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# Distribution of Endotracheally Instilled Surfactant Protein SP-C in Lung-Lavaged Rabbits

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**ABSTRACT. In lung-lavaged surfactant-deficient rabbits ($n = 6$) requiring artificial ventilation, porcine surfactant was instilled endotracheally. This resulted in improvement of lung function so that the animals could be weaned off artificial ventilation. The animals were killed 4½ h after surfactant administration and the porcine surfactant protein was localized in the lung with a MAb. We found surfactant protein in all lobes of the lung but the distribution was not homogeneous. Surfactant protein C was found in less than 15% of the alveolar spaces and in less than 1% of the bronchi. (*Pediatr Res* 29: 178–181, 1991**

**Abbreviations**

**PEEP, positive end-expiratory pressure**
**SP-B, surfactant protein B**
**SP-C, surfactant protein C**

The endotracheal instillation of surfactant improves lung function in premature newborn babies with surfactant deficiency. However, the immediate beneficial effects of surfactant therapy are sometimes followed by a relapse several hours later. Factors responsible for the relapse include patency of the ductus arteriosus (1) and inhibition of surfactant activity by proteins leaking into the alveolar space (2). Another factor that may be important is the distribution in the lung of the administered surfactant. The distribution of instilled surfactant has been subsequently measured by analysis of different components of instilled surfactant in alveolar wash and in lung tissue (3–7). However, the localization and distribution of the instilled surfactant at the microscopic level in lung tissue has not been reported. In our study, an immunohistochemical technique was used to localize the surfactant-associated protein, SP-C, with the aid of a MAb, after a clinical response.

## MATERIALS AND METHODS

*Animals.* the experiments were performed under approved institutional animal care protocols with concern for animal welfare.

Healthy 3-mo-old rabbits ($n = 12$) with a body weight of 2.73 ± 0.11 kg (mean ± SD) were treated as we described before (8). In short: after anesthesia (sodium pentobarbital 30 mg/kg body weight i.v.), the animals were put into the supine position on a heated mattress. the left carotid artery and right jugular vein were cannulated. Subsequently, the animals were intubated by means of a tracheostomy. The rabbits were then paralyzed (pancuronium bromide 0.1 mg/kg body weight) and artificially ventilated (fraction of inspired oxygen 1.0) using an Amsterdam infant ventilator MK III (Hoek Loos Co., Schiedam, The Netherlands) set in the volume-controlled mode. Tidal volume was set to 8 mL/kg body weight at a rate of 60 breaths/min. Severe respiratory failure was induced by lung lavage as follows: NaCl 0.9% (35 mL/kg body weight, at a temperature of 38°C) was injected slowly endotracheally and gently withdrawn and this lavage was repeated four times with an interval of 5 min. The total lavage procedure took 25 min. While the lung lavage procedure was being performed, tidal volume was increased from 8 to 10 mL/kg body weight with a concomitant stepwise increase in PEEP from 0 at the start to 10 cm $H_2O$ at the end of the last lung lavage. Thirty min after the lung lavage, the PEEP was decreased to 5 cm $H_2O$. Surfactant was administered endotracheally in six rabbits 45 min after the lung lavage procedure. We used Curosurf (Chiesi Co., Parma, Italy), a phospholipid fraction isolated from pig lungs (9). This phospholipid fraction, which also contained 1–2% of the hydrophobic surfactant-associated proteins, SP-B and SP-C, was suspended in saline and given at a dose of 100 mg phospholipids/kg body weight. The phospholipid concentration of Curosurf is 80 mg/mL. During the instillation procedure, the position of the animals was changed according to Adams *et al.* (10) so that 30% of the total dose was administered to the right lower part of the lung, 30% to the left lower part, 20% to the left upper part, and 20% to the right upper part. The same procedure was followed in the six control animals except that they did not receive any surfactant or its vehicle. The total time of artificial ventilation after surfactant treatment was 4 h: 2 h after surfactant instillation we started a weaning procedure in all animals; the PEEP was decreased to 2.5 cm, 1 h thereafter the PEEP was discontinued, and in the final hour of artificial ventilation the fraction of inspired oxygen was stepwise lowered to 0.21. No additional pancuronium bromide was administered after this point to allow the rabbits to resume breathing spontaneously. After 30 min of spontaneously breathing in room air, the animals were killed with an overdose of pentobarbital.

*Lung function.* Blood samples for the determination of arterial $P_{O_2}$, $P_{CO_2}$, and pH were drawn from the left carotic artery. Samples were analyzed using an ABL II blood gas analyzer (Radiometer Co., Copenhagen, Denmark).

After completion of the experiments, pressure-volume characteristics of the excised lungs were obtained using the following

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procedure: The lungs were deflated in a vacuum jar and then placed in an air-tight box and the trachea was connected to a spirometer outside the box. Volume and pressure changes were recorded over a 20-min period in which the pressure in the box was first lowered to −30 cm $H_2O$, followed by a rise to atmospheric pressure. Static compliance at 30 cm $H_2O$, the stability index according to Gruenwald (11), and the expansion index according to Clements *et al.* (12) were determined from these measurements.

*Preparation and selection of MAb.* Immunization of BALB/c mice was performed with Curosurf by intraperitoneal injection in complete Freunds' adjuvant, followed after 1 mo by a booster given by intravascular injection. Four d later, the spleens of the animals were removed and a cell suspension prepared. The hypoxanthine phosphoribosyltransferase negative myeloma cell line X63 was used as the fusion partner, using the fusion procedure of Kennett (13) with some modifications (14). The fusion mixture was divided over four 96-well cluster trays, which yielded 10–20 hybridomas per well. These hybridomas were recloned and the supernatants were screened for the desired properties: reaction with the porcine SP-B or SP-C and no cross-reaction with the rabbit surfactant-associated proteins.

The screening procedure was carried out directly on frozen tissue specimens from normal pigs, with rabbit lung tissue as a negative control (14). In short, tissue sections were fixed in acetone for 10 min, air dried, and washed in PBS. Subsequently, undiluted hybridoma culture supernatant was pipetted on top of the wet sections. As a second step reagent, horseradish peroxidase-conjugated rabbit anti-mouse Ig (Dakopatts, Copenhagen, Denmark) was used, and 3-amino-9-ethyl-carbozole was the chromogen in the subsequent peroxidase reaction. A positive reaction was indicated by a reddish brown deposit. The specimens were counterstained with hematoxylin. The results were evaluated by light microscopy. Out of the 400 clones that were screened, one clone produced a supernatant that reacted with the alveolar surface of pig lung and not with rabbit lung. In the porcine lung, we found a clear brown staining of clumped material along the walls of the patent airspaces and not in the interstitium. Also, no reaction could be observed intracellularly. The supernatant containing this MAb was named PORSU-1 and used in our study.

The reaction specificity of PORSU-1 was assessed by an ELISA (sensitivity in the ng range), using two isolated porcine surfactant-associated proteins as substrate (15). These proteins are hydrophobic and consist of an 8.7-kD polypeptide (SP-B) (16) and a 4.2-kD lipopeptide (SP-C) (17). The proteins were solubilized in methanol and put into 96-well vinyl assay plates (Costar, Badhoevedorp, The Netherlands) at a concentration of 10 ng protein/well. Thereafter, the methanol was evaporated under nitrogen. After blocking excess protein binding sites in the coated wells by incubation with PBS containing 5% (vol/vol) FCS, PORSU-1 or an irrelevant MAb was added. Any binding of the surfactant-associated proteins with PORSU-1 or with the irrelevant MAb was visualized with a second step antibody preparation, horseradish peroxidase-conjugated rabbit anti-mouse Ig (Dakopatts) and orthophenyl diamine as a chromogen. All antibody incubations took 90 min and between the different steps washing was performed with PBS. A clearly positive staining was only seen when PORSU-1 was added to the plates that were coated with the 4.2-kD lipopeptide. In this way, the specificity of PORSU-1 for porcine SP-C was established. The irrelevant MAb was not reactive with the 4.2-kD lipopeptide nor with the 8.7-kD polypeptide.

*Immunohistochemical staining of lung tissue specimens.* Frozen lung tissue blocks that were stored at −80°C were cut into 6-$\mu$m thick sections. The area around the tissue was dried and the tissue was fixed for 10 min in 0.3% $H_2O_2$ in methanol. Twenty-five $\mu$L of PORSU-1 was pipetted on top of the wet sections. After incubation for 30 min in a humidified atmosphere at room temperature, the specimens were washed three times in PBS for 5 min, followed by the same staining technique as was used in the selection procedure of the MAb. For light microscopic study, the sections were mounted in Kaiser glycerol gelatin (Merck, Darmstadt, West Germany).

*Quantification of distribution of instilled surfactant.* The lung tissue specimens were studied by light microscopy at a magnification of 100×. A 42-point multipurpose counting grid (18) was placed in the ocular of the microscope. Using this grid, it was possible to score the presence of surfactant in the different structures of the microscopic specimen: each of the 42 points was identified as to structure (aerated alveoli, collapsed alveoli, bronchi, blood vessels, and cartilage) and the presence of surfactant. "Surfactant positive points" were counted for each structure in the specimens. These points were counted in patent airspaces and in collapsed areas separately.

To determine the number of readings per specimen that were required to obtain a representative score of the presence of surfactant in the lung tissue, 10 successive readings on a specimen were performed. The score of the surfactant positive points of each reading was averaged with the score of the previous reading. The average of the summated scores and the number of the readings were plotted on an average summation graph (19). After five readings, the average of the mean score was not changed by the summation of the 6th and subsequent readings. We therefore performed five readings per lung specimen of each sample site of all animals. Consequently, 2100 points were scored for each animal.

*Statistical analysis.* All data are reported as mean ± SD unless stated otherwise. Differences of means were tested by the Mann-Whitney U-test. A $p$ value of $\leq 0.05$ was considered to be statistically significant.

## RESULTS

*Blood gases.* Values of arterial $P_{O_2}$, $P_{CO_2}$, and pH are shown in Figure 1. Surfactant instillation resulted in a rise in $P_{O_2}$ from $10.5 \pm 4.1$ to $47.2 \pm 11.3$ kPa ($p < 0.05$) and a decrease in $P_{CO_2}$ from $5.5 \pm 0.5$ to $4.3 \pm 0.5$ kPa ($p < 0.05$). In control animals, no change in arterial $P_{O_2}$ was observed and during the whole experiment the arterial $P_{O_2}$ remained significantly lower. When the PEEP was lowered, arterial $P_{CO_2}$ gradually increased in the control animals with a concomitant fall in pH and at a PEEP of 0 cm $H_2O$ all animals died. The surfactant-treated animals reestablished spontaneous breathing, and in room air they maintained normal blood gases ($P_{O_2}$ $6.7 \pm 2.2$, $P_{CO_2}$ $5.4 \pm 1.0$ kPa, and pH $7.39 \pm 0.04$).

*Pressure-volume characteristics of lung.* In Table 1, the static lung compliance, lung stability according to Gruenwald (11), and expansion index according to Clements *et al.* (12) are presented.

*Quantification of distribution of instilled surfactant.* SP-C was visible in the lung sections of all surfactant-treated animals and not in the controls. It was found focally in aerated lung parts filling up the entire alveolar space or as a condensed layer along alveolar walls, but predominantly in collapsed alveolar spaces. SP-C was found in less than 15% of the alveoli and less than 1% of the bronchi. There were large variations in the presence and distribution pattern of SP-C between the treated rabbits. The amount of SP-C in the different parts of the lung is presented in Figure 2.

## DISCUSSION

The aim of the study was to determine the distribution of endotracheally instilled surfactant in lung-lavaged rabbits. We hypothesized that a natural component of surfactant would be the best indicator for the localization of surfactant. We therefore chose the small hydrophobic polypeptide to use as a "surfactant marker." Although we have no definite proof, we assume that this surfactant polypeptide is not separated easily from the phos-

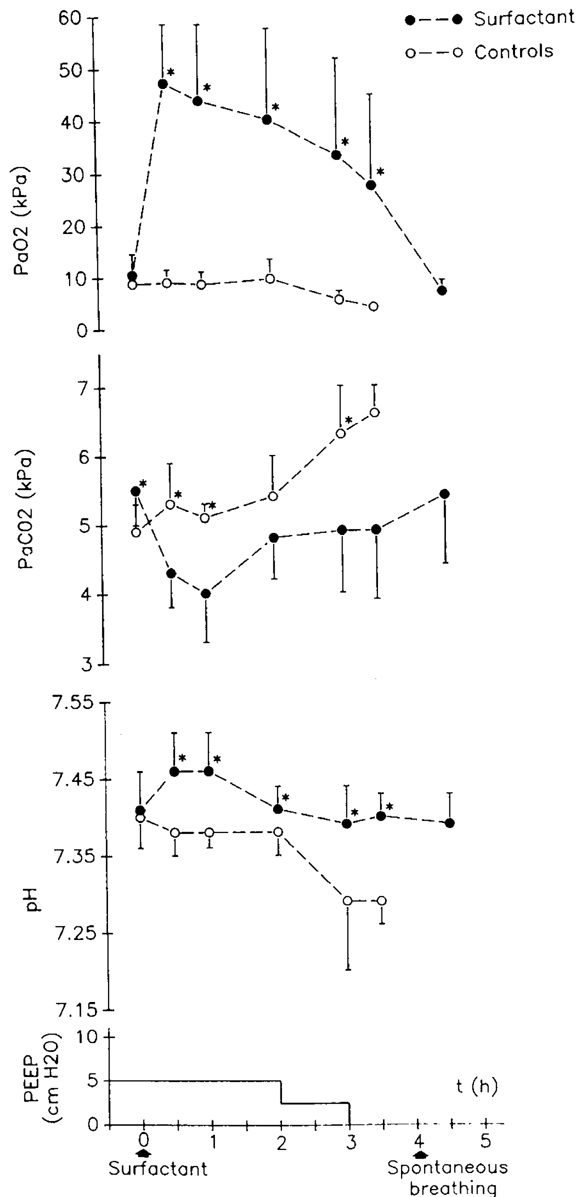


Fig. 1. Arterial $P_{O_2}$, $P_{CO_2}$, and pH of lung-lavaged rabbits before and after the endotracheal administration of porcine surfactant. Symbols for the surfactant-treated and control animals are given in the figure. The gradual decrease of PEEP is given in the lower panel.

Table 1. *Static lung compliance in vitro, stability index according to Gruenwald (11), and expansion index according to Clements et al. (12) (mean ± SD) of lung-lavaged rabbits of which six had been treated with porcine surfactant and six served as controls*

| Group | Static compliance ($mL/cm\ H_2O/kg$ body wt) | Stability index (mL/mL) | Expansion index (%) |
|---|---|---|---|
| Surfactant-treated rabbits ($n = 6$) | 0.66 ± 0.10 | 1.08 ± 0.15 | 66 ± 11 |
| Controls ($n = 6$) | 0.34 ± 0.17 | 0.77 ± 0.10 | 41 ± 3 |
| $p$ value | 0.05 | <0.05 | <0.05 |

pholipid fraction because it has a high affinity for the phospholipids (20) and special techniques are required to separate it from the surfactant lipids (15).

Other investigators have raised polyclonal antisera to detect surfactant apoprotein in lung tissue and isolated type II cells (21–23). In this study, we prepared a MAb that reacted specifically

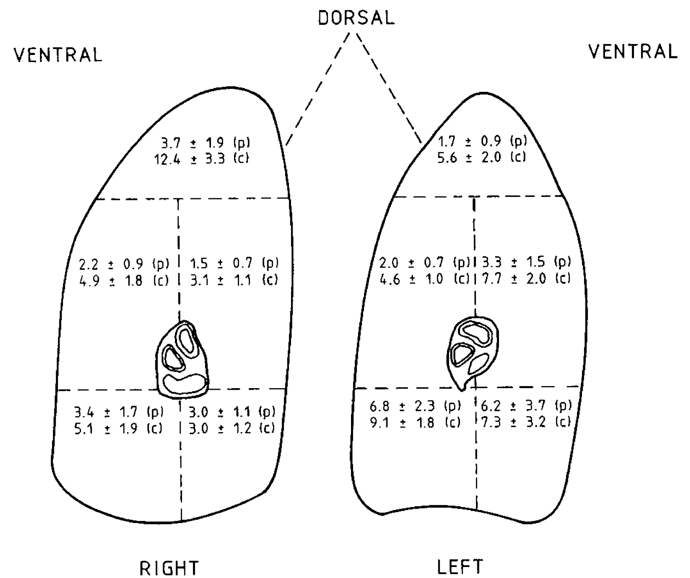


Fig. 2. Relative distribution of porcine SP-C over patent ($p$) and collapsed ($c$) areas in the different lung parts, expressed as number of surfactant positive points (mean ± SEM), in lung-lavaged rabbits that were treated with porcine surfactant endotracheally.

with the 4.2-kD surfactant lipopeptide (SP-C) of the porcine surfactant preparation but not with the endogenous rabbit surfactant. We showed that surfactant treatment of the lung-lavaged rabbits was effective in improving lung function. We therefore assumed that if SP-C remained closely associated with the instilled surfactant phospholipids we would find a uniform distribution of SP-C along the alveolar lining of most of the lung. However, the results of the immunohistochemical staining of the lung tissue clearly demonstrated that this was not the case. We found a large variation in the presence and distribution of the SP-C between the surfactant-treated rabbits. Surprisingly, SP-C was localized in less than 15% of the alveolar spaces, yet it was possible to wean the rabbits off the artificial ventilation, with subsequent spontaneous breathing in room air. Inhomogenous distribution of substituted surfactant phospholipids has been experimentally shown before (6, 24). Recently, we and others have shown in rabbits that the distribution is dependent on the volume of the instilled surfactant (25, 26). In this study, the nonuniform type of lung injury induced by repeated lung lavage might have contributed to the inhomogenous distribution of SP-C, but it is possible that a part of the instilled surfactant had been cleared from the lung.

Clearance of surfactant from the lung takes place via the airways, by phagocytosis, enzymatic degradation within the alveolus, and transport by lymphatic ducts, but the major pathway probably is uptake by the type II alveolar cells, with subsequent reutilization (27). Uptake of surfactant phospholipids by alveolar cells was demonstrated for endotracheally administered tracer amounts of surfactant (4, 28–31) and recently for treatment doses of surfactant (8, 32). Bartussio *et al.* (33) demonstrated in 3-d-old rabbits that SP-C is taken up by the lung tissue similarly to phospholipids (33). These authors showed that the uptake of SP-C is a rapid process.

If uptake of SP-C along with the phospholipids occurred also in our animals, this might explain the small amount of porcine surfactant polypeptide that we could localize 4½ h after instillation. As we stated above, there was sufficient alveolar surfactant during the spontaneous breathing, after weaning from the artificial ventilation, and it was possible that after uptake of the instilled porcine surfactant the phospholipids were resecreted into the alveolar space in a way similar to endogenous surfactant (29, 30). If this was true, then the porcine SP-C was not resecreted

along with the phospholipids and therefore metabolized via another still unknown pathway.

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