Compositional and functional changes of pulmonary surfactant in a guinea-pig model of chronic asthma

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Recent studies have found that severe surfactant dysfunction occurs during an asthma attack, but the changes in surfactant in a guinea-pig model of chronic asthma have not been studied. We therefore analysed the surfactant recovered from guinea-pigs after repeated inhalation of ovalbumin to see if the surfactant recovered from chronic asthmatic lungs would be intrinsically altered. Guinea pigs immunized through repeated inhalation of aerosolized ovalbumin (OA) were exposed to the antigen once a week for a month. Twenty-four hours after the last challenge the alveolar wash was recovered. We calculated saturated phosphatidylcholine (Sat-PC) and total protein (TP) pool sizes in alveolar spaces. Surfactant subtype conversion of large aggregate surfactant (LA) to small aggregate surfactant was studied in vitro by means of the surface area cycling technique. The phospholipid composition of LA was analysed by thin layer chromatography and the surface activity of LA was also determined. We found decreased surfactant pool sizes, decreased ratio of Sat-PC to TP in alveolar lavages in asthma groups, and surface activity of the surfactant recovered from asthmatic lungs to be inferior to that of the controls. Accelerated surfactant subtype conversion in vitro was also noted in the lungs of asthmatic animal models. In addition, the changes in phospholipid compositions which were similar to the pattern of acute lung injury suggested that alveolar inflammation might be involved in the pathogenesis of chronic asthma. These results indicate that surfactant is intrinsically abnormal in chronically asthmatic lungs.

Key words: alveolar inflammation; surface tension; surfactant subtype conversion.

Introduction

Although numerous previous studies have revealed that bronchial asthma is an inflammatory disease of the airway, the pathogenesis of asthma has not been understood completely. Evidence from autopsy studies suggests that the pathological changes seen in asthma occur in both the large and small airways (1). Hamid et al. also studied the small airways and parenchyma of resected lung specimens from patients with asthma and demonstrated that there was a similar but more severe inflammatory process present in the peripheral compared with the central airways of patients with asthma (2). Recent physiological evidences also suggest that the peripheral airways are the major site of airway obstruction and account for the majority of the airway hyper-responsiveness in patients with asthma (3,4).

Pulmonary surfactant is a highly surface active material which is mainly synthesized by alveolar type II cells and prevents alveolar collapse by reducing surface tensions at the air–liquid interface. Surfactant also covers the surface of small airways and plays a significant role in the patency of small airways (5–7). Kurashima et al. (8) first reported in adult asthmatics that nebulization of surfactant-TA resulted in dramatic improvement of vital capacity (VC) and forced expiratory volume in 1 sec (FEV1), suggesting surfactant dysfunction in the asthmatic lungs. Recently, Liu et al. demonstrated that a rapid inactivation of surfactant developed within 30 min when the intra-muscularly immunized guinea-pig was challenged with antigen (9). They found a similar phospholipid concentration and composition in immunized and challenged animals and controls, and speculated that severe surfactant dysfunction could be due to the proteins leaked into the airways.

More recently, Kurashima et al. analysed surface activity of sputum from acute asthmatic patients and demonstrated that surface properties of airway fluid deteriorated in the early phase of the asthma attack, but were ameliorated in the recovery phase, which suggested that changes in airway surfactant may be partly involved in the pathogenesis of
airway obstruction during an asthmatic attack (10). The changes of surfactant in patients or animal models of chronic asthma however, has not been studied extensively.

We previously reported that guinea-pigs sensitized by repeated inhalation of ovalbumin (OA) aerosols exhibited not only prolonged two-peaked eosinophil infiltration in the airway (6 and 24 h after the antigen challenge), but also late-phase airway narrowing and post-late-phase airway hyper-responsiveness, similar to the asthmatic response in human atopic asthma (11,12). In the present study, we therefore analysed surfactant recovered from lungs in guinea-pigs following repeated inhalation of ovalbumin to see if the surfactant in chronic asthmatic lungs would be changed intrinsically.

Material and methods

SENSITIZATION AND ANTIGEN PROCEDURE

This study protocol was reviewed and approved by the Dokkyo University School of Medicine Committee on Animal Care, and complies with NIH guidelines for animal care. Female Hartley guinea-pigs ranging in weight from 250 to 300 g were sensitized by exposure to an aerosol of OA solution (1% in sterile saline) for 10 min a day for 10 consecutive days as described previously (11,12). A selective H1 antagonist, d,1-chlorpheniramine maleate (10 mg kg$^{-1}$) was administrated intra-peritoneally 15 min before the OA inhalation to avoid fatal anaphylactic reaction from day 6. Seven days after the end of the 10-day exposure of the antigen, the animals were subjected to antigen inhalation and the antigen inhalation was repeated every 7 days.

EXPERIMENTAL PROTOCOLS

Guinea-pigs were divided into four separate groups as follows (Fig. 1):

(i) immunized with OA exposure and challenged with OA (OA-OA);
(ii) immunized with OA and ‘challenged’ with saline; (OA-S);
(iii) ‘immunized’ with saline and ‘challenged’ with OA (S-OA);
(iv) ‘immunized’ with saline and ‘challenged’ with saline (S-S).

At day 45, the guinea-pigs were placed in an acrylic cylinder which completely covered their heads. Then, through the cylinder, they were exposed to an aerosol of 1% OA or saline for 1 min using a DeVilbiss 646 nebulizer (DeVilbiss Co, Somerset, PA, U.S.A.) driven by compressed air at 51 min$^{-1}$. Respiratory resistance (Rrs) was measured by a modification of a forced oscillation technique described by Mead (13) in inhalation-sensitized guinea-pigs and the animals that failed to develop more than 100% increase of baseline Rrs immediately after the challenge were excluded from this study. Twenty-four hours after the challenge, each animal was killed by intraperitoneal injection of pentobarbital (100 mg kg$^{-1}$ body weight) followed by transection of the abdominal aorta.

ISOLATION OF SURFACTANT

After opening the thorax completely, the animal was tracheostomized and lavaged with saline at 4°C as reported previously (14,15). The efficiency for recovery of surfactant by the lavage procedure was evaluated by separately analysing eight sequential lavages.

Surfactant in alveolar washes was separated into large and small aggregate surfactants by centrifugation (14,15). Briefly, the pooled alveolar washes for each animal were first centrifuged at 140 g for 10 min to remove cell debris. The recovered cells were analysed on total and differential cell counts. The supernatant was then centrifuged at 40 000 g for 15 min at 4°C. The pellet from the 40 000 g centrifugation was suspended in saline and layered over 0–8 M sucrose in saline. After centrifugation at 40 000 g for 15 min, the pellicle floating on the sucrose solution was aspirated and recentrifuged at the same g force for 15 min. Recovered surfactant as the pellet was designated as large aggregate surfactant (LA) and suspended in 0–1–0–2 ml of saline. The supernatant from the first 40 000 g centrifugation was designated as small aggregate surfactant (SA).

Large aggregate surfactant isolated from three guinea-pigs was combined for the measurement of surfactant subtype conversion, minimal surface tension and phospholipid composition because of the small surfactant pool sizes.

SURFACTANT ANALYSIS

To determine the saturated phosphatidylcholine (Sat-PC) pool size, aliquots of alveolar washes were extracted with organic solvent (16) and treated with osmium tetroxide (17), and the quantity of Sat-PC recovered from alumina columns was measured by phosphorus assay (18). The amount of total protein was measured by the method of Lowry et al. (19). Phospholipid compositions of lipid extracted samples of large aggregate surfactant recovered from animals were determined by two dimensional thin-layer chromatography (20).

SURFACTANT SUBTYPE CONVERSION IN VITRO

Surface area cycling was performed as described previously (14,21–23). Briefly, an aliquot of LA isolated from each
group of animals was resuspended in Tris buffer (0.15 M NaCl, 0.01 M Tris HCl, 1 mM CaCl$_2$, 1 mM MgSO$_4$ and 0.1 mM EDTA at pH 7.4). Each LA (0.5 mg as total lipids) was mixed with 2 ml of Tris buffer in a capped polystyrene tube (Becton Dickinson, Lincoln Park, NJ, U.S.A.). The polystyrene tubes were attached to the disk of a Rototorque rotator (TAITEC, Japan). The tubes were then rotated for 3 h at 40 rpm at 37°C in order to increase the surface area from 1-1 to 9.0 cm$^2$ twice in each cycle. The contents of tubes were centrifuged at 40,000 g for 15 min at 4°C. The quality of LA in the pellet and the residual SA in the supernatants converted from LA were measured by phosphorous assay of lipid extracts (16,19).

**MINIMAL SURFACE TENSION MEASUREMENTS**

The large aggregate surfactants (LA) have good surface activities when tested in vitro are functional as surfactant when tested in vivo (23). Large aggregate surface activities were tested in this study. A pulsating bubble surfactometer (PBS, Electronetics, Amherst, NY, U.S.A.) was used for minimal tension measurements (24). The concentrations of LA were adjusted to 1-25 or 2-5 mg of lipids per milliliter and applied to the measurements. The bubble pulsates at a rate of 20 cycles min$^{-1}$ and the value at minimal bubble size (ST min$^{-1}$) during 15 min of pulsation was assessed.

**DATA ANALYSIS**

All values are expressed as means ± SEM. The significance of differences between means of the groups were tested by analysis of variance with the Bonferroni correction. P-values less than 0.05 were considered to indicate statistical significance.

**TABLE 1. Body weights, cell fractions in BAL fluids**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body weights (g)</th>
<th>Total cells ($\times 10^6$)</th>
<th>% Eosinophils</th>
<th>% Neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>OA-OA (n = 15)</td>
<td>484 ± 8</td>
<td>7.9 ± 1.3*</td>
<td>32.0 ± 2.2*</td>
<td>17.3 ± 1.4*</td>
</tr>
<tr>
<td>OA-SAL (n = 15)</td>
<td>473 ± 5</td>
<td>5.8 ± 0.7*</td>
<td>20.0 ± 2.1*</td>
<td>2.3 ± 0.5*</td>
</tr>
<tr>
<td>SAL-OA (n = 15)</td>
<td>490 ± 4</td>
<td>2.3 ± 0.3</td>
<td>5.0 ± 0.9</td>
<td>3.0 ± 1.0</td>
</tr>
<tr>
<td>SAL-SAL (n = 15)</td>
<td>482 ± 7</td>
<td>2.2 ± 0.5</td>
<td>4.0 ± 1.0</td>
<td>3.0 ± 1.8</td>
</tr>
</tbody>
</table>

Value are means ± SEM.

*P < 0.01 OA-OA, OA-SAL vs. SAL-OA, SAL-SAL; **P < 0.01 OA-OA vs. OA-SAL, SAL-OA, SAL-SAL.

**Results**

**DESCRIPTION OF ANIMALS AND RECOVERED CELL FRACTIONS IN BAL FLUIDS**

The bronchial lavage (BAL) fluid from animals in the OA-OA and OA-S groups contained increased total cells, eosinophils and neutrophils compared with those from control animals (OA-OA, OA-S vs. S-OA, S-S, P < 0.01; Table 1). The alveolar washes recovered from OA-OA animals also contained a higher percentage eosinophils and neutrophils than those from OA-S (P < 0.01).

**ALVEOLAR WASHES AND POOL SIZES**

To quantify the surfactant pool sizes in asthmatic animals, alveolar lavage technique is critical. We were concerned whether surfactant could be recovered consistently by alveolar washes from an animal model of bronchial asthma. Therefore, eight sequential alveolar washes from the animals in four studied groups were analysed separately for Sat-PC content (data not shown). The shapes of the washout curves were similar in OA-immunized and non-immunized guinea-pigs, indicating that the alveolar wash procedure was similarly effective for animals in all groups. For all subsequent results with alveolar washes, five repetitive washes were performed and pooled as reported previously (14,15).

Alveolar Sat-PC pools in OA-OA and OA-S animals were significantly smaller than those in S-OA and S-S animals (P < 0.01, Table 2). Alveolar LA pools in OA-OA, OA-S groups were also smaller than those in S-OA and S-S groups [P < 0.01, Fig. 2(b)]. Per cent LA was similar in all four groups [Fig. 2(a)]. Total protein contents in alveolar washes was increased four-fold in the OA-OA group, and two-fold in the OA-S group relative to the non-immunized group (OA-OA > OA-S > S-OA, S-S, P < 0.001, Table 2). The ratios of Sat-Pc to total protein contents were also smaller in asthma groups than those in control groups (OA-OA > OA-S > S-OA, S-S, P < 0.01, Table 2).

**Table 2. Surfaceactant analysis**

<table>
<thead>
<tr>
<th>Groups</th>
<th>SAT-PC in AW (µmol kg$^{-1}$)</th>
<th>TP (mg kg$^{-1}$)</th>
<th>SAT-PC/TP (µmol mg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OA-OA</td>
<td>3.85 ± 0.2*</td>
<td>20.7 ± 2.7**</td>
<td>0.224 ± 0.12*</td>
</tr>
<tr>
<td>OA-SAL</td>
<td>4.00 ± 0.2*</td>
<td>10.9 ± 1.3*</td>
<td>0.437 ± 0.206*</td>
</tr>
<tr>
<td>SAL-OA</td>
<td>4.44 ± 0.2*</td>
<td>8.8 ± 0.4</td>
<td>1.062 ± 0.265</td>
</tr>
<tr>
<td>SAL-SAL</td>
<td>4.56 ± 0.5</td>
<td>4.3 ± 0.5</td>
<td>1.176 ± 0.505</td>
</tr>
</tbody>
</table>

Value are means ± SEM (n = 5).

*P < 0.01 OA-OA, OA-SAL vs. SAL-OA, SAL-SAL; **P < 0.001 OA-OA vs. OA-SAL, SAL-OA, SAL-SAL.

SAT-PC: saturated phosphatidylcholine; TP: total protein; AW: alveolar wash.
SURFACTANT SUBTYPE CONVERSION IN VITRO

Cycling for 180 min resulted in about 45% conversion of the LA from OA-immunized guinea-pigs but only ~38% of the surfactant from non-immunized animals converted to SA (OA-OA, OA-S > S-OA, S-S, P < 0.01, Fig. 2(c)).

PHOSPHOLIPID COMPOSITION

The phospholipids recovered by alveolar wash from OA-OA and OA-S animals contained a lower proportion of phosphatidylcholine, phosphatidylglycerol and a higher proportion of phosphatidylinositol, than did the S-OA, S-S animals (P < 0.01, Table 3). A proportion of lysophosphatidylethanolamine in asthmatic animals tended to be higher than that in controls. The compositions of the phospholipids for OA-OA, and OA-S guinea-pigs were very similar. These patterns of phospholipid compositions in asthmatic animals were quite similar to those seen in animal models in acute lung injury (25).

SURFACE TENSION MEASUREMENTS

The mean minimal surface tension of LA recovered from asthmatic and control animals are shown in Fig. 3. Although the mean minimal surface tensions at 2.5 mg ml⁻¹ lipids were not different between the surfactant recovered from OA-OA and OA-S and that from S-OA and S-S groups, the animals in the OA-OA and OA-S groups showed significantly higher minimal surface tensions at 1.25 mg ml⁻¹ lipid than did the S-OA and S-S animals (P < 0.01).

Discussion

We found decreased surfactant pool sizes and the ratio of Sat-PC to total protein in alveolar washes in asthmatic animals compared with those in controls. Since leaked serum proteins can inhibit surfactant function directly (15,26), it is probable that surfactant dysfunction could occur in the lungs of the asthmatic animal models. The surface activity for the surfactant recovered from the asthmatic animals were also inferior to those from control animals in our study. Therefore, the increased leaked proteins, decreased surfactant pool size and the inferior surface activity of the surfactant itself could further limit surfactant function in the asthmatic lungs. Pulmonary surfactant is not only important for the stability of the alveoli, but also for small airway function (5–7). The patency of small airway could be dependent on the properties of surfactant (6,7). The surfactant film at the alveolar air/liquid interface is partly extruded into adjacent conducting airways during expiration. The extruded dysfunctional surfactant would result in the airway closure which is often seen in asthmatic lungs (27).

One possible explanation for the decreased surfactant pool size in asthma groups could be due to the increased phospholipases. Increased levels of phospholipase A2 have been found in BAL fluids from patients with bronchial asthma (28). The enzyme catalyses the hydrolysis of phospholipids in membranes of epithelial cells. Moreover, surfactant phospholipids could be catalysed into lysophosphatidylethanolamine and palmitic acid by phospholipase A2. Superoxides, proteinases from eosinophils, like myeloperoxidase, eosinophil cationic proteins which are abundant in...
The lungs with bronchial asthma might be also able to damage the surfactant.

Surfactant exists mainly in two general subtypes: LA composed of lamellar bodies, tubular myelin and loose lipid arrays that has good biophysical and in vivo function, and SA in vesicular forms with poor function (5–7). LA is a newly secreted material from type II cells and is thought to be a source of the surface films at the air–liquid interface (29). This film is then refined to increase the concentration of Sat-PC through changes in surface area with breathing, and then small vesicles, primarily containing lipids, re-enter the hypothesis for recycling or catabolism. The conversion of surfactant from LA to SA can be also reproduced in vitro by using a surface area cycling technique (22,30). The converted SA in vitro does not function as surfactant in vivo or in vitro (23). Using this technique, Gross et al. (22,31) studied the conversion from LA to SA in vitro. Ueda et al. previously reported that the rate of in vitro conversion of LA to SA was more rapid for surfactant from preterm animals than from surfactant from mature or adult animals in sheep (32). The rate of the conversion also increased when the LA were recovered from animals with lung injury (21). The rate of form conversion is now thought to be one of the factors governing the size of functional surfactant pools in the lungs. We also found increased surfactant subtype conversion for the surfactant recovered from the animals in asthma groups compared with the surfactant from controls. The accelerated surfactant subtype conversion would be the another explanation for the decreased functional surfactant pool sizes in the asthmatic lungs.

The rate of in vitro conversion of LA to SA is regulated by multiple factors. Conversion is more rapid for LA from humans and animals with lung injury (15,27). Ventilation style could also affect the form conversion in preterm lambs and a rabbit model of acute lung injury (33,34). The conversion is thought to be mediated by a serine protease, referred to ‘surfactant convertase’ (22). Recently, Gross et al. proposed that the surfactant convertase is a carboxylesterase (35). The conversion of LA to SA is inhibited by SP-A (21,36). Veldhuizen et al. found that SP-B was degraded during the conversion from LA to SA (37), suggesting that SP-B may be the substrate for ‘surfactant convertase’. We do not have the accurate assays for SP-A, SP-B and ‘convertase’ in the BAL fluids of guinea-pigs, but we also demonstrated the elevated minimal surface tension for the surfactant recovered from asthmatic animals. Taken together, compositional changes, i.e. decreased SP-A and SP-B, or increased ‘convertase’ activity could exist in the surfactant system for asthmatic lungs.

In our study, the accelerated conversion rate in asthma models did not reflect the percentage of LA in vivo as several studies have reported before in acute lung injury (20,25,31). For example, there was a five-fold increase in the ratio of SA to LA in N-nitroso-N-methylurethane-injured rabbits due to the more rapid surfactant subtype conversion. The explanation for this discrepancy might be due to the differences of the degree of the lung injury. If the recycling or catabolic pathway (i.e. type II cells) is not damaged severely in acute lung injury, the accumulation of non-functional surfactant could not occur.

Liu et al. recently reported surfactant dysfunction when the immunized guinea-pig was challenged with OA (9). Although they could not find any changes in

### Table 3. Phospholipid composition of pulmonary surfactant (% of total phospholipid)

<table>
<thead>
<tr>
<th>Groups</th>
<th>PC</th>
<th>PG</th>
<th>PI</th>
<th>PE</th>
<th>LPC</th>
<th>SM</th>
<th>PS</th>
</tr>
</thead>
<tbody>
<tr>
<td>OA-OA</td>
<td>77·6 ± 1·3*</td>
<td>8·0 ± 0·3*</td>
<td>8·4 ± 0·9*</td>
<td>3·4 ± 0·1</td>
<td>1·1 ± 0·2</td>
<td>0·9 ± 0·1</td>
<td>0·8 ± 0·2</td>
</tr>
<tr>
<td>OA-SAL</td>
<td>76·4 ± 1·1*</td>
<td>8·2 ± 0·2*</td>
<td>8·6 ± 0·3*</td>
<td>3·6 ± 0·4</td>
<td>1·2 ± 0·1</td>
<td>1·0 ± 0·2</td>
<td>0·9 ± 0·2</td>
</tr>
<tr>
<td>SAL-OA</td>
<td>80·4 ± 0·6</td>
<td>9·6 ± 0·3</td>
<td>3·9 ± 0·3</td>
<td>3·5 ± 0·2</td>
<td>0·8 ± 0·1</td>
<td>1·2 ± 0·1</td>
<td>0·8 ± 0·1</td>
</tr>
<tr>
<td>SAL-SAL</td>
<td>80·9 ± 0·4</td>
<td>9·8 ± 0·2</td>
<td>3·9 ± 0·3</td>
<td>3·4 ± 0·2</td>
<td>0·8 ± 0·1</td>
<td>1·1 ± 0·1</td>
<td>0·8 ± 0·1</td>
</tr>
</tbody>
</table>

Value are means ± SEM (n = 5).

*P < 0·01 OA-OA, OA-SAL vs. SAL-OA, SAL-SAL.

PC: phosphatidylcholine; PG: phosphatidylglycerol; PI: phosphatidylinositol; PE: phosphatidylethanolamine; LPC: lysophosphatidylcholine; SM: sphingomyelin; PS: phosphatidylserine.

Fig 3. Minimal surface tension of surfactant recovered from asthmatic and control animals. Although the mean minimal surface tensions at 2·5 mg ml⁻¹ lipids were not different between OA-OA and OA-S groups and S-S and S-OA groups, surfactant from animals in OA-OA and OA-S groups showed significantly higher minimal surface tensions at 1·25 mg ml⁻¹ lipid than in S-S and S-OA groups (n = 5 for each measurements; P < 0·01).

■: 1·25 mg l⁻¹ lipid; □: 2·5 mg ml⁻¹ lipid.
Surface changes in bronchial asthma

In summary, we demonstrated the intrinsic changes of surfactant in the asthmatic lungs. Surfactant pool size decreased and surface activity of surfactant itself recovered from asthmatic lungs was inferior to that of surfactant from controls. Accelerated subtype conversion in vitro also suggested compositional changes of surfactant. The changes of phospholipid compositions also suggested the alveolar inflammation. Further studies to clarify the pathogenesis of these intrinsic changes of surfactant will be needed to understand the mechanisms of ‘airway and alveolar inflammation’ in bronchial asthma.

Acknowledgements

We are grateful to Mr Takashi Namatame for excellent technical assistance.

References