Effects of various forms of surfactant protein C on tidal volume in ventilated immature newborn rabbits

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PULMONARY SURFACTANT IS PRIMARILY composed of phospholipids and surfactant proteins (SPs) (17) and reduces surface tension of the air-liquid interface by forming a surface film (28, 31). Hydrophobic SPs (SP-B and SP-C) are important for facilitating surface adsorption of phospholipid molecules and for promoting tidal volume (17, 20). The regular form of SP-C is known to be monomeric, with a hydrophobic valine-rich α-helix at the carboxy terminal (15–17). The amino terminal part of regular SP-C is hydrophilic, but in most species the two cysteine residues are attached to the palmitoyl groups via thioester bonds with an overall stoichiometry ratio of close to 1:1 between the cysteine residues and the palmitoyl groups (6, 16). However, the relationship between molecular configuration and the physiological function are not yet completely understood.

Two types of abnormal SP-Cs are known to accumulate in the lungs of patients with pulmonary alveolar proteinosis (PAP). One type is monomeric but poor in palmitoyl groups, and the other type is dimeric and also deficient in palmitoyl groups (32, 34, 39). Information on the relationship between the configuration and function of SP-C may be obtained from analyses of these abnormal SP-Cs. Numerous investigations on surfactants consisting of synthetic lipids and SPs or the analogs have recently been conducted to develop artificial surfactant for therapeutic use (8, 27). The techniques used in these investigations can be applied to analysis of SP-C function. In the present study, several reconstituted surfactants (RSs), consisting of synthetic phospholipids, normal SP-B, and abnormal SP-Cs from PAP patients, were administered to surfactant-deficient immature newborn rabbits. Tidal volumes of the animals were then measured under pressure-controlled ventilation to evaluate the physiological function of abnormal SP-Cs.

METHODS

Preparation of MNS, Normal SP-B, and Normal SP-C

Bronchoalveolar lavage (BAL) fluid from recently slaughtered pigs was centrifuged (150 g for 10 min) to remove cell debris, and the supernatant was further centrifuged (2,000 g for 1 h, 4°C). Modified natural surfactant (MNS) consisting of 98.0% phospholipids, 0.9% other lipids, and 1.1% hydrophobic SPs (0.37% SP-B and 0.72% SP-C) was obtained from the pellet by extraction with chloroform-methanol solution (2:1 vol/vol), by washing with 0.5% saline, and by acetone precipitation. More precise chemical compositions and isolation methods for MNS are presented elsewhere (20, 21).

MNS was dissolved again in chloroform-methanol (1:1 vol/vol) containing 0.1 N hydrochloric acid at 5%. From this solution, we could separate two types of protein fractions by using Sephadex LH-60 column chromatography (Pharmacia Biotechnology, Uppsala, Sweden) (7) with the aid of ultraviolet (280 nm) absorbance using a spectrophotometer (U-2000, Shimadzu, Kyoto, Japan) at each fraction.

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Preparation of Abnormal SP-Cs

Bal fluid was obtained from therapeutic lung lavage of two PAP patients admitted to Kanazawa University Hospital. The chloroform-soluble fraction was obtained in the same manner as used for the preparation of MNS. This fraction was further separated into pulmonary alveolar proteinosis SP-B (SP-Bpap) and dimeric (dSP-Cpap) and monomeric forms of SP-C (mSP-Cpap) under the same methods used for isolation of nSP-C (32, 34). Purity and molecular weight were determined according to the methods described above. Amount of palmitoyl groups in nSP-C and abnormal SP-Cs were quantified using gas chromatography after hydrolysis with 0.1 mol/l of potassium hydroxide (6) and by methylation with boron fluoride-methanol (25).

Preparation of RSs

First, a synthetic phospholipid mixture (SPL) was prepared by mixing synthetic dipalmitoylphosphatidylcholine (Sigma Chemical, St. Louis, MO), synthetic dioleoylphosphatidylcholine (Sigma Chemical), and egg yolk phosphatidylglycerol (Sigma Chemical) at a weight ratio of 6:2:2 in chloroform-methanol (95:5) solution (36). The basic mixture was prepared using gas-phase protein sequencer (PSPQ-10, Shimazu, Kyoto, Japan) after blotting on a polyvinylidene difluoride membrane (Sequi-Blot, 0.2 μm, Bio-Rad Laboratories, Richmond, CA) according to the method of Towbin et al. (38). The relative amount of protein on tricine SDS-PAGE was determined by use of a lane and spot analyzer (AE-6920, ATTO, Tokyo, Japan). The concentration of proteins was determined according the micro-Kjeldahl method (37).

Secondary Structure of Various SP-Cs

The circular dichroism (CD) spectra were obtained using a spectropolarimeter (J-710, JASCO, Tokyo, Japan) in a 0.2-mm cell at 25°C. Various SP-Cs (75 μg) were mixed with SPL (750 μg). These mixtures were dried and suspended in 1 ml of phosphate buffer (50 mmol/l, pH 6.0) (18). Two series of 10 scans from 240 to 200 nm were averaged, and protein-free SPL spectra were subtracted to yield the protein spectra. CD spectra were expressed as mean residue ellipticity calculated by protein concentration and estimation of the mean molecular weight of amino acid residues as 115. These spectra were analyzed for secondary structure with the aid of a neural network computer program (k2d, kindly distributed by M. A. Andrade through the World Wide Web; http://www.embl-heidelberg.de/~andrade/k2d.html) comprising a database of weights and a recall program for determining α-helix and β-sheet structure based on these weights (1, 12).

Animal Experiment 1

Validation of MNS and immature newborn rabbit model. Twenty immature newborn rabbits were delivered by hysteroctomy from three pregnant does at a gestational age at between 26 days 22 h and 27 days 5 h (term = 31 days). Animals were tracheotomized under anesthesia with intraperitoneal pentobarbital sodium (0.5 mg) and randomly assigned to MNS (n = 10) or nontreated (n = 10) groups. MNS group animals were administered 100 μl of MNS suspension (50 mg/ml) via tracheal cannula before taking their first breath, whereas control group animals were administered nothing. Animals were then transferred to a system of multiple-body plethysmographs kept at 37°C (20). After all litttermates were prepared, animals were relaxed with intraperitoneal suxamethonium bromide (0.02 mg) and subjected in parallel to pressure-controlled ventilation. The respirator unit (Servo 900B, Siemens-Elema, Solna, Sweden) delivered 100% oxygen at 40 breaths/min with a 50% inspiration time.

The peak inspiratory pressure (PIP) was first raised to 30 cmH2O for 1 min to facilitate distribution of administered materials, then lowered to 25 cmH2O for 15 min, and then to 20 cmH2O and 15 cmH2O for 5 min each. Finally, PIP was again increased to 25 cmH2O for 5 min. No end-expiratory pressure was applied to the ventilatory circuit. Individual tidal volumes were recorded at the end of each 5-min interval by using a pneumotachograph system described elsewhere (20). Electrocardiograms were recorded immediately after tidal volume measurements, and animals showing QRS complexes at a frequency of over 100 beats/min were considered survivors. After the animals were killed by an overdose of pentobarbital, the abdomen was opened to inspect the diaphragm for evidence of pneumothorax. Animals with pneumothorax were excluded from statistical analyses.

Animal Experiment 2: Function of nSP-C, mSP-Cpap, and dSP-Cpap

nSP-C series. Thirty-one immature newborn rabbits were delivered from five pregnant does and randomly assigned to groups receiving four types of RSs containing nSP-C at concentration of 0% (basic mixture, n = 7), 1% (n = 7), 2% (n = 10), or 3% (n = 7). Animals were administered 100 μl of each
one of the RS suspensions (50 mg/ml) via the tracheal cannula and were ventilated in the same manner as in animal experiment 1. Tidal volumes at a PIP of 25 cmH2O were measured four times, and the last value (30 min after start of the ventilation) was used for comparisons. Animals with pneumothorax were excluded from statistical analyses.

*mSP-Cpap* series. Thirty-one immature newborn rabbits were delivered from five pregnant does and randomly assigned to groups receiving RSs (50 mg/ml) containing mSP-Cpap at concentrations of 0% (basic mixture, n = 7), 1% (n = 7), 2% (n = 10), or 3% (n = 7). The experiment was performed in a manner identical to the nSP-C series.

*dSP-Spap* series. Twenty-four immature newborn rabbits were delivered from four pregnant does and randomly assigned to groups receiving RSs (50 mg/ml) containing dSP-Spap at concentrations of 0% (basic mixture, n = 7), 1% (n = 7), or 2% (n = 10). The experiment was performed in a manner identical to the nSP-C series.

Statistical Analysis

Data for tidal volume and surface tension were expressed as median and range (parentheses). Intergroup differences were examined by using Kruskal-Wallis test followed by Fisher’s exact test. Levels of P < 0.05 were considered statistically significant.

RESULTS

Characterization of Hydrophobic SPs

Tricine SDS-PAGE of the various SP-Cs is shown in Fig. 1A. Under the nonreducing condition, the molecular weight of nSP-C, dSP-Cpap, and mSP-Cpap were 4.9 kDa, 8.5 kDa, and 4.2 kDa, respectively. Under the reducing condition, the dSP-Cpap demonstrated a prominent staining band at 4.4 kDa and a faint one at 8.5 kDa, indicating the dimeric nature of dSP-Cpap. Tricine SDS-PAGE of nSP-B and SP-Bpap is shown in Fig. 1B. Under the nonreducing condition, nSP-B (lane 1) revealed a major band at 21–22 kDa and a faint one at 16 kDa. Under the reducing condition, the major band migrated to 11 kDa, but the faint band remained at 16 kDa. A similar band was also found in the MNS prepared after isolation by sucrose-gradient centrifugation according to Frosolono et al. (10) (data not shown). The amino terminal sequence of the major band at 21–22 kDa in the nonreducing condition represented Phe-Pro-Ile-Pro-Leu-Pro-Phe-X-Trp-Leu- (X was supposed to be “Cys”), which agreed with the result of the previous report (7). Densitometry indicated that the purity of the nSP-B was 84–85%. Two major bands were demonstrated for SP-Bpap, one at 21 kDa and the other at 30 kDa. Under the reducing condition, the major bands migrated to 10 kDa with a faint band at 16 kDa. Western blotting using anti-SP-B antibody is shown in Fig. 1C. The major bands of nSP-B (21 kDa) and SP-Bpap (21–30 kDa) demonstrated the binding to anti-SP-B antibody. Their faint bands at 16 kDa did not bind with antibody. A faint binding at 10 kDa was found in the blot of dSP-Cpap, indicating the contamination with SP-B monomer. On the basis of densitometry, the contamination was calculated to 1.5%.

Weight ratio of SP-Bpap, dSP-Cpap, and mSP-Cpap obtained from BAL fluid of PAP patients was 27:23:50. Relative contents of palmitoyl groups in dSP-Cpap and mSP-Cpap were 42 and 38% of that contained in the identical weight of nSP-C, respectively.

Pulsating-Bubble Measurement

Findings of dynamic surface tensions are shown in Table 1. Both γmin and γmax of the basic mixture composed of SPL and nSP-B were significantly lower than those of SPL, indicating that nSP-B was functional. Similar findings were obtained on SPL supplemented with nSP-C only, of which γmin was, however, significantly higher than that of MNS. Neither nSP-B nor nSP-C alone could constantly lower γmin values of SPL to <5 mN/m. The RS consisting of SPL and both nSP-B and nSP-C demonstrated the same γmin and γmax values as MNS.

Secondary Structure of Various SP-Cs

CD spectra of nSP-C demonstrated minima at 208 and 222 nm (Fig. 2), a result characteristic of α-helix (11). The spectra of mSP-Cpap revealed smaller minima at these wavelengths than nSP-C. The minima of
dSP-Cpap were somewhat smaller than those of mSP-Cpap (Fig. 2). The k2d program indicated that the α-helical content of the nSP-C was larger than those of mSP-Cpap or dSP-Cpap (Table 2).

Animal Experiment 1

Two of 10 animals died in the nontreated group, whereas all animals in the MNS group survived until the end of experiment. No pneumothorax was observed in the nontreated group, whereas 2 of the 10 animals in the MNS group were excluded because of pneumothorax. Body weight of all animals was 33.6 ± 3.4 g, without significant differences between the MNS and nontreated groups. As shown in Fig. 3, median tidal volume of the nontreated group was <2 ml/kg in four measurements at a PIP of 25 cmH2O, whereas the values of the MNS group was >24 ml/kg (P < 0.01 vs. nontreated group).

Animal Experiment 2

nSP-C series. Body weight of all animals was 33.6 ± 3.5 g, without significant differences among the four groups. All animals survived until the end of the experiment, and no cases of pneumothorax were observed. Changes in tidal volume are shown in Fig. 4. Tidal volumes with the basic mixture were larger than those seen in the nontreated group of animal experiment 1 at all PIPs (P < 0.01). At a PIP of 25 cmH2O, tidal volumes of animals receiving the RSs containing nSP-C at 2 or 3% were significantly larger than those receiving basic mixture (nSP-C at 0%). At PIPs of 15 and 20 cmH2O, tidal volumes of animals administered RS containing SP-C at 3% were significantly larger than those receiving basic mixture.

mSP-Cpap series. Body weight of all animals was 30.4 ± 4.0 g, without significant differences among the four groups. All animals survived until the end of the experiment, but one animal receiving the RS containing mSP-Cpap at 2% was excluded because of pneumothorax. Changes in tidal volumes are shown in Fig. 5. At a PIP of 25 cmH2O, median tidal volumes of all groups were only ~10 ml/kg.

dSP-Cpap series. Body weight of all animals was 30.2 ± 4.7 g, without significant differences among the three groups. All animals survived until the end of the experiment, and no cases of pneumothorax were observed. Changes in tidal volumes are shown in Fig. 6. At a PIP of 25 cmH2O, median tidal volume of the animals receiving the RS containing 2% dSP-Cpap was nearly twice that of those receiving basic mixture (P < 0.05).

DISCUSSION

In animal experiment 1, tidal volumes of the MNS group were significantly larger than those of the nontreated group. Although difference in tidal volumes might have been exaggerated by relationships between alveolar opening pressures and PIPs, the same pressure-controlled ventilation used in the present study has been used previously to evaluate the physiological activity of surfactant preparations (7, 20, 21). Sucrose-gradient centrifugation, which has been used to isolate

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SPL only</th>
<th>nSP-B</th>
<th>nSP-C</th>
<th>γmin (mN/m)</th>
<th>γmax (mN/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPL only</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>23 (18–28)*</td>
<td>61 (48–66)*</td>
</tr>
<tr>
<td>Basic mixture</td>
<td>+</td>
<td>0.7%</td>
<td>–</td>
<td>4.6 (1.5–8.6)†</td>
<td>29 (26–40)†</td>
</tr>
<tr>
<td>RS containing nSP-C (1%)</td>
<td>+</td>
<td>0.7%</td>
<td>1.0%</td>
<td>3.1 (0.9–4.4)†</td>
<td>31 (28–33)†</td>
</tr>
<tr>
<td>SPL with nSP-C (3%)</td>
<td>+</td>
<td>–</td>
<td>3.0%</td>
<td>7.8 (4.2–13)‡‡</td>
<td>35 (28–44)‡‡</td>
</tr>
<tr>
<td>MNS</td>
<td></td>
<td>0.37%</td>
<td>0.72%</td>
<td>2.0 (0.7–4.3)†</td>
<td>30 (26–33)†</td>
</tr>
</tbody>
</table>

Values are median (range) of 8 measurements. SPL, synthetic phospholipid mixture; nSP-B and nSP-C, normal SP-B and normal SP-C, respectively; γmin and γmax, minimum and maximum surface tension after 5 min of pulsation, respectively; RS, reconstituted surfactant; MNS, modified natural surfactant. Phospholipid concentration was 10 mg/ml. *P < 0.05 vs. MNS. †P < 0.05 vs. SPL.

![Graph](https://www.jap.org/vol94/april03/fig2.png)

Fig. 2. Circular dichroism spectra of nSP-C (●) and SP-Cs derived from pulmonary alveolar proteinosis lung in monomeric (mSP-Cpap, □) and dimeric forms (dSP-Cpap, ▲).

Table 2. Secondary structure of various SP-Cs assessed by use of circular dichroism

<table>
<thead>
<tr>
<th>Protein</th>
<th>α-Helix</th>
<th>β-Sheet</th>
<th>Random Coil</th>
</tr>
</thead>
<tbody>
<tr>
<td>nSP-C</td>
<td>0.59</td>
<td>0.08</td>
<td>0.34</td>
</tr>
<tr>
<td>mSP-Cpap</td>
<td>0.41</td>
<td>0.11</td>
<td>0.47</td>
</tr>
<tr>
<td>dSP-Cpap</td>
<td>0.38</td>
<td>0.07</td>
<td>0.55</td>
</tr>
</tbody>
</table>

nSP-C, normal SP-C derived from porcine lungs; mSP-Cpap and dSP-Cpap, monomeric and dimeric forms of SP-C derived from lungs of patients with pulmonary alveolar proteinosis, respectively.
“complete” surfactant (10, 20, 21), was omitted from the present study. Lipid composition of MNS, therefore, may differ from that of complete pulmonary surfactant. Another difference between complete surfactant and MNS is the absence of SP-A and SP-D in MNS given that extraction with organic solvents results in removal of hydrophilic proteins (17, 20). However, we have found that this MNS promotes tidal volumes in surfactant-deficient immature newborn rabbits to a similar extent as complete surfactant (21). From these perspectives, we believe that the physiological activity of surfactant preparations can be assessed using the protocols of the present study and that MNS is a reasonable source for hydrophobic SPs.

An RS consisting of the same phospholipids as used in the present study and porcine SP-B (0.7%) and porcine SP-C (1.4%) could improve blood-gas findings in rats with acute lung injury in our previous experiment (36). Although the reconstituted materials without SP-B exhibit some surface activity (21, 26), SP-B is believed to represent an indispensable component to optimize the surfactant activity (21, 27). In the present study, the pulsating-bubble measurements revealed that surface tension of SPL was significantly decreased by adding nSP-B or nSP-C. However, surface tension did not decrease to the same level as MNS by either of the two SPs alone. Apparent molecular size, amino terminal sequence, and findings under Western blot-

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**Fig. 3.** Tidal volumes (TV) of immature newborn rabbits at various peak inspiratory pressures (PIP) in animal experiment 1. ▼, Not given any material (nontreated group, n = 10); ▲, treated with modified natural surfactant (MNS group, n = 8). Values are medians (ranges). *P < 0.05 vs. nontreated group. Some ranges are hidden by symbols.

**Fig. 4.** TV in the nSP-C series of animal experiment 2 under PIP of 15 cmH₂O (triangles), 20 cmH₂O (circles), and 25 cmH₂O (squares). Immature newborn rabbits were administered reconstituted surfactants (RSs) consisting of basic mixture (mixture of synthetic phospholipids and normal SP-B) and 0–3% nSP-C. RS without nSP-C (0%) corresponds to the basic mixture (open symbols). Values represent medians (ranges); n = 7–10 animals per RS. Some ranges are hidden by symbols. *P < 0.05 vs. basic mixture.

**Fig. 5.** TV in the mSP-Cpap series of animal experiment 2 under PIP of 15 cmH₂O (triangles), 20 cmH₂O (circles), and 25 cmH₂O (squares). Immature newborn rabbits were administered RSs consisting of basic mixture and 0–3% mSP-Cpap. RS without mSP-Cpap (0%) corresponds to the basic mixture (open symbols). Values represent medians (ranges); n = 7–10 animals per RS. Some ranges are hidden by symbols.

**Fig. 6.** TV in the dSP-Cpap series of animal experiment 2 under PIP of 15 cmH₂O (triangles), 20 cmH₂O (circles), and 25 cmH₂O (squares). Immature newborn rabbits were administered RSs consisting of basic mixture and 0–2% dSP-Cpap. RS without dSP-Cpap (0%) corresponds to the basic mixture (open symbols). Values represent medians (ranges); n = 7–10 animals per each RS. Some ranges are hidden by symbols. *P < 0.05 vs. basic mixture.
ting of nSP-B in the present study were consistent with those previously reported for regular porcine SP-B (7, 17, 33). In the present experiment, the basic mixture, i.e., mixture of SPL and nSP-B (0.7%), increased the tidal volume of immature newborn rabbits from <2 ml/kg to ~7.7 ml/kg. This only modest improvement may be because the SP-B concentration was lower than that used in a previous study (2.0%) (7). The concentration of SP-B used in our experiment may have been suboptimal, so that a larger tidal volume could be obtained by using a higher concentration. It was reported that addition of SP-B at a concentration of 2.0% reduced the γmin of an artificial surfactant, including phospholipids and an analog of SP-C, to nearly the same value as that of another surfactant preparation (8). Baatz et al. (2) reported that dimeric SP-C from bovine lung predominantly consisted of β-sheets. In contrast, Creuwels et al. (5) reported that the dimeric SP-C was helical. Both studies demonstrated that dimeric SP-C improved surface activity of synthetic phospholipid mixtures. In the present experiment, the α-helical content of mSP-Cpap was similar to that of dSP-Cpap, although both values were lower than that of nSP-C. The palmitoyl content of mSP-Cpap was also similar to that of dSP-Cpap. The functional efficiencies, however, were clearly different in the present study. Data from the present study indicate that surfactant function is influenced not only by the α-helical content and degree of palmitoylation of SP-C but also by the presence of dimeric SP-C.

Surfactant forms surface film at the air-liquid interface, which is thought to be consisting of a phospholipid monolayer at the most superficial part and bilayers in the underlying part (22, 28, 31). Some researchers have speculated that palmitoylation of SP-C may link the monolayer to a neighboring bilayer and link two bilayers together by anchoring palmitoyl groups and the α-helix to different layers, because the palmitoylated SP-C analog demonstrated improved adsorption of surface-associated phospholipid layers to the air-liquid interface during surface expansion (13). Wang et al. (40) reported that regular SP-C facilitated the adsorption of both synthetic phospholipids and phospholipids isolated from natural surfactant better than depalmitoylated SP-C. The surface adsorption rate of surfactant molecules is an important factor in physiological activity (20, 31). Nonionic polymers such as dextran and polyethylene glycol are known to improve the surface adsorption rate of surfactant preparations, and presumably these polymers pull neighboring phospholipid layers together by removing water molecules (23, 24). Taking all these possibilities into consideration, we believe that each of two α-helix structures in the dSP-Cpap molecule anchored to different lipid layers and linked them together, improving surface adsorption and physiological function despite the reduced content of palmitoyl groups.

The helical structure of regular SP-C is reportedly able to unfold and form aggregates with β-sheets, leading to formation of amyloid fibrils (14, 35). Such fibrils have been found in BAL fluid from a PAP patient (14). In the present experiment, neither polymeric forms nor aggregates of SP-C with higher molecular weights could be found on the tricine SDS-PAGE, probably because only the chloroform-soluble fraction was used for isolation of abnormal SP-Cs. The palmitoyl content of mSP-Cpap was found to be 38% of that of nSP-C. The mSP-Cpap fraction could thus consist of either a largely monopalmitoylated species or a mixture of nonpalmitoylated and dipalmitoylated species. The respective concentrations of these three species were not determined. Nevertheless, results of the animal experiment indicated the importance of the palmitoyl content of nSP-C. We used SPL for the lipid component of
RSs, with synthetic surfactants in mind. We may therefore need to examine whether the same results could be obtained by using the phospholipids isolated from natural surfactant (40). Although many areas remain to be examined, we conclude that monomeric and less-palmitoylated SP-C derived from PAP patients lacks the ability to enhance tidal volume of immature newborn rabbits. Palmitoylation is important for the function of monomeric SP-C. Dimeric SP-C appearing in PAP patients, however, shows physiological function despite a deficiency in palmitoyl groups.

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