potentially important therapeutic strategy for the treatment of airway remodeling in asthma. In this study, we found that apigenin inhibited TGF-β1-induced proliferation of ASMCs. Previous studies showed that apigenin-treated osteosarcoma cells displayed obvious arrest of cells in the G0/G1 phase after 24 h [19]. Here, we found that apigenin increased the percentage of ASMCs at G0/G1 phase and correspondingly decreased their percentage at the SandG2/M phase. To explore the molecular mechanism underlying the G1 phase arrest, we investigated the regulatory proteins which control the G1 checkpoint in the cell cycle. In the present study, we found that G1 phase arrest by apigenin was associated with the downregulation of cyclin-D1, a G1 cyclin, and the upregulation of $p21^{Cip1}$. These data suggest that apigenin treatment inhibited TGF-β1-induced ASMCs proliferation by inhibiting cell cycle progression.

ASMC migration is not only essential for development of respiratory system but also important for airway remodeling in asthma [20, 21]. Previous studies showed that ASMCs migration toward the airway epithelium in response to inflammatory mediators such as TGF-β1 contributes to the airway remodeling [22-24]. It is interesting that apigenin inhibited human umbilical vein endothelial cells (HUVECs) migration, compared with the control [25]. Here, we found that TGF-β1 significantly increased ASMCs migration, whereas, apigenin significantly suppressed TGF-β1-induced ASMCs migration.

TGF-β1 is a potent inducer of a hypertrophic and hypercontractile ASM phenotype [26]. The classical Smads pathways (receptor-mediated Smads, Smad 2/3), mitogen-activated protein kinase (MAPK) as well as nuclear factor kappa B (NF-κB) signaling, have been reported to be involved in the modulation of ASMC proliferation and migration induced TGF-β1 [2, 27, 28]. Recent studies have shown that exogenously applied TGF-β alone promoted a contractile ASM phenotype through the activation of Smad signaling [29, 30]. In addition, Chen et al. showed that triptolide inhibits TGF-β1-induced cell proliferation in rat ASMCs by suppressing Smad signaling [31]. In line with these results, in the current study, we found that TGF-β1 treatment stimulated phosphorylation of Smad 2 and Smad 3, however, apigenin prevented TGF-β1-induced phosphorylation of Smad 2 and Smad 3 in ASMCs. These data suggest that apigenin inhibited the proliferation and migration of TGF-β1-stimulated ASMC by inhibiting Smad signaling pathway.

In conclusion, this study demonstrated that apigenin inhibited the proliferation and migration of TGF-β1-stimulated ASMCs by inhibiting Smad signaling pathway. These data might provide useful information for treating asthma and show that apigenin has potential for attenuating airway remodeling.

Acknowledgements

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Disclosure of conflict of interest

None.

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