1,25-$(OH)_2D_3$ participates and modulates airway remodeling by reducing MGP and TGF-β1 expression in TNF-α-induced airway smooth muscle cells

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Abstract

Background. Asthma has been proven to be a respiratory disorder that is characterized by the airway remodeling, airway inflammation and reversible airway obstruction. The 1,25-hydroxyvitamin D_3 (1,25-$(OH)_2D_3$) plays critical roles in delaying remodeling.

Objectives. To investigate the effects of 1,25-$(OH)_2D_3$ on the airway remodeling in tumor necrosis factor α (TNF-α)-induced airway smooth muscle cells (ASMCs).

Materials and methods. The human ASMCs were divided into a blank control group (without treatment), a TNF-α group (treated with 10 ng/mL TNF-α) and a 1,25-$(OH)_2D_3$+TNF-α group (pre-treated with 10^{-7} M 1,25-$(OH)_2D_3$, then with 10 ng/mL TNF-α). The MTT assay was used to evaluate cell proliferation. Matrix Gla protein (MGP) and transforming growth factor β1 (TGF-β1) were examined using western blot assay.

Results. The TNF-α treatment significantly increased ASMCs proliferation and enhanced MGP and TGF-β1 expression compared to a blank control group ($p < 0.05$). The 1,25-$(OH)_2D_3$ treatment (1,25-$(OH)_2D_3$+TNF-α group) significantly inhibited cell viability (0.83 ±0.01), compared to that in the TNF-α group (0.92 ±0.01) ($p < 0.05$). The 1,25-$(OH)_2D_3$ treatment significantly downregulated MGP expression (0.61 ±0.02), compared to that of the TNF-α group (1.51 ±0.35) ($p < 0.05$). The 1,25-$(OH)_2D_3$ treatment significantly reduced TGF-β1 expression (0.69 ±0.17), compared to that of the TNF-α group (1.6 ±0.18) ($p < 0.05$).

Conclusions. The 1,25-$(OH)_2D_3$ could participate and modulate airway remodeling by reducing MGP and TGF-β1 expression in TNF-α-induced ASMCs. This study provided therapeutic insight and theoretical basis for clinical research.

Key words: TGF-β1, TNF-α, 25-hydroxyvitamin D_3, airway smooth muscle cells

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Background

Asthma has been proven to be a respiratory disorder that is characterized by airway remodeling, airway inflammation and reversible airway obstruction.[1,2] The airway obstruction and airway remodeling demonstrates many characteristics, including the enhancement of vascular permeability, the promotion of angiogenesis, and hypertrophy and hyperplasia of the airway smooth muscle cells (ASMCs).[3,4] In the occurrence and development processes of asthma, the ASMCs convert from the contractile type to synthetic type. Meanwhile, the ASMCs could synthesize and release the cytokines and inflammatory factors, and accelerate the airway remodeling in the asthma processes.[5] The tumor necrosis factor α (TNF-α), a kind of pro-inflammatory cytokine, could induce the formation of synthetic ASMCs.[6] Therefore, in this study, the TNF-α was administered to ASMCs to establish a synthetic ASMCs model of asthma.

The 1,25-hydroxyvitamin D_3(1,25-$(OH)_2D_3$) could assist the balance of calcium and phosphate levels, promote the calcium absorption, and – ultimately – keep the bone health.[7] Studies by Hall and Agrawal and Chen et al. reported that deficiency of (1,25-$(OH)_2D_3$) is positively correlated with the enhanced morbidity of asthma.[8,9] A few recent studies also discovered that (1,25-$(OH)_2D_3$) could directly inhibit the proliferation and migration of ASMCs in vitro.[10,11] The matrix Gla protein (MGP) is a specific biomarker for the synthetic smooth muscle cells, and could reflect the status and the amounts of the ASMCs.[12] Recently, plenty of cytokines or growth factors secreted by the inflammatory cells have been proven to be associated with the proliferation of ASMCs – especially for the transforming growth factor β1 (TGF-β1), which participates in the airway remodeling and the asthma-associated inflammation.[13,14]

Objectives

In the present study, we administered TNF-α to ASMCs to mimic the inflammatory condition of the airways injured in asthma, and aimed to evaluate the effects of (1,25-$(OH)_2D_3$) on proliferation of ASMCs as well as explore the associated mechanisms.

Materials and methods

Cell culture and trial grouping

The human ASMCs were purchased from Shanghai BioLeaf BioTech. Co. Ltd. (Cat. No. SX-C1160; Shanghai, China) and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco BRL. Co. Ltd., Grand Island, USA), containing 10% fetal bovine serum (FBS; Gibco BRL. Co. Ltd.) at 37°C and 5% CO_2. After passaging the cells for 3–6 generations, ASMCs were digested and prepared to the single-cell suspension, and seeded into 96-well plates (Corning-Costar, Corning, USA). The ASMCs were then divided into 3 groups, including a blank control group (without any treatment), a TNF-α group (ASMCs treated with TNF-α at final concentration of 10 ng/mL) and a 1,25-$(OH)_2D_3$+TNF-α group (ASMCs pre-treated with 10^{-7} M 1,25-$(OH)_2D_3$ for 1 h, and then treated with TNF-α at a final concentration of 10 ng/mL). The TNF-α was purchased from the PeproTech Co. Ltd. (Rocky Hill, USA). The 1,25-$(OH)_2D_3$ was purchased from Sigma-Aldrich (St. Louis, USA).

This study has been approved by the Ethical Committee of Tianjin Union Medicine Centre (Tianjin, China).

MTT assay

Cell proliferation of ASMCs was determined using MTT detection kit (Cat. No. M1020; SolarBio, Beijing, China), according to the manufacturer's protocol. In brief, the ASMCs (at a density of 3×10^3 cells/well) were cultured in 96-well plates and treated as previously described for 24 h. The MTT was added into the 96-well plates (20 μL per well) and incubated for 4 h at 37°C. Then, 150 μL dimethyl sulfoxide (DMSO; Cat. No. D8371; SolarBio) was added to each well to dissolve the formed crystal in the wells. The absorbance for each well at the wavelength of 490 nm was measured by employing the microplate reader.

Western blot assay

The ASMCs were lysed using Lysis Buffer (Cat. No. P0013C; Beyotime Biotechnology, Shanghai, China) according to the manufacturer's instruction. Lysates were separated using 15% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE; Amresco, Inc., Solon, USA) and electro-transferred onto polyvinylidene difluoride (PVDF; Millipore, Boston, USA). Then, PVDF membranes were blocked in phosphate-buffered saline (PBS) using 5% defatted milk supplemented with 0.05% Tween-20 (Beyotime Biotechnology). The PVDF membranes were then treated with rabbit antihuman MGP polyclonal antibody (1:3000, Cat. No. ab86233; Abcam, Cambridge, USA), rabbit antihuman TGF-β1 polyclonal antibody (1:3000, Ca. No. ab92486; Abcam) and rabbit antihuman β-actin polyclonal antibody (1:2000, Cat. No. ab82275; Abcam) at 4°C overnight. Subsequently, PVDF membranes were incubated using 1:2000 horseradish peroxidase (HRP)-conjugated goat antirabbit immunoglobulin G (IgG) (Cat. No. ab6721; Abcam) at room temperature for 2 h. The western blot bands were visualized with BeyoECL Plus kit (Cat. No. P0018S; Beyotime Biotechnology). Finally, the images of western blot bands were captured and analyzed with Image Pro Plus v. 6.0 software (Media Cybernetics, Inc., Bethesda, USA).

Statistical analysis

The data were analyzed using GraphPad Prism software v. 6.0 (GraphPad Software, San Diego, USA). The continuous variables were represented as mean ± standard deviation (SD) and analyzed using analysis of variance (ANOVA) test validated with Tukey's post hoc test. The assumption for the normality and the homogeneity of variances necessary for the comparisons of means were analyzed and tested with Kolmogorov–Smirnov test and Levene's test, respectively (all with normality and homogeneity in this study). The statistical significance was defined when $p < 0.05$. All experiments and tests were conducted at least 6 times.

Results

1,25-$(OH)_2D_3$ inhibited the TNF-α-induced ASMCs proliferation

The results showed that the cell viability for TNF-α-treated ASMCs (0.92 ±0.01) was significantly higher compared to the blank control group (0.69 ±0.02) (Fig. 1, $p < 0.05$). However, the 1,25-$(OH)_2D_3$ treatment (1,25-$(OH)_2D_3$+TNF-α group) markedly inhibited the cell viability (0.83 ±0.01), as compared to the TNF-α group (0.92 ±0.01) (Fig. 1, $p < 0.05$).

1,25-$(OH)_2D_3$ downregulated MGP expression in TNF-α stimulated ASMCs

Western blot analysis was conducted to evaluate the expression of MGP (Fig. 2A). The results indicated that MGP expression in the TNF-α group (1.51 ±0.35) was significantly higher compared to the blank control group (0.17 ±0.04) (Fig. 2B, $p < 0.05$). However, the 1,25-$(OH)_2D_3$ treatment (1,25-$(OH)_2D_3$+TNF-α group) markedly downregulated the MGP expression (0.61 ±0.02) compared the TNF-α group (1.51 ±0.35) (Fig. 2B, $p < 0.05$).

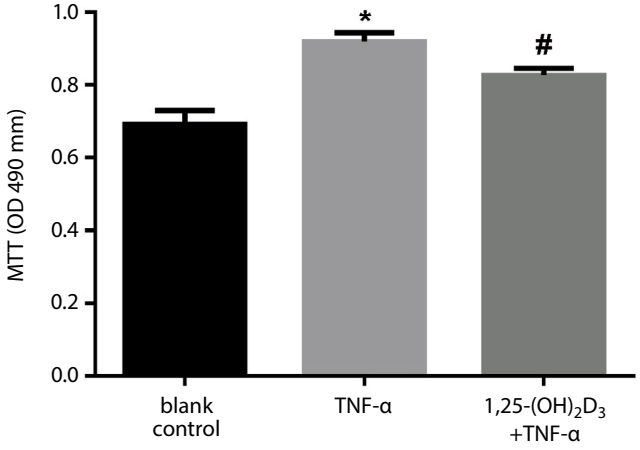

Fig. 1. Evaluation for the effects of 1,25-$(OH)_2D_3$ on the cell proliferation of airway smooth muscle cells (ASMCs) (n = 6 for all groups)

* $p < 0.05$ compared to blank control group; # $p < 0.05$ compared to tumor necrosis factor α (TNF-α) group.

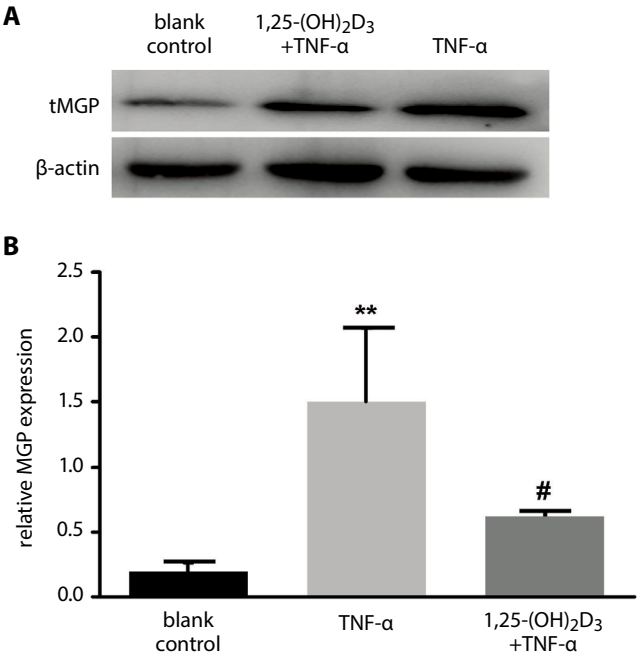

Fig. 2. Determination for the matrix Gla protein (MGP) expression in tumor necrosis factor α (TNF-α)-induced airway smooth muscle cells (ASMCs), using western blot analysis (n = 6 for all groups). A. Western blot images; B. Statistical analysis for the MGP expression

** $p < 0.05$ compared to blank control group; # $p < 0.05$ compared to TNF-α group; tMGP – total MGP.

1,25-$(OH)_2D_3$ reduced the TGF-β1 expression in TNF-α-stimulated ASMCs

The TGF-β1 expression was also determined using the western blot analysis (Fig. 3A). The findings showed that TGF-β1 expression was significantly higher in TNF-α group (1.6 ±0.18) compared to the blank control group (0.59 ±0.06) (Fig. 3B, $p < 0.05$). Meanwhile, the 1,25-$(OH)_2D_3$ treatment (1,25-$(OH)_2D_3$+TNF-α group) markedly reduced the TGF-β1 expression (0.69 ±0.17) compared to the TNF-α group (1.6 ±0.18) (Fig. 3B, $p < 0.05$).

Discussion

According to the report published by Fanta in 2009, approx. 300 million people worldwide suffer from asthma.[15] Although some advances in research on asthma have been made, the present therapeutic strategy is limited to controlling the symptom, without complete cure. The airway remodeling in the asthma patients is thoroughly studied, and the ASMCs hyperplasia and phenotype transformation are the basic characteristics of the airway remodeling.[16] The ASMCs are present as the contractile type in normal lung tissue, while presenting as the synthetic

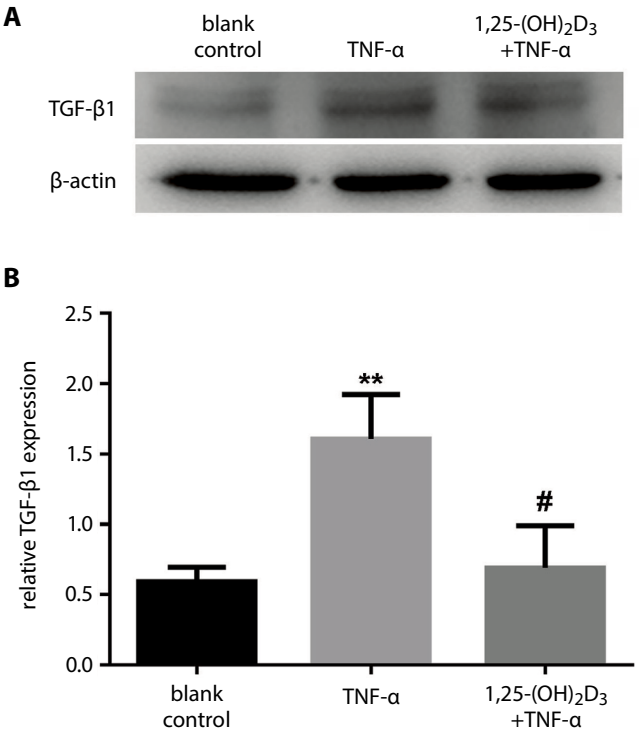

Fig. 3. Effects of 1,25-$(OH)_2D_3$ treatment on the transforming growth factor β1 (TGF-β1) expression determined with western blot analysis (n = 6 for all groups). A. Western blot images for TGF-β1. B. Statistical analysis for the TGF-β1 expression

* $p < 0.05$ compared to blank control group. # $p < 0.05$ compared to TNF-α group; TNF-α – tumor necrosis factor α

type or as contractile-synthetic transformation type in the asthma lung tissue.[17] The synthetic-type ASMCs can synthesize and release the immunoregulatory factors and chemical substances, and are characterized by higher proliferative activity.[18] Therefore, inhibiting the transformation of ASMCs from the contractile type into synthetic type is the key strategy for preventing and treating the asthma.

The 1,25-$(OH)_2D_3$ plays a critical role in counteracting the airway inflammation and regulating the immune response in vivo.[19,20] In recent years, 1,25-$(OH)_2D_3$ has been proven to play a critical role in modulating airway remodeling; however, only a few studies were conducted on the effects of 1,25-$(OH)_2D_3$ on the contractile-synthetic transformation of ASMCs.[1,21] Other studies also demonstrated that the administration of 1,25-$(OH)_2D_3$ could inhibit the airway hyperresponsiveness, lower the immunoglobulin E (IgE) levels and alleviate airway eosinophilia, all of which are mediated by cytokines and growth factors such as MGP and TGF-β1.[19,22]

Therefore, in this study, we evaluated the expression of MGP and TGF-β1 in TNF-α-stimulated ASMCs to verify the effects of 1,25-$(OH)_2D_3$ on the airway remodeling. The MGP is a vitamin K-dependent molecule, with higher expression in the lung tissue.[23] Our findings showed that under the TNF-α treatment, the ASMCs were transformed into the synthetic type, which is consistent with the study by Dogan et al.[24] However, the 1,25-$(OH)_2D_3$ treatment significantly reduced the TGF-β1 expression in TNF-α-stimulated ASMCs, which suggests that 1,25-$(OH)_2D_3$ could inhibit the transformation of ASMCs from contractile type into the synthetic type. The TGF-β1 is a cytokine characterized by powerful biological activity and powerful functions.[25] Studies by Yeganeh et al. and Chen et al. also reported that TGF-β1 is a critical risk factor for the abnormality of airway smooth muscle structures and dysfunction, and is overexpressed in the development and progression of asthma.[26,27] In the present study, we discovered that 1,25-$(OH)_2D_3$ treatment could significantly reduce the expression of TGF-β1 in the TNF-α-stimulated ASMCs. This result suggests that the 1,25-$(OH)_2D_3$ could suppress the release of TGF-β1 and inhibit the airway remodeling.

Limitations

Some limitations of the study should be mentioned. Firstly, the correlation between the two 1,25-$(OH)_2D_3$-associated cytokines, MGP and TGF-β1, has not been clearly elucidated. Secondly, the effects of 1,25-$(OH)_2D_3$ on the airway remodeling have not been verified and the associated mechanisms have not been fully clarified. In an upcoming study, the correlation between MGP and TGF-β1 should be determined using Pearson's coefficient analysis, and the effects of 1,25-$(OH)_2D_3$ on the airway remodeling should be investigated further.

Conclusions

The 1,25-$(OH)_2D_3$ could participate in and modulate the airway remodeling in the TNF-α-treated ASMCs. It not only inhibited the cytokines-induced phenotypic transformation of ASMCs, but also reduced the secretion of inflammatory factors, both of which are the potential strategy for alleviating the airway remodeling. This study provided the therapeutic insight and theoretical basis for the clinical research.

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