Original Article

Suppressive effects of the expectorant drug ambroxol hydrochloride on quartz-induced lung inflammation in F344 rats

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Abstract: Surfactant proteins (SPs) are essential to respiratory structure and function. The expectorant drug ambroxol hydrochloride is clinically prescribed to stimulate pulmonary surfactant and airway serous secretion. Therefore, ambroxol hydrochloride may affect SP production and pulmonary inflammation. Lung toxicity of fine particles of various materials has been examined previously in our *in vivo* bioassay using the intratracheal (i.t.) instillation approach. In the present study, we evaluated modulatory effects of ambroxol hydrochloride on quartz-induced lung inflammation in F344 rats. Male 6-week-old F344 rats were exposed by i.t. instillation to 2 mg of quartz particles suspended in 0.2 mL of saline. Ambroxol hydrochloride was administered at 0, 12, and 120 ppm in rat basal diet for 28 days, and then formalin-fixed paraffin-embedded lung, liver, and kidney samples were prepared. No changes in general condition, body and organ weights, or food consumption upon exposure to quartz were noted. The mean ambroxol intake in rats of the 12 ppm group was comparable to the human conventional dose. Histopathology of lung lesions was evaluated, and the degree of inflammation was scored. At 120 ppm, ambroxol hydrochloride significantly decreased individual lung inflammation scores for pulmonary edema and lymph follicle proliferation around the bronchiole, as well as the total inflammation score, in quartz-treated rats. Expression of SP-C in the type II alveolar cells and macrophages was greater in inflammatory lesions than in non-inflamed areas. Ambroxol treatment did not affect expression of SP-B and SP-C. In conclusion, we demonstrated that ambroxol hydrochloride relieves quartz-induced lung inflammation. (DOI: 10.1293/tox.2016-0050; J Toxicol Pathol 2017; 30: 153–159)

Key words: quartz, rat, lung inflammation, surfactant protein, ambroxol hydrochloride, instillation

Introduction

Pulmonary surfactant proteins play a key role in modulating the interfacial properties of surfactant phospholipids, leading to a complex and dynamic cycle of material at the air-liquid interface of the alveoli[1]. Of the 4 subtypes of surfactant proteins (SPs), SP-A and SP-D probably play more important roles in host defense mechanisms, whereas SP-B and SP-C are crucial in lowering surface tension in the lung[1, 2]. SP-A, SP-B, and SP-D are synthesized in the alveolar type II epithelial cells and Clara cells, whereas SP-C expression is restricted to the type II cells[2, 3]. In our previous experiments, SP-B and SP-C were found to be strongly expressed in lung hyperplasias and adenomas, which suggested that expression of these proteins is probably associated with lung tumorigenesis[2].

The expectorant drug ambroxol hydrochloride is clinically prescribed to stimulate pulmonary surfactant and airway serous secretion, to enhance airway ciliary movement, and to facilitate removal of sputum[4, 5]. Some studies have reported that ambroxol treatment regulated SP production and lowered pulmonary inflammation. SP-B protein expression in the whole lung was increased in SD rats that received three administrations of 75 mg/kg ambroxol hydrochloride with a 12-h interval between intraperitoneal injections[6]. It has also been demonstrated that two intravenous injections of 20 mg/kg ambroxol hydrochloride with a 6-h interval stimulated synthesis and secretion of exogenous pulmonary surfactant in the lungs and exerted a protective effect in SD rats against pneumonia induced by *Pseudomonas aeruginosa*[7]. Paraquat-induced lung damage was reduced by a 28-day consecutive administration of ambroxol at a dose of 35 mg/kg[8]. However, there have been no studies that have explored potential effects of ambroxol on instilled particle-induced lung inflammation in F344 rats.

Lung toxicity of fine particles of various materials was examined previously in our *in vivo* bioassay by using an intratracheal (i.t.) instillation approach[9–11]. In human cases, coal miners or building construction workers, who are exposed to quartz dust, demonstrate obstructive and restrictive loss of lung capacity[12] as well as chronic obstructive pulmonary disease[13, 14]. This is associated with inflammatory cell responses characterized by alveolitis with recruitment of inflammatory cells, particularly neutrophils, and may result in pulmonary fibrosis and impaired lung function[15]. Intratracheal instillation of quartz into rats produces inflammatory reactions followed by histological changes characteristic of lung fibrosis[16], similar to pathological changes in humans. At a dose of 2 mg/rat i.t., fine particles of quartz caused severe inflammatory changes in the lung characterized by neutrophil infiltration and edema after 28 days[10].

In the present study, we evaluated modulatory effects of ambroxol hydrochloride on quartz-induced lung inflammation in F344 rats.

Materials and Methods

Chemicals

Ambroxol hydrochloride (CAS23828-92-4) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Quartz particles (DQ-12) smaller than 7 μm in diameter were obtained from Deutsche Montan Technologie GmbH (Germany). Unlike conventional non-crystalline quartz that resembles amorphous silica, DQ-12 has a crystalline form. Distribution of DQ-12 particle sizes is as follows: 10% < 0.6 μm, 50% < 1.1 μm, 90% < 2.3 μm; BET surface area, 9.4 m^2/g; density, 2.6 g/mL[17]. Physiological saline (Otsuka Isotonic Sodium Chloride Solution; Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan) was used as the vehicle for quartz particles.

Animals

Male F344/DuCrlCrj rats (4 weeks old) were purchased from Charles River Laboratories Japan (Atsugi, Japan) and maintained in the Division of Animal Experiments of the Life Science Research Center at Kagawa University according to the institutional animal care guidelines. The protocol of the experiment was approved by the Animal Care and Use Committee of Kagawa University. Rats were housed in polycarbonate cages with white wood chips for bedding under controlled conditions of humidity (60 ± 10%), lighting (12-h light/dark cycle), and temperature (24 ± 2°C) and given free access to drinking water and the basal diet CE-2 (CLEA Japan Inc., Tokyo, Japan).

Experimental design and tissue samples

Twenty male 6-week-old F344/DuCrlCrlj rats were randomly divided into 4 groups of 5 rats each. On Day 0 of the experiment, the rats received a single i.t. instillation of 2 mg quartz suspended in 0.2 mL of saline (Groups 2, 3, and 4) under anesthesia. An anesthetized rat was placed on a table and fixed with three elastic bands hooked under the anterior teeth. Intratracheal instillation was performed by using a specially designed aerosolizer (PennCentury, Philadelphia, PA, USA) after wiping the oral mucosa with a cotton swab. The top of the insufflator was appropriate in shape to allow spray application of particles. Ambroxol hydrochloride was mixed in the basal diet and administered to the rats of Groups 3 and 4 at doses of 12 and 120 ppm, respectively, for 4 weeks. The concentration of ambroxol hydrochloride was set around the human conventional dose of 45 mg/day[5], which corresponds to 12 ppm (for calculations, we used an average human body weight of 50 kg, rat body weight of 200 g, and daily food consumption of 15 g in rats). Lung, liver, and kidney samples were obtained from rats sacrificed on Day 28. At autopsy, the lungs, trachea, and heart were removed and infused through the trachea with 10% phosphate buffered formalin, rinsed in the same fixative, and immersed in the fixative for approximately 48 h. Lung, liver, and kidney tissues were then routinely embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H.E.) for histopathological examinations.

Histopathological analysis

Inflammatory changes in the lungs were histopathologically examined and scored for the following parameters[9, 10, 18]: neutrophil infiltration in the walls and spaces of the alveoli, macrophage infiltration in the alveoli, pulmonary edema, pulmonary fibrosis, granuloma, and lymph follicle proliferation around the bronchioles alveoli. Severity of changes was determined with a point system as follows: 0, no change; 1, weak; 2, moderate; 3, severe. All parameters were summed in each group, and the total values were compared among groups. Immunohistochemical staining processes from deparaffinization to counterstaining with hematoxylin were performed with an automated immunohistochemical stainer (Ventana HX Discovery system; Ventana Medical Systems, Tucson, AZ, USA). Lung tissue sections were heated in CC1 buffer (Ventana Medical Systems, Tucson, AZ, USA) for the standard 60-min antigen retrieval protocol for SP-B and in CC2 buffer (Ventana Medical Systems, Tucson, AZ, USA) for the mild 30-min antigen retrieval protocol for SP-C. Thereafter, the sections were incubated with primary antibodies against SP-B (bs-1034R, rabbit polyclonal, 1:100 dilution, 12-h incubation, Bioss Inc., Woburn, MA, USA) and SP-C (sc-13979, rabbit polyclonal, 1:50 dilution, 1-h incubation, Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Incubation with a biotinylated goat anti-rabbit IgG secondary antibody (Vector Laboratories, Inc., Burlingame, CA, USA) was performed for 30 min. Expression of SP-B and SP-C was evaluated as absent (−), weak (+), moderate (++), or strong (+++) compared with the signal from a histopathologically normal area.

Statistics

Data are expressed as the mean ± standard deviation (S.D.). Body and organ weight data were analyzed by Dunnett's multiple comparison test. Inflammation scores were analyzed by Steel's multiple comparison test. A statistical

Table 1. Final Body and Organ Weights of Rats Treated with Quartz and Ambroxol Hydrochloride

Group	Treatment Quartz	Treatment Ambroxol (ppm)	No. of rats	Final body weight (mean ± S.D., g)	Lung Absolute (g)	Lung Relative (%)	Liver Absolute (g)	Liver Relative (%)	Kidneys Absolute (g)	Kidneys Relative (%)
1	−	0	5	239.3 ± 4.6	1.05 ± 0.09	0.44 ± 0.03	8.68 ± 0.34	3.62 ± 0.09	1.61 ± 0.04	0.67 ± 0.01
2	+	0	5	231.8 ± 7.5	1.19 ± 0.23	0.52 ± 0.11	8.14 ± 0.41	3.51 ± 0.07	1.55 ± 0.05	0.67 ± 0.03
3	+	12	5	228.9 ± 11.3	1.28 ± 0.10	0.56 ± 0.03	8.11 ± 0.38	3.54 ± 0.09	1.55 ± 0.10	0.68 ± 0.02
4	+	120	5	227.4 ± 6.2	1.20 ± 0.10	0.53 ± 0.04	8.38 ± 0.22	3.69 ± 0.07	1.52 ± 0.07	0.67 ± 0.02

Quartz (+): the rats were intratracheally instilled with 2 mg quartz suspended in 0.2 mL of saline. Relative organ weights are indicated as percentage values of the final body weights.

Table 2. Food Consumption and Daily Ambroxol Hydrochloride Intake

Group	Treatment Quartz	Treatment Ambroxol (ppm)	No. of rats	Food consumption (g/day) Week 1	Week 2	Week 3	Week 4	Overall	Ambroxol hydrochloride intake (mg/kg/day)
1	−	0	5	12.1	13.8	13.9	14.2	13.5	0
2	+	0	5	12.2	13.6	13.4	13.0	13.1	0
3	+	12	5	11.9	13.5	13.1	13.1	12.9	0.91
4	+	120	5	12.0	13.2	13.0	13.2	12.8	9.08

Quartz (+): the rats were intratracheally instilled with 2 mg quartz suspended in 0.2 mL of saline.

Table 3. Inflammation Scores in the Lung

Group	Treatment Quartz	Treatment Ambroxol (ppm)	No. of rats	Neutrophil infiltration In the wall	Neutrophil infiltration In the alveolar spaces	Histiocytic macrophage infiltration in the alveoli	Pulmonary edema	Pulmonary fibrosis	Granuloma	Lymph follicle proliferation around the bronchiole	Total score
1	−	0	5	0.0	0.0	0.2 ± 0.4	0.0	0.0	0.0	0.4 ± 0.5	0.6 ± 0.9
2	+	0	5	2.0 ± 0.7	3.0 ± 0.0	3.0 ± 0.0	1.6 ± 0.5	0.8 ± 0.8	1.4 ± 0.5	1.6 ± 0.5	13.4 ± 2.5
3	+	12	5	2.0 ± 0.0	3.0 ± 0.0	3.0 ± 0.0	1.2 ± 0.4	1.0 ± 0.7	1.2 ± 0.4	1.8 ± 0.4	13.2 ± 1.8
4	+	120	5	1.2 ± 0.8	2.0 ± 1.2	2.2 ± 0.8	0.4 ± 0.5*	0.0	0.6 ± 0.5	0.4 ± 0.5*	6.8 ± 3.8*

Quartz (+): the rats were intratracheally instilled with 2 mg quartz suspended in 0.2 mL of saline. Mean ± S.D. Significant differences from Group 2 (Steel test, *$P<0.05$).

analysis system (EXSUS version 8.0, CAC Croit Corporation, Tokyo, Japan) was used. Data were considered to be significantly different if $P<0.05$.

Results

There were no remarkable differences in general condition, body weights, or food consumption between treated and control rats. Final body and organ weights are summarized in Table 1. There were no treatment-related changes in the weight of the lung, liver, or kidney (Table 1). Food consumption and calculated ambroxol hydrochloride intake are summarized in Table 2. Intake of ambroxol hydrochloride was dose dependent. In the present study, the mean ambroxol intake by Group 3 rats at 12 ppm (0.91 mg/kg/day) was proportionally comparable to the conventional dose of 45 mg/day in human subjects (assuming a mean human body weight of 50 kg); so, this means that rats were exposed to a sufficient and translationally relevant amount of ambroxol

hydrochloride. Inflammation scores in the lung are summarized in Table 3. Inflammation scores of pulmonary edema and lymph follicle proliferation around the bronchiole, as well as total scores, were significantly lower in quartz-exposed rats that received 120 ppm ambroxol hydrochloride (Group 4) than in quartz-exposed animals, which were not treated with ambroxol hydrochloride (Group 2) (Fig. 1). Meanwhile, the inflammation scores in the latter group and in rats that received 12 ppm ambroxol hydrochloride (Group 3) were not statistically different. No significant histopathological findings were noted in the liver or kidney. Immunohistochemistry results are summarized in Table 4. SP-B expression was strongly induced (+++) in the bronchiolar epithelial cells, whereas the mucus in the alveoli, type II alveolar cells, and macrophages were all weakly positive (+) (Fig. 2). The level of SP-B expression in the type II alveolar cells was slightly increased in quartz-exposed animals compared with that in control animals. Furthermore, SP-C expression was stronger in the type II alveolar cells (+++)
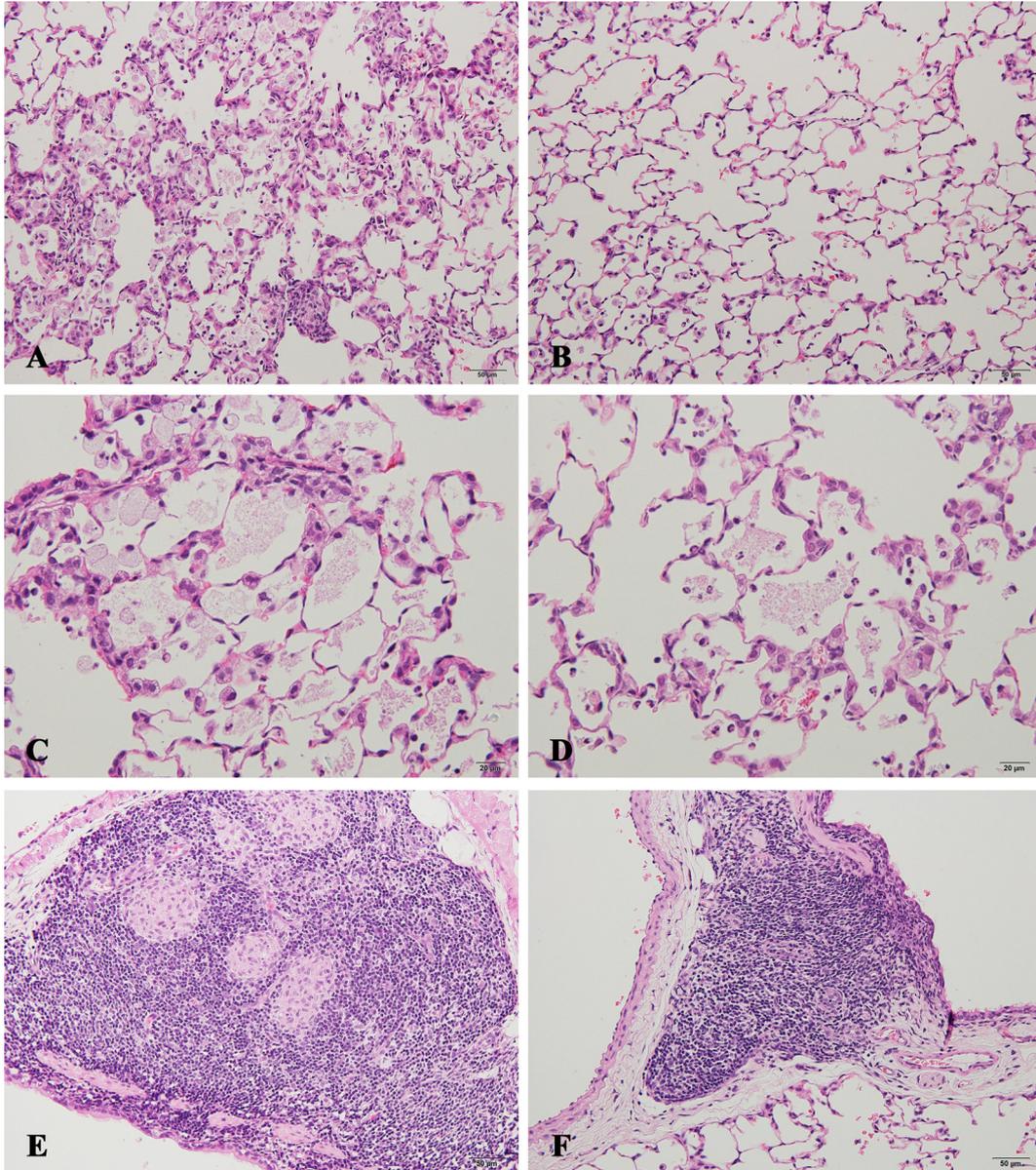
Fig. 1. Histopathological findings in inflammatory lesions induced by quartz in the F344 rat lung (H.E.). A, C, and E, Group 2 (0 ppm ambroxol hydrochloride); B, D, and F, Group 4 (120 ppm ambroxol hydrochloride) (×400). Note the neutrophil and histiocytic macrophage infiltration (A and B), pulmonary edema (C and D), and lymph follicle proliferation around the bronchiole (E and F). Treatment with 120 ppm ambroxol hydrochloride decreased each of these inflammation signs.

and macrophages (++) within inflammatory lesions than in intact control areas (Fig. 3). Ambroxol treatment did not affect expression SP-B and SP-C.

Discussion

In the present study, inflammation induced by i.t. treatment with quartz was significantly decreased by a supraclinical dose of ambroxol. At this dose level, there was no concern about the toxicity of ambroxol treatment, since it has been reported that 28-day repeated oral administration of 500 mg/kg ambroxol hydrochloride did not cause any

toxic effects in rats[19]. It was confirmed immunohistochemically that expression of inducible nitric oxygen synthase (iNOS) and heme oxygenase-1 (HO-1) increases in the lung following quartz i.t. instillation[20, 21]. In the *in vitro* experiments, NR8383 macrophages released reactive oxygen species (ROS), interleukin-1 beta (IL-1β), and tumor necrosis factor alpha (TNFα) due to the exposure to quartz[21]. Thus, lung inflammation induced by quartz was suggested to involve both oxidative stress and the pro-inflammatory signaling pathway involving nuclear factor kappa-B (NF-κB)[22]. An anti-inflammatory effect could be expected by inhibiting generation of reactive oxygen and nitrogen species or

Table 4. Summary of Immunohistochemistry Data Regarding the Expression Levels of Surfactant Protein-B (SP-B) and Surfactant Protein-C (SP-C)

Group	1	2	3	4
Treatment				
Quartz	−	+	+	+
Ambroxol hydrochloride (ppm)	0	0	12	120
No. of animals	5	5	5	5
SP-B				
Inflammatory lesions				
Mucus in the alveoli	NA	+	+	+
Type II alveolar cells	NA	+	+	+
Macrophages	NA	+	+	+
Noninflammatory area				
Type II alveolar cells	−	+	+	+
Bronchiolar epithelial cells	+++	+++	+++	+++
SP-C				
Inflammatory lesions				
Mucus in the alveoli	NA	++	++	++
Type II alveolar cells	NA	+++	+++	+++
Macrophages	NA	++	++	++
Noninflammatory area				
Type II alveolar cells	+	+	+	+
Bronchiolar epithelial cells	−	−	−	−

Quartz (+): the rats were intratracheally instilled with 2 mg quartz suspended in 0.2 mL of saline. NA, not applicable; −, negative; +, weakly positive; ++, moderately positive; +++, strongly positive.

inflammatory mediators released by quartz application. Ambroxol administration has been reported to suppress the lipopolysaccharide-induced production of TNF-α, IL-1β, interleukin-6 (IL-6), superoxide, hydrogen peroxide, and nitric oxide derived from rat macrophages *in vitro*[23]. In rats with paraquat-induced lung fibrosis, the protective effect of ambroxol was histologically prominent and presumably mediated by its free radical scavenging and antioxidant activity[8]. The results of our study of the effects of ambroxol treatment on quartz-induced lung inflammation in rats are consistent with these observations. The anti-inflammatory effect of ambroxol may be also associated with the reduced oxidative stress or inhibition of inflammatory cytokine production. It has been reported that ambroxol reduced adhesion of viscous sputum and enhanced the movement of the cilia in the leporine trachea and tracheal mucosa exposed to sulfur dioxide[24]. Increases in output volume and phosphatidylcholine content in the respiratory tract fluid were also noted in rabbits treated with ambroxol[25]. Thus, if ambroxol treatment stimulates clearance of the airways, it probably promotes draining of quartz particles from the respiratory tract, thereby removing the cause of the inflammatory response. Furthermore, this suggests that internal use of ambroxol hydrochloride may prevent or considerably reduce potential lung damage in workers who are exposed to dust containing quartz or other fine particles.

In the present experiment, we demonstrated that expression levels of SP-B and SP-C were increased in the inflammatory lesions. SP-B and SP-C have physicochemical properties that promote reduction of the surface tension of biological interfaces[3]. SP-B inhibited lipopolysaccharide-induced nitric oxide production by rat alveolar macrophages[26]

and may play an important role against oxidative stress in the lung. A phospholipid preparation containing recombinant SP-C improved oxygenation and reduced formation of hyaline membranes in a rat-lung-lavage model of acute lung injury[27]. Thus, SP-C may contribute to amelioration of pulmonary function. Although a higher dose of ambroxol (75 mg/kg i.p., twice a day) had been shown to increase expression levels of SP-B and SP-C for a short period (36 h)[6], we did not observe significant changes in SP-B and SP-C expression following ambroxol treatment in the present study. This conflict might be referred from differences in the administration procedure. Mild increases in surfactant protein expression as a result of a gentle rise in ambroxol blood concentration with dietary administration were considered undetectable by immunohistochemistry. Thus, the exact mechanisms of suppressive effects of ambroxol administration on lung inflammation induced by quartz in F344 rats remain unclear.

In conclusion, we demonstrated a suppressive effect of ambroxol hydrochloride on quartz-induced lung inflammation. Thus, intake of ambroxol hydrochloride may potentially be recommended to prevent or minimize lung damage in individuals whose occupations involve exposure to dust containing quartz or other fine particles.

Disclosure of Potential Conflicts of Interest: The authors declare that there are no conflicts of interest associated with this manuscript.
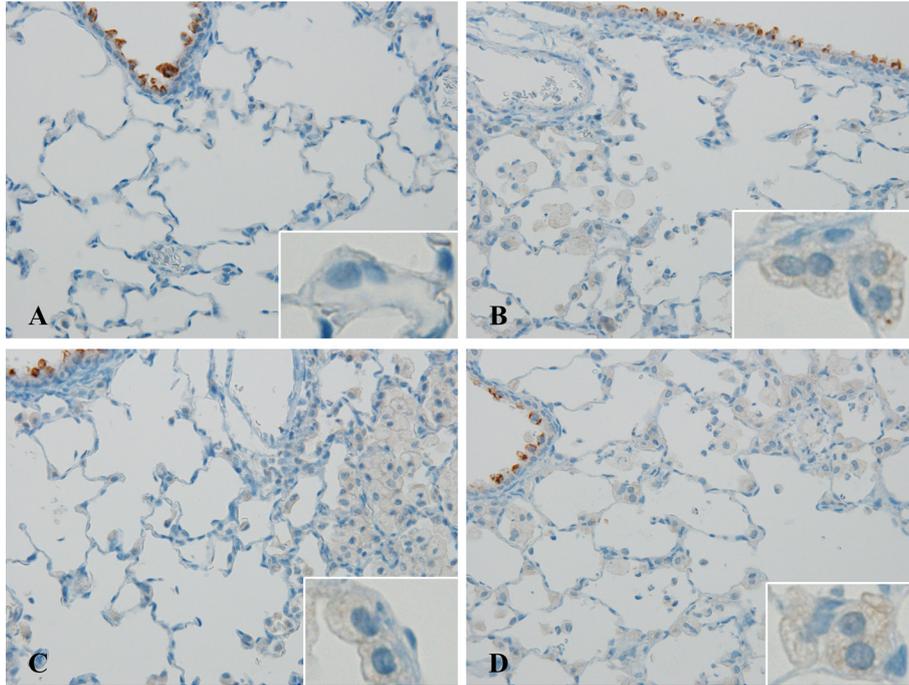
Fig. 2. Immunohistochemistry of SP-B expression in the F344 rat lung after quartz exposure. A, Group 1 (control); B–D, Groups 2–4 (i.t. instillation of quartz plus 0, 12, or 120 ppm ambroxol hydrochloride, respectively) (×400). Note the weak positivity in the type II alveolar cells (B–D, insert ×1,000), in the mucus in the alveoli, and in macrophages (B–D). Strong positivity was observed in bronchiolar epithelial cells (A–D). There were no differences in SP-B expression levels between the treatment groups.
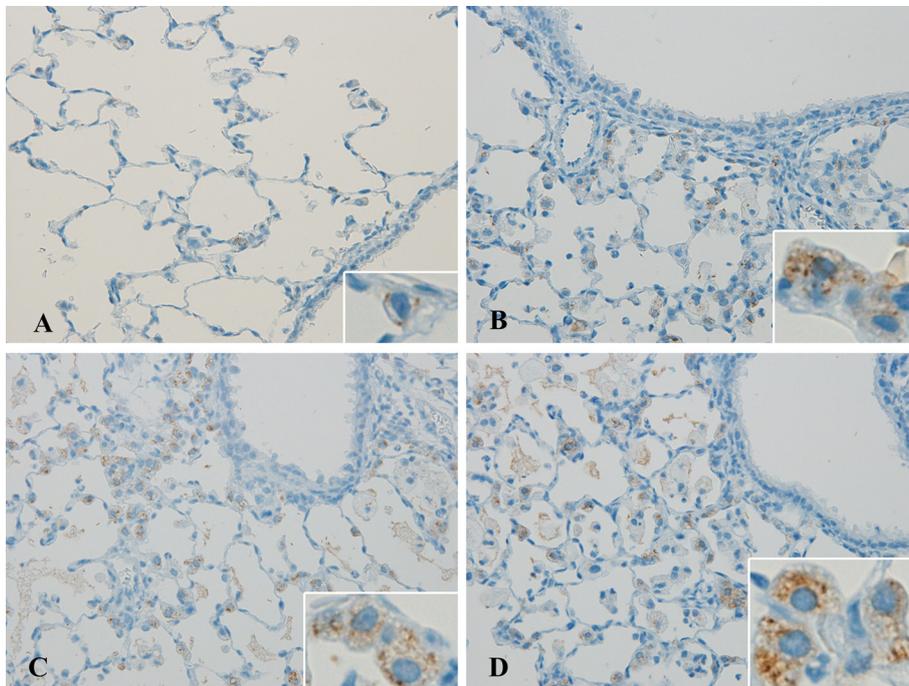

Fig. 3. Immunohistochemistry of SP-C expression in the F344 rat lung after quartz exposure. A, Group 1 (control); B–D, Groups 2–4 (i.t. instillation of quartz plus 0, 12, or 120 ppm ambroxol hydrochloride, respectively) (×400). Note the weak positivity in the type II alveolar cells outside the inflamed area (A, insert ×1,000) and moderate positivity in the mucus in the alveoli and in macrophages (B–D). Strongly positivity was observed in the type II alveolar cells within the inflammatory lesions (B–D, insert ×1,000). There were no differences in SP-C expression levels between the treatment groups.

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