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Protective effect of pulmonary surfactant on elastase-induced emphysema in mice

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ABSTRACT: The aim of this study was to obtain some evidence of a protective role for pulmonary surfactant in the pathogenesis of emphysema.

Firstly, we developed a quick and easy method to treat mice with a series of intratracheal instillations. Subsequently, three groups of mice were treated as follows: two groups received intratracheal instillations with pancreatic elastase (1.8 mg·kg^{-1} BW) followed after 3, 48 and 96 h in one group (El/Surf group) by intratracheal administration of surfactant (100 mg phospholipid·kg^{-1} BW), and in the other group by instillations with saline (El/s group). The third group of control mice was treated with saline followed by three doses of surfactant (s/Surf group). After eight weeks, the mice were killed and emphysema was measured by calculating the mean linear intercepts (Lm) of airspaces. The Lm values in the different groups were statistically tested for differences by the Mann-Whitney test.

Instillation of pancreatic elastase (El/s group) resulted in an evenly distributed increase in Lm compared with the control group. Administration of surfactant in elastase-treated mice (El/Surf group) resulted in a statistically significant inhibition of airspace enlargement. Although the Lm in the El/Surf group was still higher than in the control group, analysis of histograms of Lm values per field of examination revealed that the Lm distribution in the former group was similar to that of the s/Surf group. The El/s group, on the contrary, showed the presence of many fields with enlarged air spaces. Repeated instillations with saline and/or surfactant had no effect on the Lm.

We conclude that the significant inhibition of elastase-induced airspace enlargement by surfactant treatment, reported here, fully supports our earlier hypothesis concerning a protective effect of pulmonary surfactant in the pathogenesis of emphysema.

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Pulmonary emphysema, a major public health problem, is characterised by destruction of alveolar walls and enlargement of the airspaces distal to the terminal bronchiole. To study the pathogenesis of emphysema, animal models have been developed [1].

It became evident that emphysema develops after intratracheal instillation of elastolytic enzymes, suggesting that degradation of elastine by these enzymes is an important step in the pathogenesis of emphysema. However, in addition to elastolytic enzymes and their reported inhibitors, evidence accumulates that other factors may play a role in the pathogenesis of emphysema. We hypothesised that the type II cell or its secretory product, pulmonary surfactant, may protect the lung against elastolytic enzyme-induced injuries [2].

We previously reported the incidence of lower numbers of type II cells in lungs of emphysema patients [2] and argued that these findings were in line with a decreased protective role of the type II cell or its secretory product, pulmonary surfactant, in the pathogenesis of emphysema. In the present study, we will focus on the latter aspect, *i.e.* the role of pulmonary surfactant in the development of emphysema. Pulmonary surfactant is a surface active material, composed of phospholipids (about 90%) and proteins, that forms a monolayer at the air liquid interface [3]. A major function of pulmonary surfactant is regulation of the surface tension, but there is also evidence that

surfactant plays a role in the immune defence of the lung and has antioxidant capacities (see reviews by VAN GOLDE *et al.* [3] and MORTON [4]). As a result of intensive research there is increasing understanding of the functions, components and metabolism of pulmonary surfactant, summarised in a number of recent reviews [4–8]. As mentioned previously [2], we believe that some of the known functions or properties of pulmonary surfactant may protect the lung parenchyma from emphysematous lesions induced by elastolytic enzymes. To investigate the role of pulmonary surfactant in the pathogenesis of emphysema, we developed a method to treat mice with a series of endotracheal instillations, and studied the effect of exogenous surfactant administration on pancreatic elastase-induced airspace enlargement.

Materials and methods

Animals and intratracheal instillations

Adult female, inbred, Swiss-type (CPB-S) mice (aged 3–4 months), weighing 30.9±2.5 g (mean±SD) were used for intratracheal instillations. The mice were anaesthetised by CO_2 asphyxiation, and held in an almost upright position by suspending the animal on its front teeth. The tongue was extended from the mouth using forceps, and the light tip of a flexible cold light fibreoptic was placed against the skin at throat level. By looking into the mouth, the white cartilageous rings of the trachea could be seen in an otherwise transparent rose surrounding. When the mice started to gasp for breath, the trachea was quickly intubated with a 20 gauge, blunt needle attached to an automatic Hamilton syringe (CR-700), and 50 µl of reagent (see below) or saline was instilled into the lungs. The mice were kept in this upright position for a few seconds until they regained consciousness.

Treatment and study design

Emphysema in mice was induced by intratracheal instillation of pancreatic elastase. Porcine pancreatic elastase (144 $U \cdot mg^{-1}$) was purchased from Calbiochem (La Jolla, CA, USA) and given in doses of 1.8 $mg \cdot kg^{-1}$ BW, based on data in the literature [9]. To study the effect of surfactant, mice were treated with natural sheep surfactant (100 mg phospholipid·kg^{-1} BW), isolated from lung lavage fluid as described previously [10]. These natural surfactant fractions contained the surfactant-associated protein A (SP-A) as well as the hydrophobic surfactant-associated proteins B and C (SP-B AND SP-C).

In a series of pilot experiments, two groups of mice were given intratracheal instillations with elastase at t=0 h, followed in one group by instillations with saline at t=3, 48 and 96 h. Control groups consisted of animals that received saline at t=0 h, followed at t=3, 48 and 96 h by instillations with saline or surfactant, or of untreated animals. The final assay to assess the effect of pulmonary surfactant on the development of elastase-induced emphysema was subdivided in two successive experiments. For each experiment the mice were randomly divided into three groups. Two groups received intratracheal instillations with elastase at t=0 h, followed after 3, 48 and 96 h in one group by intratracheal administrations of surfactant (El/Surf group, n=12), and in the other group by administrations of saline (El/s group, n=13). The third group consisted of control animals which were treated with saline at t=0 h, followed by three doses of sheep surfactant at t=3, 48 and 96 h (s/Surf group, n=12).

Morphometric evaluation of emphysema

After eight weeks, the animals were killed, exsanguinated by intracardiac puncture, and the lungs with the trachea were carefully excised. By intratracheal intubation, the lungs were immediately inflated with Bouin's fixative at a pressure of 25 cmH_2O for 2 h. The lungs were stored in the fixative for 24 h, and then dehydrated and embedded in paraffin.

Frontal sections through each complete pair of lungs were cut at 5 µm and sections of three different levels were stained with haematoxylin and eosin (HE). The severity of emphysema was evaluated by calculating the mean linear intercepts (Lm) of the airspaces, since the Lm is the most sensitive morphometric parameter for experimental emphysema in animals [11]. All measurements were performed by one person without knowledge of prior treatment. The Lm was calculated according to DUNNILL [12] and THURLBECK [13] using the light microscope with a ×25 objective lens and a ×8 eyepiece lens that contained a crossed hairline of known length. For each pair of lungs, three sections were examined and the Lm was calculated for the left (one lobe) and right (four lobes) lungs separately, and for the upper and lower parts of the right lung separately. We did not determine the Lm separately for each of the four right lung lobes, since they were not all measurable in most cross sections. Per section, we always evaluated 10 randomly selected fields per lobe (or lung part). The whole lung Lm values represent the mean of the left and right lung Lm values, the latter being the mean of the values calculated for the upper and lower parts. To obtain an impression of the variation in airspace enlargement without averaging effect, we also analysed the changes in airspaces by histograms of the Lm (per 15 µm steps) per field of examination. This is a modification of the method described by MCCARTNEY *et al.* [14]. All fields examined in one frontal section of each pair of lungs were included for this analysis.

Data analysis

The Lm values were statistically analysed by using the non-parametric Mann-Whitney test in which a p value <0.01 was considered to be significant. The level of 0.01 was chosen to correct for multiple testing.

The histograms were tested for significant differences using the Kolmogorov-Smirnov two-sample test.

Results

Intratracheal instillation technique and the induction of emphysema

The CO_2 anaesthetic followed by intratracheal instillation was well-tolerated by the mice; within a few minutes after the instillation the mice were awake and walking around. Instillations with the reagents (saline, elastase, or sheep surfactant diluted in saline), to which india ink was added, revealed that the volume necessary for a good spreading was 50 µl (data not shown). We did not observe any mortality due to instillations with 1.8 $mg \cdot kg^{-1}$ BW of pancreatic elastase. We sacrificed some animals 3 and 7 days after instillation with elastase or saline. In agreement with findings of other investigators [15, 16] we observed some haemorrhage, oedema and infiltration of the lungs with polymorphonuclear leucocytes (PMN) and macrophages at 3 days, but at 7 days most of these initial reactions seemed to have disappeared. These reactions were most evident after elastase instillations, but light reactions were also present after saline instillations. Inflammatory reactions were not evident at the time the mice were killed for morphometric evaluation of emphysema. At that time the group mean total body weight of the mice having received elastase (36.6±3.4 g) (mean±SD) was no different from that of the controls (34.2±4.0 g). The pancreatic elastase-induced lesions in mice were predominately characteristic of panlobular emphysema, but centrilobular emphysema was usually also observed.

Since repeated intratracheal instillations are not usual in experimental emphysema, we performed a series of pilot experiments in which we showed that the severity of the elastase-induced emphysematous lesions, as assessed by the Lm of the airspaces, was not influenced by subsequent instillations with saline (table 1). The development of emphysema after elastase treatment is revealed by the statistically higher Lm values in the elastase-treated group of mice compared to control groups. Statistical analysis also revealed that there were no differences in Lm values between the different control groups, indicating that repeated instillations with saline and/or surfactant had no effect on the Lm.

Following intratracheal elastase instillations, we observed the presence of emphysematous lesions in all lung lobes. Figure 1 shows the elastase-induced increase in Lm values (mean±SEM, n=12) for the right and left lung, and for the right upper lung parts and lower lung parts separately. For all lobes or parts of the lungs, the increase in Lm in elastase-treated mice compared to the mice treated with saline was statistically significant ($p \leq 0.001$ by Mann-Whitney test). Within both groups, the group mean Lm values were not significantly different between the different lung parts; although, within one single pair of lungs the lesions were sometimes more severe in one lobe than another.

Table 1. – Effects of (repeated) intratracheal instillations on mean linear intercepts (Lm) of air spaces in mice

	Lm	n
Elastase groups		
Elastase*	61.3±8.9	9
Elastase*/saline**	62.2±7.8	9
Control groups		
Saline***	44.9±5.3	9
Saline*/surfactant**	44.1±3.0	9
Untreated controls	43.5±4.1	4

Whole lung values of mean linear intercepts (Lm) presented as group means (±SEM). The number of animals per group is indicated by n. The Lm values of the elastase-treated groups of mice were statistically different from those of all the control groups ($p<0.01$). *: administered at t=0 h; ** administered at t=3, 48 and 96 h; ***: administered at t=0, 3, 48 and 96 h.

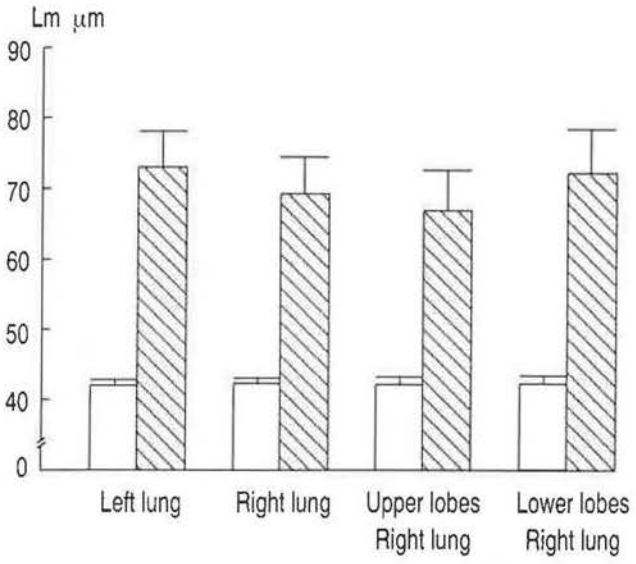

Fig. 1. – Distribution of airspace enlargement in elastase-treated mice lungs. Mean linear intercepts (Lm) of airspaces (mean±SEM) for two groups of 12 mice treated with saline (unshaded bars) or elastase (shaded bars). Within the groups, the Lm values were not statistically different between the different lung lobes or lung parts, but the differences between the elastase- and saline-treated group were always statistically significant.

Effect of surfactant instillation on elastase-induced emphysema

The effect of surfactant instillations on the development of elastase-induced emphysema was studied in two successive experiments, which revealed the same results. Figure 2 shows the compilated whole lung Lm-values for each pair of lungs of the two experiments. The mean Lm value for the group treated with elastase followed by saline (El/s group) was 69.5±4.5 (SEM) μm (range 48.6–95.8 μm). The Lm values in this group were significantly ($p<0.0001$) higher than those in the control group that received saline and surfactant (s/Surf group). The mean Lm value for this control group was 41.8±0.6 μm, (range 38.9–45.6 μm). The group treated with natural sheep surfactant 3, 48 and 96 h after instillation of elastase (El/Surf group) had a mean whole lung Lm value of 54.4±4.9 μm, (range 42.8–92.1 μm). The Lm values in the El/Surf group were significantly lower ($p=0.004$) than those in the El/s group. Despite this significant inhibition of elastase-induced airspace enlargement, the Lm values of the El/Surf group were still higher and significantly different from those in the s/Surf group ($p=0.0001$). The higher mean Lm value in the El/Surf group is largely due to the exceptionally high Lm values (92.1 and 88.1 μm) in 2 of the 12 pairs of lungs of this group (fig. 2) compared to the other whole lung Lm values of this group (42.8–53.6 μm).

Microscopic examination of lungs of mice from the El/s group usually showed the presence of extended emphysematous lesions (fig. 3a).

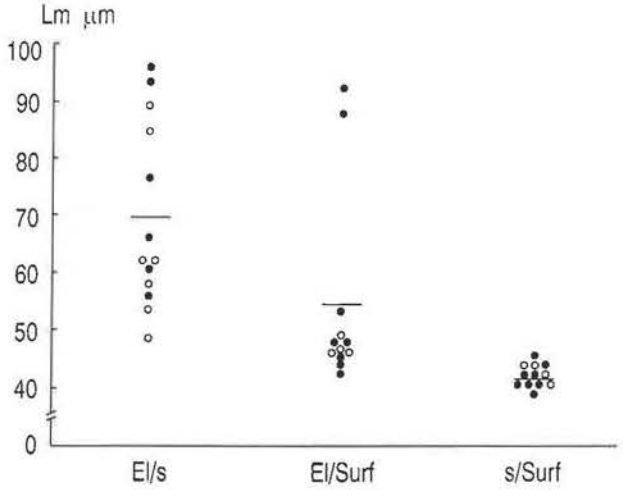

Fig. 2. – Effect of intratracheally instilled surfactant (three times at 3, 48 and 96 h) on elastase-induced emphysema in mice. The results of two successive experiments (A and B) are presented as individual whole lung mean linear intercept (Lm) values (●: A; ○: B) a well as the group means (lines) compilated from the two experiments. The Lm values of the surfactant-treated group (El/Surf group) were statistically different from those in the saline treated group (El/s group) ($p=0.004$) and the control group (s/Surf group) ($p<0.0001$). El/s: lungs treated with elastase followed by saline; El/Surf: lungs treated with elastase followed by natural sheep surfactant; s/Surf: lungs treated with saline followed by natural sheep surfactant.

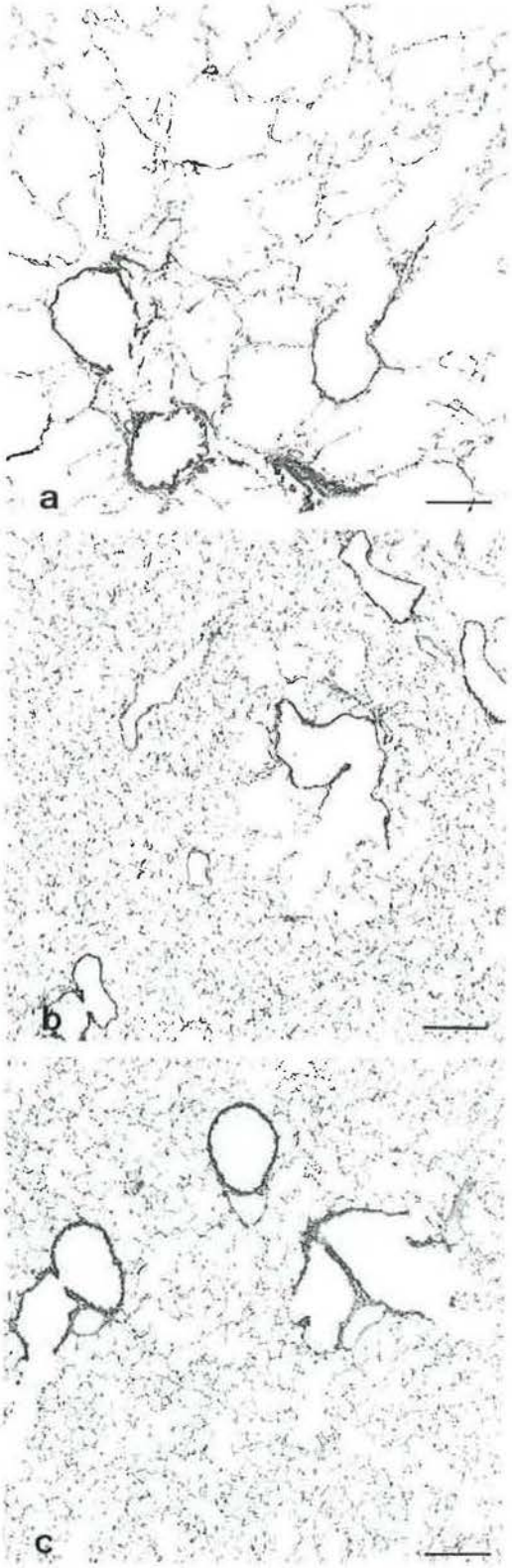

Fig. 3. – Micrographs of lung tissue sections, stained with haematoxylin and eosin. a: Extended emphysematous lesions in mouse lung treated with elastase and saline (El/s group). b: Normal lung parenchyma with some focal and limited emphysematous lesions in mouse lung treated with surfactant following elastase administration (El/Surf group). c: Control mouse lung to which saline and surfactant were administered (s/Surf group). Bars 200 μm.

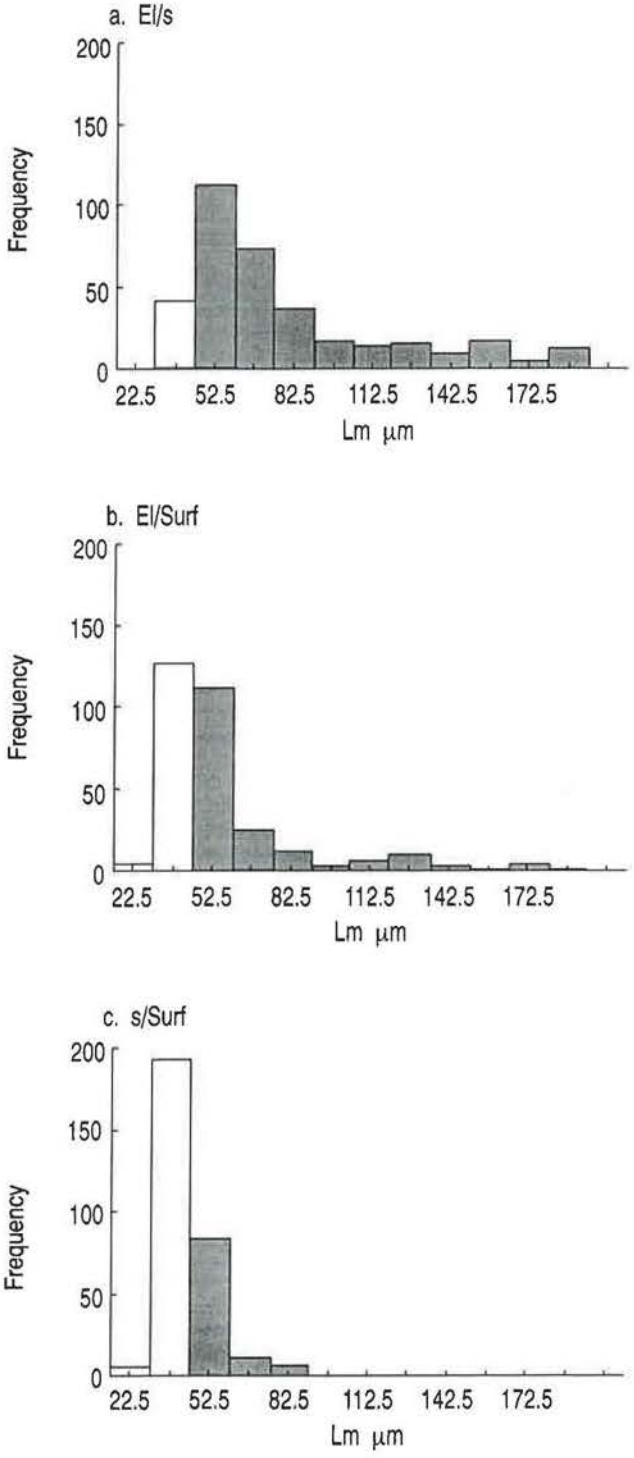

Fig. 4. – Histograms of mean linear intercept (Lm) distributions in the different treatment groups. All fields examined in one frontal section of each complete pair of lungs were included for this analysis. Fields having a Lm equal to or lower than the mean Lm value plus one standard deviation assessed in untreated controls animals (UC-Lm) are represented by unshaded bars, and fields with a Lm higher than the UC-Lm are represented by shaded bars. The Lm frequencies in the El/s group differed statistically from those in the s/Surf control group between 30–120 μm, whereas the El/Surf group differed statistically from the s/Surf group only in the 30–45 μm category. See legend to figure 2 for explanation of group treatment.

We also observed some emphysematous lesions in most of the lungs of mice in the El/Surf group, but these lesions were usually focal (fig. 3b), and large areas of the lungs from the El/Surf group appeared to be not very different from saline-treated lungs (fig. 3c).

Some morphometric information on emphysematous lesions in individual fields of examination is provided by the Lm frequency histograms (fig. 4), which show the frequency distributions of the Lm calculated per field of examination (one frontal section from each pair of lungs per animal per group, approximately 30 fields of examination). The Lm distribution in the s/Surf group (fig. 4c) was not significantly different from that in untreated controls (Kolmogorov-Smirnov test, data not shown), the majority of the fields having a low Lm, equal or lower than the mean Lm value plus one standard deviation assessed in untreated control animals (UC-Lm). For each group fields with Lm values ≤ UC-Lm (±1 SD) are represented by unshaded bars, those with Lm values >UC-Lm (±1 SD) by shaded bars. The Lm distribution in the El/s group (fig. 4a) revealed that more than 85% of the examined fields had a Lm higher than the UC-Lm, and that a considerable number of fields had very high Lm values. In the El/Surf group (fig. 4b) only about 50% of the fields examined had a Lm higher than the UC-Lm. The highest Lm values observed in the El/Surf group were not lower than in the El/s group, but Lm values >120 μm in this group were all present in the two pairs of lungs with the high mean Lm values. Analysis by the Kolmogorov-Smirnov test for two-samples revealed that the range in which the El/s group and the s/Surf group were significantly different ($p<0.005$) was between 30 and 120 μm, whereas the El/Surf group and the s/Surf group differed only between 30 and 45 μm. The El/s group and the El/Surf group were significantly different ($p<0.005$) between 30 and 105 μm, the maximum differences in cumulative proportion being between 30 and 60 μm.

Discussion

The aim of this study was to obtain evidence of a protective role for pulmonary surfactant in the pathogenesis of emphysema. For this purpose, we developed methods to treat mice with intratracheal instillations. The simple method used for anaesthesia, *i.e.* CO_2 asphyxiation, enabled us to treat larger numbers of mice with a series of intratracheal instillations of reagents within 0 and 96 h, without overburdening the animals or investigators. This CO_2 anaesthetic also provided the advantage that the mice breathed rather deep at the time of instillation, whereas for instance Nembutal hampered breathing following instillation, which also resulted in high mortality. The events occurring in the lung after administration of pancreatic elastase are described in a number of studies, as reviewed by SNIDER *et al.* [1]. The hamster is most widely used in animal models of emphysema, but mice

and rats have also been employed [9, 11, 17, 18]. The present study was performed in an inbred strain of Swiss mice, which were also used for our extensive studies on lung morphology and function [19, 20]. An advantage of using murine lungs for the morphometric evaluation of emphysematous lesions is that a single pair of lungs can easily be evaluated as a whole. Furthermore, animal size and cost facilitate studies of larger groups. VALENTINE *et al.* [9] also provided evidence that in the mouse, endotracheal administration of porcine pancreatic elastase results in elastine breakdown and induces alveolar destruction with subsequent alveolar enlargement. In contrast to VALENTINE *et al.* [9] who found parenchymal damage most frequently in the lower half of the lung lobes, we found a rather even distribution of the emphysematous lesions. This was probably caused by better spreading of the elastase bolus in our experiments, due to deeper and better breathing of the mice at the time of instillation. The use of the automatic Hamilton syringe, which injected the reagents rather forcefully, *i.e.* in microdrops, might also have facilitated spreading.

Inhibition of elastase-induced emphysema was formerly demonstrated by treatment with elastase inhibitors several hours before the administration of elastase [21, 22]. We chose to administer surfactant after elastase administration because surfactant given before or simultaneously with the elastase might become inactivated by the elastase [23, 24]. We instilled the first dose of surfactant 3 h after elastase instillation, since destruction of the original surfactant lining layer and loss of surface activity appear to occur at that time [23, 25]. It has also been noted that by then elastolytic-enzyme-induced injuries have lead to the presence of degradation products, cellular exudate and oedema [16, 23, 26] in the alveolar spaces, *i.e.* agents known to impair the biophysical activities of surfactant [27–29]. We repeated the surfactant treatment at 48 and 96 h, *i.e.* during the period that an abundance of surfactant inhibitors were likely to be present in the alveolar space. For control instillations to surfactant treatment we used saline and not albumin or serum proteins, although they might be present in low concentrations in our surfactant preparations, since these substances may impair surfactant biophysical activity [27]. Pilot studies, which we performed (data not shown), showed that instillations with albumin did not inhibit the development of elastase-induced emphysema.

This is the first study demonstrating a protective effect of surfactant on elastase-induced emphysema. Treatment with exogenous surfactant at 3, 48 and 96 h after elastase instillation clearly reduced the elastase-induced airspace enlargement. This inhibition of emphysema development was found in two successive experiments; the mean whole lung Lm values were 53 and 79% lower in the El/Surf group than in the El/s group. Analyses of the compilated data of these two experiments by the Mann-Whitney test revealed that the differences in whole lung Lm values were statistically significant. Despite this significant inhibition, we observed some focal and limited emphysematous lesions in most of the lungs of mice of the El/Surf group by light microscopy. An impression of the lesions throughout the lungs (without the averaging effect as in case of whole lung Lm values) was given by histograms of Lm frequency. These histograms of Lm frequency clearly demonstrated that the Lm distribution of the El/Surf group was similar to that of the s/Surf control group, whereas the El/s group showed the presence of many fields with high or very high Lm values. In the El/Surf group only 2 of the 12 animals showed the presence of extended emphysematous lesions, resulting in an exceptionally high mean whole lung Lm value. This apparent lack of protection by surfactant treatment in these lungs might be caused by incomplete replacement of a well-functioning surfactant lining layer. We do not exactly know what may cause incomplete replacement, but individual prolonged presence or higher concentrations of surfactant inhibitors due to injury and oedema may play a role.

As for the mechanism(s) of surfactant protection, we assume that the exogenous surfactant normally substitutes the elastase-destroyed original surfactant lining layer, and prohibits the development of elastase-induced emphysema by just fulfilling its normal functions. It is likely that this effect might be enhanced by repeated doses of surfactant, at least initially. A pilot experiment (with two mice) indicated that a single administration 3 h after elastase treatment was not as effective. We do not know if further administrations, *i.e.* 4 days after elastase treatment, would further enhance the effect of surfactant treatment. We hypothesise that the type II cells, the producers of surfactant and progenitors of the complete alveolar epithelium [30], may only initially not be able to cope with the speed of surfactant destruction by elastase.

Further studies will be necessary to elucidate the precise mechanism(s) by which surfactant protects the lungs against the development of emphysematous lesions. However, based on current knowledge of the surfactant system, we feel that some or all of the following mechanisms may play a role in the protective effect. Firstly, it seems likely that prohibition of overstretching of lung parenchyma by surface active surfactant prevents injuries that may result in emphysematous lesions. Also, in case of elastolytic enzyme-induced injuries, overstretching may interfere with necessary repair processes to prevent emphysema. Secondly, the surfactant lining layer may act as a barrier against elastolytic enzymes from the alveolar lumen by providing antielastase activity, effected by surfactant-associated proteins such as SP-B [31], surfactant phospholipids [32] or association with other elastase inhibitors, such as α_1-proteinase inhibitor [33]. The antioxidant activities of pulmonary surfactant [34] may add to the antielastase capacity of the lung, since it is known that elastase inhibitors are inactivated by oxidation [35, 36]. The effect of surfactant on the immune response [37–39] may also result in a lower burden of elastolytic enzymes in the lung. Thirdly, it

has been reported that the major surfactant associated protein, SP-A, has some specific biological functions (see reviews by POSSMAYER [6] and WEAVER and WHITSETT [8]). Some of these functions such as its ability to attenuate the inhibitory effect of serum proteins [40, 41], might add to the protective effect of pulmonary surfactant against the development of emphysematous lesions. Our further studies may give more insight in the role of SP-A. In this respect, note that SP-A was present in the natural surfactant fractions that we used in the present study, but is usually eliminated from therapeutic surfactant fractions to reduce possible risks of immune responses. Furthermore, it is reported that impairment of surfactant biophysical activity after exposure to neutrophilic elastase is mainly due to proteolytic cleavage of SP-A [24, 29].

In conclusion, the present study shows a significant inhibition of emphysema development by surfactant instillations given in three doses during the first four days after treatment with pancreatic elastase. These findings fully support our earlier hypothesis [2] concerning a protective effect of pulmonary surfactant, and thus the type II cell, in the pathogenesis of emphysema. It seems likely that surfactant dysfunctioning may also be one of the important factors in the development of emphysematous lesions in humans. Our findings and conclusions concur with the suggestion that the air lung interface is the site of initiation of the destructive changes of elastase-induced emphysema [42], and with data indicating that elastolytic enzymes are capable of inhibiting surfactant function [23–25, 29].

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References

1. Snider GL, Lucey EC, Stone PI. – Animal models of emphysema. *Am Rev Respir Dis,* 1986; 133: 149–169.
2. Otto-Verberne CJM, Ten Have-Opbroek AAW, Willems LNA, *et al.* – Lack of type II cells and emphysema in human lungs. *Eur Respir J,* 1991; 4: 316–323.
3. Van Golde LMG, Batenburg JJ, Robertson B. – The pulmonary surfactant system: biochemical aspects and functional significance. *Physiol Rev,* 1988; 68: 374–455.
4. Morton NS. – Exogenous surfactant treatment for the adult respiratory distress syndrome? A historical perspective. *Thorax,* 1990; 45: 825–830.
5. Hawgood S, Clements JA. – Pulmonary surfactant and its apoproteins. *J Clin Invest,* 1990; 86: 1–6.
6. Possmayer F. – The role of surfactant-associated proteins. *Am Rev Respir Dis,* 1990; 142: 749–752.
7. Wright JR. – Clearance and recycling of pulmonary surfactant. *Am J Physiol,* 1990; 259: L1–L12.
8. Weaver TE, Whitsett JA. – Function and regulation of expression of pulmonary surfactant-associated proteins. *Biochem J,* 1991; 273: 249–264.
9. Valentine R, Rucker RB, Chrisp CE, Fisher GL. – Morphological and biochemical features of elastase-induced emphysema in strain A/J mice. *Toxicol Appl Pharmacol,* 1983; 68: 451–461.
10. Otto-Verberne CJM, Ten Have-Opbroek AAW, Balkema JJ, Franken C. – Detection of the type II cell or its precursor before week 20 of human gestation, using antibodies against surfactant-associated proteins. *Anat Embryol,* 1988; 178: 29–39.
11. Eidelman DH, Bellofiore S, Chiche D, Cosio MG, Martin JG. – Behavior of morphometric indices in pancreatic elastase-induced emphysema in rats. *Lung,* 1990; 168: 159–169.
12. Dunnill MS. – Quantitative methods in the study of pulmonary pathology. *Thorax,* 1962; 17: 320–328.
13. Thurlbeck WM. – Measurement of pulmonary emphysema. *Am Rev Respir Dis,* 1967; 95: 752–764.
14. McCartney ACE, Fox B, Partridge TA, *et al.* – Emphysema in the blotchy mouse: a morphometric study. *J Pathol,* 1988; 156: 77–81.
15. Kuhn III C, Slodkowska J, Smith T, Starcher B. – The tissue response to exogenous elastase. *Bull Eur Physiopathol Respir,* 1980; 16 (Suppl.): 127–137.
16. Morris SM, Stone PhJ, Snider GL, Albright JT, Franzblau C. – Ultrastructural changes in hamster lung four hours to twenty four days after exposure to elastase. *Anat Rec,* 1981; 201: 523–535.
17. Busch RH, Lauhala KE, Loscutoff SM, McDonald KE. – Experimental pulmonary emphysema induced in the rat by intratracheally administered elastase: morphogenesis. *Environ Res,* 1984; 33: 497–513.
18. Starcher B, Williams I. – The beige mouse: role of neutrophil elastase in the development of pulmonary emphysema. *Exp Lung Res,* 1989; 15: 785–800.
19. Ten Have-Opbroek AAW. – The structural composition of the pulmonary acinus in the mouse. A scanning electron microscopical and developmental-biological analysis. *Anat Embryol,* 1986; 174: 49–57.
20. Ten Have-Opbroek AAW. – Lung development in the mouse embryo. *Exp Lung Res,* 1991; 17: 111–130.
21. Rudolphus A, Kramps JA, Dijkman JH. – Effect of human antileucoprotease on experimental emphysema. *Eur Respir J,* 1991; 4: 31–39.
22. Stone PJ, Lucey EC, Vicra GD, *et al.* – $Alpha_1$-protease inhibitor moderates human neutrophil elastase-induced emphysema and secretory cell metaplasia in hamsters. *Eur Respir J,* 1990; 3: 673–678.
23. Parra SC, Gaddy LR, Takaro T. – Early ultrastructural changes in papain-induced experimental emphysema. *Lab Invest,* 1980; 42: 277–289.
24. Pison U, Tam EK, Caughey GH, Hawgood S. – Proteolytic inactivation of dog lung surfactant-associated proteins by neutrophil elastase. *Biochym Biophys Acta,* 1989; 992: 251–257.
25. Sanderson RJ, Gaddy L, Parra S, Takaro T. – Alterations in stress distributions around interalveolar pores after exposure to papain in dogs. *Am Rev Respir Dis,* 1981; 123: 327–332.
26. Morris SM, Kagan HM, Stone PhJ, Snider GL, Albright JT. – Ultrastructural changes in hamster lung 15 min to 3 h after exposure to pancreatic elastase. *Anat Rec,* 1986; 215: 134–143.
27. Seeger W, Stohr G, Wolf HRD, Neuhof H. – Alteration of surfactant function due to protein leakage: spacial interaction with fibrin monomer. *J Appl Physiol,* 1985; 58: 326–338.
28. O'Brodovich HM, Weitz JI, Possmayer F. – Effect of fibrinogen degradation products and lung ground substance function. *Biol Neonate,* 1990; 57: 325–333.
29. Ryan SF, Ghassibi Y, Liau DF. – Effects of activated polymorphonuclear leukocytes upon pulmonary

surfactant *in vitro*. *Am J Respir Cell Mol Biol*, 1991; 4: 33–41.
30. Ten Have-Opbroek AAW. – The development of the lung in mammals: an analysis of concepts and findings. *Am J Anat*, 1981; 162: 201–219.
31. Emrie PA, Shannon JM, Mason RJ, Fisher JH. – cDNA and deduced amino acid sequence of the rat hydrophobic pulmonary surfactant-associated protein, SP-B. *Biochym Biophys Acta*, 1989; 994: 215–221.
32. Walsh RL, Dillon T, Scicchitano R, McLennan G. – Interaction of surfactants with the heparin and heparan sulphate inhibition of human leukocyte elastase. *Am Rev Respir Dis*, 1991; 143: A326.
33. Tuttle WC, Jones RK. – Flourescent antibody studies of alpha$_1$-antitrypsin in adult human lung. *Am J Clin Pathol*, 1975; 64: 477–482.
34. Matalon S, Holm BA, Baker RR, Whitfield MK, Freeman BA. – Characterization of antioxidant activities of pulmonary surfactant mixtures. *Biochym Biophys Acta*, 1990; 1035: 121–127.
35. Ruldolphus A, Heinzel-Wieland R, Stolk J, *et al.* – Non-oxidizable recombinant antileukoprotease (rALP) variants are better inhibitors of elastase-induced emphysema than native rALP. *Am Rev Respir Dis*, 1991; 143: A321.
36. Gadek JE, Pacht ER. – The protease-antiprotease balance within the human lung: implications for the pathogenesis of emphysema. *Lung*, 1990; (Suppl.), 552–564.
37. Van Iwaarden F, Welmers B, Verhoef J, Haagsman HP, Van Golde LMG. – Pulmonary surfactant protein A enhances the host-defense mechanism of rat alveolar macrophages. *Am J Respir Cell Mol Biol*, 1990; 2: 91–98.
38. Richman PS, Batcher S, Catanzaro A. – Pulmonary surfactant suppresses the immune lung injury response to inhaled antigen in guinea-pigs. *J Lab Clin Med*, 1990; 116: 18–26.
39. Wilsher ML, Parker DJ, Haslam PL. – Immunosuppression by pulmonary surfactant: mechanisms of action. *Thorax*, 1990; 45: 3–8.
40. Cockshutt AM, Weitz J, Possmayer F. – Pulmonary surfactant-associated protein A enhances the surface activity of lipid extract surfactant and reverses inhibition by blood proteins *in vitro*. *Biochemistry*, 1990; 29: 8424–8429.
41. Venkitaraman AR, Hall SB, Whitsett JA, Notter RH. – Enhancement of biophysical activity of lung surfactant extracts and phospholipid- apoprotein mixtures by surfactant protein A. *Chem Phys Lipids*, 1990; 56: 185–194.
42. Weinbaum G, Marco V, Ikeda T, *et al.* – Enzymatic production of experimental emphysema in the dog. Route of exposure. *Am Rev Respir Dis*, 1974; 109: 351–357.