



Effect of *Astragali-Cordyceps Mixture* on TGF- β /Smad signal pathway in the lung of asthma airway remodeling

Gui-xin Gao^{a,b}, Qing-mei Li^a, Hua-hao Shen^{a,*}

^a Department of Respiratory Medicine, Second Hospital of Medical School of Zhejiang University, Institute of Respiratory Diseases, Zhejiang University, 88 Jiefang Road, Hangzhou 310009, China

^b Department of Oncology Medicine, Shandong Provincial Hospital affiliated to Shandong University, Jinan 250021, China

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ABSTRACT

Aim of the study: We try to find out the influence of traditional Chinese Medicine *Astragali-Cordyceps Mixture* (ACM) on TGF- β /Smad signal pathway in the lung of asthma airway remodeling.

Materials and methods: Mice were sensitized and challenged by OVA to establish a model of asthma. To assess the effects of ACM on the mice, animals of the ACM groups were treated with ACM. Data were achieved by using techniques as follow: counting cell number of BALF, assaying the amount of collagen deposition by Masson's staining, performing RT-PCR and immunohistochemistry for mRNA and protein expression of TGF- β 1, Smad3 and Smad7.

Results: The depositions of collagen in airway wall greatly increased at the model group compared with that of the normal group. In contrast, these decreased at the ACM groups. As compared with the control group, TGF- β 1 expression also decreased at both mRNA and protein level at the ACM-M group versus increased both at the model group. Whereas, Smad7 significantly decreased only at the model group and partly restored at the ACM-M group.

Conclusions: ACM greatly improves the symptoms of asthma airway remodeling by inhibiting the expression of TGF- β 1 and upregulating the amount of Smad7.

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1. Introduction

Asthma is a chronic inflammatory disease characterized by airway infiltration of inflammatory cells inducing airway hyper-responsiveness (AHR) and airway remodeling. The airway remodeling is one of the pathophysiological characteristics of asthma, and its main pathological changes include subepithelial fibrosis formation and increased collagen deposition on airway wall. The direct consequence of the airway remodeling is persistent AHR and irreversible airway obstruction leading to a chronic and obstinate asthma with pulmonary function depression.

Transforming growth factor β 1 (TGF- β 1) is considered as the principal regulatory factor of asthma airway remodeling, which directly influences deposition of collagen promoting fibrosis formation in the airway wall. Recently, TGF- β 1/Smad signal pathway was found to be one of the important mechanisms for signal conduction in asthma airway remodeling. The Smad proteins are homologs of both the drosophila protein, mothers against decapentaplegic (MAD) and the *Caenorhabditis elegans* protein SMA. The name is a combination of the two. Smad proteins are the downstream sig-

nal regulatory proteins of TGF- β receptor complex consisting of receptor regulation Smad protein (R-Smad), common regulation Smad (Co-Smad) and inhibitory Smad protein (I-Smad). As the important members of TGF- β signal transducing system, Smad proteins directly transport signals from cell membrane to nucleus, and mediate intracellular TGF- β signal transduction regulating cell proliferation, transformation, synthesis, secretion and apoptosis. After being phosphorylated by the activated TGF- β 1 receptor type I, Smad2 and Smad3 form a hetero-complex with Co-Smad (Smad4), and transfer into cellular nucleus activating DNA transcription to regulate the target gene expression. On the other hand, Smad6 and Smad7 can block the transcription induced by TGF- β through inhibiting its signal conduction. Therefore, it would be very helpful for the treatment of asthma airway remodeling to block the intracellular TGF- β 1 signal transduction for fibroblast (FB) proliferation and collagen synthesis.

To date, there has been a growing interest in herbal products in Traditional Chinese Medicine, which is considered to be effective to treat asthma. *Astragali-Cordyceps Mixture* (ACM), a Chinese traditional medicine, composed of astragali and Chinese caterpillar fungus, is developed based on the Chinese traditional medicine theory and clinic experience. One function of this drug is to benefit lung and kidney. ACM has been proved to be a novel promising approach for the treatment of asthma, which can efficiently relieve symptoms

* Corresponding author. Tel.: +86 571 87767122; fax: +86 571 87767122.
E-mail address: hh.shen@yahoo.com.cn (H.-h. Shen).

Table 1
Summary of experimental treatments.

Groups	Mouse number	Sensitization	Challenge	Treatments	
				Reagents	Dose
Normal	8	Physiological saline	Physiological saline	Physiological saline	0.5 ml d ⁻¹
Model	8	OVA + alum	2.5% atomized OVA	Physiological saline	0.5 ml d ⁻¹
ACM-L	8	OVA + alum	2.5% atomized OVA	<i>Astragali-Cordyceps Mixture</i>	3.3 g kg ⁻¹ d ⁻¹
ACM-M	8	OVA + alum	2.5% atomized OVA	<i>Astragali-Cordyceps Mixture</i>	6.5 g kg ⁻¹ d ⁻¹
ACM-H	8	OVA + alum	2.5% atomized OVA	<i>Astragali-Cordyceps Mixture</i>	13 g kg ⁻¹ d ⁻¹
Budesonide	8	OVA + alum	2.5% atomized OVA	Budesonide atomized liquid	10 ml d ⁻¹ (0.5 mg/ml)

Abbreviations:

Normal: mice were sensitized and challenged with saline.

Model: mice were sensitized and challenged with Ova.

ACM-L: mice were treated with ACM in low dose.

ACM-M: mice were treated with ACM in middle dose.

ACM-H: mice were treated with ACM in high dose.

Budesonide: mice were treated with Budesonide.

and reduce the frequency of asthma attacks. In this study, how ACM acts on asthma airway remodeling was explored by determining its effect on TGF- β 1/Smad signal pathway.

2. Materials and methods

2.1. Preparation of *Astragali-Cordyceps Mixture*

Astragali-Cordyceps Mixture (ACM) was provided by Zhejiang Provincial Hospital of Traditional Chinese Medicine. ACM composed of astragali and Chinese caterpillar fungus (they are in the ratio of 10:3), was made at different concentration suspensions (0.16 kg l⁻¹, 0.325 kg l⁻¹, 0.65 kg l⁻¹).

2.2. Mice

Six to eight weeks old male C57BL6 mice weighing 18–22 g were purchased from Shanghai Experimental Animal Center, Chinese Academy of Sciences (SEACCAS) and raised in specific pathogen free (SPF) conditions in the Experimental Animal Center, Zhejiang Academy of Medical Science. The present study was performed according to the internationally accepted principles for laboratory animal use and care as found in the US guidelines (NIH publication #85-23, revised in 1985). Forty-eight mice were randomly divided into 6 groups (8 mice per group) and administered drug as summarized in Table 1. Two hours before challenging, mice were treated with ACM in half, equal, or double dose used in human (ACM-L, ACM-M and ACM-H groups) or physiological saline (normal and model groups) by intragastric administration once a day till the last challenge. Mice treated with 10 ml 0.5 mg/ml budesonide (Budesonide group) by atomization were used as a drug control (Shen and Wang, 2005; Shen et al., 2008).

2.3. Sample collection

After 48 h of last OVA treatment, mice were anesthetized and sacrificed. For the TGF- β 1 mRNA assay, a piece of tissue was sampled from the right lung of each mouse and stored in liquid nitrogen, and the rest was fixed with 4% formaldehyde solution and sliced. The slices were routinely prepared and used for detecting collagen deposition in airway, assaying protein expression of TGF- β 1, Smad3 and Smad7.

2.4. Determination of collagen deposition in lung airway

Masson trichrome stain was used here to detect collagen deposition in the lung tissues according to previously reports (Vanacke

et al., 2001; Henderson et al., 2002). After staining, collagen area on the basal membrane of airway in the lung tissues was analyzed by Leica-Qwin image-processing system (Leica Imaging Systems, Bensheim, Germany) using the affix color model. The result was expressed as Wac/Pbm ($\mu\text{m}^2/\mu\text{m}$) (collagen area on the basal membrane of airway/the length of the airway epithelial).

2.5. Detection of TGF- β 1 mRNA expression in lung tissue

A reverse transcription polymerase chain reaction (RT-PCR) method was employed to determine mRNA expression of TGF- β 1 in the lung tissues. Two pairs of primers were designed and synthesized at Shanghai Sangon Biological Engineering Technology & Services Co., Ltd., China, and used for detecting TGF- β 1 mRNA versus β -actin as a reference (Table 2).

Total RNA was extracted from lung tissues with Trizol reagent (Invitrogen, USA) according to the manufacture's instruction. The first strand cDNA was synthesized using a reverse transcription kit (Shanghai Sangon). A 50 μ l PCR reaction mixture contained 5 μ l 10 $\times$ Taq Buffer, 4 μ l 25 mM MgCl₂, 4 μ l 2.5 mM dNTP. Forward and backward primers (TGF- β 1 or β -actin) 2 μ l each, cDNA template 2 μ l, Taq polymerase (Shanghai Sangon) 0.5 μ l. PCR reaction was performed with a DNA thermal cycler (Perkin Elmer, USA) by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 20 s, and elongation at 72 °C for 30 s after denaturing at 95 °C for 5 min, and a final extension at 72 °C for 7 min. Five microliter of PCR products were applied to agarose gel electrophoresis at 5 V/cm for 1 h using 4 μ l DNA marker as a reference, and scanned by a IS1000 gel image analysis system (Alpha Innotech, USA) for gray scale analysis. Expression of TGF- β 1 mRNA in each sample was expressed as a ratio of TGF- β 1 gray value to β -actin ones.

2.6. Detection of TGF- β 1, Smad3 and Smad7 protein expression in the lung tissue

Protein expressions of TGF- β 1, Smad3 and Smad7 were detected by immunohistochemistry (IHC) using the corresponding EnVision™ IHC detection kit plus the enhanced kit (Dako, Denmark) following the manufacturer's instructions. The positive cells

Table 2
Primers for TGF- β 1 and β -actin detection.

Target gene	Primers	Sequence	Product (bp)
TGF- β 1	Forward	5'-CTGAGTGGCTGCTTTTG-3'	436
	Backward	5'-TTGCTGTACTGTGTGCC-3'	
β -Actin	Forward	5'-GGGACG ACATGGAGAAGA-3'	196
	Backward	5'-ACGACCAGAG GCATACAG-3'	

showed yellow or brown articles or clumps in the cytoplasm with a blue colored cellular nucleus. Using the Leica-Qwin image analysis system (Leica Imaging Systems, Bensheim, Germany), the optical density values were measured under 200× objective lens of each slice for quantitative analysis. The final optical density (OD) value is the OD value difference between the positive part to be concluded and the background part.

2.7. Statistical analysis

Data are expressed as mean ± SE. SPSS 13.5 software was used for statistical analysis. Differences between groups were tested using a one-way analysis of variance (ANOVA).

3. Results

3.1. Influence of ACM on collagen deposition in the asthma airway remodeling

As shown in Table 3 and Fig. 1, there was a little collagen deposition (0.312 ± 0.08) in airway wall as well as blood vessel surrounding in the normal mice, and the deposition increased significantly ($p < 0.01$) with an extensive distribution in airway wall and blood vessel surrounding in the asthma model mice (0.756 ± 0.14). Compared with the model group, collagen deposition in the mice treated with middle (0.55 ± 0.106) and high (0.527 ± 0.1) dose ACM was found lessened significantly ($p < 0.01$), which was more in ACM treated mice than in budesonide treated mice (0.405 ± 0.08), while the low dose ACM treatment (0.635 ± 0.11) showed less effect on the deposition ($p < 0.05$). There is no significant deviation between the middle and high dose ACM groups. These results suggest that the middle dose ACM has the best curative effect and is the best curative dose. Therefore, the middle dose will be the default dose otherwise mentioned specially (this group will be called the ACM-M group).

Table 3

Effect of drugs on airway collagen deposition.

Groups	Collagen deposition ($\mu\text{m}^2/\mu\text{m}$)
Normal	$0.312 \pm 0.08^{**}$
Model	0.756 ± 0.14
ACM-L	$0.635 \pm 0.11^*$
ACM-M	$0.55 \pm 0.106^{**}$
ACM-H	$0.527 \pm 0.1^{**}$
Budesonide	$0.405 \pm 0.08^{**}$

Abbreviations:

Normal: mice were sensitized and challenged with saline.

Model: mice were sensitized and challenged with Ova.

ACM-L: mice were treated with ACM in low dose.

ACM-M: mice were treated with ACM in middle dose.

ACM-H: mice were treated with ACM in high dose.

Budesonide: mice were treated with Budesonide.

* Compared with the model group $p < 0.05$.

** Compared with the model group $p < 0.01$.

3.2. Influence of ACM-M on TGF- β 1 expression in mouse lung tissue

As shown in Figs. 2 and 3, expression of TGF- β 1 mRNA in lung tissue of the asthma model mice (0.720 ± 0.147) increased significantly ($p < 0.01$) compared with that of the normal mice (0.221 ± 0.065), and its expression was depressed by *Astragali-Cordyceps Mixture* (0.614 ± 0.091 ; $p < 0.05$). In consequence, TGF- β 1 protein was found to express in various cells of lung including airway epithelial cells, fibroblasts, smooth muscle cells, vascular endothelial cells as well as the infiltrative inflammatory cells in model mice, while there was a little expression of TGF- β 1 protein in the normal mice. The positive area ratio (19.16 ± 3.18) and optical density (0.29 ± 0.047) of TGF- β 1 were much higher than that of the normal mice (0.016 ± 0.005 and 0.081 ± 0.021 ; $p < 0.01$) indicating its expression increased significantly in lung of the asthma model mice. In the ACM-M group mice, TGF- β 1 protein was only detected in fibroblasts, smooth muscle cells,

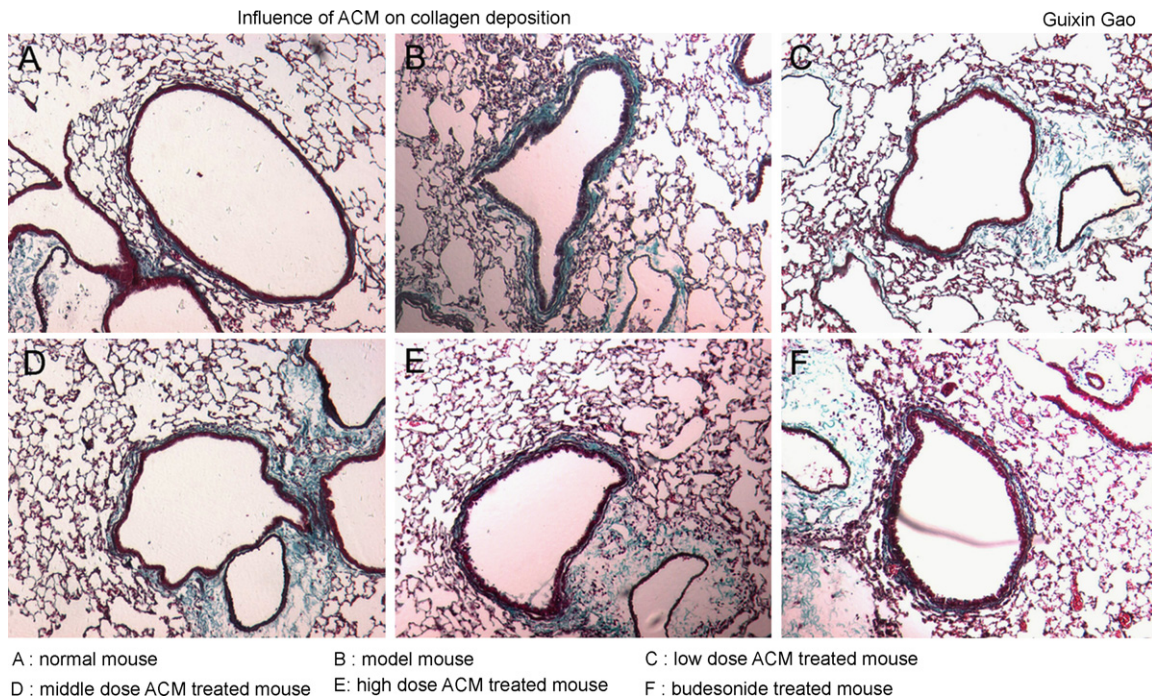
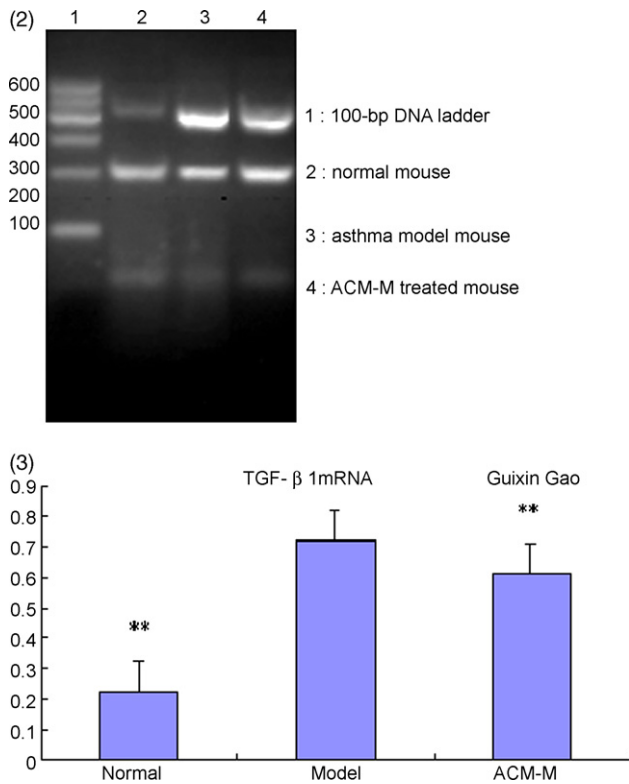


Fig. 1. Influence of ACM on collagen deposition in the asthma airway remodeling. Lung tissue sections obtained from mice on 48 h after last OVA challenge were stained with Masson trichrome stain (100×, Fig. 1). After staining, collagen area on the basal membrane of airway in the lung tissues was analyzed by Leica-Qwin image-processing system using the affix color model. The result was expressed as (collagen area on the basal membrane of airway/the length of the airway epithelial) (Table 3). Data are expressed as means ± SEM, $n = 8$. ** $p < 0.01$ vs. the model group, * $p < 0.05$ vs. the model group.



Figs. 2 and 3. Influence of ACM on TGF-β1 mRNA expression in mouse lung tissue. A reverse transcription polymerase chain reaction (RT-PCR) method was employed to determine mRNA expression of TGF-β1 in the lung tissues. Expression of TGF-β1 mRNA in each sample was expressed as a ratio of TGF-β1 gray value to β-actin ones (Figs. 2 and 3). Data are expressed as means $\pm$ SEM, $n=8$. ** $p<0.01$ vs. the model group. Normal: mice were sensitized and challenged with saline; Model: mice were sensitized and challenged with OVA; ACM-M: mice were treated with ACM in middle dose. (1) 100-bp DNA ladder. (2) Mice were sensitized and challenged with saline. (3) Mice were sensitized and challenged with OVA. (4) Mice were treated with ACM in middle dose.

vascular endothelial cells and a few inflammatory cells with a significantly decreased positive area ratio (8.31 ± 1.93) and optical density (0.17 ± 0.03) of TGF-β1 compared with the asthma model mice, but failed to be detected in airway epithelial cells (Figs. 4 and 5).

3.3. Influence of ACM-M on Smad3 and Smad7 expression in mouse lung tissue

Smad3 protein was hardly detected in lung tissue of the normal mice. In contrast, its expression increased significantly in the asthma model mice, Smad3 was found to express mainly in airway epithelial cells, fibroblasts, and vascular endothelial cells with a much higher positive area ratio (10.05 ± 1.14) and optical density (0.28 ± 0.021) than that of the normal mice (0.004 ± 0.0008 and 0.011 ± 0.003). The similar expression pattern of Smad3 was found in the ACM-M group mice, and its positive area ratio (9.498 ± 0.924) and optical density (0.264 ± 0.031) showed no statistics difference from that of the asthma model mice suggesting *Astragali-Cordyceps Mixtura* had little effect on Smad3 expression (Figs. 6 and 7).

Smad7 expressed mainly in epithelial cells and fibroblasts, and less in vascular endothelial cells in the normal mice. In the asthma model mice, the expression of Smad7 was only occasionally detected in epithelial cells with a significantly lower positive area ratio (0.0128 ± 0.002) and optical density

(0.09 ± 0.013) compared with the normal mice (10.40 ± 1.75 and 0.38 ± 0.027 ; $p<0.01$) indicating the expression of Smad7 was depressed greatly. Expression of Smad7 protein was found to increase obviously in the ACM-M group mice with a much higher positive area ratio (5.678 ± 1.07) and optical density (0.24 ± 0.038) compared with the asthma mice ($p<0.01$) suggesting that *Astragali-Cordyceps Mixtura* recovered the expression of Smad7 protein (Figs. 8 and 9).

3.4. Correlation analysis

The correlation analysis showed that collagen deposition had a notable positive correlation with mRNA expression ($r=0.758$, $p<0.01$), positive area ratio ($r=0.802$, $p<0.01$), and optical density ($r=0.793$, $p<0.01$) of TGF-β1, as well as positive area ratio ($r=0.797$, $p<0.01$), and optical density ($r=0.775$, $p<0.01$) of Smad3. However, collagen deposition showed a notable negative correlation with positive area ratio ($r=0.814$, $p<0.01$), and optical density ($r=0.846$, $p<0.01$) of Smad7.

4. Discussion

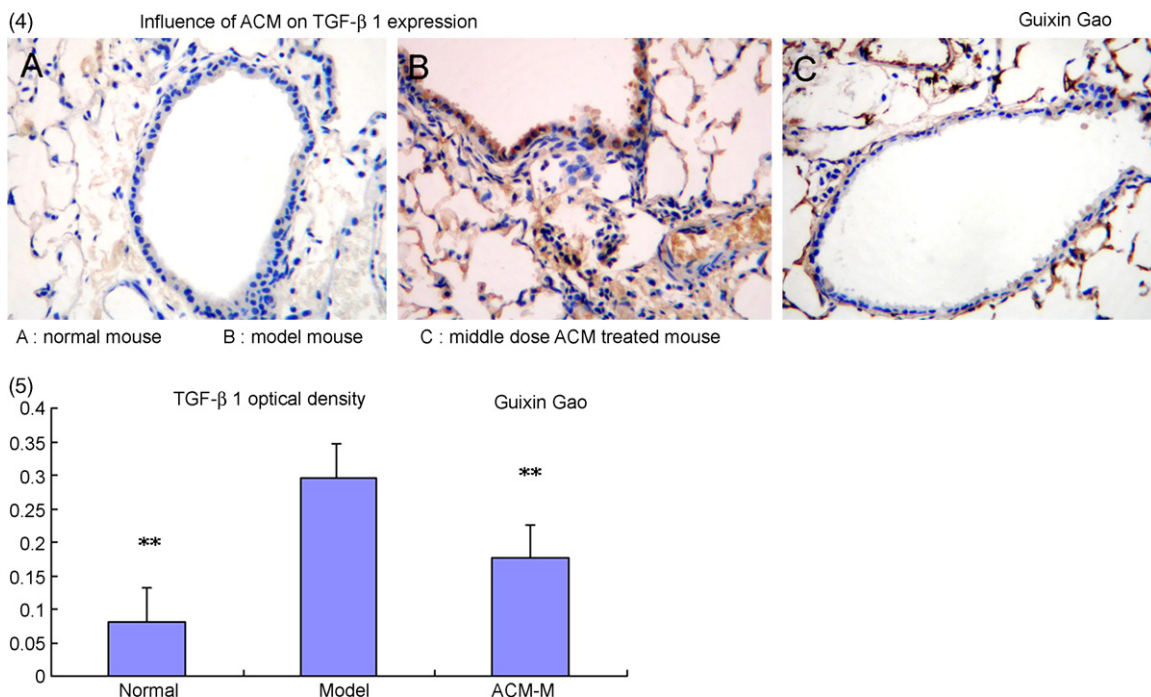
According to previous study, airway remodeling was induced after long-term repeated antigen stimulation in sensitized mice. Our data show that the collagen deposition strikingly reduced after either of three different doses ACM treatment. Although the curative effect of ACM-M and ACM-H is better than the ACM-L, there is no difference between ACM-M and ACM-H. Secondly, the curative effect of ACM does not infinitely increase with its doses. Therefore, it is important to figure out the optimal dose of ACM. According to our result, the middle dose ACM has the best curative effect and the middle dose was the best curative dose. The study will discuss the effect and mechanism of the middle dose of ACM.

By now, TGF-β/Smad signal pathway is found to be an important mechanism to induce asthma airway remodeling. Thus, blocking of TGF-β1 intracellular signal transduction to repress collagen synthesis would be very helpful for the treatment of asthma airway remodeling.

Cytokines play key roles in the airway remodeling caused by repair following injury, among which transforming growth factor β1 (TGF-β1) is thought as the major regulatory factor of asthma airway remodeling because it shows the strongest stimulation on extracellular matrix (ECM) accumulation. TGF-β1 is one of the structure related growth factor family expressed in various lung cells such as alveolar macrophage, fiber archeocyte, endothelial cells, smooth muscle cells etc. TGF-β1 has been shown to make effects in airway epithelium fibrosis as follow (Ward et al., 2003; Duvernelle et al., 2003; Charkir et al., 2003). Therefore, inhibiting production of TGF-β1 or blocking its bioactivity to depress collagen formation in airway is considered as one of the potential ways for the treatment of airway remodeling.

In this study, expression of TGF-β1 in the mucous membrane of normal airway was detected at a rather low level, which is coincident with the previous reports (Vignola et al., 1997; Hoshino et al., 1998). These results demonstrated that TGF-β1 is an important factor involved in growth regulation of normal cells.

Minshall et al. (1997) reported that TGF-β1 mRNA and its immunological activity beneath the mucosa of asthma patients were obviously higher than the controls, and the same was found in epithelium fibrosis. Both of which were directly correlated with severity of asthma detected by hybridization in situ and immunohistochemistry. Redington et al. (1997) and Luttmann et al. (1998)



Figs. 4 and 5. Influence of ACM on TGF- β 1 expression in mouse lung tissue. Immunohistochemistry (IHC) was employed to determine protein expression of TGF- β 1 in the lung tissues. Using the image analysis system, the optical density value was measured under 200 $\times$ objective lens of each slice for quantitative analysis of TGF- β 1 protein expression (Figs. 4 and 5). Data are expressed as means $\pm$ SEM, $n=8$. ** $p < 0.01$ vs. the model group. Normal: mice were sensitized and challenged with saline. Model: mice were sensitized and challenged with OVA. ACM-M: mice were treated with ACM in middle dose.

also detected the higher expression of TGF- β 1 in alveolar washing and sputum of asthma patients, respectively. It suggested that TGF- β 1 play an important role in asthma airway remodeling. In our study, TGF- β 1 expression was shown to be significantly higher than that of the normal group, with a direct correlation of collagen deposit. By treated with ACM, as the TGF- β 1 decreased, the deposit of collagen was reduced as well. Collectively, our data suggest that the ACM could lessen the collagen deposition by inhibiting TGF- β 1 expression.

Smad proteins are important members of TGF- β signal transducing system discovered recently. Smad proteins are the downstream signal regulatory proteins of TGF- β receptor complex, and participate in regulations of cell proliferation, transformation, synthesis, secretion and apoptosis, which directly transport signals from cell membrane to nucleus, and mediate TGF- β intracellular signal transduction. They are divided into receptor regulation Smad protein (R-Smad), common regulation Smad (Co-Smad) and inhibitory Smad protein (I-Smad). It was demonstrated that Smad3 of R-Smad and Smad7 of I-Smad are involved in TGF- β signal transduction, playing an important role in asthma pathogenesis especially in process of airway remodeling.

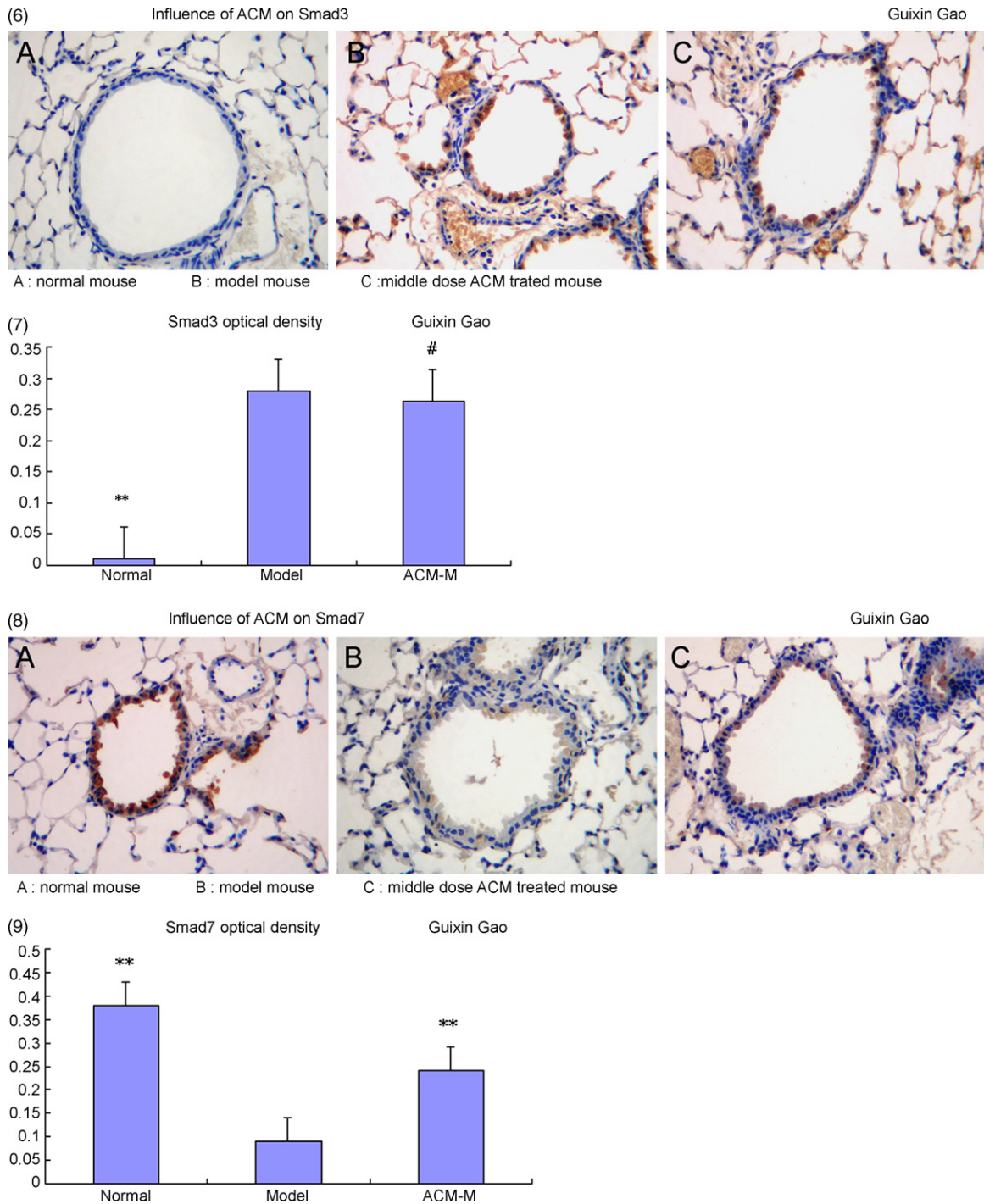
In our study, Smad7 expressed mainly in endothelial cells, fibroblasts and some vascular endothelial cells in the normal mice, was found to decrease significantly in the asthma airway remodeling mice, which coincide with previous reports (Nakao et al., 2002). Moreover, the increased expression of TGF- β 1 in the asthma model mice suggested that the deficient expression of Smad7 weakened its inhibitory feedback effect. In addition, the negative correlation of Smad7 with collagen deposition provided a clue that TGF- β 1 might take part in asthma airway remodeling through the Smad7 signal transducing pathway. In contrast, Smad3, which hardly expressed in the normal mice, was shown to

increase significantly in parallel with TGF- β 1 expression level in the asthma airway remodeling (Rosendahl et al., 2001) suggesting Smad3 have a synergistic effect with TGF- β 1 in airway remodeling. Meanwhile, expression of Smad3 is positive correlation with collagen deposition suggesting that the Smad3 signal pathway might be an important way to participate in asthma airway remodeling.

Meanwhile, the expression of Smad7 was obviously upregulated in ACM group and was negatively correlated with collagen deposition; and there were no differences between ACM group and the model group in the expression of Smad3. It suggested that ACM could reduce collagen deposition by increasing expression of Smad7 rather decreasing expression of Smad3 to block TGF- β signal conduction.

In traditional Chinese medical theory, asthma is considered deficient in the root and excess in the tip. Insufficiency of both lung and kidney is the root. So it is an ultimate cure to nourish lung and kidney to relieve wheezing. ACM, a Chinese traditional medicine, composed of astragali and Chinese caterpillar fungus, is developed based on the Chinese traditional medicine theory and clinic experience. One function of this drug is to benefit lung and kidney. ACM has been proved to be a novel promising approach for the treatment of asthma, which can efficiently relieve symptoms and reduce the frequency of asthma attacks. In clinical practice, no side effects are observed in patients with long period administration of ACM.

In this study, the ACM could lessened collagen deposition efficiently by inducing Smad7 expression and blocking TGF- β 1 pathway to control the development of asthma airway remodeling. This explores an alternative way to prevent and control the irreversible injury of lung function, and to improve asthma turnover, and the asthma patients' quality of life.



Figs. 6–9. Influence of ACM on Smad3 and Smad7 expression in mouse lung tissue. Immunohistochemistry (IHC) was employed to determine protein expression of Smad3 and Smad7 in the lung tissues. Using the image analysis system, the optical density value was measured under 200 $\times$ objective lens of each slice for quantitative analysis of Smad3 (Figs. 6 and 7) and Smad7 (Figs. 8 and 9) protein expression. Data are expressed as means $\pm$ SEM, $n=8$. ** $p<0.01$ vs. the model group; * $p<0.05$ vs. the model group. Normal: mice were sensitized and challenged with saline. Model: mice were sensitized and challenged with OVA. ACM-M: mice were treated with ACM in middle dose.

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