Pulmonary Surfactant Protein A Modulates the Cellular Response to Smooth and Rough Lipopolysaccharides by Interaction with CD14

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Pulmonary surfactant protein A (SP-A) plays an important part in Ab-independent host defense mechanisms of the lung. In this study we investigated how SP-A interacts with distinct serotypes of bacterial LPS and modulates LPS-elicited cellular responses. SP-A bound to rough forms but not to smooth forms of LPS. In the macrophage-like cell line U937, SP-A inhibited mRNA expression and secretion of TNF-α induced by smooth LPS, but rough LPS-induced TNF-α expression was unaffected by SP-A. When U937 cells and rat alveolar macrophages were preincubated with SP-A, smooth LPS failed to induce TNF-α secretion, whereas rough LPS-induced TNF-α secretion was modestly increased. To clarify the mechanism by which SP-A modulates LPS-elicited cellular responses, we further examined the interaction of SP-A with CD14, which is known as a major LPS receptor. Western blot analysis revealed that CD14 was one of the SP-A binding proteins isolated from solubilized U937 cells. In addition, SP-A directly bound to recombinant soluble CD14 (rsCD14). When rsCD14 was preincubated with SP-A, the binding of rsCD14 to smooth LPS was significantly reduced but the association of rsCD14 with rough LPS was augmented. These results demonstrate the different actions of SP-A upon distinct serotypes of LPS and indicate that the direct interaction of SP-A with CD14 constitutes a likely mechanism by which SP-A modulates LPS-elicited cellular responses.


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Pulmonary surfactant is a mixture of lipids and proteins that acts to keep alveoli from collapsing during expiration (1). Surfactant protein A (SP-A)³ is the most abundant hydrophilic glycoprotein in the surfactant (2). It belongs to the collectin subgroup of the C-type lectin superfamily, along with surfactant protein D (SP-D), bovine conglutinin, mannose-binding protein (MBP), and the protein CL43 (3). These proteins possess similar characteristic structures consisting of a short inter-subunit disulfide forming N-terminal region, a collagen-like domain, a neck domain, and the carbohydrate recognition domain (CRD) (2). SP-A may function as an autocrine regulator of phospholipid homeostasis within the alveolar space (2). In addition, SP-A plays an important role in host defense mechanism of the lung. SP-A binds to alveolar macrophages with high affinity (4) and promotes chemotaxis (5) and phagocytosis of microbial species including Staphylococcus aureus (6, 7), Herpes simplex virus type I (8), type A Hemophilus influenza (9), Mycobacterium tuberculosis (10), and Klebsiella (11). SP-A also interacts with Pneumocystis carinii (12). The protein can enhance FcR- and CR1-mediated phagocytosis by human monocyte-derived macrophages (13). Recent studies have led the contradictory conclusions for the SP-A-mediated modulation of cytokine expression in immune cells (14, 15). The precise functions of SP-A in Ab-independent immune surveillance and its mechanism of action remain to be resolved.

LPS is a major constituent of the outer membrane of Gram-negative bacteria. Most enteric bacteria express smooth LPS, which is composed of O-Ag, complete core oligosaccharides, and the endotoxin-principal region, lipid A. There are also rough mutants such as Ra, Rb, Rc, Rd, and Re strains which lack O-Ag but possess lipid A and progressively shorter core oligosaccharides (16). A number of Gram-negative bacteria colonizing the surfaces of the respiratory tract express a rough LPS phenotype (17). Direct interaction between SP-A and rough LPS has been suggested (18), but its physiological and functional significance has not been clearly defined.

LPS has been known to activate macrophages and to induce a variety of mediators including TNF-α, IL-1, IL-6, IL-8, IL-12, migration inhibitory factor, chemokine, IFN, eicosanoids, and reactive oxygen (19, 20). The response to physiological amounts of LPS by reactive cells depends on membrane CD14 (mCD14) (20), which is a 55-kDa glycoprotein that is GPI-anchored to the plasma membrane of myeloid cells. A soluble form of the protein (sCD14) is also found in plasma and facilitates the responsiveness of cells to LPS (21, 22). However, the mechanism by which the LPS signal is transduced across the cell membrane remains unknown. It has been considered that the role of mCD14 is to bind LPS and the subsequent interaction with an unidentified transmembrane protein is required for transmitting LPS signal (23). Recently, it has been suggested that Toll-like receptor 2 is a direct mediator of signaling by LPS (24).

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3 Abbreviations used in this paper: SP-A, surfactant protein A; hSP-A, human SP-A; CRF, collagenase-resistant fragment; CRD, carbohydrate recognition domain; SP-D, surfactant protein D; MBP, mannose binding protein; LBP, LPS binding protein; mCD14, membrane CD14; sCD14, soluble CD14; rsCD14, recombinant soluble CD14, PVDF, polyvinylidene difluoride; Re LPS, LPS derived from S. minesota strain Re 595 (Re mutant); RcLPS, LPS derived from E. coli strain J5 (Rc mutant).
SP-A interacts with LPS and CD14

Materials and Methods

Reagents and cells

Smooth LPS (Escherichia coli O26:B6, O111:B4), rough LPS (Salmonella minnesota Re595, E. coli 35), and polymyxin B-agarose were purchased from Sigma (St. Louis, MO). Lipid A from S. minnesota Re595 was obtained from List Biologic Laboratories (Campbell, CA). Succinimidobiotin (Sulfo-NHS-biotin) was from Pierce (Rockford, IL), and DNA polymerase and BSA were obtained from New England Biolabs (Beverly, MA). 15% glycerol was from the Health Science Research Resources Bank (Osaka, Japan). L929 cells were kindly provided by Dr. Kazuko Kajiyama (Chugai Pharmaceutical, Tokyo, Japan). The cells were maintained in RPMI 1640 medium (Nissui, Tokyo, Japan) containing 10% FCS.

SP-A

Surfactant was isolated from bronchoalveolar lavage fluids of patients with alveolar proteinosis as described previously (25). After the surfactant was delipidated with 1-butanol (26), SP-A was purified from the delipidated surfactant by manganese-Sepharose 6B column chromatography followed by gel filtration over a Bio Gel A5m column (BioRad Laboratories, Richmond, CA) (27). The collagenase-resistant fragment (CRF) of human SP-A was prepared as described previously (28).

Monitoring and removal of endotoxin in SP-A preparations

Endotoxin in SP-A preparations was removed by polymyxin B-agarose in the presence of octyl-β-D-glucoside as described by Mclntosh et al. (15). Endotoxin levels in untreated or polymyxin-treated SP-A preparations were 55 pg or below 0.5 pg/μg of protein, respectively, when measured by chromogenic assay using Limulus amebocyte lysate system (ENDOSPECY; Seikagaku Kogyo, Tokyo, Japan). Polymyxin-treated SP-A was used for the experiments performed in this study.

Binding of SP-A to LPS

LPS (5 μg/well) or lipid A in 20 μl of ethanol was placed in microwell plates (Immulon 1B; Dynex Laboratories, Chantilly, VA), and the solvent evaporated in ambient air. After the nonspecific binding was blocked with 20 mM Tris buffer (pH 7.4) containing 0.15 M NaCl, 5 mM CaCl2, and 1 mg/ml of BSA (buffer A), various concentrations of human SP-A (hSP-A) or CRF in 50 μl of the buffer A were added and incubated at 37°C for 5 h. The wells were then washed with buffer A, and 20 μg/ml anti-human SP-A IgG (50 μl/well) in PBS containing 0.1% (v/v) Triton X-100 and 3% (v/v) skim milk (buffer B) was added and incubated for 1 h, followed by the incubation with HRP-labeled anti-rabbit IgG (1:1000) for 1 h. After washing the wells with PBS containing 0.1% (v/v) Triton X-100, the peroxidase reaction was finally performed using o-phenylenediamine as a substrate. The binding of SP-A to LPS was detected by measuring absorbance at 492 nm.

Aggregation of LPS

LPS aggregation was conducted by a modified method based on that described by van Iwaarden et al. (18). LPS or lipid A (25 μg/ml) in 20 mM Tris buffer (pH 7.4) containing 0.15 M NaCl was probe-sonicated and incubated with or without hSP-A (20 μg/ml) for 5 min at room temperature. After the initial absorbance reading at 400 nm by a Hitachi U-2000 spectrophotometer (Tokyo, Japan), CaCl2 was added to a final concentration of 1 mM at a time of 3 min and the absorbance was further measured until a time of 10 min.

TNF-α mRNA expression

U937 cells (0.5 × 10^6) were obtained to differentiate to macrophages by incubation with 10 nM PMA for 24 h. The cells were further incubated in the absence of PMA for 24 h in RPMI 1640 medium containing 10% FCS. The indicated amounts of LPS were added to the cell culture and incubated in the absence or presence of SP-A for 2 h at 37°C with 5% CO2. After incubation, total cellular RNA was isolated from the cells by acid guanidium thiocyanate-phenol-chloroform method (29). A cDNA pool was obtained from 1 μg of RNA using 20 U Superscript II Reverse Transcriptase (Life Technologies, Grand Island, NY) and 0.5 mM dNTPs (30). The region of cDNA for human TNF-α was amplified from the cDNA pool derived from U937 cells by PCR using 2.5 μM specific primers (5′-AACG CTTGAGCCCATGTTGT-3′ as a sense primer and 5′-CAGATAGATGGGCTCATTAC-3′ as an antisense primer). PCR was performed in the presence of 2 mM MgCl2, 0.2 mM dNTPs, 1.25 U Taq polymerase, and 1 μg of 1′10-pC12F. After the initial incubation at 95°C for 5 min, 25 cycles of amplification were conducted with denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 2 min. PCR products (330 bp) were electrophoresed on a 1.0% agarose gel, and the purified DNA was amplified by a Bio-Imaging Analyzer BAS 2000 (Fuji Photo Film, Tokyo, Japan). TNF-α mRNA was expressed as a relative ratio compared with the radioactivity of a control PCR product derived from the cells that had been incubated in the absence of LPS and SP-A.

TNF-α secretion

TNF-α secretion into medium was measured using an L929 cell bioassay performed by a modified method based on that described by Flick et al. (30). The L929 cells were seeded into 96-well plates (6 × 10^3/well) in 100 μl/well of RPMI 1640 containing 10% FCS and 2 μg/ml actinomycin D (Sigma). Dilutions of standard rTNF-α (1–50 pg/ml) (PeproTech, Rocky Hill, NJ) or samples (1:10 and 1:50) in a volume of 100 μl/well were added and the cells were cultured at 37°C with 5% CO2 for 18 h. The wells were then washed with water, and 100 μl/well of assay medium containing 33% acidic acid was added to extract the retained crystal violet. The absorbance at 570 nm was finally measured.

Effect of SP-A upon LPS-induced TNF-α expression in U937 cells

Differentiated U937 cells (0.5 × 10^6) were incubated with 1–1000 ng/ml of smooth or rough LPS in the presence of 0–50 μg/ml of hSP-A for the indicated time. After the incubation, the medium was collected and TNF-α secretion was measured using an L929 cell bioassay as described above. TNF-α mRNA expression in U937 cells was also evaluated by RT-PCR as described above. In some experiments, SP-A was preincubated with the cells before the addition of LPS and then TNF-α secretion was measured.

Binding of SP-A to U937 cells

The binding study was adapted from that described for type II cell binding (27). Rat SP-A was iodinated by the method of Bolton and Hunter (31) using Bolton-Hunter reagent (Amersham, Arlington Heights, IL). The specific radioactivity ranged from 490 to 518 cpm/ng, and >84% of the radioactivity was precipitated by treatment with 10% (v/v) TCA. For the binding assay, differentiated U937 cells (0.5 × 10^6) in RPMI 1640 containing 10% FCS were incubated with various concentrations of the labeled protein at 37°C for 5 h. The cell monolayers were then washed with ice-cold 20 mM Tris buffer (pH 7.4) containing 0.1 M NaCl, 2 mM CaCl2, and 1 mg/ml BSA and were finally dissolved in 2 ml of 0.1 M NaOH. The amount of the labeled protein was determined using a gamma-radiation counter.

Biotinylation and solubilization of U937 cells

Differentiated U937 cells (150 × 10^6) were harvested, washed with ice-cold PBS (pH 7.4) by centrifugation at 1000 rpm, and incubated for 30 min at room temperature in 0.5 ml PBS containing N-hydroxysulfosuccinimidobiotin (Suclo-NHS-biotin) (Pierce) at a final concentration of 0.5 mg/ml. The cells were then washed with PBS to remove excess biotin, and the final cell pellet was solubilized by the incubation on ice for 30 min with 2 ml of 50 mM HEPES buffer (pH 7.4) containing 0.15 M NaCl, 1% Nonidet P-40, 1 mM PMSF, 1 mM EDTA, 1 μg/ml pepstatin A, 100 μg/ml benzamidine,
2 μM phosphoramidon, 10 μg/ml trypsin inhibitor, and 1 μg/ml aprotinin. Whole cell extracts were then centrifuged for 10 min at 400 x g, and the supernatant was further centrifuged for 30 min at 100,000 x g to separate detergent-insoluble fraction from detergent-soluble membranes.

**Isolation and Western blot analysis of SP-A binding proteins on U937 cells**

To prepare a hSP-A affinity column, 2.5 mg of hSP-A in 0.1 M NaHCO₃ buffer (pH 8.3) containing 0.5 M NaCl was coupled with 0.5 g of CNBr-activated Sepharose 4B gel according to the manufacturer’s instruction (Pharmacia Biotech). The fraction solubilized from biotinylated cell membranes described above, which had been diluted with 10% volume of binding buffer (20 mM Tris (pH 7.4) containing 0.15 M NaCl, 5 mM CaCl₂, and 0.1% Nonidet P-40), was applied to hSP-A affinity column at 4°C. After the column was washed with the binding buffer, the bound materials were eluted with 20 mM Tris buffer (pH 7.4) containing 0.15 M NaCl, 10 mM EDTA, and 0.1% Nonidet P-40. The eluate was then concentrated and dialyzed against 5 mM Tris buffer (pH 7.4). All the procedures described above were performed under sterile conditions. To visualize the SP-A binding proteins isolated from U937 cells, the eluted samples were electrophoresed under reducing conditions and transferred to polyvinylidene difluoride (PVDF) membrane. After blocking PVDF membranes with the buffer B, the membranes were incubated with HRP-conjugated streptavidin D (1:1000) for 20 min. The membranes were washed, followed by the peroxidase reaction using diaminobenzidine tetrahydrochloride as a substrate. For the Western blot analysis, the PVDF membranes on which the SP-A binding proteins were transferred were incubated with either 20 μg/ml of mAb to CD14 or control mouse serum (1:500) for 90 min. The membranes were then washed with blocking buffer, followed by the incubation with HRP-labeled anti-mouse IgM (1:500) for 1 h. After the incubation, the membranes were washed with PBS containing 0.1% (v/v) Triton X-100. They were then incubated with chemiluminescence reagent (NEN) and the proteins were visualized on an x-ray film.

**Binding of SP-A to rsCD14**

Expression and purification of recombinant human soluble CD14 (rsCD14) from the culture supernatant of *Pichia pastoris* will be described elsewhere (S. Nomura and T. Muta, unpublished data). For the ligand blot analysis, 5 μg/lane of rsCD14 was electrophoresed under reducing conditions and transferred to PVDF membrane. After nonspecific binding was blocked with buffer B, the membrane was incubated with either 20 μg/ml of hSP-A or 50 μg/ml of buffer B at room temperature overnight. The membranes were then washed with buffer B and incubated with anti-SP-A IgG (20 μg/ml) for 90 min, followed by the incubation with HRP-labeled anti-rabbit IgG (1:1000) for 75 min. SP-A that had bound to the PVDF membrane was visualized by using chemiluminescence reagent (NEN) as described above. rsCD14 transferred to the membrane was also visualized by Coomassie brilliant blue staining.

To examine the concentration-dependent binding of SP-A to CD14, 50 μl/well of sterile BSA or rsCD14 at a concentration of 10 μg/ml in 5 mM Tris buffer (pH 7.4) was coated onto microtiter wells and incubated at room temperature for 5 h. Nonspecific binding was blocked with 10 mM HEPES buffer (pH 7.4) containing 0.15 M NaCl, 5 mM CaCl₂, and 5% (v/v) BSA (buffer C) and the indicated concentrations of hSP-A in 50 μl of buffer C were added and incubated at 37°C overnight. The wells were then washed with buffer B. Anti-SP-A IgG (20 μg/ml) was added (50 μg/ml) and incubated at 37°C for 90 min, followed by the incubation with HRP-labeled anti-rabbit IgG (1:1000) for 75 min. The wells were washed with PBS containing 0.1% (v/v) Triton X-100 and the peroxidase reaction was performed using o-phenylenediamine as a substrate. The binding of hSP-A to rsCD14 was detected by measuring absorbance at 492 nm.

**Binding of rsCD14 to LPS**

Two micrograms per well of smooth or rough LPS in 20 μl of ethanol was added into microtiter wells and air-dried. Nonspecific binding was blocked with buffer C. rsCD14 (5 μg/ml) in buffer C containing 2% (v/v) human serum in the absence or the presence of hSP-A (50 μg/ml) was preincubated at 37°C for 1 h. The preincubated protein mixtures (50 μg/ml) were then added into the wells and further incubated with solid phase LPS at 37°C overnight. The binding of rsCD14 to LPS was detected by using enzyme-linked, monoclonal antisa (1:400) raised against recombinant CD14 expressed in *Escherichia coli* as a fusion protein with GST and HRP-labeled anti-rabbit IgG (1:1000).

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**FIGURE 1.** SP-A and its CRF bind to lipid A and Re LPS. Five micrograms per well of lipid A (ΔA), Re LPS (■), Rc (J5) LPS (●), *E. coli* O26:B6 (□), or O111:B4 (○) was coated onto microtiter wells and incubated with the indicated concentrations of hSP-A (A) or its CRF (B) for 5 h at 37°C. The binding of the proteins to lipids was detected using anti-human SP-A IgG as described under Materials and Methods. The data shown are mean ± SE of three experiments. *p < 0.02; **p < 0.05 when compared with the binding of SP-A to O111:B4.

**Effect of SP-A upon LPS-induced TNF-α secretion in alveolar macrophages**

Rat alveolar macrophages were obtained from bronchoalveolar lavage fluids of Sprague Dawley rats. The lungs were lavaged with PBS containing 0.5 mM EDTA. Isolated macrophages (1.0 x 10⁷/well in a 96-well plate) were incubated overnight in RPMI 1640 medium containing 10% FCS. Then, macrophages were incubated in RPMI medium containing 10% FCS in the presence of the absence of 10 μg/ml of hSP-A for 1 h, and then further incubated with 2 ng/ml of LPS for 5 h. TNF-α secretion into medium was measured using a L929 cell bioassay as described above.

**Results**

The association of SP-A with LPS

To clarify the interaction of SP-A with endotoxin, we initially examined the direct binding of SP-A to distinct structural types of LPS coated onto microtiter wells (Fig. 1A). hSP-A bound to lipid A and rough strains of LPS. The binding of hSP-A to Rc strain (*E. coli* J5) of rough LPS was significant but weaker than that to Re strain (*S. minnesota* Re595). In contrast, hSP-A did not show any significant binding to either of two strains of smooth LPS (*E. coli* O26:B6 or O111:B4). The CRF of hSP-A, which consists of the neck plus the CRD of SP-A, also bound to lipid A and Re LPS although its binding appeared weaker than that of hSP-A (Fig. 1B).
In this assay, when 5 μg of smooth LPS (O26:B6), Re LPS and lipid A had been adsorbed onto microtiter wells, chromogenic assay revealed 350 ± 29 ng (mean ± SE, n = 3) of smooth LPS, 325 ± 30 ng of Re LPS and 280 ± 18 ng of lipid A were present in the wells after the washing procedures. Thus, the solid phase ligands were similar in the amounts (by weight) bound to the microtiter wells. Therefore, these results indicate the different interactions of SP-A with smooth and rough LPS and further demonstrate that the region of the neck plus CRD is directly involved in the binding of SP-A to lipid A and rough LPS.

To confirm the interaction of hSP-A with LPS in solution, aggregation experiments were also performed. Sonicated LPS or lipid A which was incubated alone in the presence of Ca²⁺ did not significantly increase the absorbance at 400 nm (the absorbance at 10 min; 0.001 for O26:B6 or Re LPS, 0.002 for lipid A). When smooth LPS (O26:B6) was co-incubated with hSP-A, the absorbance increased slightly (0.013), but its level was below that of the self-aggregation of hSP-A (0.021). In comparison, a significant level of aggregation was observed by the co-incubation of Re LPS with hSP-A (0.041), and a remarkable increase of absorbance was obtained when lipid A was used instead of LPS (0.078). The data obtained from aggregation experiments are consistent with the results obtained by van Iwaarden et al. (18) and clearly demonstrate that SP-A binds and causes aggregation of fluid phase lipid A and rough LPS but not of smooth LPS.

Collectively, the results indicate that SP-A recognizes rough LPS but not smooth LPS in fluid phase as well as in solid phase.

**The effect of SP-A upon LPS-induced TNF-α expression in U937 cells**

We next examined whether SP-A acted differently upon the cellular responses to the distinct serotypes of LPS. The human macrophage-like cell line, U937, was differentiated and incubated with LPS in the absence or the presence of hSP-A for 2 h, and relative TNF-α mRNA levels were compared (Fig. 2A). Endotoxin-free hSP-A by itself did not induce TNF-α expression. When the cells were incubated with 10 ng/ml smooth LPS (O26:B6) in the presence of 20 μg/ml hSP-A, the TNF-α mRNA level induced by smooth LPS was significantly decreased. However, hSP-A failed to alter TNF-α mRNA expression induced by Re LPS. The secretion of TNF-α into media was also determined by L929 cell bioassay when differentiated U937 cells were incubated with LPS for 5 h. Consistently, hSP-A dramatically diminished TNF-α secretion induced by smooth LPS but did not attenuate Re LPS-stimulated TNF-α secretion (Fig. 2B).

We also examined the effect of hSP-A that had been treated at 100°C for 5 min. The differentiated U937 cells were incubated with 10 ng/ml smooth LPS in the presence of 20 μg/ml untreated or heat-treated hSP-A. While untreated hSP-A reduced TNF-α secretion to the level of 8% of that induced by smooth LPS, the level of TNF-α secretion in the presence of heat-treated hSP-A was 107%. The results indicate that heat treatment of SP-A completely destroys the inhibitory activity and support the idea that the SP-A protein affects the cellular responses to LPS.

The inhibitory effect of hSP-A upon smooth LPS-induced TNF-α secretion was clearly dependent upon the SP-A concentrations (Fig. 3A). Concentrations as low as 2 μg/ml hSP-A completely blocked TNF-α secretion stimulated by 10 ng/ml smooth LPS. We also examined the effect of hSP-A upon various concentrations of LPS (Fig. 3B). When 1–100 ng/ml smooth LPS was co-incubated with 20 μg/ml hSP-A, TNF-α secretion was completely inhibited. However, the inhibitory effect of hSP-A upon very high amounts of smooth LPS was apparently weak. hSP-A reduced TNF-α secretion stimulated by 1 μg/ml smooth LPS by only 40%. Increasing the SP-A concentration up to 50 μg/ml did not alter the degree of the inhibitory effects of SP-A. In contrast to smooth LPS, we found that 20 μg/ml hSP-A did not attenuate TNF-α secretion stimulated by either low (1 ng/ml) or high (1 μg/ml) concentration of Re LPS. These results emphasize the different actions of SP-A upon the cellular responses to distinct strains of LPS, and suggest the possibility that SP-A may prevent CD14-dependent responses to smooth LPS because the response to low but not high concentrations of LPS depends on CD14 (20, 32).

**The interaction of SP-A with U937 cells affects cellular responses to LPS**

SP-A does not bind smooth LPS but alters cellular response to smooth LPS. Therefore, we speculated that the interaction of SP-A with U937 cells might alter the cellular responses to LPS. This idea initially led us to estimate the binding of SP-A to differentiated cells.
U937 cells. $^{125}$I-labeled SP-A was found to bind differentiated U937 cells with high affinity (Fig. 4). The apparent $K_d$ calculated by Klotz plot was $2.8 \times 10^{-9}$ M and the binding sites was $5.9 \times 10^4$ per cell, assuming a multimeric molecular mass of $6.5 \times 10^5$ for SP-A (33). The binding of SP-A to U937 cells was time-dependent, and its binding reached saturation at $37^\circ$C. When $1 \mu g/ml$ $^{125}$I-labeled SP-A was incubated with the cells, 50-fold excess unlabeled SP-A reduced $^{125}$I-labeled SP-A binding to the level of 5.5% of control binding.

We next investigated whether the binding of SP-A to the cells affected subsequent cellular responses to LPS. Differentiated U937 cells were preincubated with or without $20 \mu g/ml$ hSP-A for 2 h before LPS treatment. The cells were subsequently washed with medium three times to remove unbound hSP-A and then incubated with 10 ng/ml LPS in the absence or the presence of hSP-A for 5 h. As we described above, co-incubation of hSP-A with smooth LPS (O26:B6) completely inhibited TNF-α secretion (Fig. 5). When the cells were preincubated with hSP-A for 2 h and unbound hSP-A was washed out, no TNF-α was secreted by O26:B6 LPS added subsequently ($p < 0.002$, compared with O26:B6 LPS alone without pretreatment). This finding demonstrates that the inhibitory effect of SP-A persists even after its removal from the medium. In comparison with smooth LPS, TNF-α secretion induced by rough (Re) LPS was not attenuated by co-incubated hSP-A, and it was found that after preincubating the cells with hSP-A before the addition of Re LPS, TNF-α secretion was significantly increased ($p < 0.01$, compared with Re LPS alone without pretreatment). To confirm that unbound SP-A was effectively removed from the medium by the washing procedures, we determined the amount of SP-A remaining in the medium. When differentiated U937 cells were preincubated with or without 20 mg/ml hSP-A and washed three times, the cells were subsequently incubated for 5 h with or without 20 µg/ml hSP-A and washed three times. The cells were subsequently incubated for 5 h with or without 20 µg/ml of hSP-A in the absence or the presence of 10 ng/ml smooth (O26:B6) or Re LPS. TNF-α secretion was measured by L929 bioassay and expressed as percent of LPS-stimulated TNF-α secretion. The mean value of TNF-α secretion induced by smooth or Re LPS in the absence of SP-A was 825 or 544 pg/ml (100%), respectively. The data shown are mean ± SE from three separate experiments with duplicate samples.
FIGURE 6. Isolation of the SP-A binding proteins from solubilized U937 cell membranes by SP-A affinity column and immunoblot analysis using anti-CD14 Ab. Differentiated U937 cells were labeled with 125I-hydroxy-sulfosuccinimidobiotin, solubilized, and applied to an affinity column covalently linked with hSP-A. The eluted proteins that had bound to the SP-A affinity column were electrophoresed and transferred to PVDF membrane, and were visualized using avidin-HRP (lane a). The membranes were also probed with anti-CD14 Ab (lane b) or control mouse serum (lane c), followed by the incubation with HRP-labeled anti-mouse IgM Ab.

FIGURE 7. SP-A binds to rsCD14. A, Ligand blot analysis of rsCD14. Five micrograms of rsCD14 was electrophoresed and transferred to PVDF membrane. rsCD14 on the membrane was visualized by Coomassie brilliant blue staining (lane a). The membrane was also incubated with hSP-A (lane b) or BSA (lane c) and probed with anti-SP-A IgG, followed by the incubation with HRP-labeled anti-rabbit IgG. B, Concentration-dependent binding of hSP-A to rsCD14. Fifty microliter aliquots of 10 μg/ml BSA (□) or rsCD14 (●) were coated onto microtiter wells and incubated with the indicated concentrations of hSP-A at 37°C overnight. The binding of hSP-A to rsCD14 was detected using anti-SP-A IgG as described in Materials and Methods. The presented data are mean ± SE of three experiments.

The interaction of SP-A with CD14 alters the binding of CD14 to LPS

For the purpose of clarifying the mechanism by which SP-A modulates LPS-induced cellular responses, we then investigated whether SP-A affects the interaction of CD14 with LPS. After rsCD14 was preincubated with hSP-A for 1 h, the mixture of rsCD14 and hSP-A was further incubated with smooth (O26:B6) or rough LPS coated onto the wells, and the rsCD14 that bound to the solid phase LPS was detected using anti-CD14 Ab. Preincubation of rsCD14 with hSP-A significantly reduced the binding of rsCD14 to smooth LPS (Fig. 8). In contrast, the binding of rsCD14 to Re LPS was increased by the preincubation of rsCD14 with hSP-A. The results obtained from Fig. 8 are quite consistent with those demonstrating that preincubation of U937 cells with SP-A decreases cellular responses to smooth LPS but increases the responses to rough LPS (see Fig. 5). Therefore, these data support the idea that the principal mechanism of SP-A-mediated cellular responses is that the direct interaction of SP-A with CD14 alters the subsequent binding of LPS to CD14. Smooth LPS, which cannot bind to SP-A, may fail to interact with CD14 when SP-A has bound to CD14, whereas rough LPS, which binds to SP-A, may effectively associate with CD14 via an SP-A-CD14 complex.
alveolar macrophages in the same manner as that of macrophage-demonstrate that SP-A modulates LPS-elicited responsiveness of

By the preincubation of macrophages with hSP-A, TNF-α-mediated modulation of LPS-elicited responses may depend on the

extent of SP-A to LPS (see Fig. 1). The presence of SP-A appears to correlate well with that of the binding
temperature of rsCD14 and hSP-A was further incubated with smooth (O26:B6) or Re LPS coated onto microtiter wells. The binding of rsCD14 to LPS was then detected using polyclonal antisera to rsCD14 as described in Materials and Methods. The data presented are mean ± SE of four experiments. *, p < 0.05 when compared with rsCD14 binding to smooth LPS in the absence of hSP-A; **, p < 0.02 when compared with rsCD14 binding to Re LPS in the absence of hSP-A.

The effect of SP-A on LPS-induced TNF-α expression in alveolar macrophages

To confirm that the functions of SP-A examined with U937 cells were relevant to alveolar environment, we investigated the effect of SP-A on rat alveolar macrophages (Fig. 9). SP-A has been previously shown to bind alveolar macrophages with high affinity (4). Consistently, hSP-A exhibited a significant inhibitory effect on smooth LPS-induced TNF-α secretion by alveolar macrophages. By the preincubation of macrophages with hSP-A, TNF-α secretion induced by O26:B6 or O111:B4 was reduced to 54.1 or 59.3% of that induced by LPS alone, respectively. In contrast, hSP-A failed to inhibit rough LPS-stimulated cellular responses. hSP-A did not affect Re LPS-induced TNF-α secretion and dramatically increased Re LPS-induced TNF-α secretion. These results clearly demonstrate that SP-A modulates LPS-elicited responsiveness of alveolar macrophages in the same manner as that of macrophage-like cell line U937 cells. In addition, the rank order of TNF-α secretion (O26:B6, O111:B4 < Re < Rc) elicited by LPS in the presence of SP-A appears to correlate well with that of the binding extent of SP-A to LPS (see Fig. 1A). Taken together, these results also support the idea that when SP-A has bound to CD14, SP-A-mediated modulation of LPS-elicited responses may depend on the affinity between SP-A and LPS.

Discussion

This study confirmed the binding of SP-A to lipid A and rough LPS. The binding of SP-A to Re LPS was more apparent than that to Rc LPS which possesses longer core oligosaccharides than Re LPS. SP-A failed to bind to smooth LPS. Steric constraints of the large O-Ag (O-antigen polysaccharides) and complete core oligosaccharides of smooth LPS may prevent SP-A binding to the lipid component. Interaction with rough LPS appears to be an important property of the collectin family, because SP-D and MBP also bind to rough strains of Gram-negative bacteria (35, 36). van Iwaarden et al. (18) also demonstrated the binding of hSP-A to rough LPS, but the present experiments were performed with hSP-A in which contaminating endotoxin had been removed by polymyxin B-agarose. This treatment is considered to be effective to remove endotoxin without loss of well defined SP-A functions. When analyzed by SDS-PAGE, polymyxin-treated hSP-A migrated at a position identical to untreated hSP-A and formed the usual covalent oligomers under nonreducing conditions. The level of the recognition by anti-SP-A Ab, the activities of binding lipids and interacting with alveolar type II cells were equal for treated and untreated proteins. In addition, we confirmed that 20 μg/ml of untreated hSP-A also bound to solid phase Re LPS in our assay (the final absorbance at 492 nm was 1.504 ± 0.135 (mean ± SE, n = 3)). Therefore, the present study clearly demonstrates the interaction of endotoxin-free hSP-A with LPS, and further indicates that the region of the neck plus CRD is directly involved in the LPS binding. However, this may not account for all the LPS binding because the binding of CRF to LPS was significantly weaker than that of SP-A.

SP-A inhibited TNF-α mRNA expression and TNF-α secretion induced by smooth LPS but not by rough LPS. It is unlikely that rough LPS acts more potently than smooth LPS, because the concentration-dependent stimulation by smooth and rough LPS generated almost equal amounts of TNF-α in differentiated U937 cells in this study. Furthermore, the TNF-α secretion stimulated by 100 ng/ml smooth LPS was completely blocked by 20 μg/ml SP-A, while that by 1 ng/ml rough LPS was not significantly inhibited. Approximately 2 mol equivalents of octadecameric SP-A (33) are required to neutralize 1 mol equivalent of smooth LPS in our assays. However, SP-A at molar ratio of 75:1 fails to block rough LPS-stimulated TNF-α secretion. Taken together, the inhibitory effect of SP-A is quite restricted to smooth LPS-induced cellular responses.
To understand the mechanism by which SP-A specifically prevents smooth LPS-induced TNF-α expression, several possible interactions were considered. Clearly, SP-A does not affect the cellular responses by binding directly to smooth LPS. The mode of action of SP-A is different from MBP which has been shown to inhibit TNF-α secretion induced by streptococcal cell wall polysaccharides, rhamnose glucosyl polymers (RGP), by its direct binding to RGP (37). Another possible mechanism may be that SP-A interferes with the functions of the serum components such as LPS binding protein (LBP) (38), sCD14 (22), and septicin (39), which have been shown to accelerate LPS-induced cellular responses. sCD14 and LBP are necessary for neutrophil responses to low concentrations of smooth LPS but LBP alone is sufficient for response to rough LPS (21). However, in the current study, the interaction of SP-A with the serum components in media does not appear to be a primary mechanism, because the inhibitory effect of SP-A persists even after this protein is removed from the media. Cell-bound SP-A is quite sufficient to modulate LPS-elicited cellular responses.

The present study clearly indicates that the interaction of SP-A with cells alters the cellular responses to LPS. Furthermore, we show that SP-A binds to CD14. Because SP-A does not associate with smooth LPS, it is reasonable to propose that the binding of SP-A to mCD14 may prevent smooth LPS from binding to mCD14, and consequently the smooth LPS-elicited cellular response is inhibited. This idea is clearly supported by the present results demonstrating that the binding of rsCD14 to smooth LPS is significantly decreased by the preincubation of rsCD14 with SP-A.

In contrast, the interpretation of the results obtained with SP-A and rough LPS seems more complex. Our data suggest that preincubation of SP-A with CD14 accelerates the binding of CD14 to rough LPS and increases rough LPS-mediated cellular responses. At present, we speculate that the interaction of rough LPS with CD14 is accelerated in the presence of SP-A which is a ligand for both rough LPS and CD14. The idea that SP-A can simultaneously cross-link with both rough LPS and CD14 is also supported by the results demonstrating that the domains of the SP-A molecule involved in the binding to rough LPS are different from those required for the binding to CD14 (H. Sano and Y. Kuroki, manuscript in preparation). Recent observations indicate that LPS which has bound to mCD14 activate the cells via subsequent interactions with unidentified transmembrane components (23, 40). Recently, human Toll-like receptor-2 and murine Toll-like receptor-4 each have been implicated in signaling by LPS and CD14 (24, 41, 42). An SP-A-rough LPS-mCD14 complex might effectively transfer rough LPS to another acceptor required for signaling. This idea may be analogous to the model of LBP-LPS-mCD14 ternary complex (43). Further studies are required to clarify the precise mechanism by which SP-A modulates the LPS signal transduction.

Two distinct proteins isolated from U937 cells have been identified as SP-A or Clq/MBL/SP-A receptor (44, 45), suggesting the existence of multiple receptors for SP-A. We also found several SP-A binding proteins isolated from solubilized U937 cells by SP-A affinity column, and further indicated that mCD14 was one of them. Isolation and investigation of the functions of unidentified SP-A receptors could also enable us to understand the precise mechanism of SP-A-mediated immune responses.

Although several serum molecules which affect LPS-induced cellular responses have been well studied, little is known about the components which exist in the alveolar liquid layer and modulate LPS-induced responses of alveolar macrophages. Furthermore, although CD14 has been well known as a major LPS receptor, the possible ligands for CD14 have not been fully investigated. This study highlights that SP-A can bind to CD14, and affects cellular responses to LPS in alveolar macrophages. Because a number of Gram-negative bacteria colonizing the respiratory tract do not express O-Ag or a part of the core oligosaccharides (17), the interaction of SP-A with rough LPS is likely to be important within the lung. The lipid A parts of E. coli and S. minnesota examined in this study are similar to those of important Gram-negative lung pathogens such as Pseudomonas aeruginosa and Haemophilus influenzae, indicating that SP-A may also interact with the clinical isolates of the pathogens which contain rough LPS. The stark contrast of SP-A binding to distinct serotypes of LPS is consistent with the recent findings that SP-A enhanced phagocytosis of rough strain but not of smooth strain of bacteria by macrophages (46). The interaction of SP-A with both CD14 and rough LPS may also participate in the clearance of rough LPS, because the internalization of LPS is also initiated by anchoring to mCD14 (43). TNF-α is a proinflammatory cytokine that plays a central role in many human diseases and it exhibits both beneficial and pathological effects. Overproduction of TNF-α by LPS-activated macrophages could potentially worsen the diseased state. Thus, SP-A-mediated down-regulation of TNF-α expression induced by smooth LPS may play an important dampening role in controlling inflammation.

In this study lipid-free SP-A inhibits smooth LPS-elicited cellular responses. Because SP-A interacts with lipids and is thought to exist as a lipoprotein in the alveoli, it is important to determine whether a similar effect of SP-A can be seen in the presence of surfactant lipids. We are now investigating the effects of hydrophobic surfactant components upon the response of U937 cells to LPS and SP-A.

The lungs appear to remain in a relatively uninflamed state despite the daily exposure to ambient air that contains significant numbers of microorganisms and their derivative products such as LPS. Expression of SP-A is enhanced in response to intratracheal instillation of smooth LPS (47, 48), indicating that the lung is highly resistant to the pathological effects of LPS. However, neutrophils and macrophages removed from the alveolar environment are exquisitely sensitive to LPS. One idea emerging from this study is that SP-A functions under ordinary conditions as a potent anti-inflammatory agent either sequestering microorganisms or their products or effecting an anti-inflammatory response on the target cells. On the other hand, SP-A concentrations in bronchoalveolar lavage fluids decrease in patients with acute lung injury (49) and bacterial pneumonia (50). It is unclear why the different results are obtained in humans and animal models. This may implicate complicated responses in the disease states of human. The reduction of SP-A concentrations in human alveolar fluids may also be a consequence of consumption of SP-A that has interacted with bacteria and their derivatives. Another interpretation is that TNF-α released by activated macrophages in patients with acute lung injury and pneumonia might reduce SP-A levels because TNF-α suppresses SP-A synthesis in type II cell-like cell lines (51).

In conclusion, this study demonstrates that SP-A exhibits different interactions with distinct serotypes of LPS and affects differently their elicited cellular responses. We propose that the modulation of LPS-induced cellular responses by SP-A occurs by the direct interaction of SP-A with CD14.

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