

Original Article

Matrine inhibits TGF- β 1-induced proliferation and migration of airway smooth muscle cells by suppressing the MAPK signaling pathway

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Abstract: Airway smooth muscle (ASM) mass is increased in asthma, and ASM cells (ASMCs) from patients with asthma are hyperproliferative. Previous studies have demonstrated that matrine is able to effectively ameliorate the progression of asthma; however, the effects of matrine on the proliferation and migration of ASMCs remain unclear. Therefore, the present study aimed to investigate the effects of matrine on the proliferation and migration of ASMCs, and to explore the possible underlying mechanisms. Human ASMCs cultures were pretreated with several concentrations of matrine prior to stimulation with transforming growth factor- β 1 (TGF- β 1). Cell proliferation was evaluated using the Cell Counting kit-8 assay, and flowcytometry was used to determine the effects of matrine on cell cycle progression and apoptosis. Furthermore, the expression levels of phosphorylated p38, extracellular signal-regulated kinases (ERK)1/2 and c-Jun N-terminal kinases (JNK) were determined by western blot analysis. The results demonstrated that matrine significantly inhibited TGF- β 1-induced ASMC proliferation and migration. In addition, the cell cycle was blocked at G1/S-interphase following treatment with matrine. However, matrine did not affect the apoptotic rate of ASMCs. Western blotting revealed that pretreatment with matrine reduced TGF- β 1-induced expression of phosphorylated p38, ERK1/2 and JNK in ASMCs. In conclusion, matrine may inhibit TGF- β 1-induced proliferation and migration of ASMCs via suppression of the mitogen-activated protein kinase signaling pathway.

Keywords: Matrine, airway smooth muscle cells, proliferation, migration, mitogen activated protein kinase signaling pathway

Introduction

Asthma is an inflammatory disease of the airway, which exhibits a poor response to current therapeutic strategies and affects >300 million individuals worldwide [1]. Asthma is characterized by chronic airway inflammation, a variable degree of reversible airway obstruction and airway remodeling [2]. Common characteristics of airway remodeling in asthma include epithelial shedding, goblet cell hyperplasia, subepithelial fibrosis alongside abnormal extracellular matrix (ECM) deposition, airway wall thickening, and angiogenesis of the bronchial vasculature [3]. In addition, the proliferation and migration of airway smooth muscle cells (ASMCs) is a critical factor in the pathogenesis of asthma. It has previously been reported that the proliferation of ASMCs is increased in patients with asthma

and in animal models of allergic airway inflammation [4, 5].

Transforming growth factor- β (TGF- β) is commonly associated with asthma. Previous studies have reported that TGF- β 1 expression is up-regulated in bronchial biopsies from patients with asthma, and is able to stimulate the growth of human ASMCs [6, 7]. Therefore, inhibiting TGF- β 1-induced proliferation of ASMCs may be considered a therapeutic strategy for the treatment of asthma.

Matrine, which is one of the main alkaloid components extracted from Sophora, bitter beans, broad beans and other leguminous Sophora root plants, is a type of tetracyclic quinolizidine alkaloid. Matrine has been used to treat inflammatory diseases, including enteritis and hepati-

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Table 1. Value of cell proliferation optical density

Group	24 h	48 h	72 h
Control group	0.447 ± 0.023	0.431 ± 0.035	0.433 ± 0.043
TGF-β1	0.631 ± 0.029	0.811 ± 0.032	0.933 ± 0.045
TGF-β1+matrine 1 nM	0.619 ± 0.038	0.735 ± 0.053	0.867 ± 0.067
TGF-β1+matrine 10 nM	0.503 ± 0.024	0.592 ± 0.047	0.711 ± 0.061
TGF-β1+matrine 50 nM	0.433 ± 0.024	0.456 ± 0.062*	0.501 ± 0.077*

*compared with control group, P<0.05.

Table 2. Percentage of cell cycle phase distribution after treatment (%)

Group	S phase	G2/M phase
Control group	7.21 ± 1.82	3.21 ± 1.62
TGF-β1	24.96 ± 1.54	17.52 ± 2.75
TGF-β1+matrine 1 nM	20.81 ± 1.92	16.45 ± 2.16
TGF-β1+matrine 10 nM	13.67 ± 2.01	11.63 ± 2.76
TGF-β1+matrine 50 nM	10.42 ± 2.04*	8.39 ± 3.08*

*compared with control group, P<0.05.

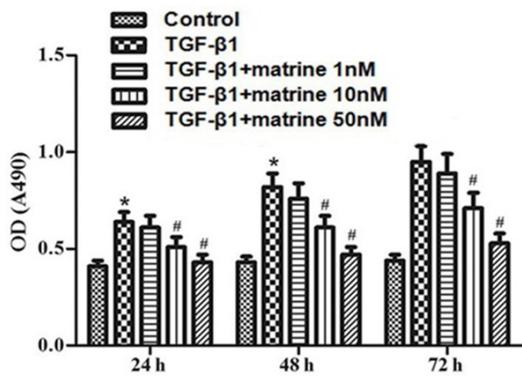


Figure 1. Matrine inhibits cell proliferation in transforming growth factor-β1 (TGF-β1)-stimulated airway smooth muscle cells (ASMCs). ASMCs were cultured with matrine at different doses (1, 10 and 50 nM) 30 min prior to TGF-β1 (10 ng/ml) stimulation. Cell proliferation was examined at 24, 48 and 72 h after TGF-β1 stimulation by Cell Counting kit-8 assay. All experiments were repeated at least three times. Data are presented as the mean ± standard deviation. *P<0.05, compared with the control group; #P<0.05 compared with the TGF-β1 group. OD, optical density.

tis [8, 9]. Matrine exerts anti-inflammatory effects by inhibiting the activation of inflammatory signals and the production of proinflammatory mediators [10, 11]. Furthermore, matrine is able to inhibit proliferation and migration, and induce apoptosis. Previous studies have demonstrated that matrine may significantly suppress eosinophil infiltration and airway hy-

perresponsiveness in asthmatic mice, and decrease the inflammatory response of tracheal epithelial cells [12]. In addition, matrine has been shown to inhibit ovalbumin-induced increases in airway resistance and eosinophilia in the lung tissue of asthmatic mice [3]. However, the effects of matrine on the proliferation

and migration of ASMCs remain unclear. Therefore, the present study investigated the effects of matrine on the TGF-β1-induced proliferation and migration of ASMCs, and explored the possible underlying molecular mechanisms.

Materials and methods

Cell culture

Human ASMCs were isolated and cultured from the bronchi of resected unused lung tissue obtained from healthy transplant donors. Smooth muscle was microdissected and enzymatically digested with collagenase and elastase (both purchased from Sigma-Aldrich, St. Louis, MO, USA) to generate cell suspensions which were then cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 U/ml streptomycin (all Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C with 5% CO₂ in a humidified incubator. ASMCs from passages 3-8 were used for further experimentation. The study was approved by the ethics committee of Medical College of Qingdao University (Qingdao, China).

ASMC proliferation assay

Cell Counting kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used to assess the rate of cellular proliferation. Briefly, human ASMCs were plated into 96-well plates at a seed density of ~2×10³ cells/well in 100 μl culture medium. The cells were starved in serum-free medium overnight, and were pre-incubated with matrine (1, 10 and 50 nM; Sigma-Aldrich) or dimethyl sulfoxide (DMSO; Sigma-Aldrich) vehicle for 30 min prior to stimulation with TGF-β1 (10 ng/ml; Sigma-Aldrich) for 24, 48 or 72 h (Table 1). Subsequently, 10 μl CCK-8 solution was added to each well, and the plates were incubated for

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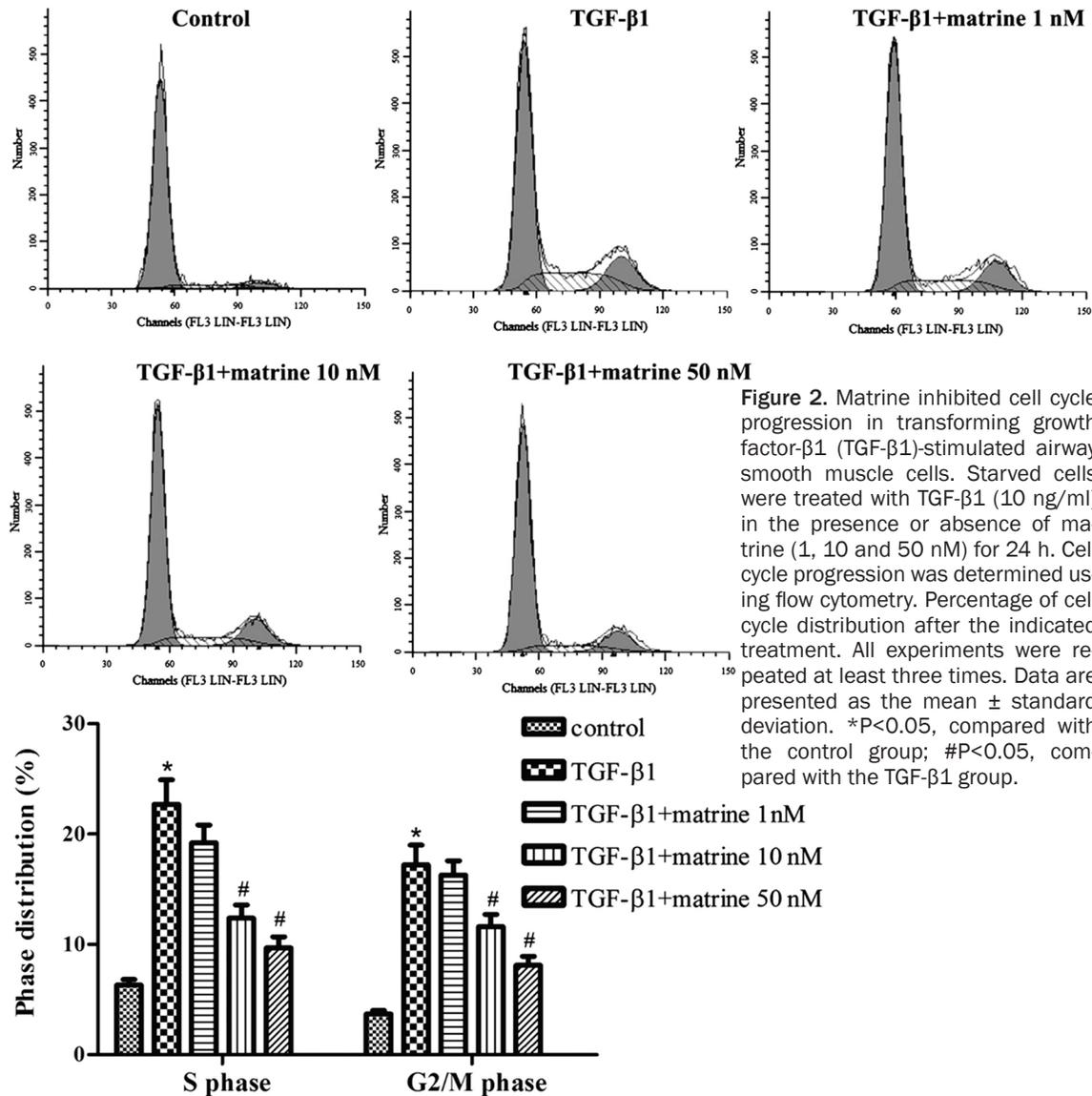


Figure 2. Matrine inhibited cell cycle progression in transforming growth factor-β1 (TGF-β1)-stimulated airway smooth muscle cells. Starved cells were treated with TGF-β1 (10 ng/ml) in the presence or absence of matrine (1, 10 and 50 nM) for 24 h. Cell cycle progression was determined using flow cytometry. Percentage of cell cycle distribution after the indicated treatment. All experiments were repeated at least three times. Data are presented as the mean ± standard deviation. *P<0.05, compared with the control group; #P<0.05, compared with the TGF-β1 group.

1 h at 37°C. Absorbance at 490 nm was measured using a microplate reader (Spectra Max 190; Takara Biotechnology Co., Ltd., Dalian, China). All experiments were conducted in triplicate.

Determination of cell cycle progression by flow cytometry

Cell cycle analyses were performed as previously described, with a few modifications [13]. A total of 24 h following treatment with matrine and TGF-β1 (10 ng/ml), the cells were trypsinized (Sigma, St. Louis, MO, USA) (Table 2). The pellets were suspended in phosphate-buffered saline (PBS) and fixed with 70% cold

ethanol at -20°C. The following day, cells were washed with citrate phosphate buffer, followed by PBS, prior to incubation with RNase solution (100 μg/ml; Sigma-Aldrich) for 30 min at 37°C. Subsequently, the cells were incubated in propidium iodide (PI) solution (100 μg/ml in PBS) at room temperature for 30 min. Cell cycle progression was detected using flow cytometry (FC 500; Beckman Counter, Inc., Brea, CA, USA). The experiment was repeated three times.

Apoptosis assay

Briefly, the treated cells were washed with PBS and centrifuged at 200×g for 5 min. Then an Annexin V-Fluorescein Isothiocyanate (FITC)

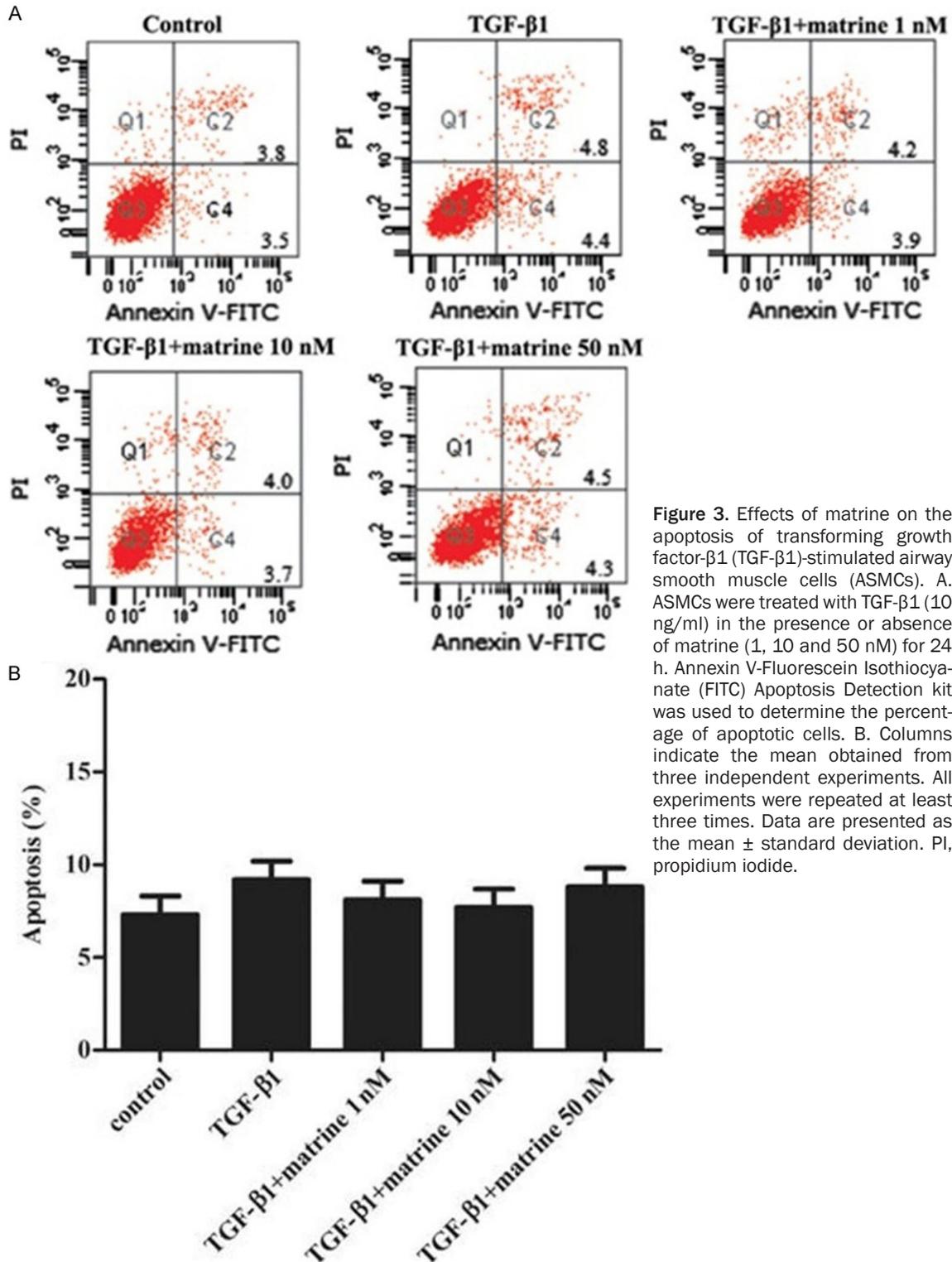


Figure 3. Effects of matrine on the apoptosis of transforming growth factor-β1 (TGF-β1)-stimulated airway smooth muscle cells (ASMCs). **A.** ASMCs were treated with TGF-β1 (10 ng/ml) in the presence or absence of matrine (1, 10 and 50 nM) for 24 h. Annexin V-Fluorescein Isothiocyanate (FITC) Apoptosis Detection kit was used to determine the percentage of apoptotic cells. **B.** Columns indicate the mean obtained from three independent experiments. All experiments were repeated at least three times. Data are presented as the mean ± standard deviation. PI, propidium iodide.

Apoptosis Detection kit (BioTeke Corporation, Beijing, China) was used to determine the percentage of apoptotic cells in the cultures. The cells were then trypsinized (Sigma-Aldrich) and suspended in 500 μl of binding buffer

containing 5 μl Annexin V-FITC and 5 μl PI (Abcam, Cambridge, UK). The cell pellet was resuspended in propidium iodide (PI) staining buffer (Sigma-Aldrich) for 15 min at 25°C, and the cells were analyzed using flow cytometry.

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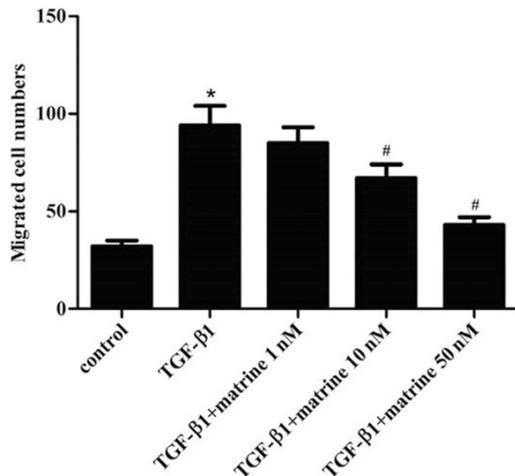


Figure 4. Effects of matrine on cell migration in transforming growth factor- β 1 (TGF- β 1)-stimulated airway smooth muscle cells (ASMCs). ASMCs were treated with TGF- β 1 (10 ng/ml) in the presence or absence of matrine (1, 10 and 50 nM) for 24 h. Cell migration was measured by Transwell analysis. All experiments were repeated at least three times. Data are presented as the mean \pm standard deviation. * P <0.05, compared with the control group; # P <0.05, compared with the TGF- β 1 group.

Annexin V-positive and PI-negative cells were detected, indicating the presence of apoptotic cells.

ASMC migration assay

The migration assay was performed using a Transwell system. The lower compartment was filled with 0.5 ml DMEM containing 1% FBS with TGF- β 1 (10 ng/ml) alone or together with matrine (1, 10 and 50 nM). ASMCs (1×10^5) were resuspended in 0.1 ml DMEM and were placed in the upper chamber of the Transwell plate. Cells were incubated for 24 h in a humidified atmosphere containing 5% CO₂ at 37°C. ASMCs were then fixed with 4% paraformaldehyde and stained with trypan blue for 10 min. ASMCs on the upper surface of the filter were mechanically removed using a cotton swab, and the migrated cells were determined by counting the cells that had migrated to the lower side of the filter using a microscope (CX21; Olympus Corporation, Tokyo, Japan). Six randomly selected fields were counted, and each sample was assayed in triplicate.

Western blot analysis

Total cellular proteins were extracted using a lysis buffer (Takara Biotechnology Co., Ltd.),

and protein concentration was determined using Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). A total of 30 μ g protein were fractionated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and were transferred onto polyvinylidene difluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). The membranes were then blocked, and probed with the following mouse monoclonal primary antibodies overnight at 4°C: Anti-extracellular signal-regulated kinases (ERK) (1:1,500; sc-514302), anti-phosphorylated(p)-ERK (1:1,500; sc-7383), anti-p38 (1:1,500; sc-81621), anti-p-p38 (1:1,500; sc-7973), anti-c-Jun N-terminal kinases (JNK) (1:1,000; sc-7345), anti-p-JNK (1:1,000; sc-6254) and anti-glyceraldehyde3-phosphate dehydrogenase (GAPDH) (1:1,000; sc-365062) (all purchased from Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Subsequently, the membranes were washed with TBST buffer (Beyotime, Nantong, China) three times and incubated with goat anti-mouse monoclonal horseradish peroxidase-conjugated IgG (1:5,000; sc-2005; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Both the primary and secondary antibodies were acquired from mouse monoclonal antibody. The blots were then washed in Tris-Buffered Saline with Tween 20 (Beyotime Institute of Biotechnology, Haimen China) and visualized using enhanced chemiluminescence (Pierce; Thermo Fisher Scientific, Inc.). The blots were semi-quantified using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA) and protein expression levels were normalized to GAPDH.

Statistical analysis

SPSS 13.0 (Chicago, IL, USA) statistical software was used. The statistical data are presented as mean \pm standard deviation. Statistical analyses of the data were performed by one-way analysis of variance for multiple comparisons, followed by the least significant difference test for comparisons between groups. P <0.05 was considered to indicate a statistically significant difference.

Results

Cell proliferation

Matrine suppresses TGF- β 1-induced ASMC proliferation. The present study examined the effects of matrine on the proliferation of AS-

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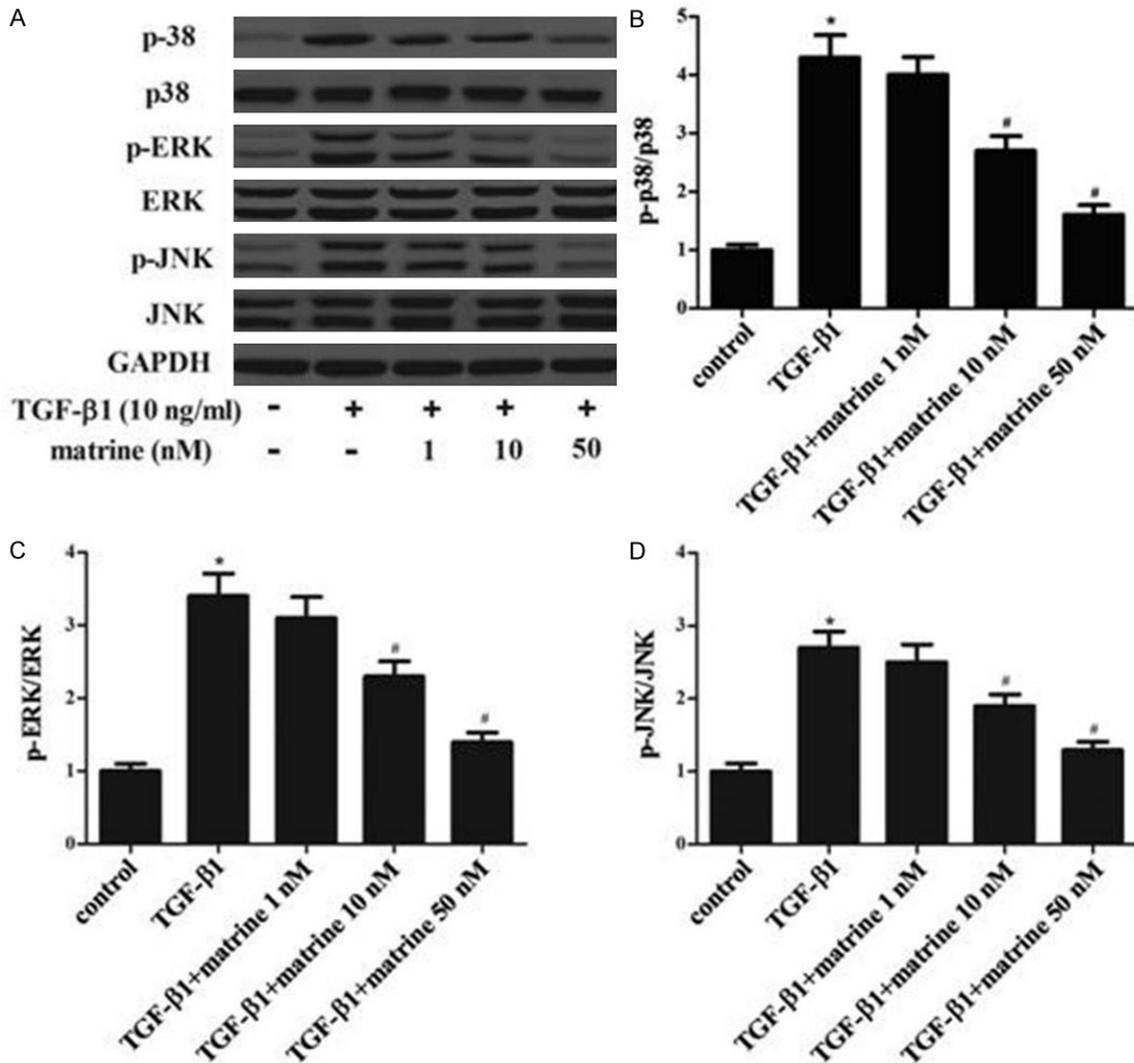


Figure 5. Effects of matrine on mitogen-activated protein kinase (MAPK) expression in transforming growth factor-β1 (TGF-β1)-stimulated airway smooth muscle cells (ASMCs). (A) ASMCs were pretreated with or without various concentrations of matrine for 30 min, and stimulated with 10 ng/ml TGF-β1 for 30 min. The protein expression levels of phosphorylated (p)-p38, total p38, p-extracellular signal-regulated kinases (ERK), total ERK, p-c-Jun N-terminal kinases (JNK) and total JNK were detected by western blot analysis. Relative protein expression levels of (B) p-p38, (C) p-ERK and (D) p-JNK were semi-quantified using Image-Pro Plus 6.0 software and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). All experiments were repeated at least three times. Data are presented as the mean ± standard deviation. *P<0.05, compared with the control group; #P<0.05, compared with the TGF-β1 group.

MCs. ASMCs exhibited various increases in cellular proliferation following stimulation with TGF-β1 for 24, 48 and 72 h, compared with the PBS control (P<0.05). However, treatment with matrine significantly inhibited the effects of TGF-β1 on proliferation, in a dose-dependent manner (Figure 1).

Cell cycle distribution

Effects of matrine on cell cycle progression and apoptosis. As determined using flow cytometry

with PI staining, treatment with TGF-β1 markedly decreased the percentage of ASMCs at G₀/G₁ phase, and correspondingly increased the percentage of cells at S and G₂/M phase, compared with the control group. However, matrine significantly inhibited this event (Figure 2).

Effects on apoptosis

Subsequently, the effects of matrine on the apoptosis of TGF-β1-stimulated AMSCs were investigated. As shown in Figure 3, neither TGF-

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β 1 nor matrine affected the apoptotic rate of ASMCs, and the percentage of apoptotic cells did not differ significantly between the groups.

ASMC migration

Matrine suppresses TGF- β 1-induced ASMC migration. The present study evaluated the effects of matrine on ASMC migration with the use of a Boyden chamber assay. ASMCs were pre-incubated with matrine or DMSO vehicle for 30 min prior to stimulation with TGF- β 1 (10 ng/ml), and the number of migrated cells was counted. As shown in **Figure 4**, stimulation with TGF- β 1 increased ASMC migration by ~124%, compared with the PBS control ($P < 0.05$). However, matrine significantly suppressed TGF- β 1-induced ASMC migration, compared with the TGF- β 1 group ($P < 0.05$).

Effects on MAPK expression

Effects of matrine on mitogen-activated protein kinase (MAPK) expression in TGF- β 1-induced ASMCs. To further elucidate the mechanism underlying matrine-inhibited ASMC proliferation and migration induced by TGF- β 1, the effects of matrine on MAPK expression were determined. As shown in **Figure 5**, the protein expression levels of p-p38, p-ERK1/2 and p-JNK were significantly increased in ASMCs following stimulation with TGF- β 1, compared with the PBS control ($P < 0.05$). However, pretreatment with matrine reduced TGF- β 1-induced expression of p-p38, p-ERK1/2 and p-JNK.

Discussion

The present study demonstrated that matrine significantly inhibited TGF- β 1-induced ASMC proliferation and migration. In addition, cell cycle progression was blocked at G₁/S-interphase following treatment with matrine. Pretreatment with matrine also reduced the TGF- β 1-induced expression of p-p38, p-ERK1/2 and p-JNK in ASMCs.

Previous studies have indicated that TGF- β 1 has an important role in ASMC proliferation in asthmatics. Studies have demonstrated that TGF- β 1 was able to increase proliferation in serum-free conditions, and further enhance serum-induced proliferation of confluent ASMCs [7]. Furthermore, reports have demonstrated that anti-TGF- β significantly reduced peribronchiolar ECM deposition, mucus production

and the proliferation of ASMCs in asthmatic mice [14]. Consistent with these results, the present study demonstrated that TGF- β 1 increased ASMC proliferation in a time-dependent manner; however, matrine significantly inhibited the effects of TGF- β 1. Since proliferation and apoptosis are believed to contribute to airway smooth muscle remodeling in asthma, the present study investigated the effects of matrine on ASMC apoptosis. Matrine was revealed to have no effect on the apoptosis of ASMCs. It has previously been reported that matrine suppresses proliferation and induces apoptosis in human cholangiocarcinoma cells [15]. In addition, matrine is able to inhibit the growth and induce apoptosis of osteosarcoma cells in vitro [16]. However, the results of the present study indicated that matrine inhibits cell growth and cell cycle progression without inducing cell death. These discrepancies may be due to different effects of matrine between tumors and ASMCs, or matrine may have a different impact on specific signal factors in ASMCs.

Increased migration of ASMCs has also been hypothesized to participate in airway remodeling in asthma [13]. Previous studies have suggested that the migration of ASMCs toward airway epithelium in response to inflammatory mediators, including TGF- β 1, contributes to airway remodeling [17, 18]. In the present study, matrine significantly inhibited TGF- β 1-induced migration of ASMCs. Therefore, in accordance with previous findings [19], these results suggested that matrine may inhibit TGF- β 1-induced migration of human ASMCs.

The MAPK signaling pathway has an important role in cell proliferation and migration [20, 21]. The MAPK family consists of ERK, p38 and JNK [22, 23]. Phosphorylation of ERK1/2 has been reported to mediate ASMC proliferation [24, 25], whereas the phosphorylation of JNK and p38 are activated by various non-specific stimuli [26]. It has been reported that TGF- β 1-induced proliferation of ASMCs is associated with increased expression of p-ERK1/2, p-p38 and p-JNK [7]. Consistent with these results, the present study demonstrated that TGF- β 1 was able to significantly increase the expression levels of p-ERK1/2, p-p38 and p-JNK in ASMCs. It has previously been suggested that matrine is able to reduce the phosphorylation

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of MAPK [27]. In the present study, matrine significantly reduced TGF- β 1-induced expression of p-ERK1/2, p-p38 and p-JNK in ASMCs. Therefore, it may be hypothesized that matrine inhibits the TGF- β 1-induced proliferation and migration of ASMCs by suppressing the MAPK signaling pathway.

In conclusion, the present study demonstrated that matrine is able to inhibit TGF- β 1-induced proliferation and migration of ASMCs by suppressing the MAPK signaling pathway. These results provide evidence suggesting that matrine may be an effective candidate for the systemic treatment of asthma airway remodeling.

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Disclosure of conflict of interest

None.

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