

# Effects of arsenic trioxide (As<sub>2</sub>O<sub>3</sub>) on airway remodeling in a murine model of bronchial asthma

Zi-ping Xu, Jian-min Huo, Yu-lan Sang, Jian Kang, and Xue Li

**Abstract:** We investigated the effects of arsenic trioxide (As<sub>2</sub>O<sub>3</sub>) as a possible approach for preventing airway remodeling in a murine model of bronchial asthma induced by ovalbumin (OVA) challenge. Forty Balb/c mice were randomly assigned to 1 of 4 groups (10 mice/group) as follows: controls (challenged with sterile saline inhalation only); OVA-challenged, no treatment; OVA-challenged, treated with dexamethasone; and OVA-challenged, treated with As<sub>2</sub>O<sub>3</sub>. All mice were sensitized by intraperitoneal injection with 10% OVA at 2 weeks prior to saline or OVA inhalation challenge. Challenges were for 8 weeks. After OVA challenge, typical asthma-like morphology changes in the bronchi and lung tissues were observed by hematoxylin–eosin staining and pulmonary function indices were reduced compared with controls. Changes in pulmonary indices and lung tissues were similar in the dexamethasone and As<sub>2</sub>O<sub>3</sub> groups and were in between those of the untreated and control groups. Compared with the untreated group, transforming growth factor  $\beta$ 1, vascular endothelial growth factor, and matrix metalloproteinase-9 protein levels and mRNA expression were decreased in lung tissues of the dexamethasone and As<sub>2</sub>O<sub>3</sub> groups. Our results suggest that steroids and As<sub>2</sub>O<sub>3</sub> can inhibit airway remodeling in chronic asthma by mechanisms related to inhibiting the expression of the 3 aforementioned mediators.

**Key words:** chronic asthma, airway remodeling, arsenic trioxide, inflammation, steroid-resistant asthma.

**Résumé :** Nous avons examiné les effets du trioxyde d'arsenic (As<sub>2</sub>O<sub>3</sub>) comme approche pour prévenir le remodelage des voies respiratoires dans un modèle d'asthme bronchique induit par une provocation à l'ovalbumine (OVA) chez la souris. Quarante souris Balb/c ont été réparties au hasard dans l'un des quatre groupes (10 souris/groupe) : contrôle (provoqué par une inhalation à la saline seulement) ; provocation à l'OVA, sans traitement ; provocation à l'OVA, traité à la dexaméthasone ; et provoqué à l'OVA, traité à l'As<sub>2</sub>O<sub>3</sub>. Toutes les souris ont été sensibilisées par une injection intrapéritonéale d'OVA 10 %, deux semaines avant la provocation par inhalation de saline ou d'OVA. Les provocations ont duré 8 semaines. À la suite de la provocation à l'OVA, des changements morphologiques des bronches et des tissus pulmonaires typiques de l'asthme étaient observés par une coloration à l'HE, et les indices de la fonction pulmonaire étaient réduits comparativement aux contrôles. Les changements dans les indices de la fonction pulmonaire et les tissus bronchiques étaient similaires dans les groupes traités à la dexaméthasone ou à l'As<sub>2</sub>O<sub>3</sub>, et se situaient à mi-chemin entre les groupes sans traitement et contrôle. Le niveau des facteurs TGF- $\beta$ 1 et VEGF et de la métalloprotéase MMP-9, ainsi que l'expression de leur ARNm étaient diminués dans les tissus pulmonaires des groupes traités à la dexaméthasone ou à l'As<sub>2</sub>O<sub>3</sub> comparativement au groupe sans traitement. Nos résultats suggèrent que les stéroïdes et l'As<sub>2</sub>O<sub>3</sub> peuvent inhiber le remodelage des voies respiratoires dans l'asthme par l'intermédiaire de mécanismes reliés à l'expression des 3 médiateurs sus mentionnés.

**Mots-clés :** asthme chronique, remodelage des voies respiratoires, trioxyde d'arsenic, inflammation, asthme résistant aux stéroïdes.

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## Introduction

Bronchial asthma is a chronic inflammatory state of the airways characterized by airway hyperreactivity, reversible air flow obstruction, and airway remodeling. Airway remodeling

causes basement membrane and smooth muscle thickening that interferes with lung function and makes asthma difficult to manage clinically (Barnes 2003; Mauad et al. 2007). Airway remodeling is associated with severe asthma (Chetta et al. 1997; Benayoun et al. 2003) and is thought to be a significant

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pathological consequence of asthma (Halwani et al. 2010). Thus, prevention and reversal of airway remodeling is of great clinical significance. However, it is unclear whether airway remodeling can truly be prevented or reversed, what the exact relationship is between airway remodeling and airway responsiveness, and what is the most effective clinical treatment of remodeling.

The Global Initiative for Asthma (GINA) regimen, including inhaled glucocorticosteroids for the treatment of asthma, has achieved good efficacy in more than 90% of patients (Bateman et al. 2008). Glucocorticoids suppress the chronic inflammatory state underlying asthma. However, the use of inhaled glucocorticosteroids for the control of asthma symptoms may lead to local and systemic adverse reactions (Lipworth 1999). In addition, about 0.1%–1.0% of asthma patients are resistant to steroids, so that they cannot use inhaled glucocorticosteroids to control their asthma symptoms (Barnes 2004). Therefore, it is necessary to search for additional ways to control the airway remodeling and changes in respiratory function that occur in asthma.

The use of arsenic for treating diseases, including asthma, dates back to 200 BC according to the first book of Chinese traditional medicine, *Shen Nong Cao Jing* (Liu et al. 2008). Its most recent clinical use has been to treat refractory acute promyelocytic leukemia (Platanias 2009). In recent years, a small number of animal studies have been undertaken on the effects of arsenic trioxide ( $As_2O_3$ ) in the treatment of asthma. Zhou et al. (2006), in an ovalbumin (OVA)-sensitized murine model of bronchial asthma, showed that  $As_2O_3$  decreased OVA-specific IgE production and ameliorated allergen-induced airway hyperresponsiveness and inflammation. Chu et al. (2010) also used OVA-sensitized mice and found in their study that  $As_2O_3$  alleviated airway hyperresponsiveness and asthma-associated eosinophilia. While both studies found that  $As_2O_3$  could decrease airway hyperresponsiveness in an animal model of acute allergic asthma, there have been no studies of the effects of  $As_2O_3$  on airway remodeling, and no studies of its effects in chronic, rather than acute, asthma.

In the current exploratory study, we sought to determine whether  $As_2O_3$  treatment could decrease airway remodeling in chronic asthma. For this study, we used a mouse model of OVA-induced chronic bronchial asthma, and compared lung tissue morphological changes and pulmonary function indices with and without  $As_2O_3$  treatment with those seen using treatment with dexamethasone (DXM): a glucocorticoid previously shown to have some efficacy for ameliorating structural changes associated with airway remodeling (Hoshino et al. 2001). We also evaluated the protein and mRNA expression in lung tissues of 3 substances previously shown to be involved in airway remodeling, transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) (Minshall et al. 1997), vascular endothelial growth factor (VEGF) (Lee et al. 2004), and matrix metalloproteinase-9 (MMP-9) (Kelly et al. 2000; Mattos et al. 2002). Our results show that  $As_2O_3$  had effects on airway remodeling, airway hyperresponsiveness, and the 3 mediators examined that were almost identical to those seen with DXM.

## Materials and methods

### Mice, drugs, reagents, and equipment

Forty 8-week-old healthy female BALB/c mice (mean body mass of  $18 \pm 2$  g) were provided by the Beijing Vital River

Experimental Animal Technology, Ltd. Mice were maintained in a barrier facility. Our experiments were approved and supervised by our institutional animal care and use committee.

The injectable  $As_2O_3$  solution was from Harbin Yida Pharmaceutical Co., Ltd. (Harbin, China). The injectable DXM solution was from Xinzheng Corporation (Tianjin Pharmaceutical Group Co., Ltd., Xinzheng, China). Egg OVA (grade V) was from Sigma (St. Louis, Missouri, USA) and was packaged in China. Methacholine (mACh) was from Beijing Bestlab Hi-Tech Co., Ltd. (Beijing, China). MMP-9 and VEGF rabbit polyclonal sera were purchased from Wuhan Boster Biological Engineering Co., Ltd. (Wuhan, China). TGF- $\beta$ 1 rabbit polyclonal serum was purchased from Santa Cruz Biotechnology Co. (Santa Cruz, California, USA), and TRIzol reagent was from Invitrogen Corporation (Carlsbad, Calif.). The primers for MMP-9, VEGF, and TGF- $\beta$ 1 were synthesized by Invitrogen, Shanghai (China) branch.

A plexiglas inhalation box (30 cm  $\times$  30 cm  $\times$  20 cm) was manufactured by us. A PARI BOY N Type 085 jet pump for inhalation was from the PARI China Company (Beijing, China). An AniRes 2005 small animal lung function system included an RES1030 signal conditioner, an AniRes animal lung function analysis system, and an RES3020 animal ventilator (Beijing Bestlab Hi-Tech Co., Ltd.).

## Experimental methods

### Protocol for induction and treatment of bronchial asthma

Mice were randomly divided into 4 OVA-sensitized groups (10 mice/group), 1 group without, and 3 groups with an additional OVA inhalation challenge, as follows: controls (no OVA challenge); OVA-challenged controls (no further treatment); OVA-challenged + DXM; and OVA-challenged +  $As_2O_3$ . All animals had free access to water and food. Prior to the inhalation challenges, all mice were sensitized to OVA by 1 intraperitoneal (i.p.) injection administered in the following manner: a sterile solution of 1  $\mu$ g of OVA and 2 mg of  $Al(OH)_3$  (alum; used as an adjuvant) per millilitre of saline was prepared, after which 1 mL of this solution was injected i.p. We began inhalation challenges 2 weeks later. The mice were placed in an inhalation box and challenged with inhalation of either nebulized saline (control) or nebulized OVA (OVA-challenged groups) (Tsuchiya et al. 2003). For the OVA challenge, 1% OVA solution was used (1 mg of OVA dissolved in 100 mL of distilled water). The nebulized inhalation procedure was performed for 30 min 2 times per week for 8 weeks. Body masses were measured before each inhalation. Mice in the DXM treatment group received i.p. injections (2 mg DXM/kg body mass), as did the mice in the  $As_2O_3$  treatment group (4 mg  $As_2O_3$ /kg), before each nebulized inhalation. The concentration of DXM was based on previous studies with mice (Pitchford et al. 2004). The concentration of  $As_2O_3$  was also based on previous studies with mice (Zhou et al. 2003, 2004).

### Pulmonary function testing

To test pulmonary function, a small animal whole body plethysmographic lung function system was used that included an RES1030 signal conditioner, an AniRes animal lung function analysis system, and an RES3020 animal ventilator. Pulmonary function was measured at the end of the study, before the mice were sacrificed, in the following manner.

After anesthetization with an i.p. injection of 0.4% pentobarbital solution (~40 mg/kg body mass), tracheal intubation was performed, mice were placed in the body scan box, and the parameters of the animal ventilator were set at a tidal volume of 10 mL/kg, an inspiratory time (Ti)/expiratory time (Te) ratio of 15:10, and a respiratory rate of 90 breaths/min. mACh was diluted with phosphate buffer (0.01 mol/L PBS, pH 7.4) and 0.1 mL of a mACh solution (increasing from 0.00625 to 0.05 mg/kg concentration) was injected through the external jugular vein at intervals of approximately 5 min. The next higher dose was given after the airway resistance returned to normal. The waveform and data were recorded and airway resistance was measured.

### Sample collection

After pulmonary function was measured, mice were sacrificed and their lungs removed. Prior to sacrifice, mice were anesthetized with i.p. injections of 0.4% pentobarbital (~40 mg/kg body mass). The actual dose used depended on the mouse's condition while under anesthesia for pulmonary function studies. Mice were sacrificed by bilateral eyeball phlebotomy. The rationale for this approach was to promote rapid death after massive hemorrhage so that blood in the pulmonary circulation could be reduced as much as possible, which would facilitate observation of pathological changes in lung tissue. After sacrifice, the mice were dissected and the trachea and both lungs exposed. The airway was then filled with 1 mL of formaldehyde and the lungs removed. The right lung was harvested, fixed in 10% formalin, and the tissue was embedded in paraffin, after which sections were made and stained with routine hematoxylin–eosin (HE) staining. Tissue from the left lung was stored at  $-70^{\circ}\text{C}$  for later use.

### Airway morphometry

Comparable magnifications and voltages were selected for each tissue slice for 3 microscope fields (located in the upper, middle, and lower areas of each slice). A Motic digital medical image analysis system (Motic Med, 6.0A) was used for image acquisition, and Images Advance 3.0 graphic analysis software was used to measure the internal perimeter of bronchi (Pi; i.e., perimeter of the basement membrane), the total wall area (WAt), the smooth muscle area (WAm), and the number of bronchial smooth muscle cell nuclei ( $N$ ) in the HE-stained specimens. The measured values of WAt, WAm, and  $N$  were standardized using Pi and were recorded as WAt/Pi, WAm/Pi, and  $N$ /Pi.

### Protein expression of MMP-9, VEGF, and TGF- $\beta$ 1

Immunohistochemical staining used the streptavidin–biotin complex (SABC) method suggested by the manufacturers. As a negative control, PBS replaced the primary antibody. The samples were observed using ordinary optical microscopy for the qualitative analysis of protein expression (Wenzel et al. 2003). Five representative visual fields were randomly selected in each section ( $400\times$  magnification) and were categorized into the following 5 grades based on the intensity of staining: no staining (–), light yellow ( $\pm$ ), brown yellow (+), dark yellow ( $++$ ), and dark brown ( $+++$ ). Positive results were indicated by (+) or a higher grade. For semiquantitative analysis of protein expression, 5 visual fields were randomly selected in each stained section ( $400\times$  magnification) and the number of positive cells in each visual field was counted.

Protein expression was recorded as number of positive cells per high power field.

### mRNA expression levels of the MMP-9, VEGF, and TGF- $\beta$ 1 genes

Total RNA from lung tissue was extracted using the TRIzol extraction method. The RNA concentration was determined using GeneQuant. Total RNA was reverse-transcribed into cDNA, which was used as a template for PCR amplification. For *MMP-9*, the upstream primer sequence was 5'-CCTCTGGCATCCTCTTGTG-3'; the product size was 158 bp. The downstream primer sequence was 5'-ACGCTGGTATAAGGTGGTCT-3'; the product size was 146 bp. For *VEGF*, the upstream primer sequence was 5'-CTTCCTACAGCACAGCAGATGTGAA-3' and the downstream primer sequence was 5'-TGGTGACATGGTTAATCGGTCTTTC-3'; the product size was 366 bp. For *TGF- $\beta$ 1*, the upstream primer sequence was 5'-GCCCTGGACACCAACTATTGC-3' and the downstream primer sequence was 5'-GCTGCACTTG-CAGGAGCGCAC-3'; the product size was 335 bp. For internal reference mouse  $\beta$ -actin, the upstream primer sequence was 5'-CCTCTATGCCAACACAGTGC-3' and the downstream primer sequence was 5'-GTACTCCTGCTTGCTGATCC-3'; the product size was 212 bp. The PCR products were electrophoresed, and a UVP gel density scanner was used for a density scan of the electrophoresis bands of the specific amplification products (*MMP-9*, *VEGF*, *TGF- $\beta$ 1*, and  $\beta$ -actin genes). Labwork310 software was used to analyze the densities of the electrophoresis bands. The expression level of *MMP-9* gene mRNA was given as the ratio of its band density to that of the  $\beta$ -actin1 gene band, the expression level of *VEGF* gene mRNA was given as the ratio of its band density to that of the  $\beta$ -actin2 gene band, and the expression level of *TGF- $\beta$ 1* gene mRNA was given as the ratio of its band density to that of the  $\beta$ -actin3 gene band.

### Statistical analysis

Results are given as the mean  $\pm$  SD. Continuous variables, airway morphologic values, and the expression of *MMP-9*, *VEGF*, and *TGF- $\beta$ 1* in lung tissues were compared by one-way ANOVA. When a significant difference was found between groups, multiple comparisons of means were made using the Bonferroni approach with type-I error adjustment. Linear mixed models were used to assess the mACh concentration and group effects on inspiratory resistance, expiratory resistance, and dynamic compliance. All statistical assessments were 2-sided and evaluated at the 0.05 level of significance. Statistical analyses were conducted using SPSS 15.0 software (SPSS, Inc., Chicago, Illinois, USA).

## Results

### Survival

Three of the 40 female Balb/c mice assigned to the test groups died during the OVA sensitization period prior to inhalation challenges: 1 in the control group, 1 in the OVA-challenged untreated group, and 1 in the OVA-challenged DXM-treated group. Unfortunately, we did not do necropsies to determine the causes of death. However, having occurred during the OVA sensitization period, these deaths were not due to either the inhalation challenges or the  $\text{As}_2\text{O}_3$ /DXM treatments.

**Fig. 1.** Pulmonary function indices for female Balb/c mice. As described in the Materials and methods, mice were randomly assigned to 1 of 4 groups as follows: control (without ovalbumin (OVA) challenge;  $N = 9$ ); OVA-challenged, but otherwise untreated ( $N = 9$ ); OVA-challenged and treated with dexamethasone (DXM;  $N = 9$ ); OVA-challenged and treated with arsenic trioxide ( $As_2O_3$ ;  $N = 10$ ). Prior to inhalation challenges, mice were sensitized by an intraperitoneal injection of 1 mL OVA solution (containing 1  $\mu$ g OVA and 2 mg  $Al(OH)_3$ ); 2 weeks later, pulmonary challenges began. Pulmonary function indices were determined after 8 weeks of inhalation challenges with either OVA or sterile saline (control). These indices included (a) inspiratory resistance, (b) expiratory resistance, and (c) dynamic compliance after challenges with different concentrations of methacholine. Results are presented as the mean  $\pm$  SD. The letters a, b, and c indicate that there were statistically significant differences between the groups: “a” compared with “b”; “a” compared with “c”; and “b” compared with “c” (all  $P < 0.001$ ).

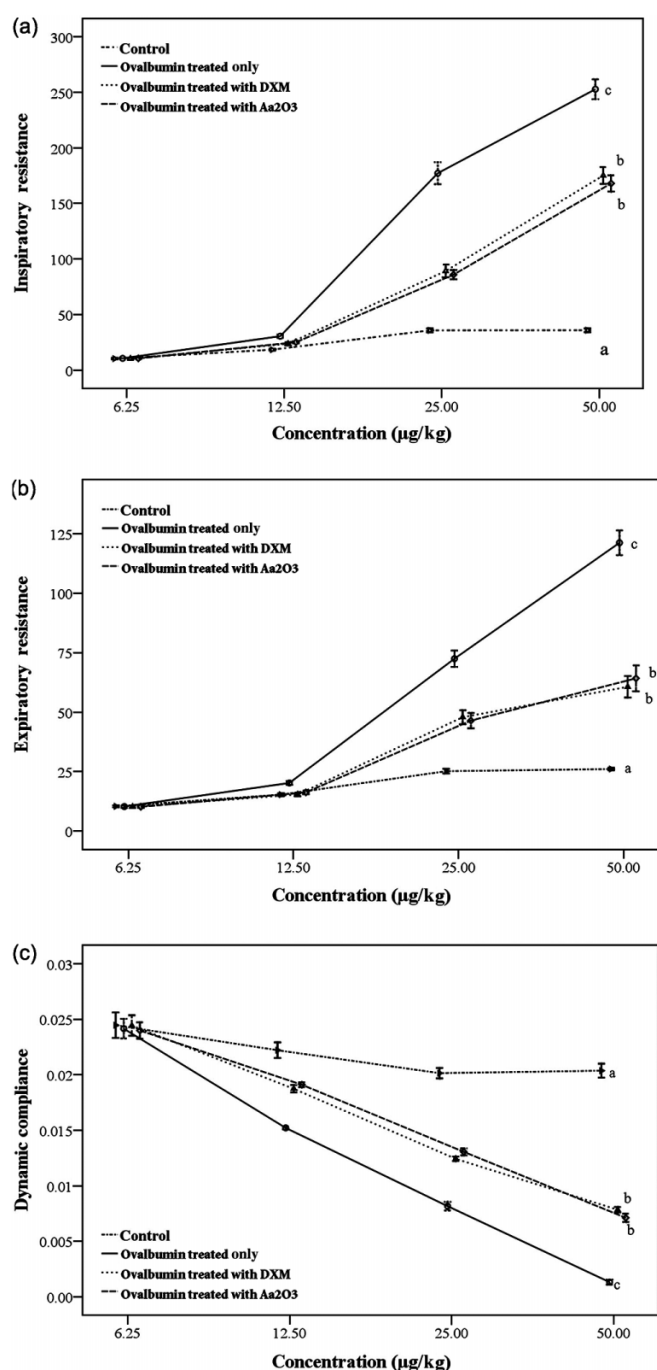
### Changes in pulmonary function

DXM and  $As_2O_3$  treatment reversed the airway hyperreactivity caused by OVA challenge to a similar extent. Figure 1 shows comparisons of inspiratory resistance ( $R_i$ ; Fig. 1a), expiratory resistance ( $R_e$ ; Fig. 1b), and dynamic compliance ( $C_{dyn}$ ; Fig. 1c) among the 4 groups. These results indicate that there were significant mACH concentration and group effects on  $R_i$ ,  $R_e$ , and  $C_{dyn}$  (all  $P < 0.001$ ). At mACH concentrations of 12.5  $\mu$ g/kg and higher, the significantly lowest  $R_i$  and  $R_e$  values (Fig. 1a and 1b) and significantly highest  $C_{dyn}$  values (Fig. 1c) were found in the control group without OVA challenge (all  $P < 0.001$ ), whereas the significantly highest  $R_i$  and  $R_e$  values and significantly lowest  $C_{dyn}$  values were found in the OVA-challenged untreated group (all  $P < 0.001$ ). The  $R_i$ ,  $R_e$ , and  $C_{dyn}$  values for both the OVA-challenged DXM-treated and OVA-challenged  $As_2O_3$ -treated groups were intermediate between these extremes. However, there were no significant differences for  $R_i$  ( $P = 0.196$ ),  $R_e$  ( $P = 1.000$ ), and  $C_{dyn}$  ( $P = 0.082$ ) between the OVA-challenged DXM-treated and OVA-challenged  $As_2O_3$ -treated groups.

### Changes in lung morphology

DXM and  $As_2O_3$  also reversed the airway remodeling caused by OVA challenge to a similar extent. In the control group, the epithelial arrangement at all levels of the bronchi was neat, the alveolar structure was intact, and the bronchial mucosa was smooth and complete (Fig. 2a). In the OVA-challenged untreated group (Fig. 2b), a large amount of inflammatory cell infiltration was seen around the bronchi, there was shedding of the bronchial mucous membrane, the bronchial wall and basement membrane were thickened, the bronchial smooth muscles were hyperplastic and hypertrophic, and the epithelial glands showed hyperplasia and mucous gland metaplasia. In the DXM (Fig. 2c) and  $As_2O_3$  groups (Fig. 2d), pulmonary changes in mice were intermediate between those of the OVA-challenged untreated group and control group. There were no differences between the DXM and  $As_2O_3$  groups (Fig. 2).

Quantitative results of airway morphologic values for the 4 groups of mice are shown (Table 1). As described in the Materials and methods, morphology values were standardized



to the internal perimeter of bronchi ( $P_i$ ), including  $W_{at}/P_i$ ,  $W_{am}/P_i$ , and  $N/P_i$ . There were significant differences in  $W_{at}/P_i$ ,  $W_{am}/P_i$ , and  $N/P_i$  among the control, OVA-challenged without further treatment, OVA-challenged DXM-treated, and OVA-challenged  $As_2O_3$ -treated groups (all  $P < 0.001$ ). The lowest  $W_{at}/P_i$ ,  $W_{am}/P_i$ , and  $N/P_i$  values were found in the control group. Compared with the OVA-challenged untreated group, significantly lower values for  $W_{at}/P_i$ ,  $W_{am}/P_i$ , and  $N/P_i$  were found in the OVA-challenged DXM-treated and OVA-challenged  $As_2O_3$ -treated groups. However, there were no significant differences in  $W_{at}/P_i$  and  $W_{am}/P_i$  between the OVA-challenged DXM-treated and OVA-challenged  $As_2O_3$ -

**Fig. 2.** Lung morphology changes in female Balb/c mice. Hematoxylin–eosin staining of bronchial and lung sections (light microscope 200× magnification) for (a) control mice, (b) ovalbumin (OVA)-challenged untreated mice, (c) OVA-challenged mice treated with dexamethasone (DXM), and (d) OVA-challenged mice treated with arsenic trioxide (As<sub>2</sub>O<sub>3</sub>).

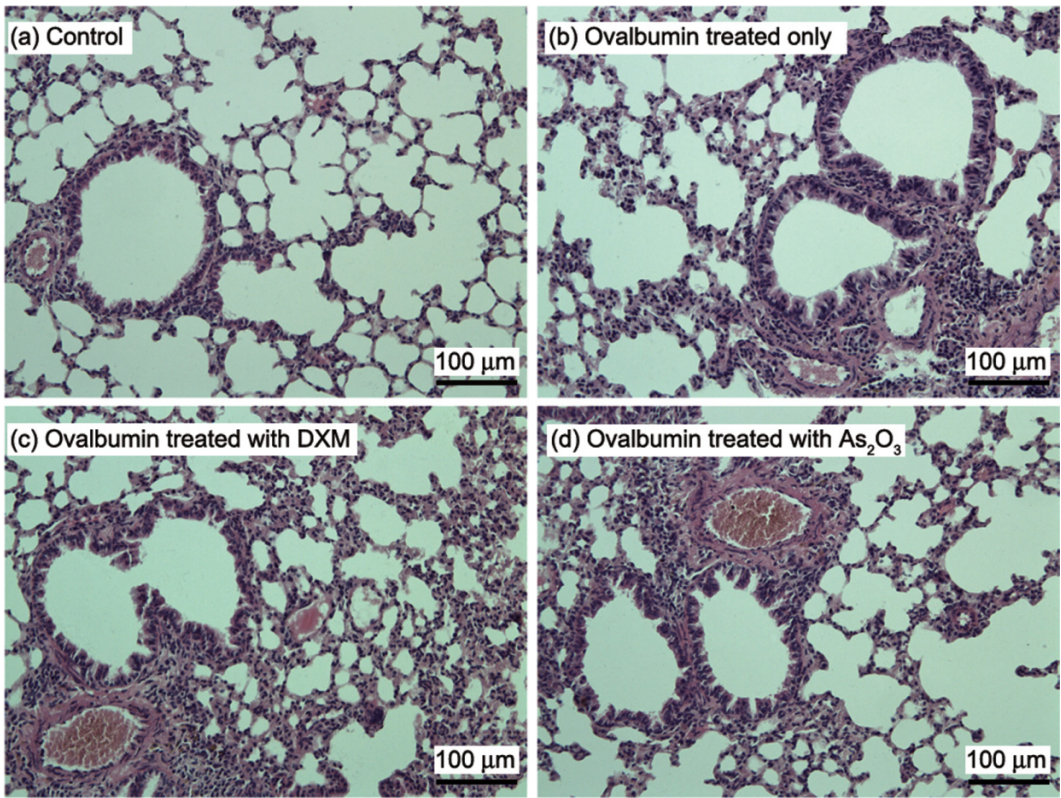


**Table 1.** Airway morphology results for OVA-challenged female Balb/c mice.

|                              | Control ( <i>N</i> = 9) | OVA-challenged without further treatment ( <i>N</i> = 9) | OVA-challenged treated with DXM ( <i>N</i> = 9) | OVA-challenged treated with As <sub>2</sub> O <sub>3</sub> ( <i>N</i> = 10) | <i>P</i> value |
|------------------------------|-------------------------|----------------------------------------------------------|-------------------------------------------------|-----------------------------------------------------------------------------|----------------|
| WAt/Pi (μm <sup>2</sup> /μm) | 35.99±2.62              | 55.93±1.76*                                              | 45.86±1.66*†                                    | 46.52±1.57*†                                                                | <0.001         |
| WAm/Pi (μm <sup>2</sup> /μm) | 1.67±0.15               | 9.19±0.54*                                               | 4.28±0.31*†                                     | 4.34±0.34*†                                                                 | <0.001         |
| <i>N</i> /Pi (no./μm)        | 0.0011±0.0002           | 0.0233±0.0007*                                           | 0.0105±0.0005*†                                 | 0.0110±0.0003*†‡                                                            | <0.001         |

**Note:** OVA, ovalbumin; DXM, dexamethasone; As<sub>2</sub>O<sub>3</sub>, arsenic trioxide; WAt, total wall area; WAm, smooth muscle area; *N*, no. of smooth muscle nuclei; and Pi, internal diameter of bronchi. *P* values were determined using ANOVA. Pair-wise multiple comparisons between groups were made using Bonferroni's test with α = 0.008 adjustment. \*, *P* < 0.001 compared with the control group; †, *P* < 0.001 compared with the OVA-challenged untreated group; and ‡, *P* < 0.001 for the OVA-challenged DXM-treated compared with the OVA-challenged As<sub>2</sub>O<sub>3</sub>-treated groups.

treated groups. However, a small but significant difference was found in *N*/Pi between the DXM-treated and As<sub>2</sub>O<sub>3</sub>-treated groups (0.0105 ± 0.0005 compared with 0.0110 ± 0.0003 no./μm, *P* = 0.001).

**Changes in lung tissue growth factor expression**

DXM and As<sub>2</sub>O<sub>3</sub> reduced, to a similar extent, the increase in MMP-9, VEGF, and TGF-β1 seen in lung tissue of OVA-challenged mice. Figure 3 shows representative photomicrographs of lung tissue sections stained for MMP-9 (Fig. 3a), VEGF (Fig. 3b), and TGF-β1 (Fig. 3c) from the 4 groups of mice. Table 2 summarizes the protein and mRNA expression of MMP-9, VEGF, and TGF-β1 in the lung tissues of these groups. There were significant differences in protein expression of MMP-9, VEGF, and TGF-β1 among the control,

OVA-challenged untreated, OVA-challenged DXM-treated, and OVA-challenged As<sub>2</sub>O<sub>3</sub>-treated groups (all *P* < 0.001). The lowest protein expression of MMP-9, VEGF, and TGF-β1 were found in the control group. Compared with the OVA-challenged untreated group, significantly lower protein expression of MMP-9, VEGF, and TGF-β1 were found in the DXM-treated and As<sub>2</sub>O<sub>3</sub>-treated groups. However, there were no significant differences in the protein expression of MMP-9, VEGF, and TGF-β1 between the DXM-treated and As<sub>2</sub>O<sub>3</sub>-treated groups (*P* > 0.05).

DXM and As<sub>2</sub>O<sub>3</sub> also decreased, to a similar extent, the increase in MMP-9, VEGF, and TGF-β1 mRNA seen in lung tissue from OVA-challenged mice. Comparable with the patterns of protein expression, the lowest expression of mRNA

**Fig. 3.** Changes in expression of matrix metalloproteinase-9 (MMP-9), vascular endothelial growth factor (VEGF), and transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) in the lung tissues of female Balb/c mice. As described in the Materials and Methods, lung tissues from the 4 groups of mice were immunohistochemically stained for the 3 proteins. Representative photomicrographs show staining for (a) MMP-9, (b) VEGF, and (c) TGF- $\beta$ 1 (light microscope 200 $\times$  magnification). DXM, dexamethasone; and As<sub>2</sub>O<sub>3</sub>, arsenic trioxide.

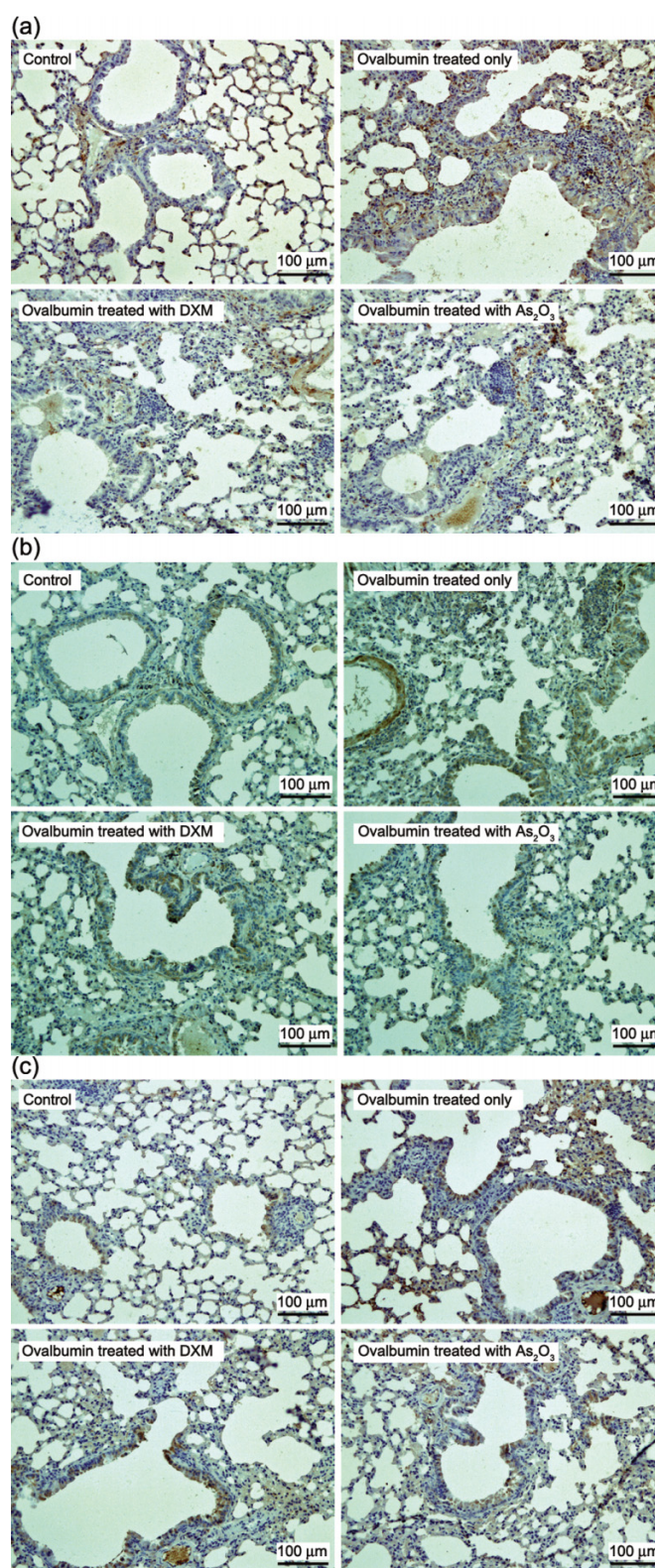
for MMP-9, VEGF, and TGF- $\beta$ 1 were found in the control group, and the highest expression of these mRNAs were found in the OVA-challenged untreated group. However, there were no significant differences in MMP-9, VEGF, and TGF- $\beta$ 1 mRNA expression between the DXM-treated and As<sub>2</sub>O<sub>3</sub>-treated groups ( $P > 0.05$ ).

## Discussion

Airway remodeling, an important pathophysiological change in chronic asthma, is associated with severe asthma (Benayoun et al. 2003; Chetta et al. 1997). Our results showed that DXM and As<sub>2</sub>O<sub>3</sub> had similar inhibitory effects on airway remodeling in a murine model of chronic asthma. Both airway hyperresponsiveness and airway pathological morphology were significantly and similarly lessened in the DXM- and As<sub>2</sub>O<sub>3</sub>-treated groups. In addition, both treatments exhibited similar inhibitory effects on the high expression of MMP-9, VEGF, and TGF- $\beta$ 1 seen in lung tissues of untreated animals. With one exception, we found no significant difference between the DXM- and As<sub>2</sub>O<sub>3</sub>-treated groups in any of their actions. The exception was that the numbers of smooth muscle nuclei (N/Pi) were slightly, but significantly, different between the As<sub>2</sub>O<sub>3</sub> and DXM groups. The extreme similarity between the responses to DXM and As<sub>2</sub>O<sub>3</sub> would seem to suggest that they act through a common downstream pathway. However, DXM and As<sub>2</sub>O<sub>3</sub> have very different initial mechanisms of action. DXM, like all steroids, binds to a cytoplasmic receptor, translocates into the nucleus, binds to DNA, and affects gene transcription. As<sub>2</sub>O<sub>3</sub> is thought to act by binding to -SH residues on a number of cellular enzymes (Platanias 2009; Miller et al. 2002).

Image analysis results suggested that after As<sub>2</sub>O<sub>3</sub> treatment, the total wall area (WAt/Pi), the smooth muscle area (WAm/Pi), and the number of bronchial smooth muscle cell nuclei (N/Pi) in the lung tissue sections of OVA-challenged mice were significantly different from those of untreated OVA-challenged mice. Immunohistochemistry and RT-PCR results also showed that the expression of MMP-9, VEGF, and TGF- $\beta$ 1 were decreased in bronchial smooth muscles, mucous epithelial cells, and alveolar epithelial cells after As<sub>2</sub>O<sub>3</sub> intervention.

Although the morphological and pulmonary function changes that occur with remodeling are well known, the critical molecular components that contribute to airway remodeling remain controversial (Barnes 2003; Mauad et al. 2007). The results of our study showed that both protein and mRNA expression levels of MMP-9, VEGF, and TGF- $\beta$ 1 were significantly higher in the lung tissues of OVA-challenged mice than those in unchallenged controls after 8 weeks of challenge during the period when chronic asthma developed. Increased expression of these factors in mice was



positively associated with airway hyperresponsiveness and airway morphological changes, including smooth muscle hypertrophy. These results demonstrated that MMP-9, VEGF, and TGF- $\beta$ 1 were involved in the development of airway remodeling in a murine model of chronic asthma.

**Table 2.** Expression of MMP-9, VEGF, and TGF- $\beta_1$  in the lung tissues of female Balb/c mice.

| A. Protein expression (%)       |                    |                                                     |                                            |                                                                       |         |
|---------------------------------|--------------------|-----------------------------------------------------|--------------------------------------------|-----------------------------------------------------------------------|---------|
|                                 | Control<br>(N = 9) | OVA-challenged without<br>further treatment (N = 9) | OVA-challenged treated<br>with DXM (N = 9) | OVA-challenged treated<br>with As <sub>2</sub> O <sub>3</sub> (N = 9) | P value |
| <b>MMP-9</b>                    |                    |                                                     |                                            |                                                                       |         |
| Macrophage                      | 4.02 ± 1.18        | 13.09 ± 2.15 <sup>†</sup>                           | 9.31 ± 1.84 <sup>†‡</sup>                  | 9.96 ± 2.43 <sup>†‡</sup>                                             | <0.001  |
| Epithelial cells                | 5.64 ± 0.96        | 21.9 ± 2.64 <sup>†</sup>                            | 8.44 ± 1.95 <sup>†‡</sup>                  | 8.96 ± 2.06 <sup>†‡</sup>                                             | <0.001  |
| <b>VEGF</b>                     |                    |                                                     |                                            |                                                                       |         |
| Smooth muscle cell              | 7.67 ± 1.17        | 34.09 ± 3.27 <sup>†</sup>                           | 14.00 ± 2.44 <sup>†‡</sup>                 | 14.50 ± 1.76 <sup>†‡</sup>                                            | <0.001  |
| Epithelial cells                | 4.62 ± 1.15        | 24.89 ± 2.32 <sup>†</sup>                           | 9.93 ± 2.04 <sup>†‡</sup>                  | 9.30 ± 1.97 <sup>†‡</sup>                                             | <0.001  |
| <b>TGF-<math>\beta_1</math></b> |                    |                                                     |                                            |                                                                       |         |
| Smooth muscle cell              | 2.22 ± 1.06        | 7.49 ± 1.12 <sup>†</sup>                            | 4.00 ± 1.04 <sup>†‡</sup>                  | 4.80 ± 1.07 <sup>†‡</sup>                                             | <0.001  |
| Epithelial cells                | 3.87 ± 1.20        | 13.71 ± 2.57 <sup>†</sup>                           | 7.84 ± 1.09 <sup>†‡</sup>                  | 8.84 ± 1.08 <sup>†‡</sup>                                             | <0.001  |
| B. mRNA expression <sup>a</sup> |                    |                                                     |                                            |                                                                       |         |
|                                 | Control<br>(N = 8) | OVA-challenged without<br>further treatment (N = 8) | OVA-challenged treated<br>with DXM (N = 8) | OVA-challenged treated<br>with As <sub>2</sub> O <sub>3</sub> (N = 8) | P value |
| <i>MMP-9</i>                    | 0.66 ± 0.25        | 2.10 ± 0.28 <sup>†</sup>                            | 1.33 ± 0.16 <sup>†‡</sup>                  | 1.42 ± 0.12 <sup>†‡</sup>                                             | <0.001  |
| <i>VEGF</i>                     | 0.92 ± 0.16        | 5.44 ± 0.50 <sup>†</sup>                            | 2.53 ± 0.28 <sup>†‡</sup>                  | 2.53 ± 0.19 <sup>†‡</sup>                                             | <0.001  |
| <i>TGF-<math>\beta_1</math></i> | 0.89 ± 0.17        | 3.69 ± 0.34 <sup>†</sup>                            | 2.09 ± 0.13 <sup>†‡</sup>                  | 2.15 ± 0.13 <sup>†‡</sup>                                             | <0.001  |

**Note:** OVA, ovalbumin; DXM, dexamethasone; As<sub>2</sub>O<sub>3</sub>, arsenic trioxide; MMP-9, matrix metalloproteinase-9; VEGF, vascular endothelial growth factor; and TGF- $\beta_1$ , transforming growth factor- $\beta_1$ . P-values were determined with ANOVA. Pair-wise multiple comparisons between groups were made using Bonferroni's test with  $\alpha = 0.008$  adjustment. <sup>†</sup>,  $P < 0.001$  compared with the control group; <sup>‡</sup>,  $P < 0.001$  compared with the OVA-challenged untreated group.

<sup>a</sup>The expression level of *MMP-9* gene mRNA is given as the ratio of its band density to that of the  $\beta$ -actin1 gene band, the expression level of *VEGF* gene mRNA is given as the ratio of its band density to that of the  $\beta$ -actin2 gene band, and the expression level of *TGF- $\beta_1$*  gene mRNA is given as the ratio of its band density to that of the  $\beta$ -actin3 gene band.

MMP-9, a type of gelatinase B, participates in the degradation of collagen and plays an important role in airway epithelium repair and remodeling. A previous study (Mattos et al. 2002) reported that in patients with moderate and severe asthma, MMP-9 activity in sputum and bronchoalveolar lavage fluid was significantly higher than that seen in healthy people.

VEGF is a potent growth factor acting on vascular endothelial cells (Lee et al. 2004). Although angiogenesis has rarely been studied in asthma and the mechanisms of angiogenesis remain unclear, studies have shown that in patients with mild, moderate, and severe asthma, vascular density (number of blood vessels per unit area) and vascular area (area of vessels located within unit area) are increased in the airway submucosa and (or) lamina propria and that the degree of these changes parallels the severity of asthma (Hoshino et al. 2001; Wilson and Li 2001).

TGF- $\beta_1$  is an important fibrosis-promoting cytokine. In the airways of asthma patients, smooth muscle cells secrete large amounts of TGF- $\beta_1$ . This secreted TGF- $\beta_1$  may further promote proliferation and hypertrophy of smooth muscle cells and goblet cells, increase collagen and fibronectin synthesis and promote their deposition in the extracellular matrix, resulting in lumen stenosis and irreversible lung function changes. TGF- $\beta_1$  mRNA expression and thickness of the basement membrane are positively associated with the severity of asthma (Minshall et al. 1997). Our results are consistent with the results of others concerning the involvement of the 3 mediators in airway remodeling.

Steroids have previously been reported to inhibit airway remodeling in chronic asthma. For example, Wang and Yang (2007) observed the effects of DXM on airway remodeling in a rat model of asthma. Their results suggested that DXM could reduce collagen deposition in the airway wall and smooth muscle proliferation, thereby inhibiting airway remodeling in asthmatic rats. Filosso et al. (2003) found that inhaled steroids effectively reduced airway inflammation and lung injury, and Kumar et al. (2003) found that DXM could inhibit airway inflammation and remodeling in a murine model of chronic asthma.

The cytokines, inflammatory mediators, and growth factors released after airway epithelial injury play an important role in asthma (Patil and Long 2010). Inhalation of steroids can cause indirect effects on airway remodeling by regulating inflammatory responses. A possible mechanism is that because steroids have antiinflammatory effects, they may prevent epithelial cell injury caused by inflammatory mediators. In addition, inhalation of steroids can downregulate the expression of inflammatory genes in airway epithelial cells.

As<sub>2</sub>O<sub>3</sub> treatment in OVA-sensitized murine models has been shown, like steroid treatment, to abrogate the airway hyperresponsiveness, inflammation, and eosinophilia associated with asthma (Zhou et al. 2006; Chu et al. 2010). Because of the involvement of MMP-9, VEGF, and TGF- $\beta_1$  in airway remodeling and the similarity in the actions of DXM and As<sub>2</sub>O<sub>3</sub> on airway hyperresponsiveness and inflammation, we hypothesized that the inhibitory effects of DXM or As<sub>2</sub>O<sub>3</sub> on airway remodeling were likely related to inhibiting MMP-9, VEGF, and TGF- $\beta_1$  expression.

Whether or not DXM and As<sub>2</sub>O<sub>3</sub> share a common downstream mechanism in lessening the airway changes seen in asthma is not known and will require further investigation. One mediator that might be investigated as a cause of the similarity between DXM and As<sub>2</sub>O<sub>3</sub> is TGF-β1, because both treatments decrease TGF-β1, and an increase in this central mediator of tissue fibrosis and remodeling can increase levels of the other 2 mediators studied (Boxall et al. 2006).

One limitation of our study is that we did not assess possible effects of As<sub>2</sub>O<sub>3</sub> on immune system function. In a biodistribution study of As<sub>2</sub>O<sub>3</sub> in mice, Burchiel et al. (2009) concluded that As<sub>2</sub>O<sub>3</sub> significantly suppressed T cell-mediated humoral (antibody formation) responses. Although the mediators examined in this study probably play no role in humoral immune responses, future studies must take into consideration possible effects on immune system function.

In addition, we did not assess possible toxic effects of As<sub>2</sub>O<sub>3</sub>, a compound known to be highly toxic. Its toxicity is very high when administered either orally or via injection and thus, its clinical application is greatly limited. The toxic dose for an adult human is 10 mg and the lethal dose is 100 mg. In our study, the dose of 4 mg/kg for mice was selected based on reference to the literature (Zhou et al. 2003; Zhou et al. 2004). However, if an equivalent dose was administered to adult humans, it would far exceed the lethal dose.

One goal of future work is to explore the minimum effective dose and the maximum safe dose. Possible long-term toxic effects of sublethal doses, such as on pulmonary tissues, must also be taken into account. However, another goal would be to look for a common pathway explaining the similar effects of DXM and As<sub>2</sub>O<sub>3</sub>. If such a common downstream pathway is found, drugs might be developed to act on this pathway and in this way avoid the toxicities of both steroids and As<sub>2</sub>O<sub>3</sub>.

## Conflict of Interest

The authors declare that there are no conflicts of interest associated with this work.

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