RESEARCH COMMUNICATION

Rat surfactant protein D enhances the production of oxygen radicals by rat alveolar macrophages

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Rat surfactant protein D (SP-D) was shown to enhance the production of oxygen radicals by rat alveolar macrophages. This enhancement, which was determined by a lucigenin-dependent chemiluminescence assay, was maximal after 18 min at an SP-D concentration of 0.2 μg/ml. Surfactant lipids did not influence the stimulation of alveolar macrophages by SP-D, whereas the oxygen-radical production of these cells induced by surfactant protein A was inhibited by the lipids in a concentration-dependent manner.

INTRODUCTION

Pulmonary surfactant is a complex of lipids, proteins and carbohydrates that lines the alveolar surfaces of the lung. Its major function is to prevent alveolar collapse at end-expiration.

One of the most abundant surfactant proteins is surfactant protein A (SP-A), which is synthesized and secreted by alveolar type II cells [1,2]. SP-A has been shown to enhance the adsorption of phospholipids to the monolayer at the air/liquid interface in the presence of surfactant protein B [3] and to regulate the homoeostasis of extracellular surfactant [4–6]. In addition, SP-A may also be involved in the host-defence system of the lung, as it has been shown to act as an opsonin for viruses [7] and to stimulate alveolar macrophages [7–9].

In 1988 another hydrophilic surfactant-associated protein was described by Persson *et al.* [10], namely surfactant protein D (SP-D). SP-D is a collagen-domain-containing glycoprotein that is synthesized and secreted by rat type II cells [10,11]. SP-D isolated from broncho-alveolar lavage is a multimeric complex of disulphide-bonded trimers comprised of apparently identical subunits, each with a molecular mass of 130 kDa [10,12]. SP-D is also a Ca^{2+}-dependent carbohydrate-binding protein and is structurally related to other C-type mammalian lectins, such as conglutinin and SP-A [13,14]. The complete cDNA coding sequence and deduced amino acid sequence of rat SP-D have recently been reported by Shimizu *et al.* [15]. The structural similarities of SP-D and SP-A raise the possibility that these proteins have related functions.

In the present study we demonstrate, using a lucigenin-dependent chemiluminescence assay, that SP-D can stimulate alveolar macrophages to generate oxygen radicals.

MATERIALS AND METHODS

Purification of SP-D

Rats were given an intratracheal instillation of silica to induce alveolar lipoproteinosis [16], and SP-D was prepared from lung lavage essentially as described by Persson *et al.* [12,17]. $BaSO_4$ powder was added to the surfactant-depleted lavage supernatant (80 mg/ml) and the solution was stirred at room temperature for 30 min. The adsorbant was collected by centrifugation at 10000 *g* and washed twice with 150 mM-NaCl/50 mM-Tris/HCl, pH 7.5 (TBS), containing 2.5 mM-sodium citrate. SP-D was eluted from the adsorbant by washing with TBS containing 200 mM-sodium citrate. The preparation was dialysed against TBS containing 2 mM-$CaCl_2$ and applied to a mannose–Sepharose 6B column [18] equilibrated with the same buffer. Proteins bound to the column were eluted with TBS containing 10 mM-EDTA. The eluted material was applied to a column of Sephacryl S-500 (Pharmacia LKB Biotechnology) and SP-D was eluted with an apparent molecular mass of 2.3 MDa.

Antisera

To generate an antiserum, 100 μg of purified SP-D was prepared in complete Freund's adjuvant and injected intramuscularly into rabbits. The initial immunization was followed by a second administration of SP-D antigen (70 μg) in incomplete adjuvant and a third administration (70 μg) without adjuvant. The IgG fraction was purified by affinity chromatography using a Protein A–Sepharose CL4B column (Pharmacia). The IgG fraction was also absorbed by passage over rat-serum-conjugated Affi-Gel 10 and 15 (Bio-Rad) columns [15]. Antibody specificity for SP-D was confirmed by immunoblot analysis [15]. $F(ab')_2$ fragments of the IgG fraction were prepared and purified as described by Parham *et al.* [19]. The fragments were dissolved in TBS (0.6 mg/ml) and stored at −20 °C.

Isolation of SP-A and surfactant lipids

SP-A was isolated from broncho-alveolar lavage fluid of patients with alveolar proteinosis as previously described [20]. SP-A was dissolved in 5 mM-Hepes buffer, pH 7.4 (1 mg/ml), and stored in small aliquots at −70 °C.

Lipids were extracted from rat pulmonary surfactant by the Bligh & Dyer technique [21], as described previously [8]. The lipids were suspended in TBS to a concentration of 1.1 mg of phospholipids/ml.

Abbreviations used: SP-A, surfactant protein A; SP-D, surfactant protein D; TBS, Tris-buffered saline (for composition and pH, see the text); PMA, phorbol 12-myristate 13-acetate.

Isolation of rat alveolar and peritoneal macrophages

Rat alveolar macrophages were isolated from male specific-pathogen-free Wistar rats (200–225 g body wt.) as described previously [8]. The macrophages were suspended in 140 mM-NaCl / 5 mM-KCl / 2.5 mM-Na_2HPO_4 / 10 mM-Hepes / 2 mM-$MgSO_4$/6 mM-glucose/2 mM-$CaCl_2$, pH 7.4, supplemented with 0.1% (w/v) gelatin (buffer A) to a concentration of 2×10^6 cells/ml.

Rat peritoneal macrophages were isolated as described previously [8]. The macrophages were suspended in buffer A to a concentration of 2×10^6 cells/ml. The peritoneal leucocytes used in the present study consisted of more than 80% peritoneal macrophages.

Chemiluminescence assay

The generation of oxygen radicals by rat macrophages was monitored by using the lucigenin-dependent chemiluminescence assay described in [8]. Briefly, the chemiluminescence response of 3.5×10^5 macrophages incubated with various concentrations of SP-D or SP-A or phorbol 12-myristate 13-acetate (PMA) in the presence of lucigenin (250 μM) was determined. The assay volume was 0.4 ml. Chemiluminescence was measured at 37 °C in a luminometer for 3 s every 2 min over a 30 min period. Except for the time course where the chemiluminescence response was depicted every 2 min, peak signals of the chemiluminescence responses were used to compare the various experimental conditions.

RESULTS AND DISCUSSION

Enhancement of the production of oxygen radicals by alveolar macrophages induced by SP-D

SP-D enhanced the production of oxygen radicals by rat alveolar macrophages (Fig. 1). The enhancement was time-dependent, starting after 6 min and reaching a maximum after 18 min. To compare the SP-A-induced stimulation of alveolar macrophages with the SP-D-mediated activation of these cells, the chemiluminescence response was recorded as a function of either the SP-A or SP-D concentration (Fig. 2). Maximal stimulation of the alveolar macrophages was observed at a concentration of 0.2 μg of SP-D/ml, whereas the SP-A-mediated chemiluminescence was initiated at 0.25 μg/ml and reached a plateau at 1 μg of SP-A/ml [8]. The concentration of SP-D present in the bronchoalveolar lavage of rats is 10 μg/rat [22]. The concentration needed for maximal activation of the alveolar macrophages is 0.2 μg of SP-D/ml. Since the alveolar aqueous phase in the rat is of the order of 100 μl, this result indicates that, *in vivo*, there may be enough SP-D present for maximal activation of alveolar macrophages. However, it should be emphasized that the actual concentration of SP-D in the alveolar aqueous phase cannot be derived from the total amount of this protein that is recovered by broncho-alveolar lavage. In this context it is important to mention that SP-D (like SP-A) is produced not only by alveolar type II cells, but also by Clara cells that are located in the bronchioli [23,24].

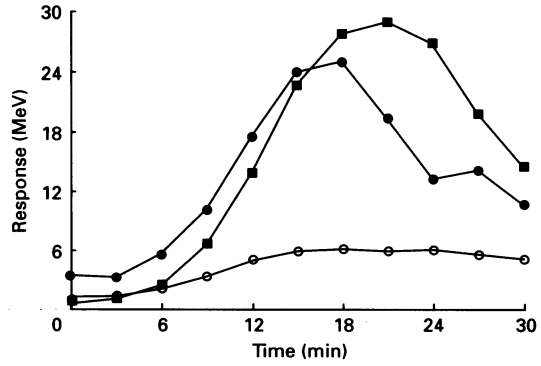

Fig. 1. SP-D stimulates oxygen-radical production by alveolar macrophages

Alveolar macrophages were incubated with buffer (○) or PMA (25 ng/ml; ■) or SP-D (0.20 μg/ml; ●) at 37 °C in the presence of lucigenin. A representative of one of three experiments is shown. The response was expressed as MeV; 1 MeV corresponds to 2×10^5 counts/min.

$F(ab')_2$ fragments of an IgG fraction directed against SP-D completely abolished the SP-D-induced production of oxygen radicals (Fig. 3), indicating that alveolar macrophages are truly stimulated by SP-D. The $F(ab')_2$ fragments directed against SP-D did not influence the SP-A-induced activation of alveolar macrophages. In addition, the $F(ab')_2$ fragments alone did not induce oxygen-radical production by the macrophages. No chemiluminescence response was detected when alveolar macrophages were incubated with either SP-A or SP-D in the presence of superoxide dismutase, an enzyme which converts superoxide into H_2O_2, indicating that both SP-A and SP-D stimulate the macrophages to produce superoxide radicals (results not shown).

Effect of SP-D on peritoneal macrophages

Table 1 shows that peritoneal macrophages, in contrast with alveolar macrophages, cannot be stimulated by SP-D to elicit a chemiluminescence response. Previously we reported that also SP-A can only stimulate alveolar macrophages to generate superoxide [8]. Whether SP-A and SP-D bind to a similar receptor on the alveolar macrophage, which is not present on other phagocytes, or whether the observed similar specificity of SP-D and SP-A for alveolar macrophages is merely coincidental, is not known, but would be an interesting topic for future research.

Influence of surfactant lipids on the activation of alveolar macrophages

We also studied the influence of surfactant lipids on the activation of alveolar macrophages by SP-A and SP-D (Fig. 4). The lipids had no effect on the SP-D-induced chemiluminescence response by rat alveolar macrophages. However, they completely inhibited the activation of alveolar macrophages by SP-A in a concentration-dependent manner, whereas surfactant lipids alone did not have an effect. The lipids used in these experiments were derived from a Bligh & Dyer [21] extract of rat lung surfactant. This extract may, in addition to lipids, contain the hydrophobic surfactant proteins B and C. To exclude the possibility that the inhibitory effects of surfactant lipids on the stimulation of macrophages by SP-A were due to these proteins instead of the lipids, control experiments were performed with a phospholipid mixture that consisted of dipalmitoyl phosphatidylcholine, egg phosphatidylcholine and egg phosphatidylglycerol (6:2:1, by wt.), which reflects the lipid composition of pulmonary surfactant [25]. The artificial lipid mixture did inhibit the SP-A-induced chemiluminescence response, but did not affect the SP-D-induced chemiluminescence response, just as was shown for the surfactant lipid extract (results not shown).

The SP-A preparation used in the present study was derived from alveolar-proteinosis patients. As has been reviewed by Hawgood [2], there are some structural differences between SP-A isolated from alveolar-proteinosis patients and rat SP-A. This could suggest that the effects observed in the present study would be restricted to human SP-A and rat alveolar macrophages.

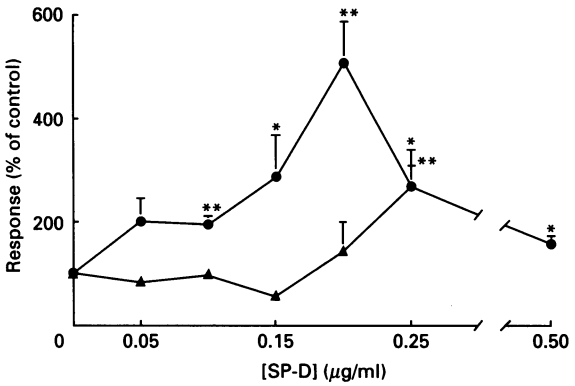

Fig. 2. Enhanced oxygen-radical production by alveolar macrophages is dependent upon the concentration of SP-D

Alveolar macrophages were incubated with various concentrations of either human SP-A (▲) or rat SP-D (●). The responses of the macrophages in the presence of either SP-A or SP-D were expressed as percentages of the chemiluminescence response to macrophages in the absence of stimuli. The observations were depicted as means ± S.E.M. for four independent experiments. Statistical significance: $*P < 0.05$ ($n = 4$); $**P < 0.01$ ($n = 4$) (Student's t test).

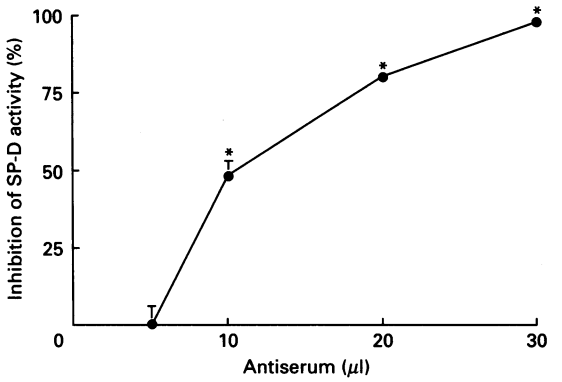

Fig. 3. SP-D stimulation of alveolar macrophages is blocked by anti-SP-D antibody

Alveolar macrophages were incubated with SP-D (0.2 μg/ml) in the presence of various concentrations of $F(ab')_2$ fragments of IgG directed against SP-D (0.6 mg/ml). The response was expressed as the percentage inhibition of the chemiluminescence response in the presence of SP-D. The observations were depicted as means ± S.E.M. for three independent experiments ($*P < 0.001$, $n = 3$).

Table 1. Cell specificity of the chemiluminescence response induced by SP-D

The responses of macrophages in the presence of stimuli, SP-A, SP-D or PMA are expressed as percentages of the response of macrophages in the absence of stimuli. The results are given as means ± S.E.M. for three experiments ($*P < 0.05$; $n = 3$; $**P < 0.01$, $n = 3$).

Stimulus added	Concn.	Response (%) Rat alveolar macrophages	Rat peritoneal macrophages
SP-D	0.2 μg/ml	360 ± 75*	134 ± 35
SP-A	5 μg/ml	388 ± 76*	138 ± 13
PMA	25 ng/ml	461 ± 45**	375 ± 29**

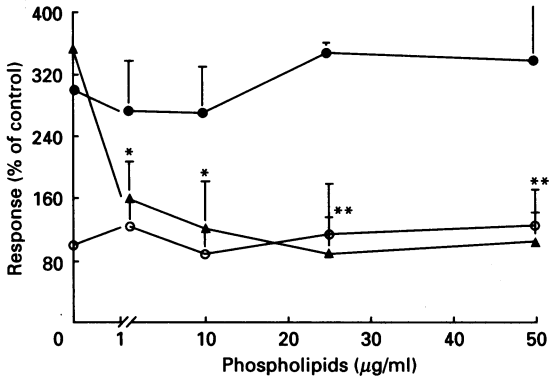

Fig. 4. Lipids attenuate the effects of SP-A, but not SP-D, upon oxygen-radical production by alveolar macrophages

Alveolar macrophages were incubated with buffer (○) or SP-A (5 μg/ml; ▲) or SP-D (0.2 μg/ml; ●) in the presence of various concentrations of surfactant lipids. The response was expressed as described in the legend of Fig. 2. The observations were depicted as means ± S.E.M. for three independent experiments ($*P < 0.05$, $n = 3$; $**P < 0.02$, $n = 3$).

However, so far no functional differences have been described between human and rat SP-A. Equal results with human and rat SP-A were obtained in studies on binding with lipids [26], their interactions with rat type II cells [27] and, most relevant for this study, their stimulation of rat alveolar macrophages [8]. Unfortunately it is very difficult to isolate pure SP-A from rats in the quantities needed for the present study. However, as additional control we performed a small number of experiments using SP-A isolated from rats. Rat SP-A (2 μg/ml) induced a response of 401 ± 55 (mean ± S.E.M.; $P < 0.01$; $n = 4$), expressed as percentage of the chemiluminescence response of macrophages in the absence of stimuli, which is similar to the response induced by human SP-A (388 ± 76; Table 1). In conjunction with the published studies on the functional similarity of human and rat SP-A mentioned above, it seems very likely that the results obtained in the present study with human SP-A can be extrapolated to rat SP-A.

SP-A is known to bind to phospholipids and to form lipid aggregates in the presence of Ca^{2+} [28–30]. Therefore a possible explanation for the observed inhibitory effect of lipids on SP-A-induced stimulation of the macrophages could be that SP-A binds to lipids and forms vesicles that prevent interaction of SP-A with the macrophages. An alternative explanation that cannot be fully excluded is that lipids compete with alveolar macrophages for the same binding site on the SP-A molecule.

SP-D cannot induce lipid aggregation [22]. In addition, approximately only 25% of the SP-D present in the bronchoalveolar lavage of rats is found associated with particulate pulmonary surfactant [22]. The weak interaction of SP-D with phospholipids may therefore explain why phospholipids do not inhibit the SP-D-induced stimulation of alveolar macrophages.

To conclude from these results that SP-D may be more important in the alveolar defence than SP-A, because SPA is inhibited by the surfactant lipids, whereas SP-D is not, would be premature. The contribution of SP-A and SP-D secreted by Clara cells [23,24] to the concentrations of these proteins in the alveoli, and the stimulation of the secretion of SP-A by γ-interferon [31], which does not result in increased surfactant lipid concentrations, are, for example, factors that may influence the balance between SP-A, SP-D and phospholipids in the alveoli. In conclusion, our findings demonstrate that SP-D can activate

alveolar macrophages to produce superoxide radicals, and may therefore play a role in the host-defence system of the lung.

This work was supported by the Dutch Asthma Foundation (Netherlands Astma Fonds) and by the National Institutes of Health Grant 29891.

REFERENCES

1. Possmayer, F. (1988) Am. Rev. Respir. Dis. **138**, 990–998
2. Hawgood, S. (1989) Am. J. Physiol. **257**, L13–L22
3. Hawgood, S., Benson, B. J., Schilling, J., Damm, D., Clements, J. A. & White, R. T. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 66–84
4. Wright, J. R. & Clements, J. A. (1987) Am. Rev. Respir. Dis. **135**, 426–444
5. Dobbs, L. G., Wright, J. R., Hawgood, S., Gonzales, R., Venstrom, K. & Nellenbogen, J. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 1010–1013
6. Rice, W. R., Ross, G. F., Singleton, F. M., Dingle, S. & Whitsett, J. A. (1987) J. Appl. Physiol. **63**, 692–698
7. Van Iwaarden, J. F., Van Strijp, J. A. G., Ebskamp, M. J. M., Welmers, A. C., Verhoef, J. & Van Golde, L. M. G. (1991) Am. J. Physiol. **261**, L204–L209
8. Van Iwaarden, F., Welmers, B., Verhoef, J., Haagsman, H. P. & Van Golde, L. M. G. (1990) Am. J. Respir. Cell Mol. Biol. **2**, 91–98
9. Tenner, A. J., Robinson, S. L., Borchelt, J. & Wright, J. R. (1989) J. Biol. Chem. **264**, 13923–13928
10. Persson, A., Rust, K., Chang, D., Moxley, M., Longmore, W. & Crouch, E. (1988) Biochemistry **27**, 8576–8584
11. Crouch, E., Rust, K., Persson, A., Mariencheck, W., Moxley, M. & Longmore, W. (1991) Am. J. Physiol. **260**, L247–L253
12. Persson, A., Chang, D., Rust, K., Moxley, M., Longmore, W. & Crouch, E. (1989) Biochemistry **28**, 6361–6367
13. Drickamer, K. (1988) J. Biol. Chem. **263**, 9557–9560
14. Thiel, S. & Reid, K. B. M. (1989) FEBS Lett. **250**, 78–84
15. Shimizu, H., Fisher, J. H., Papst, P., Benson, B., Lau, K., Mason, R. J. & Voelker, D. R. (1992) J. Biol. Chem. **267**, 1853–1857
16. Dethloff, L. A., Gilmore, L. B., Brody, A. R. & Hook, G. E. R. (1986) Biochem. J. **233**, 111–118
17. Persson, A., Chang, D. & Crouch, E. (1990) J. Biol. Chem. **265**, 5755–5760
18. Fornstedt, N. & Porath, J. (1975) FEBS Lett. **57**, 187–191
19. Parham, P. M., Androlewicz, M. J., Brodsky, F. M., Holmes, N. J. & Ways, J. P. (1982) J. Immunol. Methods **53**, 133–173
20. Haagsman, H. P., Hawgood, S., Sargeant, T., Buckley, D., White, R. T., Drickamer, K. & Benson, B. J. (1987) J. Biol. Chem. **262**, 13877–13880
21. Bligh, E. G. & Dyer, W. J. (1959) Can. J. Biochem. Physiol. **37**, 911–917
22. Kuroki, Y., Shiratori, M., Ogasawara, Y., Tsuzuki, A. & Akino, T. (1991) Biochim. Biophys. Acta **1086**, 185–190
23. Walker, S. R., Williams, M. C. & Benson, B. (1986) J. Histochem. Cytochem. **34**, 1137–1145
24. Crouch, E., Rust, K., Marienchek, W., Parghi, D., Chang, D. & Persson, A. (1991) Am. J. Respir. Cell Mol. Biol. **5**, 13–18
25. King, R. J. (1984) in Pulmonary Surfactant (Robertson, B., van Golde, L. M. G. & Batenburg, J. J., eds.), pp. 1–15, Elsevier Science Publishers, Amsterdam.
26. Kuroki, Y. & Akino, T. (1991) J. Biol. Chem. **266**, 3068–3073
27. Kuroki, Y., Mason, R. J. & Voelker, D. R. (1988) J. Biol. Chem. **263**, 17596–17602
28. King, R. J., Carmichael, M. C. & Horowitz, P. M. (1983) J. Biol. Chem. **258**, 10672–10680
29. Hawgood, S., Benson, B. J. & Hamilton, R. L. (1985) Biochemistry **24**, 184–190
30. Efrati, H., Hawgood, S., Williams, M. C., Hong, K. & Benson, B. J. (1987) Biochemistry **26**, 7986–7993
31. Ballard, P. L., Liley, H. G., Gonzales, L. W., Odom, M. W., Ammann, A. J., Benson, B., White, R. T. & Williams, M. C. (1990) Am. J. Respir. Cell Mol. Biol. **2**, 137–143

Received 7 May 1992/10 June 1992; accepted 16 June 1992