

Decrease of the surface fraction of surfactant proteins containing Clara cells and type II pneumocytes in a rat asthma model

Andreas Schmiedl^{a,b,*}, Thomas Tschernig^b, Frank Brasch^c,
R. Pabst^b, Gerhard Bargsten^a

^aDepartment I, Centre of Anatomy, Hannover Medical School, Hannover, Germany

^bDepartment II, Centre of Anatomy, Hannover Medical School, Hannover, Germany

^cInstitute of Pathology, University Hospital Bergmannsheil, Bochum, Germany

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Abstract

In asthma surfactant proteins (SP) might differ in distribution and composition and thus play a role in pathophysiology of this disease. Therefore, the well-established animal model of ovalbumin sensitized and challenged rats were used to study the distribution of surfactant proteins in Clara cells and type II pneumocytes. Serial sections of paraffin embedded lung tissue were sequentially immunostained by the avidin–biotin–peroxidase complex (ABC) technique. Antisera against SP-A, SP-B and Clara cell specific protein (CC10) were used. We determined stereologically the surface fraction of immunolabelled cells and semiquantitatively the percentage of test fields containing labelled alveolar macrophages. In allergen sensitized and provoked rat lungs: (1) the surface fraction of SP-A and SP-B positive Clara cells was significantly reduced, (2) the surface fraction of Clara cells stained with CC10 was coincided with controls, (3) the surface fraction of SP-A and not of SP-B positive type II pneumocytes decreased significantly, (4) a significantly higher percentage of test fields with SP-A labelled alveolar macrophages was evaluated. Thus, in this animal model of asthma the inflammatory process after allergen challenge is accompanied by alterations in the distribution patterns of SP in Clara cells and type II pneumocytes.

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Introduction

Pulmonary surfactant, a highly surface active complex of phospholipids and apoproteins (Clements, 1961; Clements et al., 1961; Hawgood and Clements, 1990;

Johansson et al., 1994) covers the air-liquid interphase of alveoli and small conducting airways (Bastacky et al., 1995; Gil and Weibel, 1971). Pulmonary surfactant consists of an intraalveolar and intracellular surfactant pool. Intraalveolar surfactant is present in the hypophase of alveolar and bronchiolar surface and reveals active and inactive subtypes of surfactant lipids and the surfactant specific apoproteins (SP) A, B and C, which are necessary for structural and functional integrity as well as stabilization of active surfactant components (Griese, 1999; Hawgood and Clements, 1990). The

*Corresponding author. Zentrum Anatomie, Abt. Anatomie II-4120-, Medizinische Hochschule Hannover, Carl-Neuberg-Straße 1, 30625 Hannover, Germany. Tel. +49 511 532 29 00; fax: +49 511 532 29 48.

E-mail address: schmiedl.andreas@mh-hannover.de (A. Schmiedl).

hydrophobic surfactant proteins SP-B and SP-C have only biophysical functions and are necessary for surfactant homeostasis (Goerke, 1998; Griese, 1999; Johansson et al., 1994). The hydrophilic SP-A has additional functions in innate immunity (Wright, 1997). The so-called SP-D has only immunomodulatory effects (Wright, 1997). Type II pneumocytes synthesize, store, secrete and to a large extent reuptake surfactant phospholipids and surfactant proteins (Chevalier and Collet, 1972; Voorhout et al., 1993). SP-A and SP-D are also detected in Clara cells of bronchioli (Crouch et al., 1992; Voorhout et al., 1992; Walker et al., 1986; Williams et al., 1988). Also, SP-B distribution has been described in Clara cells (Horowitz et al., 1991; Phelps and Floros 1988; Stahlman et al., 1992). Clara cells are nonciliated, nonmucous, secretory cells of the bronchiolar epithelium of distal pulmonary airways (Widdicombe and Pack, 1982). Clara cell secretory products may be responsible for the determination of the chemical and physical properties of the lining of small airways (Widdicombe and Pack, 1982). Clara cells are known to secrete a variety of proteins, whereas the Clara cell specific protein 10 (CC10), which is identical with human uteroglobin, is the major secretory protein (Singh and Katyal, 2000), which may have immunomodulatory and anti-inflammatory effects (Arsalane et al., 2000; Mukherjee et al., 1988; Shijubo et al., 1999).

An intact pulmonary surfactant system is necessary for normal respiratory function. Various diseases such as respiratory distress syndrome, pneumonia or ischemia/reperfusion injury are accompanied by surfactant abnormalities (Fehrenbach et al., 2000; Griese, 1999; Lewis et al., 1997; Ochs et al., 2000). Additionally, evidence has been increased for involvement of surfactant alterations in asthmatics during the last years (Hohlfeld et al., 2002; Hohlfeld et al., 1999; Jarjour and Enhorning, 1999; Liu et al., 1995; Liu et al., 1997). Recent studies have shown that alterations and inflammation of small airways and parenchyma of the lung contribute to asthma pathogenesis (Kraft, 1999; Kraft et al., 1996; Liu et al., 1995; Schmiedl et al., 2003).

The surfactant proteins, particularly SP-A and SP-D influence adaptive immune functions and allergic inflammation in vivo (Borron et al., 1996; Cheng et al., 1998; Wang et al., 1996, 1998). Immunomodulatory functions of SP-A and CC-10 are described. However, there is contradictory information about surfactant protein alterations in asthma. On the one side decreased levels of SP-A and SP-D were found in bronchoalveolar lavage fluid from sensitized mice with mite allergen-induced airway inflammation (Wang et al., 2001). On the other side, allergen challenge of sensitized mice has induced an increase of SP-A and SP-D immunostaining in nonciliated airway epithelial cells, while SP-B immunostaining was unchanged (Haley et al., 2002). In a murine asthma model decreased levels of SP-B and

SP-C protein and m-RNA as well as increased SP-D levels and unchanged SP-A levels were determined (Haczku et al., 2001). Finally, overexpression of IL-4 has led to increased levels of SP-A and SP-B in bronchoalveolar fluids and lung homogenates, whereas staining of type II pneumocytes was unchanged (Jain-Vora et al., 1997). Therefore, we used an immunohistochemical and stereologic approach to gain information about the distribution of CC10 in Clara cells as well as of SP-A and SP-B in type II pneumocytes and Clara cells in the Brown Norway (BN) rat model of asthma.

Material and methods

Male Brown Norway rats (mean weight 260 g) were kept under specific pathogen-free conditions and had ad libitum access to food and water. The animals were divided into two groups. Four rats served as controls and three rats were used for asthma induction.

Asthma model

Brown Norway rats were sensitized and challenged according to the asthma model of Brown Norway rats (Schuster et al., 2000):

(1) First sensitization:

1 mg ovalbumin + 200 mg Al(OH)₃ in 1 ml 0.9% NaCl solution, applied subcutaneously (sc).
 5×10^9 heat killed *Bordetella pertussis* bacilli (kindly donated by Chiron Behring, Marburg, Germany) in 0.4 ml 0.9% NaCl solution, given intraperitoneally (i.p.).

(2) Second sensitization with the same allergens one week later.

(3) Airway challenge (provocation) one further week later:

inhalation of an aerosol of 5% ovalbumin/saline solution (0.25 l/min).

To this Brown Norway rats were placed in a Plexiglas tube for 15 min. A flexible latex mask was placed over the nose and connected to a nebulizer (Pariboy; Ritzau, Starnberg, Germany).

Twenty-four hours after challenge rats were processed for light microscopy.

Control rats were either sensitized with ovalbumin but sham challenged by NaCl inhalation to imitate provocation ($n = 2$) or received H₂O (sham sensitization) and challenged with ovalbumin ($n = 2$). In both cases control rats were exposed to the same procedure as test rats but the animals were not both sensitized and challenged with ovalbumin, which is necessary for asthma induction.

Successful asthma induction by sensitization and challenge with ovalbumin was established by determination of a significant increase of leucocytes especially eosinophils in BAL (Schuster et al., 2000) and lung parenchyma 24 h after challenge as well as by evaluation of an significant increase of lung resistance immediately after challenge of sensitized rats (Schmiedl et al., 2003).

Fixation protocol

Control and challenged rats were anesthetized with ether. After opening the abdominal wall the animals were killed by aortic exsanguination and the lungs were dissected from the chest and fixed by instillation in a specific instillation device with 4% paraformaldehyde buffered in 0.1 M phosphate buffer. The instillation device was developed in our laboratory and produced in the technical department of the medical school. Specimens of lung tissue were dehydrated with a graded ethanol series and embedded in paraffine (Paraplast). Serial sections of lung tissue blocks were cut at 5 µm thickness and mounted on glass slides. After deparaffinization with xylene and rehydration, the sections were conventionally stained (*routine-stained*) with hematoxylin and eosin or used for immunostaining.

Antisera

The Clara cells were identified with a polyclonal rabbit antiserum against the Clara cell specific 10-kDa protein termed CC-10 (1:400). CC10 was kindly provided by Prof. G. Sufke, Institute for Molecular Biology and Tumor Research, University of Marburg, Germany. The polyclonal rabbit antisera against the surfactant proteins SP-A (1:400) and SP-B (1:400) were kindly provided by the Laboratories of S. Hawgood; and of J.A. Whitsett.

Immunohistochemical protocol

Serial sections of paraffine embedded lung tissue were sequentially immunostained for CC-10, SP-A, and SP-B. After removal of the paraffin wax, the sections were immunostained by the avidin–biotin–peroxidase complex (ABC) technique (Hsu et al., 1981). Briefly, following incubation with 4% nonimmune goat serum (30 min), the sections were incubated in appropriately diluted first antisera (24 h at 4 °C). Subsequently, biotinylated goat anti-rabbit IgG (1:100; 30 min) and a preformed streptavidine/biotin–peroxidase complex (30 min; both obtained from Jackson Immuno Research, West Grove, USA) served as the second and third layer of the protocol (final concentration of streptavidin 5 µg/ml and of biotin–peroxidase 0.7 µg/ml). Peroxidase activity was demonstrated by a solution containing

0.7 mM diaminobenzidine (DAB) and 0.002% H₂O₂ in 0.01 M PBS buffer, pH 7.3.

Sections were examined without counterstaining in an Orthoplan microscope (Leitz; Wetzlar, Germany) by brightfield and interference illumination. Specificity controls to exclude methodological or antibody non-specificities were performed.

Sampling

For scanning of paraffin sections we used the systematic quadrats subsampling method to generate test fields over the whole section (Muller et al., 1981). Started at a random position outside of the section, test fields were selected by moving the microscope stage at fixed intervals. Thus, each area of the section had the same chance of being evaluated.

Morphometry

Using the intersection counting method (Weibel et al., 1981) the following parameters were determined: relative surface of CC10 positive labelled Clara cells in bronchiolar epithelium (%); relative surface of SP-A positive labelled Clara cells in bronchiolar epithelium (%); relative surface of SP-B positive labelled Clara cells in bronchiolar epithelium (%); relative surface of SP-A positive labelled type II pneumocytes in alveolar epithelium (%); relative surface of SP-B positive labelled type II pneumocytes in alveolar epithelium (%). Additionally, the percentage of test fields exhibiting SP-A positive labelled alveolar macrophages (%) was determined.

Statistics

Differences of the stereological parameters between the two groups were tested for significance ($p < 0.05$) by the nonparametric Kruskal–Wallis 1-Way ANOVA on Ranks test. Mean values were given as $\pm$ SEM. All statistical analyses were performed using the Sigma Stat 2.0 and Sigma Plot 3.0 software programs (Jandel Scientific, Erkrath, Germany).

Results

Bronchiolar epithelium

The nonciliated Clara cells are unequivocally identifiable by their dome shaped projections into the lumen between ciliated epithelial cells of bronchioles in both groups (Fig. 1a, b). In the control and asthmatic group CC10 was detected only in bronchiolar Clara cells (Fig. 1b). The relative surface of CC10 positive Clara

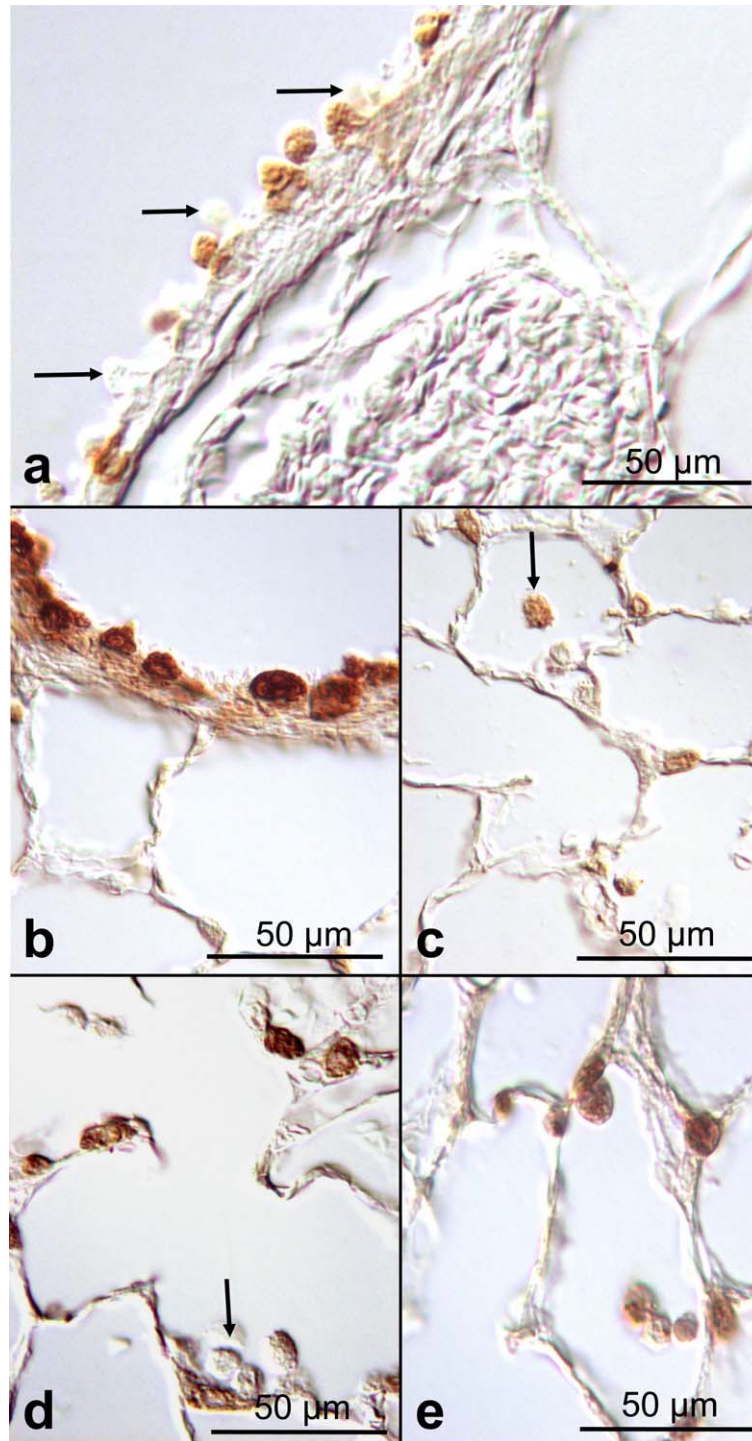


Fig. 1. Immunohistology: (a) Bronchiolar epithelium covered with Clara cells. Besides SP-A positive Clara cells, Clara cells exhibiting no labelling for SP-A are visible (arrows, interference microscopy). (b) Bronchiolar epithelium with CC-10 positive Clara cells. Alveolar epithelium shows no CC-10 positive staining (interference microscopy). (c) Alveolar epithelium with SP-A positive type II pneumocytes in lungs of the control group. Macrophages (arrows) were sporadically stained (interference microscopy). (d) Alveolar epithelium with SP-B positive type II pneumocytes in lungs of the control group. Macrophages without SP-B labelling are visible (arrow, interference microscopy). (e) Alveolar epithelium with SP-A positive type II pneumocytes in lungs of asthmatic rats. Furthermore, SP-A positive macrophages are seen in alveoli (interference microscopy).

cells was similar in the control and asthma group (46.3 ± 2.5 vs. 45.1 ± 2.0). The surfactant proteins SP-A and SP-B were detected in Clara cells of the bronchiolar

epithelium (Fig. 1a) as well as in type II pneumocytes of the alveolar epithelium (Fig. 1c–d). However, not all Clara cells were labelled with antibodies against

surfactant proteins (Fig. 1a). About 26% of the bronchiolar epithelial surface comprised of SP-A or SP-B positive Clara cells in the control group. Compared to the control group, the asthmatic group exhibited a significant decrease of bronchiolar surface associated with SP-A positive Clara cells from 26% to 14% (Fig. 2a). The bronchiolar surface fraction covered with SP-B positive Clara cells showed also a significant decrease in the asthmatic group compared to controls (Fig. 2a).

Alveolar epithelium

CC10 was never detected in alveolar epithelial cells in both groups. Type II cells of the alveolar epithelium exhibited SP-A specific labelling (Fig. 1c) as well as SP-B specific labelling (Fig. 1d) in controls and asthmatic rats. In the control group macrophages were only sporadically stained for SP-A (Fig. 1c). After asthma induction SP-A labelled macrophages were seen more frequently (Fig. 1e). Macrophages labelled with SP-B specific antibodies were only rarely seen in both groups. Macrophages without SP-B labelling predominante (Fig. 1d).

The surface fraction of SP-A stained type II pneumocytes amounted to 9% in controls and decreased significantly to 6% after allergen challenge in the asthmatic group (Fig. 2b). The relative surface of alveolar epithelium covered with SP-B positive type II pneumocytes amounted to about 9% in the control and asthmatic group (Fig. 2b).

In controls 15% of test fields exhibited SP-A positive macrophages (Fig. 2c). In asthmatic rats a significantly higher percentage of test fields with SP-A labelled alveolar macrophages was evaluated (36%).

Discussion

In this study, the distribution of SP was determined using quantitative immunohistochemistry. We found significant differences in the distribution patterns of SP containing Clara cells and type II pneumocytes after challenge of sensitized Brown Norway rats. So, the fraction of SP-A and SP-B positive Clara cells has decreased, the fraction of SP-A positive type II pneumocytes has decreased and test fields containing SP-A positive alveolar macrophages have increased (cf. Fig. 2a–c). However, the fraction of SP-B positive type II pneumocytes shows no alterations. Also, there are no alterations of the relative surface of CC-10 positive Clara cells (cf. results). These results support the hypothesis that surfactant specific apoproteins, predominantly SP-A play a role in the allergic inflammation in asthma bronchiale. SP are involved in two critical

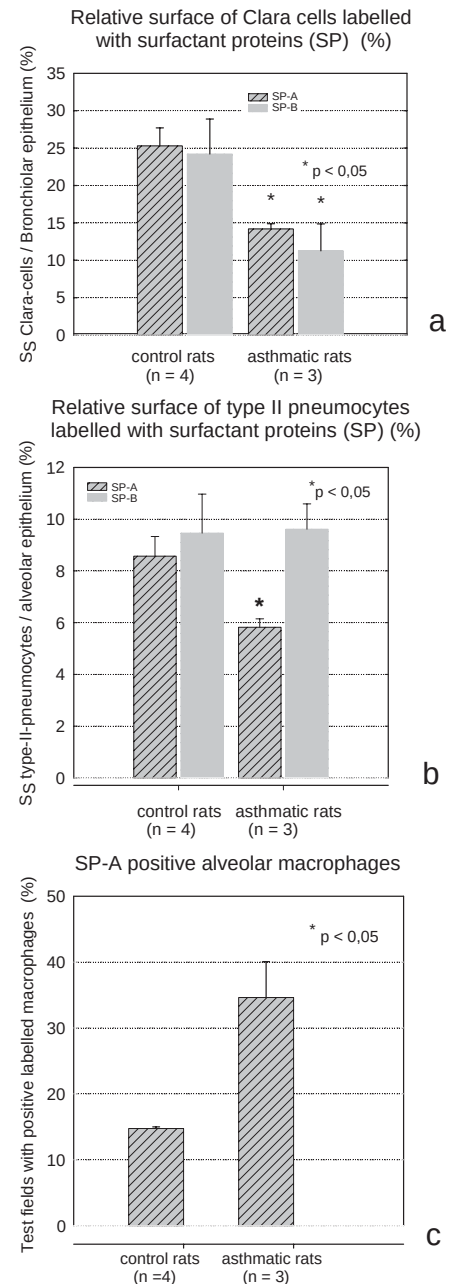


Fig. 2. Morphometry: (a) Surface fraction of SP-A or SP-B positive Clara cells related to bronchiolar epithelium. After asthma induction SP-labelling of the bronchiolar surface was significantly reduced. The used antibody dilution labelled significantly less Clara cells in the bronchiolar epithelium. * = Significant differences compared to control rats. (b) Fraction of type II pneumocytes labelled with SP related to alveolar epithelial surface. After asthma induction the surface fraction of SP-A labelled type II pneumocytes was significantly reduced compared to controls. However, the surface fraction of SP-B labelled type II pneumocytes showed no differences in both groups. * = Significant differences compared to control rats. (c) Percentage of test fields exhibiting SP-A positive alveolar macrophages. After asthma induction significant more test fields contain SP-A positive alveolar macrophages compared to controls.

processes in the lung, maintenance of alveolar surfactant function and host defense (Hawgood and Clements, 1990; Hohlfeld et al., 2002; Wang et al., 2001; Wright, 1997). SP-A as well as SP-D interact with cell surfaces of microorganism enhancing phagocytosis by macrophages or monocytes (Crouch and Wright, 2001) and could modulate immune cell functions (Wright, 1997). The role of these SP on adaptive immun functions and the allergic inflammation in asthma has been shown by in vitro (Allen et al., 1999; Madan et al., 1997a; Wang et al., 1996) and in vivo studies (Madan et al., 1997b). Changes in surfactant proteins during allergen-induced inflammatory responses are found by numerous authors. However, there are more or less discrepancies in the published results depending on differences in animal models or timing. So, in a murine model of allergic pulmonary inflammation increased immunostaining of SP-A and SP-D was found in nonciliated epithelial cells of noncartilaginous airways after aerosolized challenge (Haley et al., 2002). In contrast, SP-B immunostaining was unchanged. In a further murine model, challenging with *Aspergillus fumigatus* resulted in unaffected levels of tissue mRNA and protein of SP-A, but in decreased SP-B and SP-C messenger RNA expression (Haczku et al., 2001). In a dust mite allergen-induced model of asthma marked reductions of SP-A and SP-D in BALF (bronchoalveolar fluid) were determined by ELISA (Wang et al., 2001). Furthermore, the immunohistochemical findings of these authors exhibit a significant decrease in SP-A staining of alveolar walls, and a focal intense staining for SP-A in terminal bronchiolar epithelium where eosinophil infiltration occurred (Wang et al., 2001). Others have found that allergen challenge results in a downregulation of the human SP-C promotor, whereas no changes in the SP-B m-RNA expression, but upregulation in SP-A and predominantly SP-D were seen (Mishra et al., 2001). Lung sections and lung homogenates of transgenic mice with IL-4 expression showed increased levels of surfactant proteins A and B, and in contrast, a decreased or absent SP-B expression in hypertrophic epithelial cells of the conducting airways (Jain-Vora et al., 1997). Also, Haczku et al. have found decreased bronchoalveolar levels of SP-B protein and SP-B m-RNA after intratracheal administration of IL-4 to aspergillus-sensitized mice (Haczku et al., 2002). Our results evaluated on a rat asthma model are partly in accordance with the published data of the mentioned authors. The significant decrease in the fraction of SP-A containing Clara cells and type II pneumocytes of the alveolar epithelium may be due to a decreased synthesis or an increased secretion of SP-A in our asthma model. So, Liu and coworkers have found a significant reduced synthesis of phospholipids in type II pneumocytes isolated from immunized and challenged animals (Liu et al., 1997). The significant increase of SP-A positive macrophages may be on the

one hand an indirect indication of an intensified uptake of increased alveolar SP-A, possibly be associated with allergen, on the other hand an increase of the number of alveolar macrophages.

Our stereological and immunohistochemical findings suggest alterations in the SP-A metabolism after allergen challenge. The decrease of the fraction of SP-A positive cells and the increase of test fields with SP-A positive alveolar macrophages suggest an increased secretion of SP-A. However, our data do not allow a final evidence about an increase or decrease of SP-A metabolism. Therefore, in situ hybridization and ELISA studies are necessary.

In contrast to the findings of others (Arsalane et al., 2000; Shijubo et al., 1999), the surface fraction of CC-10 positive bronchiolar cells was comparable in both groups. The differences with others may be the result of different evaluation protocols and lung inflammation models. So, Arsalane et coworkers determined a marked decrease of the secretion and synthesis of CC-10 during LPS-induced lung inflammation using Northern blot analysis and planimetry (Arsalane et al., 2000). An decrease of CC-10 producing cells was also found in resected lung specimens without cancer of patients suffering on chronic asthma (Shijubo et al., 1999).

In this study, we presented an immunohistochemical, stereological approach to get information about alterations in the distribution of SP-containing cells not only in bronchiolar epithelium but also in alveolar epithelium in a rat asthma model. Further investigations are necessary to clarify finally alterations in SP metabolism after asthma induction.

We suggest that alterations in the metabolism of surfactant proteins contribute to surfactant dysfunction combined with elevation of inactive surfactant subtypes.

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References

- Allen MJ, Harbeck R, Smith B, Voelker DR, Mason RJ. Binding of rat and human surfactant proteins A and D to *Aspergillus fumigatus* conidia. *Infect Immun* 1999;67: 4563–9.
- Arsalane K, Broeckaert F, Knoop B, Wiedig M, Toubreau G, Bernard A. Clara cell specific protein (CC16) expression after acute lung inflammation induced by intratracheal

- lipopolysaccharide administration. *Am J Respir Crit Care Med* 2000;161:1624–30.
- Bastacky J, Lee CY, Goerke J, Koushafar H, Yager D, Kenaga L, Speed TP, Chen Y, Clements JA. Alveolar lining layer is thin and continuous: low-temperature scanning electron microscopy of rat lung. *J Appl Physiol* 1995;79:1615–28.
- Borron P, Veldhuizen RA, Lewis JF, Possmayer F, Caveney A, Inchley K, McFadden RG, Fraher LJ. Surfactant associated protein-A inhibits human lymphocyte proliferation and IL-2 production. *Am J Respir Cell Mol Biol* 1996;15:115–21.
- Cheng G, Ueda T, Nakajima H, Nakajima A, Kinjyo S, Motojima S, Fukuda T. Suppressive effects of SP-A on ionomycin-induced IL-8 production and release by eosinophils. *Int Arch Allergy Immunol* 1998;117(Suppl. 1):59–62.
- Chevalier G, Collet AJ. In vivo incorporation of choline-3 H, leucine-3 H and galactose-3 H in alveolar type II pneumocytes in relation to surfactant synthesis. A quantitative radioautographic study in mouse by electron microscopy. *Anat Rec* 1972;174:289–310.
- Clements JA. Pulmonary edema and permeability of alveolar membranes. *Arch Environ Health* 1961;2:280–3.
- Clements JA, Hustead RF, Johnson RP, Gribetz I. Pulmonary surface tension and alveolar stability. *J Appl Physiol* 1961;16:444–50.
- Crouch E, Parghi D, Kuan SF, Persson A. Surfactant protein D: subcellular localization in nonciliated bronchiolar epithelial cells. *Am J Physiol* 1992;263:L60–6.
- Crouch E, Wright JR. Surfactant proteins a and d and pulmonary host defense. *Annu Rev Physiol* 2001;63:521–54.
- Fehrenbach A, Ochs M, Warnecke T, Wahlers T, Wittwer T, Schmiedl A, Elki S, Meyer D, Richter J, Fehrenbach H. Beneficial effect of lung preservation is related to ultrastructural integrity of tubular myelin after experimental ischemia and reperfusion. *Am J Respir Crit Care Med* 2000;161:2058–65.
- Gil J, Weibel ER. Extracellular lining of bronchioles after perfusion-fixation of rat lungs for electron microscopy. *Anat Rec* 1971;169:185–99.
- Goerke J. Pulmonary surfactant: functions and molecular composition. *Biochim Biophys Acta* 1998;1408:79–89.
- Griese M. Pulmonary surfactant in health and human lung diseases: state of the art. *Eur Respir J* 1999;13:1455–76.
- Haczku A, Atochina EN, Tomer Y, Cao Y, Campbell C, Scanlon ST, Russo SJ, Enhorning G, Beers MF. The late asthmatic response is linked with increased surface tension and reduced surfactant protein B in mice. *Am J Physiol Lung Cell Mol Physiol* 2002;283:L755–65.
- Haczku A, Atochina EN, Tomer Y, Chen H, Scanlon ST, Russo S, Xu J, Panettieri Jr. RA, Beers MF. *Aspergillus fumigatus*-induced allergic airway inflammation alters surfactant homeostasis and lung function in BALB/c mice. *Am J Respir Cell Mol Biol* 2001;25:45–50.
- Haley KJ, Ciota A, Contreras JP, Boothby MR, Perkins DL, Finn PW. Alterations in lung collectins in an adaptive allergic immune response. *Am J Physiol Lung Cell Mol Physiol* 2002;282:L573–84.
- Hawgood S, Clements JA. Pulmonary surfactant and its apoproteins. *J Clin Invest* 1990;86:1–6.
- Hohlfeld JM, Ahlf K, Enhorning G, Balke K, Erpenbeck VJ, Petschallies J, Hoymann HG, Fabel H, Krug N. Dysfunction of pulmonary surfactant in asthmatics after segmental allergen challenge. *Am J Respir Crit Care Med* 1999;159:1803–9.
- Hohlfeld JM, Erpenbeck VJ, Krug N. Surfactant proteins SP-A and SP-D as modulators of the allergic inflammation in asthma. *Pathobiology* 2002;70:287–92.
- Horowitz S, Watkins RH, Auten Jr. RL, Mercier CE, Cheng ER. Differential accumulation of surfactant protein A, B, and C mRNAs in two epithelial cell types of hyperoxic lung. *Am J Respir Cell Mol Biol* 1991;5:511–5.
- Hsu SM, Raine L, Fanger H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J Histochem Cytochem* 1981;29:577–80.
- Jain-Vora S, Wert SE, Temann UA, Rankin JA, Whitsett JA. Interleukin-4 alters epithelial cell differentiation and surfactant homeostasis in the postnatal mouse lung. *Am J Respir Cell Mol Biol* 1997;17:541–51.
- Jarjour NN, Enhorning G. Antigen-induced airway inflammation in atopic subjects generates dysfunction of pulmonary surfactant. *Am J Respir Crit Care Med* 1999;160:336–41.
- Johansson J, Curstedt T, Robertson B. The proteins of the surfactant system. *Eur Respir J* 1994;7:372–91.
- Kraft M. The distal airways: are they important in asthma? *Eur Respir J* 1999;14:1403–17.
- Kraft M, Djukanovic R, Wilson S, Holgate ST, Martin RJ. Alveolar tissue inflammation in asthma. *Am J Respir Crit Care Med* 1996;154:1505–10.
- Lewis JF, Novick RJ, Veldhuizen RAW, editors. Surfactant in lung injury and lung transplantation. R.G. Landes Company, 1997.
- Liu M, Wang L, Enhorning G. Surfactant dysfunction develops when the immunized guinea-pig is challenged with ovalbumin aerosol. *Clin Exp Allergy* 1995;25:1053–60.
- Liu M, Wang L, Holm BA, Enhorning G. Dysfunction of guinea-pig pulmonary surfactant and type II pneumocytes after repetitive challenge with aerosolized ovalbumin. *Clin Exp Allergy* 1997;27:802–7.
- Madan T, Eggleton P, Kishore U, Strong P, Aggrawal SS, Sarma PU, Reid KB. Binding of pulmonary surfactant proteins A and D to *Aspergillus fumigatus* conidia enhances phagocytosis and killing by human neutrophils and alveolar macrophages. *Infect Immun* 1997a;65:3171–9.
- Madan T, Kishore U, Shah A, Eggleton P, Strong P, Wang JY, Aggrawal SS, Sarma PU, Reid KB. Lung surfactant proteins A and D can inhibit specific IgE binding to the allergens of *Aspergillus fumigatus* and block allergen-induced histamine release from human basophils. *Clin Exp Immunol* 1997b;110:241–9.
- Mishra A, Weaver TE, Beck DC, Rothenberg ME. Interleukin-5-mediated allergic airway inflammation inhibits the human surfactant protein C promoter in transgenic mice. *J Biol Chem* 2001;276:8453–9.
- Mukherjee AB, Cordella-Miele E, Kikukawa T, Miele L. Modulation of cellular response to antigens by uteroglobin and transglutaminase. *Adv Exp Med Biol* 1988;231:135–52.

- Muller AE, Cruz-Orive LM, Gehr P, Weibel ER. Comparison of two subsampling methods for electron microscopic morphometry. *J Microsc* 1981;123:35–49.
- Ochs M, Fehrenbach H, Nenadic I, Bando T, Fehrenbach A, Schepelmann D, Albes JM, Wahlers T, Richter J. Preservation of intraalveolar surfactant in a rat lung ischaemia/reperfusion injury model. *Eur Respir J* 2000;15: 526–31.
- Phelps DS, Floros J. Localization of surfactant protein synthesis in human lung by in situ hybridization. *Am Rev Respir Dis* 1988;137:939–42.
- Schmiedl A, Hoymann HG, Ochs M, Menke A, Fehrenbach A, Krug N, Tschernig T, Hohlfeld JM. Increase of inactive intra-alveolar surfactant subtypes in lungs of asthmatic Brown Norway rats. *Virchows Arch* 2003;442:56–65.
- Schuster M, Tschernig T, Krug N, Pabst R. Lymphocytes migrate from the blood into the bronchoalveolar lavage and lung parenchyma in the asthma model of the brown Norway rat. *Am J Respir Crit Care Med* 2000; 161:558–66.
- Shijubo N, Itoh Y, Yamaguchi T, Imada A, Hirasawa M, Yamada T, Kawai T, Abe S. Clara cell protein-positive epithelial cells are reduced in small airways of asthmatics. *Am J Respir Crit Care Med* 1999;160:930–3.
- Singh G, Katyal SL. Clara cell proteins. *Ann N Y Acad Sci* 2000;923:43–58.
- Stahlman MT, Gray ME, Whitsett JA. The ontogeny and distribution of surfactant protein B in human fetuses and newborns. *J Histochem Cytochem* 1992;40:1471–80.
- Voorhout WF, Veenendaal T, Kuroki Y, Ogasawara Y, van Golde LM, Geuze HJ. Immunocytochemical localization of surfactant protein D (SP-D) in type II cells, Clara cells, and alveolar macrophages of rat lung. *J Histochem Cytochem* 1992;40:1589–97.
- Voorhout WF, Weaver TE, Haagsman HP, Geuze HJ, Van Golde LM. Biosynthetic routing of pulmonary surfactant proteins in alveolar type II cells. *Microsc Res Tech* 1993;26:366–73.
- Walker SR, Williams MC, Benson B. Immunocytochemical localization of the major surfactant apoproteins in type II cells, Clara cells, and alveolar macrophages of rat lung. *J Histochem Cytochem* 1986;34:1137–48.
- Wang JY, Kishore U, Lim BL, Strong P, Reid KB. Interaction of human lung surfactant proteins A and D with mite (*Dermatophagoides pteronyssinus*) allergens. *Clin Exp Immunol* 1996;106:367–73.
- Wang JY, Shieh CC, You PF, Lei HY, Reid KB. Inhibitory effect of pulmonary surfactant proteins A and D on allergen-induced lymphocyte proliferation and histamine release in children with asthma. *Am J Respir Crit Care Med* 1998;158:510–8.
- Wang JY, Shieh CC, Yu CK, Lei HY. Allergen-induced bronchial inflammation is associated with decreased levels of surfactant proteins A and D in a murine model of asthma. *Clin Exp Allergy* 2001;31:652–62.
- Weibel ER, Taylor CR, Gehr P, Hoppeler H, Mathieu O, Maloiy GM. Design of the mammalian respiratory system. IX. Functional and structural limits for oxygen flow. *Respir Physiol* 1981;44:151–64.
- Widdicombe JG, Pack RJ. The Clara cell. *Eur J Respir Dis* 1982;63:202–20.
- Williams MC, Hawgood S, Schenk DB, Lewicki J, Phelps MN, Benson B. Monoclonal antibodies to surfactant proteins SP28-36 label canine type II and nonciliated bronchiolar cells by immunofluorescence. *Am Rev Respir Dis* 1988;137:399–405.
- Wright JR. Immunomodulatory functions of surfactant. *Physiol Rev* 1997;77:931–62.