

ORIGINAL ARTICLE

Effect of 1,25-(OH)₂D₃ (a vitamin D analogue) on passively sensitized human airway smooth muscle cells

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*Department of Respiratory Medicine, Xijing Hospital, Fourth Military Medical University, Xi'an, China***Effect of 1,25-(OH)₂D₃ (a vitamin D analogue) on passively sensitized human airway smooth muscle cells**SONG Y, QI H, WU C. *Respirology* 2007; 12: 486–494

Background and objectives: In asthma, airway smooth muscle cell (ASMC) hyperplasia plays an important role in airway remodelling. Increased expression of matrix metalloproteinases-9 (MMP-9), a disintegrin and metalloprotease 33 (ADAM33) in ASMCs are also relevant to asthmatic airway remodelling. 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃) has potent antiproliferative properties *in vitro* in various cell types; however, its role in ASMCs is not well understood. This study investigated the effect of 1,25-(OH)₂D₃ on passively sensitized human bronchial (airway) smooth muscle cell (HASM) proliferation and MMP-9 and ADAM33 expressions.

Methods: The effect of 1,25-(OH)₂D₃ on cell proliferation was examined by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium-bromide colorimetry assay; cell cycle analysis by flow cytometry; and immunocytochemical staining for proliferating cell nuclear antigen (PCNA). The expression of MMP-9 and ADAM33 in HASMCs was investigated by real-time quantitative PCR and Western Blot analysis.

Results: 1,25-(OH)₂D₃ effectively suppressed passively sensitized HASMC proliferation, proliferating cell nuclear antigen expression and G₁/S transition in HASMCs passively sensitized with asthmatic serum. Further analysis showed that 1,25-(OH)₂D₃ significantly down-regulated the expressions of protein for MMP-9 and ADAM33, as well as their mRNA levels in passively sensitized HASMCs.

Conclusions: 1,25-(OH)₂D₃ has direct inhibitory effects on passively sensitized HASMCs *in vitro*, including inhibition of cell proliferation and expression of MMP-9 and ADAM33, suggesting a possible beneficial role for 1,25-(OH)₂D₃ in preventing and treating asthmatic airway remodelling.

Key words: 1,25-dihydroxyvitamin D₃, airway, asthma, cell proliferation, smooth muscle.

INTRODUCTION

While asthma is a disorder of the conducting airways characterized by Th2-directed inflammation, a second set of mechanisms is being increasingly recognized as fundamental to disease chronicity and severity; these mechanisms are called 'remodelling'.¹

The pathological changes due to airway remodelling are significant contributors to irreversible airway obstruction and persistent airway hyperresponsiveness.² Although airway remodelling involves alterations of several cell types, airway smooth muscle cells (ASMCs) appear to play a critical role. Three-dimensional morphometric studies of asthmatic airways have shown that ASMC hyperplasia as a prominent feature and the most plausible explanation for the chronic disease process.³ In asthma, ASMCs are likely to be exposed to a range of mediators or growth factors which may promote their proliferation or/and inhibit cell apoptosis. These factors, including platelet-derived growth factor, epidermal growth factor, basic fibroblast growth factor and platelet-activating factor, are elevated in the plasma of asthmatic patients and implicated in the pathophysiological mechanisms of asthma. ASMCs undergo other

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changes associated with hyperplasia, including increased secretion of various chemokines, cytokines and growth factors and the production of extracellular matrix proteins, including matrix metalloproteinases (MMPs). MMPs are the key enzymes responsible for extracellular matrix (ECM) degradation.

In asthma, MMP-9 is the most relevant of the MMPs currently identified.⁴ The critical role of MMP-9 in airway remodelling is seen in patients with severe asthma, particularly those with irreversible airway narrowing.⁵ With high-level expression in ASMCs and lung fibroblasts,⁶ a disintegrin and metalloprotease 33 (ADAM33) has been identified as a novel asthma-susceptibility gene by genome-wide screening and association studies.⁷ Subsequently, genetic studies have provided evidence that ADAM33 plays an important role in airway remodelling.⁸ Its level of expression is associated with asthma development and severity,⁹ and it declines with therapeutic interventions.¹⁰

The steroid hormone 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃) is the active metabolite of vitamin D. It exerts most of its biological activity via a specific nuclear vitamin D receptor (VDR). In addition to its central role in bone metabolism and the immune system,¹¹ 1,25-(OH)₂D₃ has long been recognized as an important contributor to a diverse array of cellular processes, including cell growth, differentiation and apoptosis.¹² A wide spectrum of negative effects are exerted by 1,25-(OH)₂D₃, suggesting its pro-apoptotic and/or antiproliferative effects are important in normal tissue development and function, as well as in the induction of growth arrest in cancer and non-cancerous hyperproliferative disorders.¹³ To date there are no data on the regulatory effect of 1,25-(OH)₂D₃ on ASMCs.

This study investigated the effects of 1,25-(OH)₂D₃ on cell proliferation and the expression of MMP-9 and ADAM33 in passively sensitized HASMCs *in vitro*, in order to better understand the role of 1,25-(OH)₂D₃ in asthmatic airway remodelling.

METHODS

Reagents

3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium-bromide (MTT), trypsin and 1,25-(OH)₂D₃ were purchased from Sigma-Aldrich Int (St Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) was from Gibco-BRL Inc. (Carlsbad, CA, USA). Fetal calf serum (FCS) was from Hangzhou Sijiqing Biological Engineering Materials Co (Hangzhou, China). Polyclonal antibody against MMP-9 and enhance chemiluminol (ECL) kit were purchased from Santu Cruz Biotechnology Co. (Santa Cruz, California). Polyclonal antibodies against proliferating cell nuclear antigen (PCNA) and smooth muscle α -actin (α -SMA) were purchased from Boster Biotechnology Co. (Wuhan, China). And anti-ADAM33 polyclonal antibody was from R&D System Inc. (Minneapolis, MN, USA). NE-PER nuclear and cytoplasmic extraction reagents kit was purchased from Pierce Biotechnol-

ogy Inc. (Rockford, IL, USA). Human bronchial (airway) SMCs (HASMCs) were purchased from Sciencell Research Laboratories (San Diego, CA, USA).

Origin of serum

Control and asthmatic serum was prepared as described previously by Xu *et al.*¹⁴ Asthmatic serum was obtained from 20 acute asthmatic patients (12 men and eight women, aged 22–35 years). These patients had the following characteristics: (i) prior diagnosis of asthma, (ii) allergic history, (iii) no history of other respiratory diseases, and (iv) no use of corticosteroid within the past week. Control serum was obtained from 11 non-asthmatic patients (seven men and four women, aged 25–35 years). These subjects had the following characteristics: (i) no history of allergy or asthma, (ii) no other respiratory diseases, and (iii) no recent medication use. All serum was collected using a sterile technique and diluted in DMEM.

Cell culture and identification of HASMCs

Primary culture of HASMCs was maintained in DMEM supplemented with 10% FCS and 1% penicillin/streptomycin at 37°C in a humidified 5% CO₂–95% air atmosphere. Their SMC identity was verified by standard immunocytochemistry with an anti- α -SMA polyclonal antibody (1:100 dilution). Cells at passages 4–8 were used for the following experiments.

Grouping of HASMCs

For all experiments in which growth-arrested cells were required, the culture medium (DMEM, 10% FCS, antibiotics) was aspirated after HASMC adherence, and the cells were randomly divided into three groups: control, asthma and vitamin D (VD group). In the control and asthma groups, serum-free arrest medium (DMEM, antibiotics) was added. In the VD group, 1,25-(OH)₂D₃ at indicated concentrations was added, as well as the arrest medium. Following cell synchronization for 48 h, the medium of the control cultures was replaced with DMEM containing 10% control serum to stimulate cell-cycle entry, and the medium of the treated cultures in the asthma and VD groups was replaced with DMEM containing 10% asthmatic serum. The cells were then incubated for different periods for different experiments.

MTT assay

HASMCs were cultured in 96-well plates. After 48 h, 20 μ L/well of MTT was added to the culture medium. Following further incubation at 37°C, 5% CO₂ for 4 h, the culture medium was aspirated and replaced with 150 μ L/well of DMSO. Plates were then agitated for 10 min, and the absorbance (value A) was measured at a test wavelength of 492 nm in an ELISA plate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Cell cycle analysis

After culture for 24 h, HASMCs were harvested by rapid trypsinization and centrifuged at 1000 r.p.m. for 5 min. Cells were then washed by ice-cold PBS twice and fixed with ice-cold 70% ethanol overnight at 4°C. The HASMCs were stained with propidium iodide for 30 min at room temperature. The distribution of cells in phase G₀/G₁, S and G₂ was determined by flow cytometry. The proliferation index (PI) was calculated according to the equation: $PI = (S + G_2/M) / (S + G_1/G_0 + G_2/M) \times 100\%$.

Immunocytochemical staining for PCNA

HASMCs in coverslips were washed with PBS twice and fixed in 4% paraformaldehyde and then stained by standard immunocytochemical techniques. A rabbit polyclonal antibody against PCNA protein was used at a dilution of 1:100 as the primary antibody in this study. The substitution of PBS for the specific primary antibody was performed as the negative control. Five high-power sights were randomly chosen in each experiment and 500 cells were randomly counted. The percentage of cells with positive stained nuclei was calculated. The PCNA-positive rate (label index, LI) was calculated as follows: $LI = (\text{number of positive cells} / \text{total number of cells}) \times 100\%$.

Western Blot analysis

After stimulation, the whole-cell lysate of the HASMCs was obtained using NE-PER nuclear and cytoplasmic extraction reagents kit according to the instruction manual. Each extract was subjected to electrophoresis in 10% SDS-PAGE and transferred to a nitrocellulose membrane. The primary antibodies used were polyclonal antibodies of anti-MMP-9 and anti-ADAM33, both at a 1/1000 dilution. Equal sample loading was confirmed using an anti- α -SMA antibody. Immunoreactive bands were visualized by the ECL kit, and band intensities were measured with a

gel analysis system and normalized to those of α -SMA for quantitative analysis.

Real-time quantitative PCR

HASMCs were incubated as described above for 48 h, and cellular RNA was extracted with TRIZOL reagent (Invitrogen, San Diego, CA, USA) according to the manufacture's instructions. Following this, 2 μ g RNA was reversely transcribed to cDNA, and real-time quantitative PCR assays were conducted using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). PCR amplification was performed using the FastStart DNA Master SYBR Green I mixture (Roche Ltd, Basel, Switzerland). The PCR conditions for 25-hydroxyvitamin D-24-hydroxylase (CYP24) were 40 cycles of denaturation at 95°C for 20 s, annealing at 60°C for 20 s and extension at 72°C for 20 s. The PCR condition for VDR was the same as for CYP24, except 38 thermal cycles and annealing at 61°C for 20 s. The PCR conditions for MMP-9 and GAPDH comprised 30 cycles of 94°C (30 s), 60°C (1 min) and 72°C (1 min). With regard to ADAM33, its cDNA samples were amplified for 36 cycles of 95°C (15 s), 60°C (30 s) and 79°C (8 s), following three cycles of 95°C (5 s), 55°C (10 s) and 72°C (20 s). For quantification, a standard curve was generated with various dilutions of the cDNA templates. Target mRNA levels were normalized to those of the glyceraldehyde-3-phosphatedehydrogenase (GAPDH) housekeeping gene in order to compensate for differences in the mRNA levels between various samples. Oligonucleotide primers for VDR, CYP24, MMP-9, ADAM33 and GAPDH mRNA are shown in Table 1.

Statistical analysis

All experiments were carried out a minimum of three times to ensure consistent results. Datasets were expressed as mean $\pm$ SE. Inter-group comparisons were established using one-way ANOVA. Student, Newman and Keuls tests were used for multiple comparisons. Statistical significance was considered at $P < 0.05$.

Table 1 Primers used for real time PCR cDNA amplification

Gene	Primer sequence	Product size (bp)
VDR	Forward primer: 5'-TGCCTGACCCTGGAGACTTTGACC-3' Reverse primer: 5'-CATCATGCCGATGTCCACACAGCG-3'	242
CYP24	Forward primer: 5'-GGC TTC TCC AGA AGA ATG CAG GGG ATG AAG-3' Reverse primer: 5'-TGA GGC TCT TGT GCA GCT CGA CTG GAG TGA-3'	117
MMP-9	Forward primer: 5'-TTC ATC TTC CAA GGC CAA TC-3' Reverse primer: 5'-CTTGTCGCTGTCAAAGTTCCG-3'	287
ADAM33	Forward primer: 5'-CTG AGA ACT CTC ATG AGC-3' Reverse primer: 5'-GAC TCA GTC GAA CCA TAG-3'	540
GAPDH	Forward primer: 5'-GGT ATC GTG GAA GGA CTC ATG AC-3' Reverse primer: 5'-ATG CCA GTG AGC TTC CCG TTC AGC-3'	188

ADAM33, a disintegrin and metalloprotease 33; CYP24, 25-hydroxyvitamin D-24-hydroxylase; GAPDH, glyceraldehyde-3-phosphatedehydrogenase; MMP-9, matrix metalloproteinase-9; VDR, vitamin D receptor.

RESULTS

ASMC identification

Phase-contrast microscopy of the cells at confluence revealed a spindle shape, with a central oval nucleus and prominent nucleoli, as well as the typical 'hill-and-valley' appearance. In addition, nearly 100% of the cells at 4–8th passages showed positive immunocytochemical staining with an anti- α -SMA polyclonal antibody (1:100 dilution).

VDR expression in HASMCs

To verify the possible effect of 1,25-(OH)₂D₃ on HASMCs, real-time quantitative PCR was first used to determine VDR expression in HASMCs. As shown in Figure 1, passive sensitization with asthmatic serum alone did not appear to alter the mRNA level of VDR compared with that in the control cultures, but 1,25-(OH)₂D₃ pretreatment increased the mRNA level. Moreover, the mRNA encoding CYP24, a primary vitamin D-responsive gene known to be regulated by VDR,¹⁵ was also significantly up-regulated in the VD group compared with the asthma and control cultures. The results demonstrated the capacity of HASMCs to respond to 1,25-(OH)₂D₃.

1,25-(OH)₂D₃ reduces passively sensitized HASMC proliferation

Gene expression profiling studies have revealed that VDR may play a role in regulating SMC proliferation and 1,25-(OH)₂D₃ can inhibit cell proliferation in human coronary artery SMCs.¹⁶ In this study, 1,25-(OH)₂D₃ was hypothesized to inhibit HASMC proliferation and MTT assay was performed. Asthmatic serum markedly stimulated HASMC proliferation, but 1,25-(OH)₂D₃ blocked this event in a concentration-dependent manner (Fig. 2). The inhibition of 1,25-(OH)₂D₃ was barely detectable at a concentration of 10⁻¹⁰ M, which was similar to that observed physiologically in the plasma. However, it became statistically significant at 10⁻⁹ M and peaked at a concentration of 10⁻⁷ M, which was then adopted as the optimal concentration of 1,25-(OH)₂D₃ for other experiments in the present study.

Effect of 1,25-(OH)₂D₃ on the PCNA expression of passively sensitized HASMCs

Passive sensitization with asthmatic serum increased the intensity and number of PCNA-stained HASMCs, but pretreatment with 1,25-(OH)₂D₃ reduced these stimulatory effects (data not shown). Figure 3 shows the PCNA-positive rate of cultured HASMCs in different groups at 48 h. Significant augmentation in the PCNA-positive rate of cultured HASMCs was obtained in the asthma group compared with that in the control group, whereas 1,25-(OH)₂D₃ preincubation markedly decreased the increased PCNA-

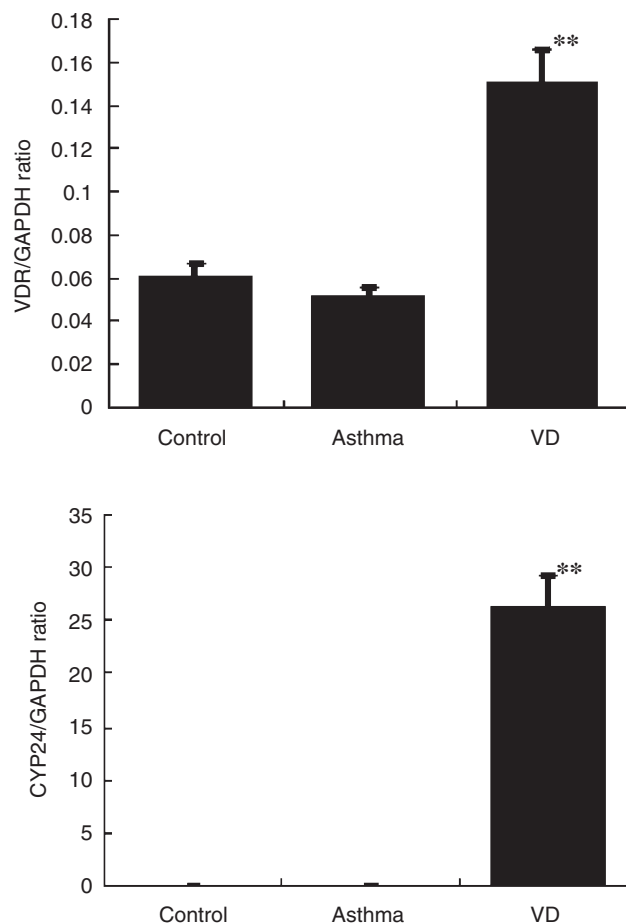


Figure 1 Expression of vitamin D receptor (VDR) and 25-hydroxyvitamin D-24-hydroxylase (CYP24) mRNA in human bronchial (airway) smooth muscle cells (HASMCs). HASMCs were synchronized with or without 10⁻⁷ M 1,25-(OH)₂D₃ for 48 h, then stimulated for 48 h with Dulbecco's modified Eagle's medium containing either asthmatic serum (for the asthma and VD group studies) or control serum (for the control group studies). Real-time quantitative PCR was performed on total-cell RNA using specific primers for VDR and CYP24. The expression level of target mRNA was normalized to the glyceraldehyde-3-phosphatedehydrogenase mRNA level. Each bar represents mean $\pm$ SE from three independent experiments (***P* < 0.05 vs asthma group). GAPDH, glyceraldehyde-3-phosphatedehydrogenase; VD, vitamin D.

positive rate. The results further supported the anti-proliferative activity of 1,25-(OH)₂D₃ in HASMCs.

1,25-(OH)₂D₃ blocks the G₁/S transition in the cell cycle of passively sensitized HASMCs

Pretreatment with 1,25-(OH)₂D₃ markedly increased the percentage of HASMCs induced by asthmatic serum at the G₀/G₁ phase and correspondingly decreased the percentage at the S and G₂/M phase (Table 2). 1,25-(OH)₂D₃ hampered the G₁/S transition of HASMCs passively sensitized by asthmatic serum.

Table 2 Effects of 1,25-(OH)₂D₃ on the cell cycle of passively sensitized human bronchial (airway) smooth muscle cells (HASMCs)

Group	G ₀ /1	S	G ₂ /M	PI
Control	73.87 ± 0.80	16.47 ± 0.69	9.67 ± 0.73	26.13 ± 0.8
Asthma	63.33 ± 0.68*	20.27 ± 1.32*	16.4 ± 1.52*	36.93 ± 0.94*
Vitamin D	69.30 ± 1.62**	14.83 ± 0.67**	15.87 ± 1.29	30.7 ± 1.62**

P* < 0.05 versus control group, *P* < 0.05 versus asthma group.

HASMCs were synchronized with or without 10⁻⁷ M 1,25-(OH)₂D₃ for 24 h and followed by stimulation for 48 h with Dulbecco's modified Eagle's medium containing asthmatic serum (for the asthma and VD group studies) or control serum (for the control group studies). The cell cycle was then assayed by flow cytometry and the proliferation index (PI) of each group was calculated. Values are presented as mean ± SE from three independent experiments.

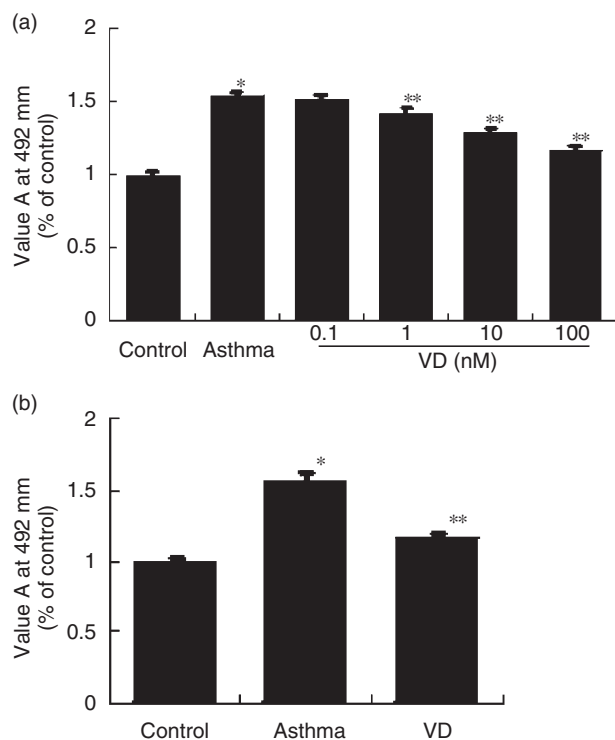


Figure 2 Inhibition of passively sensitized human bronchial (airway) smooth muscle cell (HASMC) proliferation by 1,25-(OH)₂D₃. HASMCs were synchronized with or without 1,25-(OH)₂D₃ at indicated concentrations for 48 h and followed by stimulation for 48 h with Dulbecco's modified Eagle's medium containing asthmatic serum (for the asthma and VD group studies) or control serum (for the control group studies). Then 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium-bromide assay was performed. (a) HASMCs were seeded at a density of 6 × 10³ cells/well and pretreated with increasing concentrations of 1,25-(OH)₂D₃, ranging from 10⁻¹⁰ M (similar to its physiological concentration) to ~10⁻⁷ M. (b) Cells were seeded at a density of 8 × 10³ cells/well and pretreated with 10⁻⁷ M 1,25-(OH)₂D₃. Value A was measured at 492 nm and then calculated as percentage of control (100%). Each bar represents mean ± SE from three independent experiments (**P* < 0.05 vs control group, ***P* < 0.05 vs asthma group). VD, vitamin D.

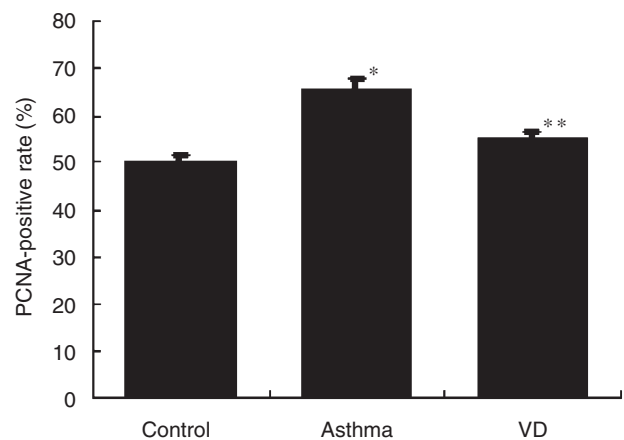


Figure 3 The calculated proliferating cell nuclear antigen (PCNA)-positive rates of cultured human bronchial (airway) smooth muscle cells (HASMCs). HASMCs were synchronized with or without 10⁻⁷ M 1,25-(OH)₂D₃ for 48 h, followed by stimulation for 48 h with Dulbecco's modified Eagle's medium containing asthmatic serum (for the asthma and VD studies) or control serum (for the control group studies). Immunocytochemical analysis for PCNA was performed. Each bar represents mean ± SE from three independent experiments (**P* < 0.05 vs control group, ***P* < 0.05 vs asthma group). VD, vitamin D.

Additionally, 2 days of treatment with asthmatic serum resulted in a significant increase in the PI of HASMCs and significant inhibition of 1,25-(OH)₂D₃. The suppression by 1,25-(OH)₂D₃ of the cell cycle was in accordance with the results of cell proliferation assay.

1,25-(OH)₂D₃ inhibits the MMP-9 and ADAM33 protein expressions in passively sensitized HASMCs

Western Blot performed on HASMC protein extracts yielded a band of protein for MMP-9 at the expected size of 62 kDa (Fig. 4), consistent with the MW of

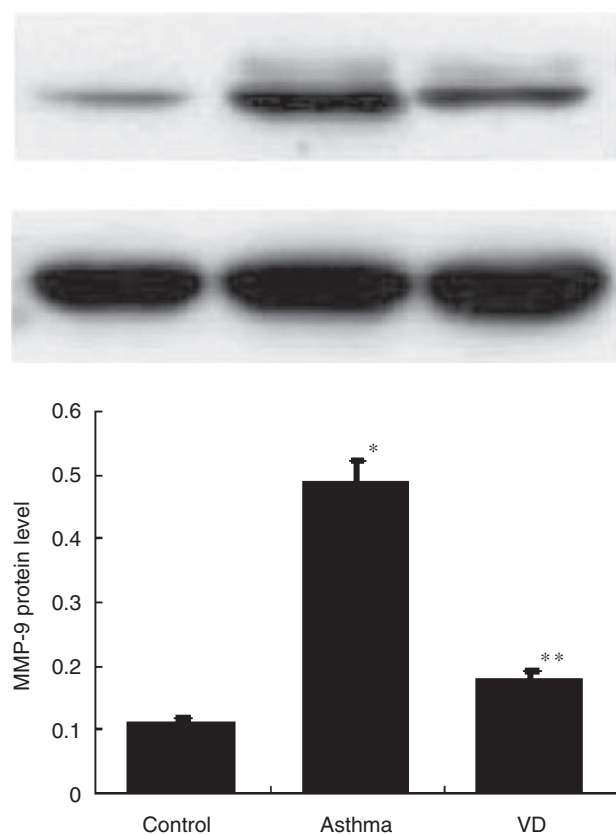


Figure 4 1,25-(OH)₂D₃ inhibits matrix metalloproteinase-9 (MMP-9) protein expression in passively sensitized human bronchial (airway) smooth muscle cells (HSMCs). HSMCs were synchronized with or without 10⁻⁷ M 1,25-(OH)₂D₃ for 48 h, followed by stimulation with Dulbecco's modified Eagle's medium containing asthmatic serum for an additional 48 h in the asthma and VD group or control serum in the control group. The total-cell lysate was prepared and Western Blot analysis was performed. The upper panel shows Western Blot gel using an anti-MMP-9 polyclonal antibody, and equal sample loading was confirmed with a smooth muscle α-actin (α-SMA) antibody. Results shown are representative of three independent experiments. The lower panel depicts the densitometric evaluation of MMP-9 protein level, normalized to the α-SMA level. Each bar represents mean ± SE from three independent experiments (**P* < 0.05 vs control group, ***P* < 0.05 vs asthma group). VD, vitamin D.

MMP-9. The results showed that stimulation with asthmatic serum induced a 4.45-fold increase in the MMP-9 protein expression in the total cell lysate compared with that in the control group, whereas 1,25-(OH)₂D₃ pretreatment attenuated its level in passively sensitized HSMCs by 63.3%.

Similarly, but less strikingly, results were duplicated on the ADAM33 protein expression. 1,25-(OH)₂D₃ caused up to 50.9% decrease in ADAM33 protein level induced by asthmatic serum (Fig. 5).

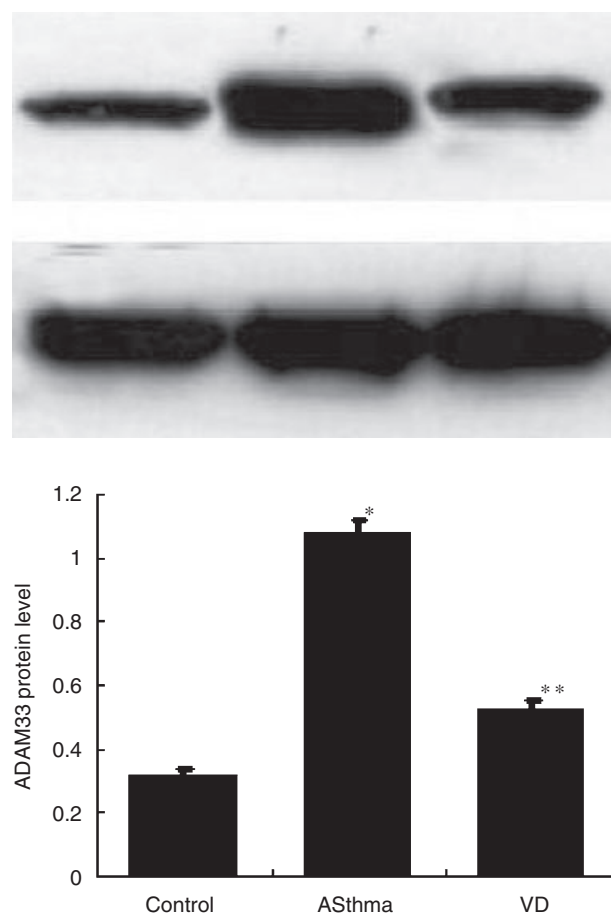


Figure 5 1,25-(OH)₂D₃ inhibits a disintegrin and metalloprotease 33 (ADAM33) protein expression in passively sensitized human bronchial (airway) smooth muscle cells (HSMCs). HSMCs were synchronized with or without 10⁻⁷ M 1,25-(OH)₂D₃ for 48 h, followed by stimulation for an additional 48 h with Dulbecco's modified Eagle's medium containing asthmatic serum (for the asthma and VD group studies) or control serum (for the control group studies). The total-cell lysate was prepared and Western Blot analysis was performed. The upper panel shows Western Blot gel using an anti-ADAM33 polyclonal antibody, and equal sample loading was confirmed with a smooth muscle α-actin (α-SMA) antibody. Results shown are representative of three independent experiments. The lower panel depicts the densitometric evaluation of ADAM33 protein level, normalized to the α-SMA level. Each bar represents mean ± SE from three independent experiments (**P* < 0.05 vs control group, ***P* < 0.05 vs asthma group). VD, vitamin D.

1,25-(OH)₂D₃ down-regulates the MMP-9 and ADAM33 mRNA levels in passively sensitized HSMCs

To support the findings that MMP-9 and ADAM33 protein levels were changed in the total lysate when treated by 1,25-(OH)₂D₃, MMP-9 and ADAM33 mRNA levels were measured using specific primers (Table 1). Consistent with the Western Blot results, the analysis

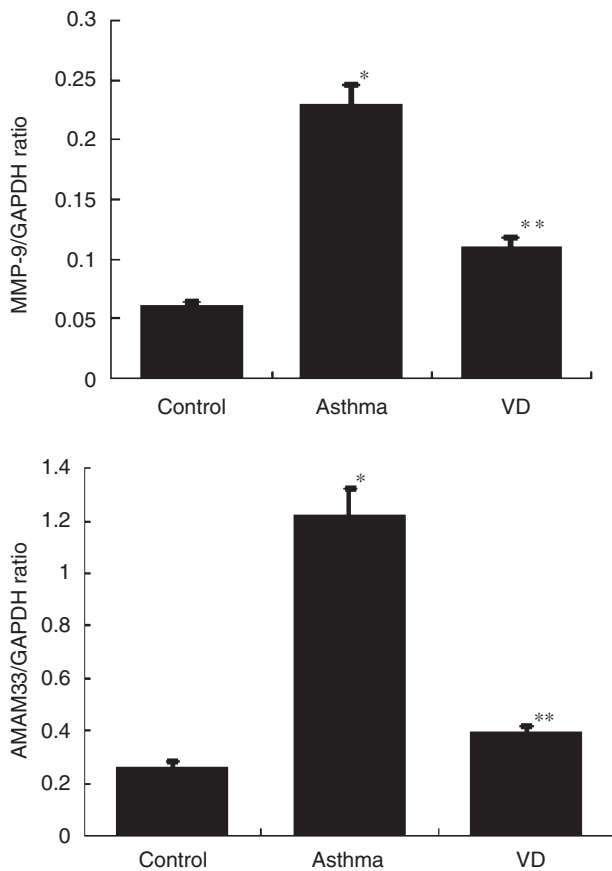


Figure 6 1,25-(OH)₂D₃ decreases the mRNA levels of matrix metalloproteinase-9 (MMP-9), a disintegrin and metalloprotease 33 (ADAM33) in passively sensitized human bronchial (airway) smooth muscle cells (HSMCs). HSMCs were synchronized with or without 10⁻⁷ M 1,25-(OH)₂D₃ for 48 h and followed by stimulation for an additional 48 h with Dulbecco's modified Eagle's medium containing asthmatic serum (for the asthma and VD group studies) or control serum (in the control group studies). Real-time quantitative PCR was performed on the total-cell RNA using specific primers for MMP-9 and ADAM33. The expression level of target mRNA was normalized to the glyceraldehyde-3-phosphatedehydrogenase (GAPDH) mRNA level. Each bar represents mean ± SE from three independent experiments (**P* < 0.05 vs control group, ***P* < 0.05 vs asthma group). VD, vitamin D.

from real-time quantitative PCR shows that 1,25-(OH)₂D₃ significantly inhibited the MMP-9 and ADAM33 mRNA levels in passively sensitized HSMCs by 52.2% and 68%, respectively (Fig. 6).

DISCUSSION

When asthma becomes more severe and chronic, it adopts different characteristics, including a degree of fixed airflow obstruction and corticosteroid refractoriness. Underlying this phenotype is airway remodeling, an increase in smooth muscle mass linked to airways hyperresponsiveness.¹⁷ Given the critical role

ASMCs play in asthmatic airway remodelling, agents that inhibit ASMC growth may be of therapeutic value. Clarification of the mechanism of ASMC proliferation in asthma is a step towards the development of new therapies. Although it is difficult to acquire ASMCs from asthmatic patients, an *in vitro* technique known as passive sensitization provides a useful model for the investigation of pathophysiological mechanisms in asthma.¹⁴

The effects of 1,25-(OH)₂D₃ are mediated mainly via interaction with VDR.¹⁸ Identification of VDR variants as a novel genetic risk factor for asthma suggests that this receptor–ligand pair may function as a regulator of asthma.¹⁹ Although 1,25-(OH)₂D₃ can be coincidentally administered to severe asthmatic patients as part of a prophylactic regimen against glucocorticoid-induced osteoporosis, the idea that it may play a beneficial role in asthmatic airway remodelling is relatively new.

There are many ways in which vitamin D may influence the remodelling of tissue, including the inhibition of MMPs,²⁰ as well as effects on the proliferation of fibroblasts and on the synthesis of collagen.²¹ These observations raise the possibility that it could influence airway remodelling. To confirm this and to identify the additional contribution of 1,25-(OH)₂D₃ in asthmatic airway remodelling, the study presented here investigated the effect of 1,25-(OH)₂D₃ on HSMCs in asthma, using an *in vitro* method of passive sensitization.

The major finding of this study was that 1,25-(OH)₂D₃ had direct antiproliferative activity in passively sensitized HSMCs. Existing data have shown that 1,25-(OH)₂D₃ has an effect of cell-cycle arrest, which is cell-type specific and characterized by a hampered G₁/S transition.²² This study extended this observation to HSMCs and showed that it could also impede the HASMC progression from G₁ to S, consequently inhibiting HASMC proliferation induced by asthmatic serum.

HSMCs are also the main cellular source of MMP-9. As an important mediator of airway remodelling, MMP-9 has been shown to possess multiple functions important for chronic airway remodelling in asthma, including initiating the degradation of ECM, enabling eosinophil migration and affecting SMC migration and proliferation.²³ Down-regulation of MMP-9 expression may be one of the mechanisms by which airway remodelling can be inhibited in asthma.²⁴ In keeping with studies showing that 1,25-(OH)₂D₃ can suppress MMP-9 expression in other cell types,^{25,26} the present study also showed that 1,25-(OH)₂D₃ had an inhibitory effect on MMP-9 mRNA and protein levels in passively sensitized HSMCs, which should be useful in controlling asthmatic airway remodelling.

There was a marked decrease in the expression of ADAM33 in passively sensitized HSMCs after pretreatment with 1,25-(OH)₂D₃. The selective expression of ADAM33 in lung mesenchymal cells, such as ASMCs and fibroblasts, and its stronger linkage and association with BHR strongly suggest that alterations in its activity may underlie abnormalities in the functions of ASMCs and fibroblasts linked to airway remodelling.²⁷ The multiple molecular actions of

ADAM33 make this molecule a strong candidate protein for the pathogenesis of airway remodelling in chronic asthma.²⁸ ADAM33 may directly impact on lung architecture and function,²⁹ due to its regulatory role in the differentiation and proliferation of the mesenchymal cells via its disintegrin domain.¹⁰ It has also been postulated that ADAM33 has important cytokine-stimulating effects by which it can indirectly influence airway remodelling.³⁰ The 1,25-(OH)₂D₃-mediated ADAM33 down-regulation may further contribute to the role of 1,25-(OH)₂D₃ in the attenuation of asthmatic airway remodelling.

The transduction pathway involved in the inhibitory role of 1,25-(OH)₂D₃ is not clear. Nuclear factor- κ B (NF- κ B) signalling pathway is extensively involved in ASMC dysregulation, in cell proliferation, and in inflammatory cytokine gene and protein expression in ASMCs.^{31,32} The VDR physically interacts with NF- κ B p65 and is directly involved in the regulation of NF- κ B activation.^{33,34} In this study, HASMCs expressed functional VDR and 1,25-(OH)₂D₃ may evoke a functional response and up-regulate VDR mRNA expression in passively sensitized HASMCs. Thus, the relationship between VDR and NF- κ B was assumed to be a possible reason for the negative effect of 1,25-(OH)₂D₃ on passively sensitized HASMCs. In other words, the up-regulation of VDR induced by 1,25-(OH)₂D₃ may have decreased NF- κ B activation in HASMCs, leading to the inhibitory effects of 1,25-(OH)₂D₃ in passively sensitized HASMCs. It might further be postulated that pretreatment with 1,25-(OH)₂D₃ could result in similar anti-proliferative effects in non-sensitized HASMCs, which would broaden the clinical applications of 1,25-(OH)₂D₃ to the treatment of COPD and other lung diseases involving hyperproliferation of ASMCs. Alternatively, the hypothesis could be made that HASMCs overexpressing VDR might exhibit anti-proliferative characteristics similar to cells pretreated with 1,25-(OH)₂D₃. Further studies are required to clarify these potential mechanisms.

The current study establishes the important role of 1,25-(OH)₂D₃ and shows that it has a potent capacity to inhibit cell proliferation and MMP-9 and ADAM33 expression in passively sensitized HASMCs. Recent studies have demonstrated the positive relationship between vitamin D and lung function.³⁵ This study is consistent with several published reports which have shown that 1,25-(OH)₂D₃ suppresses plasminogen activator inhibitor-1 in SMCs,³⁶ another essential factor for airway remodelling.³⁷ The present study presents a rationale as to how vitamin D could exert multiple inhibitory actions on HASMCs, and shows its potential beneficial effect on the alleviation of asthmatic airway remodelling and improvement of lung function.

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