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CASE REPORT

Gefitinib-induced interstitial lung disease showing improvement after cessation: Disassociation of serum markers

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Gefitinib-induced interstitial lung disease showing improvement after cessation: Disassociation of serum markers
KITAJIMA H, TAKAHASHI H, HARADA K, KANAI A, INOMATA S-I, TANIGUCHI H, SAIKAI T, ABE S. *Respirology* 2006; **11**: 217–220

Abstract: Gefitinib (ZD1839), a small-molecule epidermal growth factor receptor tyrosine kinase inhibitor, is an anticancer agent for patients with non-small cell lung carcinoma. Recently, however, as a result of accumulating evidence, it has been recognized that gefitinib can give rise to lethal lung toxicity. The authors report a case of interstitial lung disease (ILD) induced by gefitinib, which improved promptly following cessation of the administration of the agent. Clinical signs suggesting a good prognosis were noted, namely, findings similar to acute eosinophilic pneumonia on CT and a disassociation in the elevation of specific serum markers of ILD. At the time of onset of ILD, serum concentrations of surfactant protein (SP)-A and SP-D were significantly increased, whereas that of KL-6 was not increased. A previous study of three cases of lethal lung toxicity resulting from gefitinib administration revealed a significant and almost equal increase in KL-6, SP-A and SP-D. These results suggest that SP-A and SP-D may be indicators of gefitinib-induced ILD and that KL-6 is a predictor of outcome. Using a combination of these markers may help to establish a differential prognosis in patients with gefitinib-induced ILD.

Key words: gefitinib, interstitial lung disease, KL-6, lung cancer, surfactant protein-A, surfactant protein-D.

INTRODUCTION

Gefitinib (ZD1839), a small-molecule epidermal growth factor receptor tyrosine kinase inhibitor,[1,2] was approved in Japan as an anticancer agent for patients with non-small cell lung cancer in July 2002. This agent had been regarded as safe until a review of severe adverse pulmonary effects was reported by the Pharmaceuticals and Medical Devices Evaluation Center.[3] In particular, patients with gefitinib-associated interstitial lung disease (ILD) had a high mortality.[4–6] The authors describe a patient showing the characteristic findings of eosinophilic pneumonia (EP) on high-resolution CT (HRCT) and disassociation in elevations of serum markers, surfactant protein (SP)-A, SP-D and KL-6, and whose clinical outcome was favourable following cessation of the drug.

CASE REPORT

A 55-year-old woman was admitted to the authors' hospital with an 8-month history of cough. A CXR revealed massive opacification of the left lower lobe. Adenocarcinoma cells were detected by aspiration cytology from enlarged cervical lymph nodes and unilateral left pleural effusion. There were bone metastases in right 10th rib and a chest CT demonstrated an intrapulmonary metastasis in the right lung. She was finally diagnosed with advanced primary lung adenocarcinoma, clinical stage IV (T4N3M1). Oral administration of gefitinib (250 mg/day) was initiated and led to a remarkable resolution of her disease.

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After 3 months, she was readmitted to hospital with a dry cough and shortness of breath. Chest HRCT (Fig. 1) revealed ground glass attenuation (GGA) and interlobular septal thickening, distributed mainly in bilateral lower and dorsal lung fields. Bilateral pleural effusions and pericardial effusion were also observed (Fig. 1). She had fine crackles in both lung bases and her physical examination was otherwise normal. Her ESR was 25 mm/h; C-reactive protein 0.1 mg/dL; WCC 5.7×10^3 μL; AST 98 IU/L; ALT 188 IU/L; LDH 476 IU/L; SP-A 64.6 ng/mL (cut-off: 43.8 ng/mL); SP-D 491 ng/mL (cut-off: 110 ng/mL) and KL-6 230 U/mL (cut-off: 500 U/mL) (Fig. 2). Antibody titres against *Mycoplasma pneumoniae* and various other viral serologies were normal. No bacterial pathogen was cultured in the sputum. Her electrocardiogram and echo cardiogram were normal.

Gefitinib was stopped and her symptoms and radiological abnormalities improved spontaneously. Two weeks after the cessation of gefitinib, she achieved complete resolution of her HRCT abnormalities and her serum levels of SP-A and SP-D declined to nearly within reference values (Fig. 2). AST and ALT were also normalized. With such rapid improvements, BAL and lung biopsy were not performed.

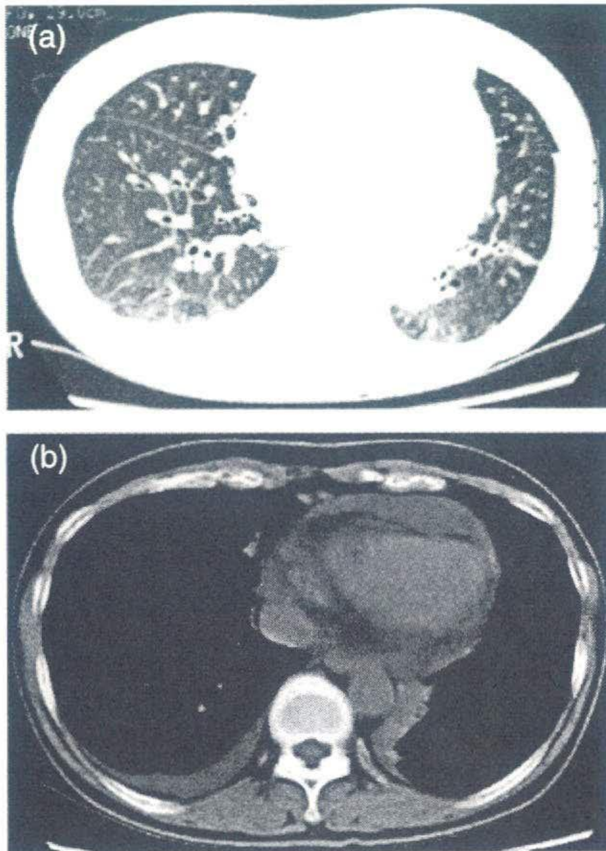

Figure 1 High-resolution CT scans at the time of onset of interstitial lung disease, demonstrating ground glass attenuation and interlobular septal thickening mainly in the lower lung fields (a), and pleuro-pericardial effusions (b).

To evaluate the cause of the high SP-A concentration, immunocytochemical analysis using human-specific anti-SP-A mAb, PE10,[11,12] was performed on the preparation used for aspiration cytology. A positive reaction for SP-A was observed in the cytoplasm of the tumour cells (Fig. 3).

DISCUSSION

The patient had a cough, slight shortness of breath and abnormalities on chest CT scans 3 months after the initiation of gefitinib. The onset was rapid and these findings disappeared quickly after the cessation of gefitinib. Serological data did not show any infectious diseases. The probability of an adverse drug reaction can be classified as definite, probable, possible, or doubtful.[13] The patient of the present report appeared to be a probable case of an adverse drug reaction against gefitinib, because her clinical reaction: (i) followed a reasonable temporal sequence after the drug; (ii) followed a recognized response to the suspected drug; (iii) improved soon after discontinuation of the drug; and (iv) could not be reasonably explained by the known characteristics of the patient's clinical state.

In general, drug-induced pulmonary disease is characterized by a wide range of pathological findings including chronic interstitial pneumonia (CIP), diffuse alveolar damage (DAD), cryptogenic organizing

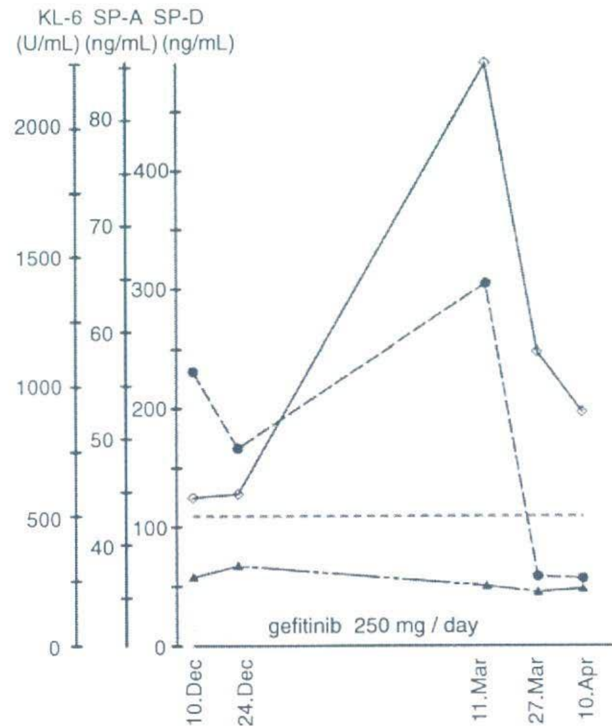

Figure 2 Serial changes in the serum concentrations of (●) surfactant protein (SP)-A, (◇) SP-D and (▲) KL-6. The assay methodology has been described for SP-A,[7] SP-D,[8] and for KL-6.[9,10] Hatched lines show the normal cut-off levels of the three markers.

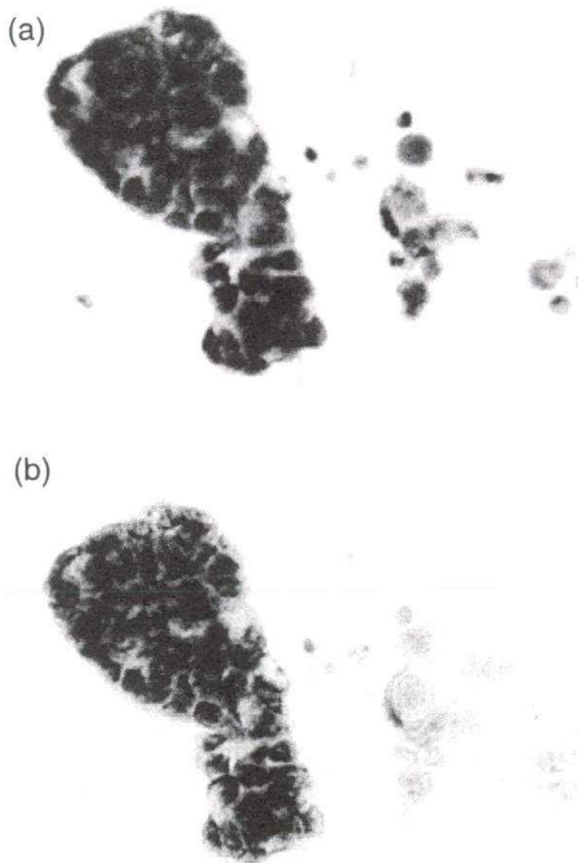

Figure 3 Tumour cells obtained with transcutaneous needle biopsy of cervical lymph nodes (a, Papanicolou staining). The same cells were destained and then immunostained with anti-human SP-A mAb, PE10, and secondary goat anti-mouse IgG alkaline phosphatase-conjugated antibody (b). Positive reactions are seen in cytoplasm of the cells (magenta colour).

pneumonia/bronchiolitis obliterans organizing pneumonia (BOOP) and EP.[14,15] Although the authors could not clarify the underlying pathohistological changes in this patient, it seemed reasonable to exclude CIP because of her rapid onset and DAD because of the spontaneous resolution of her clinical findings. Characteristics of EP on chest HRCT scans are similar to those that the patient of the present report exhibited, and are as follows: bilateral GGA, interlobular septal thickening and bilateral pleural and pericardial effusions.[16] Wandering consolidation, which is a characteristic of cryptogenic organizing pneumonia/BOOP, was not observed in the HRCT findings of this patient.

The authors recently reported a study showing that approximately 40% (5/12) of patients who developed ILD with gefitinib died as a result.[17] Two other research groups reported similar findings with CT scans showing extensive GGA bilaterally and at autopsy pathohistological findings of DAD consistent with acute CIP.[4,5] In contrast, there are patients who respond with either steroid therapy or simply by cessation of gefitinib, as in the present case.

ELISAs for SP-A, SP-D and KL-6 have been developed,[7–9] and their clinical values have been evaluated for serological examinations of ILD.[18–20] Recently, the authors reported three patients with ILD associated with gefitinib who died, each had high concentrations of these serum markers.[17] The present patient also showed high concentrations of SP-A and SP-D, whereas KL-6 was within normal limits. This discrepancy between serum markers might indicate a good prognosis. In other words, SP-A and SP-D may be common markers for DAD and EP, whereas the level of KL-6 may be of prognostic value in patients with ILD associated with gefitinib. Ohnishi and colleagues evaluated the relationship between serum KL-6 and HRCT findings in patients with drug-induced pneumonitis.[21] They showed that patients who showed DAD-like findings on HRCT ($n = 7$) presented with increased serum levels of KL-6. In contrast, patients who showed BOOP/EP-like findings on HRCT ($n = 8$) presented with KL-6 levels within the normal range. A recently reported case of EP also showed high levels of SP-A and SP-D and low levels of KL-6.[22] KL-6 was not increased in the present case and is consistent with these reports.

Increase in serum SP-A and SP-D are thought to be a combination of alveolar type II cell hyperplasia with a concomitant increase in the synthesis of SPs and vascular leakage due to a secondary disturbance in the air–blood barrier.[23] The authors of the present report speculate that the mechanism of disassociation between KL-6 and SP-A/-D is due to SP-A and SP-D being secretory proteins and KL-6 (a mucin-like high-MW human glycoprotein belonging to the MUC-1 family[10]) is basically a structural component of the cell membrane. Therefore, proteolytic cleavage of KL-6's extracellular domain would be required for leakage of KL-6s into the bloodstream. Protease activation is often associated with inflammation, as prominently activated protease promotes an inflammatory process and leads to severe respiratory failure, the elevation of KL-6 may reflect a poor prognosis.

This patient had a high concentration of SP-A prior to gefitinib treatment. The tumour cells expressed SP-A, which is produced essentially by alveolar type II cells and Clara cells in normal lungs. The increased serum concentration of SP-A was normalized following a reduction in tumour size and as such, SP-A production by tumour cells can cause elevation of serum SP-A. However, a secondary rise in SP-A may be due to gefitnib-induced ILD.

The authors' experience suggests a phenomenon of ILD caused by gefitinib, which is characterized by CT findings similar to those of EP and by a disassociation between the elevations of lung-specific serum markers.

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Monitoring markers of disease activity for interstitial lung diseases with serum surfactant proteins A and D

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Monitoring markers of disease activity for interstitial lung diseases with serum surfactant proteins A and D
TAKAHASHI H, SHIRATORI M, KANAI A, CHIBA H, KUROKI Y, ABE S. *Respirology* 2006; **11**: S51–S54
Objectives: Surfactant protein (SP) A and D are specific serum markers for interstitial lung diseases including idiopathic pulmonary fibrosis (IPF). The authors evaluated the critical roles of these markers on the prognoses of patients with IPF and the mechanisms of their elevation in sera.
Methodology: The authors evaluated the relationship between prognosis and the serum markers in 82 IPF patients. The protein content and mRNA expression of the markers were evaluated using rats with interstitial pneumonia induced by bleomycin administration.
Results: Higher levels of serum SP-D at the time of the initial visit to the Sapporo Medical University Hospital were associated with poorer prognoses, while SP-A showed no significant affect on survival. Causes of the elevation in sera were due to the acceleration of, not only production in the lungs, leakage into the circulation. The elevation was associated with alveolitis but not fibrosis.
Conclusions: SP-D is a good predictor of the prognosis in patients with IPF.

Key words: biological markers, prognosis, pulmonary fibrosis, surfactant protein A, surfactant protein D.

INTRODUCTION

Patients with severe interstitial lung diseases (ILD) represented by idiopathic pulmonary fibrosis (IPF) are known to have a poor prognosis and severe restrictive disturbance on pulmonary function tests. However, none of the serological markers without LDH activity have been effective in monitoring these diseases. LDH has been classically used as a unique marker for ILD, but its organ specificity is very low and its sensitivity is not so high for ILD. The authors previously developed novel ELISA kits for detecting serum markers, surfactant protein (SP) A and SP-D, which provide high levels of specificity and sensitivity for ILD.[1–4]

Many ILD are characterized by inflammation in the alveolar interstitium and various degrees of fibrosis. ILD are often accompanied with degeneration of the alveolar epithelium and hyperplasia of alveolar type II cells for tissue repair. The pathological changes may induce a significant alteration in surfactant metabolism, which is carried out by alveolar type II cells and alveolar macrophages. The acceleration of alveolar permeability, which also appears following interstitial inflammation, may be a cause of the elevation of serum markers.

The aims of this study were to evaluate the value of SP-A and SP-D as monitoring markers, and to elucidate the mechanisms of their elevation in sera.

METHODS

The authors conducted a retrospective study in 82 patients with IPF (65 male and 17 female) who were inpatients or outpatients at Sapporo Medical University Hospital, Sapporo, Japan between 1985 and 2003. All patients fulfilled the ATS/ERS clinical criteria.[5] Pathologic diagnoses in 29 patients were confirmed by open lung biopsy or video-associated thoracoscopic surgery. Peripheral venous blood samples, which were collected from the patients at their initial visits, were used for the KL-6, SP-A and SP-D assays. The serum samples had been stored at –80°C and then were analyzed for SP-A and SP-D using commercially available ELISA kits (SP-A: Sysmex Co., Japan; SP-D: Yamasa Co., Japan) and following the manufacturer's protocol based on previous studies.[1,6] All assays were performed in duplicate, and the results were

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given as the mean value. In immunoblot analyses, specific antibodies,[1,2,6,7] which were used for these assay systems, showed no non-specific cross-reactivity such as mannose binding proteins or other serum proteins. KL-6 was assayed with ELISA kits as previously described.[8]

LDH and ESR were measured immediately after blood sampling. Survival was defined as the time from the date of sampling to the date of death. Probability of survival was estimated by the Kaplan–Meier method.[9] Single variable survival analyses were performed with log-rank tests and multivariate regression analysis was carried out with Cox's proportional hazard model.

Specific-pathogen-free adult male Sprague-Dawley rats were used in this study. Their thoraces were irradiated with a single soft X-ray dose of 20 Gy from a linear accelerator. During the irradiation, all parts of the body other than the thorax had been protected from exposure with a lead plate. The sera from rats at each time point were collected for measurement of concentrations of SP-A and SP-D. The lungs of each rat were perfused with saline and then the lungs were lavaged. The BAL fluid was centrifuged at 250 *g* for 10 min. The lung tissue homogenate was centrifuged and its supernatant was stored. All samples were kept at −30°C for biochemical analysis. Collagen content was assessed by measuring hydroxyproline in the lungs, using a method described by Kivirikko *et al.*[10] For the isolation of total RNA, the left lung was quickly collected from the rats and frozen at −110°C. Total RNA was subjected to reverse transcriptase-polymerase chain reaction and Northern blot analysis. Autoradiographic quantitative analyses of SP-A and SP-D mRNA expression were done on a BAS system Image Analyzer.

SP-A was purified as described previously[11] and SP-D was purified by the method of Persson and colleagues.[12] An antibody against rat SP-A or SP-D was isolated from sera collected from a white New Zealand rabbit immunized with each purified surfactant protein. The measurements of SP-A[13] and SP-D[14] were performed with a sandwich ELISA.

RESULTS

The 5-year survival rate was 55.6% and the median survival period was 71 months. The median values of SP-A, SP-D, KL-6, LDH and ESR in sera from the 82 patients were 83.5 ng/mL, 253 ng/mL, 1014 U/mL, 375 IU/mL, 17.7 mm (1 h), respectively. The authors divided the patients into two groups based on the median values and evaluated the relation between serum concentration and prognosis using Kaplan–Meier survival estimation. Patients with SP-D equal to, or over, 253 ng/mL proved to have a shorter survival than did patients with the marker less than this threshold (Fig. 1). A similar effect for KL-6 was observed in patients with this marker levels equal to, or greater, than 1014 U/mL who had a poor outcome when compared with patients having a value lower than this threshold. In contrast, no significant difference was observed when LDH and SP-A were considered.

SD-rats were irradiated and then lung tissues, BAL fluid and sera were collected once a week. Interstitial inflammatory changes in alveoli were found in 4–

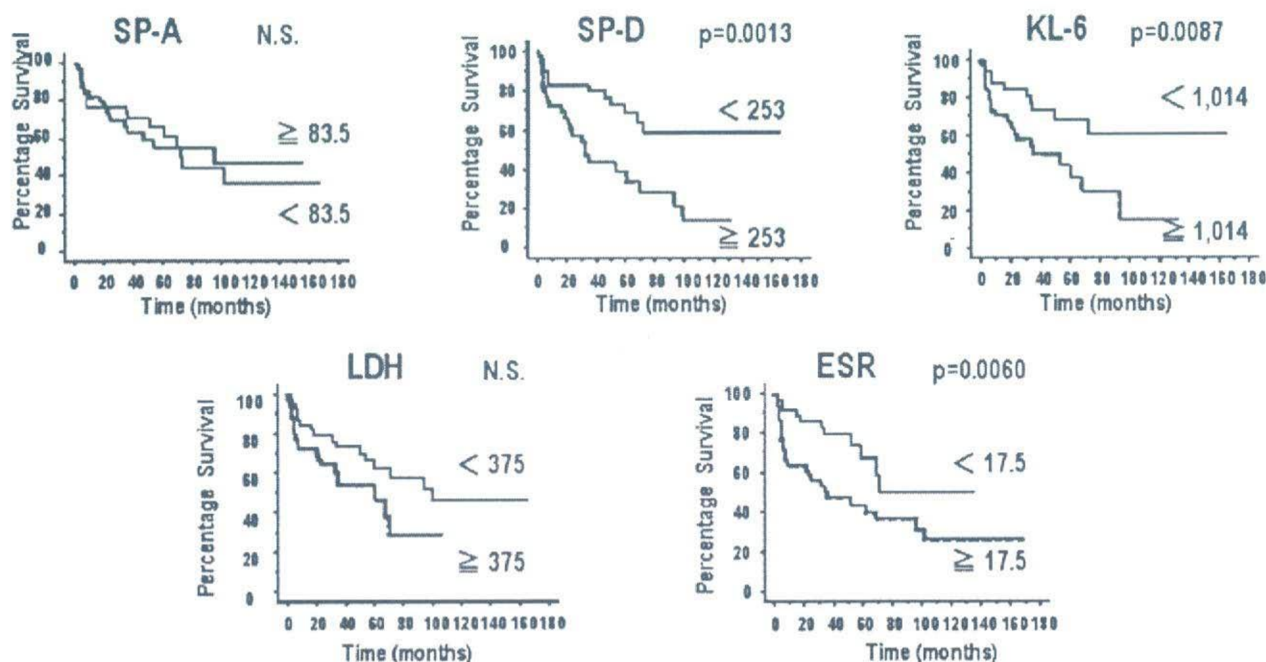

Figure 1 Probability of survival of patients with idiopathic pulmonary fibrosis showing high or low marker. All patients were divided into two groups at the time of the initial measurement by a median value of the following markers: SP-A (≥83.5 ng/mL vs. <83.5 ng/mL), SP-D (≥253 ng/mL vs. <253 ng/mL), KL-6 (≥1014 ng/mL vs. <1014 ng/mL), LDH (≥375 IU/L vs. <375 IU/L), ESR (≥17.7 mm (1 h) vs. <17.7 mm (1 h)).

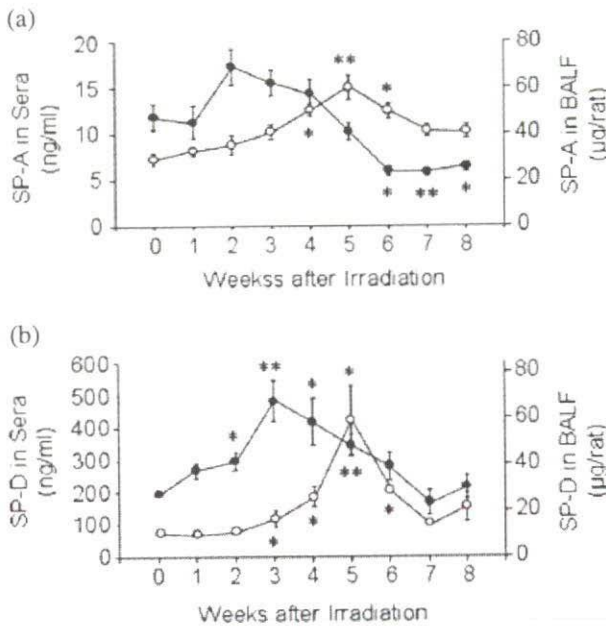

Figure 2 Serial changes of surfactant protein (SP) A and D in sera and BAL fluid. (a) SP-A and (b) SP-D in sera (open circles) showed significant increases peaking in 7 weeks, whereas those in the BAL fluid (closed circles) showed a peak of increase at a time point earlier than 7 weeks. SP-A in the BAL fluid significantly decreased during and after week 6, compared with the control at week 0. The results are expressed as the means ± standard deviation from six animals per group. $^*P < 0.05$, $^{**}P < 0.001$ compared with week 0.

5 weeks, and then mild fibrotic changes were found after 6 weeks. Protein content and mRNA expression of SP-A and SP-D increased during the period in which histologically interstitial inflammatory changes were noted. Concentrations in the BAL fluid also increased during this period, but the levels were lower than those in the period prior to histological changes (Fig. 2). Concentrations in sera increased in the period of histological changes. The significant maximum elevation was seen at 5 weeks for both proteins, which was the same to the time point of the most severe alveolitis. In contrast, the elevation in the sera after 6 weeks, when the main histological change was fibrosis, was not significant.

DISCUSSION

When all the patients were divided into two groups by the median value of each marker at the time of initial measurement, a significant difference was observed in SP-D, KL-6, LDH and ESR, with only SP-A not showing any such difference. Evaluation with Cox's Proportional Hazards Model revealed that SP-D may be the best prognostic indicator among the blood markers. Therefore, measurements of SP-D at the time of the initial diagnosis may offer valuable information establishing the prognosis of the patients.

The typical findings of pulmonary function tests (PFT) in patients with pulmonary fibrosis are consistent with restrictive impairment, of which the key variables are vital capacity (VC) and TLC. The authors previously attempted to assess whether deterioration in the PFT results could be predicted by measuring the concentrations of SP-A and/or SP-D in sera from patients with IPF.[3] The authors found that the levels of SP-D at the initial time of the study, unlike those of SP-A, correlated significantly with the velocity of decline in VC and TLC. Most subjects studied, even many who showed high levels of SP-D, did not have dyspnea at the initial time of study. Nevertheless, the results clearly indicated that high levels of SP-D are involved in subsequent declines in %VC and %TLC. Surprisingly, high levels of SP-D correlated with neither VC nor TLC at the initial time of the study, suggesting that increase of serum SP-D is not caused by the completed fibrosis, but by the ongoing epithelial damage and the successive process of fibrosis. A recent study based on multivariate analysis revealed that a change in forced VC after 6 months of follow up was an independent prognostic factor, with no additional prognostic information conferred by the histologic diagnosis, namely, IPF and non-specific interstitial pneumonia.[15] These data demonstrate the importance of the short-term change in restrictive PFT. As described above, the change in PFT can be predicted by SP-D at the initial examination. Consequently, one of the reasons why SP-D demonstrated the characteristic as a predictor of the prognosis may be the intimate association with a rapid progression of restrictive pulmonary deterioration. The fact that SP-A was not a prognostic predictor, having no association with restrictive change in PFT, would appear to corroborate this.

Surgically biopsied lung tissues from IPF patients often show regenerated hyperplastic type II cells covering the thickened fibrotic alveolar septum. SP-A is usually expressed intensively in these hyperplastic type II cells, suggesting overproduction of SP-A. However, Dr McCormack previously demonstrated that smaller ratios of SP-A to phospholipids in BAL fluid increased the relative risk for survival of IPF patients.[16] The results of the present study showed that SP-A and SP-D in the BAL fluid decreased in IPF patients, although cigarette smoke exerts a possible influence on the depletion of SP-A (unpublished data). Therefore, there is a discrepancy between the protein synthesis in epithelial cells and the concentrations in the BAL fluid. In the present study, the authors evaluated whether the discrepancy is reproducible in experimentally injured lungs. The results suggest that the overproduced SP-A and SP-D are secreted in the alveoli and then transferred immediately to the circulating blood, and that their concentration in sera increases in the presence of active inflammatory changes (alveolitis) but does not increase in the presence of only fibrosis. This suggestion is consistent with clinical data based on the findings of chest CT, that the elevation of serum SP-A and SP-D was related to the extent of ground glass opacity, but not honeycombing.[3]

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Pulmonary Surfactant Protein D Inhibits Lipopolysaccharide (LPS)-induced Inflammatory Cell Responses by Altering LPS Binding to Its Receptors*

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Pulmonary surfactant protein D (SP-D) is a member of the collectin family that plays an important role in regulating innate immunity of the lung. We examined the mechanisms by which SP-D modulates lipopolysaccharide (LPS)-elicited inflammatory cell responses. SP-D bound to a complex of recombinant soluble forms of Toll-like receptor 4 (TLR4) and MD-2 with high affinity and down-regulated tumor necrosis factor-α secretion and NF-κB activation elicited by rough and smooth LPS, in alveolar macrophages and TLR4/MD-2-transfected HEK293 cells. Cell surface binding of both serotypes of LPS to TLR4/MD-2-expressing cells was attenuated by SP-D. In addition, SP-D significantly reduced MD-2 binding to both serotypes of LPS. A chimera containing the N-terminal region and the collagenous domain of surfactant protein A, and the coiled-coil neck and lectin domains of SP-D, was a weak inhibitor of LPS-induced cell responses and MD-2 binding to LPS, compared with native SP-D. The collagenase-resistant fragment consisting of the neck plus the carbohydrate recognition domain of SP-D also was a very weak inhibitor of LPS activation. This study demonstrates that SP-D down-regulates LPS-elicited inflammatory responses by altering LPS binding to its receptors and reveals the importance of the correct oligomeric structure of the protein in this process.

Pulmonary surfactant protein D (SP-D)[3] is a member of the collectin protein family that also includes surfactant protein A (SP-A) and mannose binding lectin (1, 2). The structure of the collectins is characterized by four domains consisting of: 1) an N terminus involved in interchain disulfide bonding, 2) a collagen-like domain, 3) a coiled-coil neck domain, and 4) a carbohydrate recognition domain (CRD) (3). SP-A and mannose binding lectin contain collagenous domains consisting of 23 and 19 repeating Gly-*X*-*Y* triplets, respectively, with an interruption at the middle of the collagenous sequence (4, 5). In contrast, SP-D possesses a longer collagenous tail composed of 59 Gly-*X*-*Y* repeats without an interruption (6). These differences cause distinct oligomeric organization, with SP-A and mannose binding lectin exhibiting bouquet-like structures consisting of either six or four trimeric subunits (7) and SP-D exhibiting cruciform structures composed of four trimeric subunits (8).

Lipopolysaccharide (LPS) is a principal component of the outer membrane of Gram-negative bacteria that activates macrophages and induces a variety of inflammatory mediators, including TNF-α, IL-1, IL-6, IL-8, and interferon (9). LPS composed of *O*-antigen, core oligosaccharide, and lipid A is named smooth LPS, and LPS lacking *O*-antigen and a part of the core oligosaccharides is named rough LPS (10). Toll-like receptor 4 (TLR4) plays a critical role in recognition and signaling by LPS (11, 12). MD-2 binds directly to LPS and is required for TLR4-mediated signaling induced by LPS (13, 14). Structural examination of the TLR4-MD-2 complex revealed that MD-2 binds to the concave surface of the N-terminal and central domains of TLR4 (15). A study with recombinant soluble forms of the extracellular TLR4 domain (sTLR4) and MD-2 (sMD-2) from our laboratory indicates the importance of the N-terminal region of TLR4 in MD-2 binding (16).

Engineered genetic defects in the pulmonary collectins of mice have revealed their important functions in protecting the lung from microbial infections and inflammation. SP-D-null mice infected with group B *Streptococcus* or *Haemophilus influenza* by intra-tracheal instillation show increased inflammation and inflammatory cell recruitment in the lung (17). Increased pulmonary inflammation in LPS (*Escherichia coli* O55:B5, smooth serotype)-instilled SP-D$^{-/-}$ mice and wild-type mice was decreased by intratracheal administration of SP-D and pulmonary surfactant (18). Intratracheal recombinant SP-D prevents endotoxin shock in the newborn preterm

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[3] The abbreviations used are: SP-D, surfactant protein D; LPS, lipopolysaccharide; TLR4, Toll-like receptor 4; CRD, carbohydrate recognition domain; CRF, collagenase-resistant fragment; TNF, tumor necrosis factor; IL, interleukin; s, soluble; HEK, human embryonic kidney; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; HRP, horseradish peroxidase.

lamb (19). These *in vivo* studies provide compelling evidence that SP-D dampens pulmonary inflammation induced by LPS.

We have previously shown that SP-A modulates LPS-induced inflammatory cell responses by direct interaction with TLR4 and MD-2 (20, 21). SP-A attenuates or enhances LPS-induced inflammatory responses in a ligand-specific manner. For example, SP-A inhibits TNF-α secretion elicited by the smooth serotype of LPS, which is not a ligand for SP-A. However, SP-A can also enhance TNF-α secretion stimulated by rough LPS, which is a ligand for the protein. We have recently reported that SP-D directly binds to the extracellular domains of TLR4 and TLR2 through its CRD (22).

The purposes of this study were: 1) to determine whether SP-D binds a complex of TLR4 and MD-2 using sTLR4 and sMD-2, 2) to determine whether SP-D affects cytokine secretion and signaling induced by distinct serotypes of LPS, 3) to investigate the mechanism by which SP-D alters TLR4-mediated LPS signaling, and 4) to examine the role of the native oligomeric structure of SP-D in modulating LPS-induced inflammation. We now demonstrate that SP-D down-regulates inflammatory cell responses elicited by smooth and rough serotypes of LPS by altering LPS-receptor interaction.

EXPERIMENTAL PROCEDURES

Materials—Human embryonic kidney (HEK) 293 cells (CRL-1573) were obtained from American Type Culture Collection and were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum (Equitech-Bio Inc., Kerrville, TX). LPS types (*Salmonella minnesota* Re595, *E. coli* Rc, *E. coli* serotype O26:B6, and *E. coli* serotype O111:B4) were purchased from Sigma. Alexa488-labeled smooth LPS (*E. coli* O55:B5, molecular mass of ~10,000 Da) and Alexa488-labeled rough LPS (*S. minnesota*, molecular mass of ~3,000 Da) were purchased from Molecular Probes. Monoclonal antibodies 7A10 and 7C6 against human SP-D were prepared as described previously (23). Polyclonal antibody to sTLR4 was generated as described previously (24). Anti-His tag polyclonal antibody was purchased from Medical and Biological Laboratories. Streptavidin-agarose was purchased from Invitrogen. Anti-V5 antibody-conjugated agarose was obtained from Sigma.

SP-D—The 1.181-kb cDNA for human SP-D was inserted into a pEE14 plasmid vector, and recombinant human SP-D was expressed in CHO-K1 cells using the glutamine synthetase gene amplification system (25). CHO-K1 cells expressing human SP-D were grown in glutamate-free Glasgow minimum essential medium (Invitrogen) containing dialyzed fetal calf serum and 25 μM methionine sulfoximine for gene amplification. For protein purification, the cells were transferred into serum-free EXCELL 302 medium (SAFC Biosciences, Lenexa, KS) and incubated for 3–4 days. The medium was collected, and four additional harvests were performed, allowing 24–48 h of culture between harvests. The medium adjusted to pH 7.4 with 1 M Tris buffer (pH 9.0) was finally filtered with a 0.45-μm filter and applied to mannose-Sepharose 6B column. The proteins were eluted with 20 mM Tris buffer (pH 7.4) containing 2 M NaCl, 50 mM EDTA, and dialyzed against 5 mM Tris buffer (pH 7.4) containing 0.15 M NaCl for 2 days.

Chimeric Protein—We constructed a chimeric protein with SP-A and SP-D (A/D chimera) in which the N-terminal region and the collagenous domain of SP-D were replaced with those of SP-A, as represented schematically in Fig. 6*A*. The A/D chimera consists of Glu^{1}–Pro^{80} of SP-A and Asp^{203}–Phe^{355} of SP-D. The cDNA for the chimera was constructed by using PCR and the overlap extension method (26) using the cDNAs for SP-A and SP-D. The two primers used at the SP-A/SP-D splicing junction were 5′-GGAGGTCCCGAAGGTCTACAACG-AAGAGA-3′ and 5′-CCTCCAGGGCTTCCAGATGTTGCT-TCTC-3′. An SP-A sense and an SP-D antisense primers were used as follows: 5′-AAGCTTATGTGGCTGTGCCCTCTGG-CCC-3′ and 5′-GAACACCAGACGCTCAAGACTAGATCT-3′, respectively. The BamHI and XbaI sites were incorporated into the flanking 5′ and 3′ primers, respectively. The construct was inserted into the pEE14 plasmid vector using the BamHI and XbaI sites. The organization of the recombinant plasmid and sequence of the cDNA insert was confirmed by a combination of restriction enzyme mapping and DNA sequencing. The recombinant A/D chimera was expressed using the glutamine synthetase gene amplification system (25), and the recombinant protein was produced and purified by affinity chromatography as described above for SP-D production.

Collagenase Treatment—Because the yield of the A/D chimera is greater than that of SP-D, the chimeric protein was used to obtain the collagenase-resistant fragment (CRF). The A/D chimera was digested with collagenase III from *Clostridium histolyticum* at 37 °C for 22 h. The CRF was isolated by gel-filtration chromatography using a Superose 610/300 GL (GE Healthcare Bio-Science AB). The N-terminal sequence of the isolated CRF was determined by using an Applied Biosystems (Foster City, CA) amino acid sequencer. The sequences obtained were GPPGLP or GLPDVA, corresponding to the end of the collagenous domain of human SP-A (Gly^{75}-Prp-Pro-Gly-Lys-Pro^{80}) or the SP-A/SP-D splice junction (Gly^{78}-Lys-Pro^{80} of SP-A/-Asp^{203}-Val-Ala^{205} of SP-D), respectively. This indicates that the N-terminal region and the collagenous domain of SP-A were removed from the A/D chimera.

Endotoxin Removal—Endotoxin in the protein preparations was removed using polymixin-agarose and octyl-β-D-glucopyranoside as described elsewhere (27). The endotoxin level determined by *Limulus* amebocytes lysate assay was below 1.41 pg/μg of protein in the preparations of SP-D, the A/D chimera, and CRF.

Biotinylation—SP-D was biotinylated by using EZ-Linked sulfo-*N*-hydroxysuccinimide-LC-biotin (Pierce) according to the manufacturer's instructions.

Gel-filtration Chromatography—SP-D, the A/D chimera, and CRF were analyzed by gel-filtration chromatography using 10 × 300 mm Superose 610/300 GL column. The protein was eluted at 0.5 ml/min in PBS at 4 °C, and the elution was monitored by measuring the absorbance at 280 nm. Thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), ovalbumin (43 kDa), and ribonuclease A (13.7 kDa) from Pharmacia Biotech were used as molecular mass standards.

Western Blot—A 100-ng sample of the protein was subjected to 13% SDS-PAGE under reducing and denaturing conditions, and transferred onto a polyvinylidene difluoride membrane.

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The membrane was immunoprobed with anti-human SP-D polyclonal antibody (5 μg/ml), or anti-human SP-A polyclonal antibody (5 μg/ml), or anti-human SP-D monoclonal antibody 7C6 or 7A10 (2 μg/ml), followed by incubation with HRP-conjugated anti-rabbit IgG or HRP-conjugated anti-mouse IgG. The proteins that reacted with the antibodies were visualized by using a chemiluminescence reagent (SuperSignal, Pierce) according to the manufacturer's instructions.

Electron Microscopy—SP-D, the chimeric protein, and the CRF were diluted to 10 μg/ml in 50% glycerol and 20 mM ammonium bicarbonate, sprayed onto freshly cleaved mica, and rotary-shadowed molecules were observed under an H-7650 electron microscope (Hitachi Co. Ltd, Tokyo, Japan) operated at 75 kV.

Binding of SP-D to LPS—The binding of the biotinylated SP-D to Re595, Rc, or O26:B6 LPS (5 μg/well) coated onto microtiter wells was performed as described previously (22). To determine the effects of anti-SP-D monoclonal antibodies on the SP-D binding to LPS, the protein (2 μg/ml) was first incubated with anti-SP-D monoclonal antibody 7C6 or 7A10 at 37 °C for 1 h. The mixture of SP-D and antibody was then added to the LPS-coated wells (1 μg/well), and the mixture was incubated at 37 °C for 3 h.

Binding of SP-D to an sTLR4-sMD-2 Complex—A soluble form of recombinant extracellular TLR4 domain (sTLR4: Met^1–Lys^{631}) containing a His_6 tag at the C-terminal end and a soluble form of recombinant MD-2 (sMD-2) containing the C-terminal fusion V5 tag and a His_6 tag were expressed using a baculovirus-insect cell expression system as described previously (24). sTLR4 and sMD-2, co-expressed in insect cells, were purified from the medium using a nickel-chelating HisTrap column (HP 5 ml, GE Healthcare). The complex of sTLR4 and sMD-2 was purified by gel-filtration chromatography on a Superose 610/300 GL column as described above and eluted with an apparent molecular mass of 110 kDa.

The biotinylated SP-D (100 ng) and/or an sTLR4-sMD-2 complex (100 ng) were incubated for 2 h at 37 °C in 100 μl of 10 mM Hepes buffer (pH 7.4) containing 0.15 M NaCl, 2 mM $CaCl_2$, and 5% (w/v) bovine serum albumin (buffer A). After the incubation, the volume of the mixture was adjusted to 500 μl by the addition of the buffer A and further incubated with anti-V5 antibody-conjugated agarose for 2 h at 4 °C, with gentle shaking for immunoprecipitation. As a control experiment, affinity-purified mouse IgG2a (eBioscience, San Diego, CA) and protein G-Sepharose were used. For the affinity adsorption assay with the biotinylated SP-D, 800 ng of the biotinylated SP-D and 100 ng of an sTLR4-sMD-2 complex were incubated with streptavidin-conjugated agarose for 30 min at 4 °C. The beads were sedimented by centrifugation and washed four times with 10 mM Hepes buffer (pH 7.4) containing 0.15 M NaCl, 2 mM $CaCl_2$, and 0.1% (v/v) Triton X-100. The final pellet obtained was suspended in SDS-sample buffer and subjected to SDS-PAGE. Western blotting was performed as described above with HRP-labeled streptavidin, anti-V5 antibody, or anti-sTLR4 polyclonal antibody, to detect biotinylated SP-D, sMD-2, or sTLR4.

TNF-α Secretion from Alveolar Macrophages—Alveolar macrophages were obtained by bronchoalveolar lavage of Sprague-Dawley rats. The lungs were lavaged with pyrogen-free saline (Otsuka Pharmaceutical Co., Tokyo, Japan) containing 1 mM EDTA, and alveolar macrophages were sedimented by centrifugation at 150 × *g* for 10 min. Isolated macrophages were plated at 1×10^5 cells/well in a 96-well plate (Falcon) in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, and next incubated for 2 h at 37 °C in a 5% CO_2 atmosphere to allow adherence.

The indicated concentrations of SP-D, the chimera, or the CRF were incubated with macrophages for 30 min at 37 °C and subsequently incubated with the cells for 5 h at 37 °C, after the addition of LPS. The medium was collected and the concentrations of secreted TNF-α were determined by using an OptEIA rat TNF set (BD Pharmingen, San Diego, CA) according to the manufacturer's instructions.

NF-κB Reporter Assay—LPS-induced NF-κB activation was measured by using HEK293 cells (1×10^5 cell/well) that had been transfected with TLR4 cDNA (80 ng) and MD-2 cDNA (80 ng) together with an NF-κB reporter construct (30 ng, NF-κB-Luc, Stratagene) and a construct directing expression *Renilla* luciferase (10 ng, pRL-TK, Promega), as described previously (21).

Binding of sMD-2 to LPS—One microgram of Re595 LPS, or Rc LPS in 20 μl of ethanol, or 5 μg of O26:B6 LPS, or O111:B4 LPS, in 50 μl of distilled water were added to microtiter wells (Immulon 1B, Thermo, for rough LPS, MultiSorp, Nunc, for smooth LPS) and the solvent was evaporated in the ambient air. Nonspecific binding was blocked with 20 mM Tris buffer (pH 7.4) containing 0.15 M NaCl and 3% (w/v) bovine serum albumin. The sMD-2 (1 or 2 μg/ml) was preincubated with SP-D (20 or 40 μg/ml), the A/D chimera, or the CRF, and the mixture was added to the wells and further incubated for 3 h at 37 °C. The wells were washed with PBS containing 0.1% (v/v) Triton X-100 and 3% (w/v) skim milk, and incubated with an anti-His tag-polyclonal antibody at 37 °C for 1 h, followed by incubation with HRP-labeled anti-rabbit IgG. The peroxidase reaction was performed by using *o*-phenylenediamine as a substrate after washing the wells with PBS containing 0.1% (v/v) Triton X-100. The binding of sMD-2 to LPS was finally detected by measuring the absorbance at 492 nm.

Binding of LPS to TLR4/MD-2-expressing Cells—HEK293 cells, stably expressing TLR4 and MD-2 (293-hTLR4/MD-2-CD14) (InvivoGen, San Diego, CA), were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 10 μg/ml blasticidin, and 50 μg/ml hygromycin B. Adherent cells were harvested (~10^6/tube) and incubated for 60 min at 4 °C with 2 μg/ml of Alexa488-labeled smooth LPS (*E. coli* O55:B5), or Alexa488-labeled rough LPS (*S. minnesota*), which had been preincubated with SP-D (50 μg/ml) for 30 min at 37 °C. After the incubation, the cells were washed with PBS containing 1 mg/ml bovine serum albumin, and the cell-associated fluorescence was determined by using FACSCalibur and CellQuest software (BD, Bioscience).

Other Methods—Polyclonal antibodies against human SP-A and SP-D were raised in rabbits by immunizing with purified recombinant SP-A and SP-D, respectively (21, 22). Protein concentrations were estimated by the BCA assay (Pierce) using bovine serum albumin as a standard.

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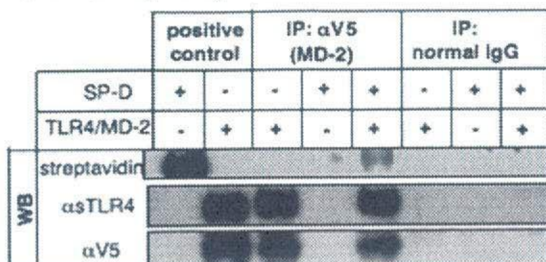

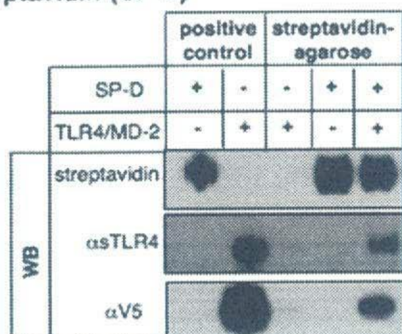

FIGURE 1. **SP-D binds to an sTLR4-sMD-2 complex.** *A*, immunoprecipitation with anti-V5 (MD-2) antibody. The isolated sTLR4-sMD-2 complex (100 ng) was incubated with biotinylated SP-D (100 ng) at 37 °C for 2 h. The sTLR4-sMD-2 complex was immunoprecipitated with anti-V5 antibody-conjugated agarose, and the immunoprecipitate was subjected to SDS-PAGE (7.5–15% polyacrylamide gel) under reducing conditions. As a control experiment, affinity-purified mouse IgG and protein G-Sepharose were used (normal IgG). The Western blot was then performed by using anti-V5 antibody for sMD-2, anti-sTLR4 polyclonal antibody for sTLR4 and HRP-streptavidin for biotinylated SP-D, respectively, as described under "Experimental Procedures." *B*, affinity adsorption assay of SP-D. Biotinylated SP-D (800 ng) was incubated with an sTLR4-sMD-2 complex (100 ng) at 37 °C for 2 h. The biotinylated SP-D was pulled down by streptavidin-conjugated agarose, and the precipitates were subjected to SDS-PAGE (7.5–15% polyacrylamide gel) under reducing conditions. The Western blot was then performed as described above.

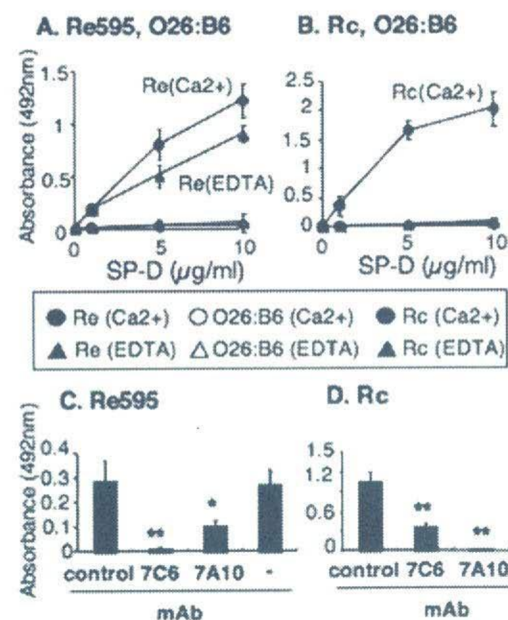

FIGURE 2. **SP-D binds to Re595 LPS and Rc LPS by different mechanisms.** *A* and *B*, the indicated concentrations of biotinylated SP-D were incubated with Re595 LPS (*A*), Rc LPS (*B*), or O26:B6 LPS (*A* and *B*) coated onto microtiter wells. Binding reactions were at 37 °C for 3 h in the presence of 2 mM $CaCl_2$ (●, Re595 LPS or Rc LPS; ○, O26:B6 LPS) or 5 mM EDTA (▲, Re595 LPS or Rc LPS; △, O26:B6 LPS) as indicated. After the incubation, the wells were washed and further incubated with HRP-conjugated streptavidin. The binding of SP-D to LPS was detected by measuring the absorbance at 492 nm, as described under "Experimental Procedures." *C* and *D*, the biotinylated SP-D (2 μg/ml), which had been preincubated with 40 μg/ml of anti-human SP-D monoclonal antibody 7C6 or 7A10, or control mouse IgG at 37 °C for 1 h, was incubated with Re595 LPS (*C*) or Rc LPS (*D*) coated onto microtiter wells at 37 °C for 3 h. After the incubation, the wells were washed and further incubated with HRP-conjugated streptavidin. The binding of SP-D to LPS was detected as described above. The data shown are the means ± S.D. from three separate experiments. *, $p < 0.05$ and **, $p < 0.01$ when compared with control IgG.

RESULTS

SP-D Binds to an sTLR4-sMD-2 Complex—We have previously shown that SP-D interacts with TLR4 (22) through the CRD of the collectin in a Ca^{2+}-dependent manner. We isolated a complex of sTLR4 and sMD-2 by gel-filtration chromatography and examined whether SP-D binds to an sTLR4-sMD-2 complex. When sMD-2 was immunoprecipitated by anti-V5 antibody-conjugated agarose, SP-D as well as sTLR4 was co-precipitated (Fig. 1*A*, WB:streptavidin and αsTLR4). The biotinylated SP-D also co-sedimented with sTLR4 and sMD-2 by an affinity adsorption assay with streptavidin-conjugated agarose (Fig. 1*B*, WB:αsTLR4 and αV5). These results indicate that SP-D can bind a complex of TLR4 and MD-2.

SP-D Binds to Re595 LPS and Rc LPS by Different Mechanisms—We have previously shown that SP-A binds to Re595 LPS and Rc LPS but not to O26:B6 LPS (20). SP-D has been reported to bind to Rc LPS and Rd LPS but not to Ra LPS and smooth LPS (28). In this study we also examined the binding of SP-D to rough LPS (Rc LPS from *E. coli* and Re595 LPS from *S. minnesota*) and smooth LPS (O26:B6 LPS from *E. coli*) coated onto microtiter wells. SP-D bound to Re595 LPS and Rc LPS in the presence of Ca^{2+} in a concentration-dependent manner (Fig. 2, *A* and *B*). SP-D failed to bind solid-phase O26:B6 LPS. The SP-D binding to Re595 LPS was not significantly attenuated by the presence of EDTA (Fig. 2*A*), whereas EDTA completely blocked the binding of SP-D to Rc LPS (Fig. 2*B*). Therefore, the SP-D binding to Rc LPS but not to Re595 LPS was Ca^{2+}-dependent.

The effect of anti-SP-D monoclonal antibodies on the LPS binding was also investigated. Antibody 7C6 completely blocked the binding of SP-D to Re595 LPS, and antibody 7A10 partially inhibited this binding (Fig. 2*C*). In contrast, antibody 7A10 but not antibody 7C6 completely inhibited the SP-D binding to Rc LPS (Fig. 2*D*). Because the epitopes for antibodies 7A10 and 7C6 are located at the CRD and the neck region, respectively (22), the results suggest that the neck region and the CRD are involved in the binding of SP-D to Re595 LPS and Rc LPS, respectively. These results are consistent with those obtained from the experiments demonstrating Ca^{2+} dependence, or independence for binding different ligands. The data confirm that rough LPS but not smooth LPS is an SP-D ligand and indicate that SP-D binds to Re595 and Rc LPS by different mechanisms.

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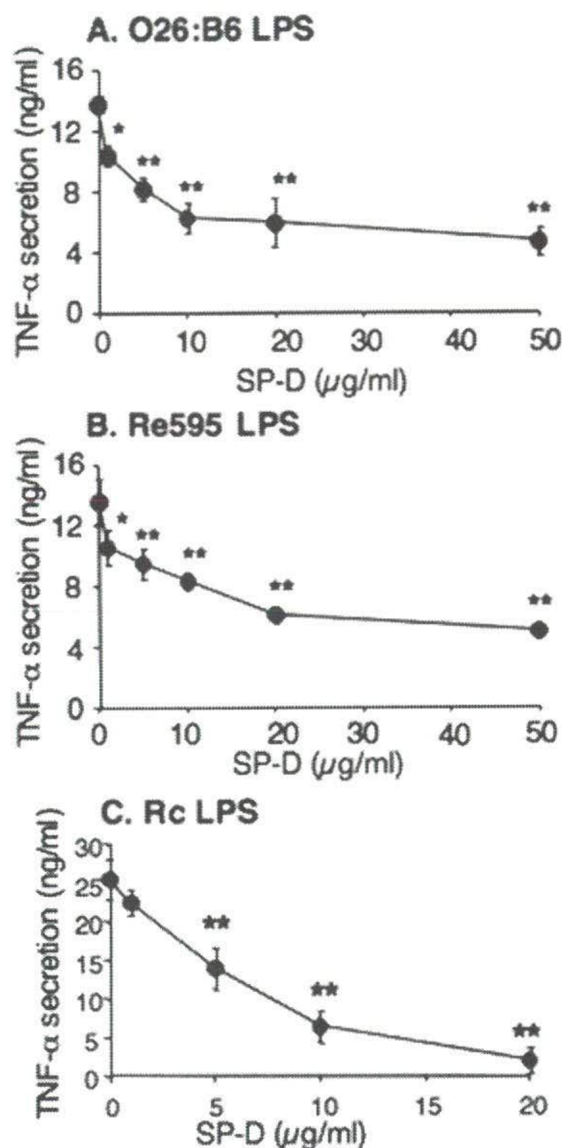

FIGURE 3. **SP-D inhibits LPS-induced TNF-α secretion from alveolar macrophages.** Rat alveolar macrophages (10^5 cells) were preincubated with the indicated concentrations of SP-D for 30 min at 37 °C in a 5% CO_2 atmosphere and were then stimulated with 100 ng/ml O26:B6 LPS (*A*), Re595 LPS (*B*), or Rc LPS (*C*) for 5 h. TNF-α secreted into the culture media was determined as described under "Experimental Procedures." The data shown are the means ± S.D. from three separate experiments. *, $p < 0.05$ and **, $p < 0.01$ when compared with LPS-induced TNF-α secretion in the absence of SP-D.

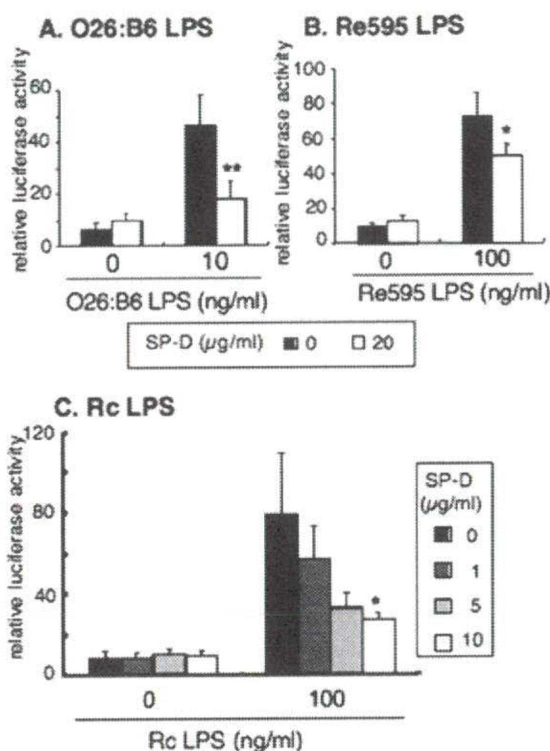

FIGURE 4. **SP-D attenuates LPS-induced NF-κB activation in TLR4/MD-2-transfected HEK293 cells.** HEK293 cells transfected with TLR4 and MD-2 were incubated with the indicated concentration of SP-D at 37 °C for 1 h in a 5% CO_2 atmosphere before adding LPS. The indicated amount of O26:B6 LPS (*A*), Re595 LPS (*B*), or Rc LPS (*C*) was then added to the wells and further incubated for 5 h at 37 °C. Luciferase activity of NF-κB activation was determined as described under "Experimental Procedures." The data shown are the means ± S.D. from three separate experiments. *, $p < 0.05$ and **, $p < 0.01$ when compared with LPS-induced NF-κB activation in the absence of SP-D.

SP-D Attenuates LPS-induced TNF-α Secretion and NF-κB Activation—SP-A, an SP-D homologue, regulates LPS-induced inflammatory responses in a ligand-specific manner. Smooth LPS is not a ligand for SP-A, but the protein potently inhibits TNF-α secretion induced by smooth LPS. Conversely, SP-A binds to rough LPS and enhances its inflammatory response (20, 21). For comparison, we next investigated whether SP-D regulates TNF-α secretion elicited by smooth and rough LPS. SP-D significantly attenuated O26:B6 LPS-stimulated TNF-α secretion from alveolar macrophages in a concentration-dependent manner (Fig. 3*A*). Unlike SP-A, SP-D down-regulated TNF-α secretion elicited by Re595 LPS and Rc LPS (Fig. 3, *B* and *C*), both of which are ligands for SP-D (Fig. 2). The inhibitory effect of SP-D appeared more potent for antagonizing the stimulation elicited with Rc LPS, than that occurring with Re595 LPS. The IC_{50}, for SP-D, was 8.16 μg/ml with O26:B6 LPS, 19.51 μg/ml with Re595 LPS, and 5.77 μg/ml with Rc LPS, respectively. We also performed the experiments with SP-A as a control. SP-A down-regulated O26:B6 LPS-induced TNF-α secretion. The results expressed as percentages of TNF-α secretion in the absence of SP-A were 23 ± 8% (the mean ± S.D., $n = 3$), 15 ± 10%, and 15 ± 3% at 1.5, 7.5, and 15 μg/ml of SP-A, respectively. In contrast, SP-A failed to attenuate Re595 LPS-stimulated TNF-α secretion. The percentages of TNF-α secretion were 110 ± 49%, 111 ± 21%, and 120 ± 43% at 1.5, 7.5, and 15 μg/ml of SP-A, respectively. These results obtained from control experiments with SP-A are consistent with our previous studies (20, 21).

We next examined the effect of SP-D on LPS-elicited NF-κB activation in HEK293 cells transfected with TLR4 and MD-2. O26:B6 LPS, Re595 LPS, and Rc LPS induced robust NF-κB-dependent luciferase activities through TLR4 and MD-2 (Fig. 4). The presence of SP-D significantly inhibited NF-κB activation

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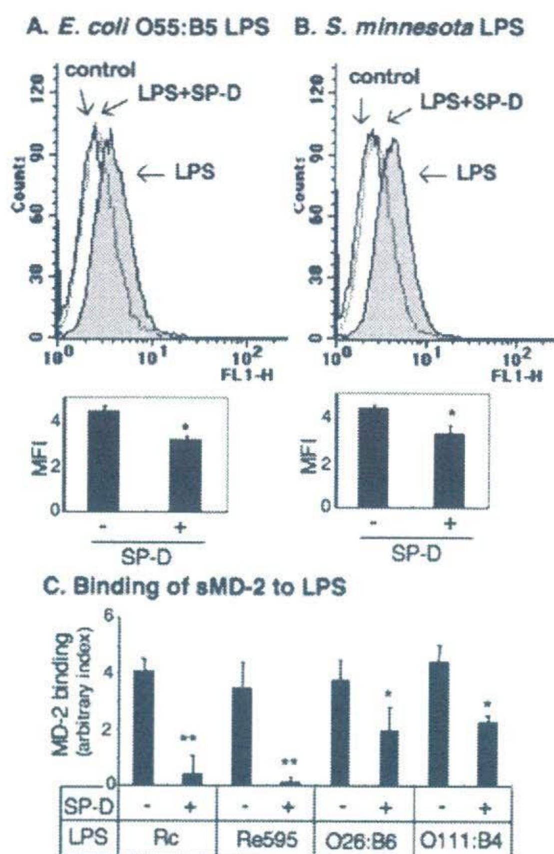
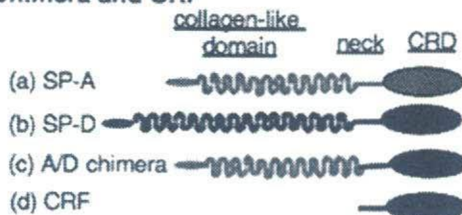
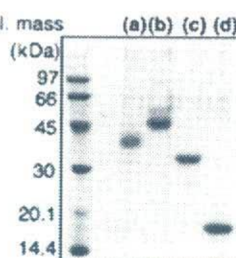


FIGURE 5. **SP-D inhibits cell surface binding of LPS to TLR4/MD-2-expressing cells and attenuates the binding of sMD-2 to LPS.** *A* and *B*, cell surface binding of LPS to TLR4/MD-2-expressing cells. HEK293 cells stably expressing TLR4/MD-2 were incubated for 60 min at 4 °C with 2 μg/ml of Alexa488-labeled *E. coli* O55:B5 LPS (*A*) or Alexa488-labeled *S. minnesota* LPS (*B*), which had been preincubated with SP-D (50 μg/ml) for 30 min at 37 °C. The binding of LPS to the cell surface was determined by flow cytometry. The *histograms* shown are representative from three experiments. The *dotted line* shows the cytometric analysis of the cells treated with SP-D plus LPS. The *gray shadow* shows the cells treated with LPS alone. The *lower panels* indicate the mean fluorescence intensity of LPS alone or LPS plus SP-D. The data shown are the means ± S.D. from three experiments. *, $p < 0.01$ when compared with SP-D (−). *C*, the binding of sMD-2 to LPS. sMD-2 (2 μg/ml) was preincubated with or without SP-D (40 μg/ml) for 1 h at 37 °C, and the mixture was further incubated for 3 h at 37 °C with Rc LPS or Re595 LPS coated onto microtiter wells. When O26:B6 LPS or O111:B4 LPS was coated onto the wells, 1 μg/ml sMD-2 was preincubated with 20 μg/ml SP-D for 1 h at 37 °C. After the incubation, the wells were washed and incubated with anti-His tag polyclonal IgG for 1 h, followed by incubation with HRP-conjugated anti-rabbit IgG for 1 h. The binding of sMD-2 to LPS was detected by measuring the absorbance at 492 nm, as described under "Experimental Procedures." The results are expressed as the means ± S.D. from three experiments. **, $p < 0.01$ and *, $p < 0.05$ when compared with SP-D (−) in each LPS.





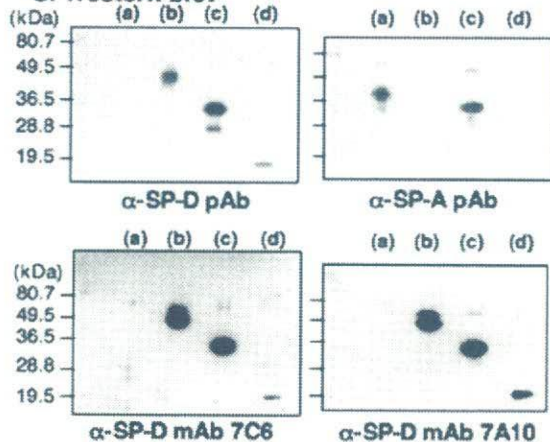


FIGURE 6. **Physical properties of the SP-A/SP-D chimera and CRF.** *A*, schematic representations of SP-A (*a*), SP-D (*b*), A/D chimera (*c*), and CRF (*d*). The domains of monomeric subunits of SP-D, SP-A, A/D chimera, and CRF are shown. Domains that are *black* are from SP-D, and domains that are *gray* are from SP-A. *B*, electrophoretic analysis of SP-A, SP-D, A/D chimera, and CRF. Two micrograms of wild-type recombinant human SP-A (*a*), wild-type recombinant human SP-D (*b*), SP-A/SP-D chimera (*c*), and CRF (*d*) were subjected to 13% SDS-PAGE under reducing and denaturing conditions, and were visualized by Coomassie Brilliant Blue staining. *C*, Western blot analysis of SP-A, SP-D, A/D chimera, and CRF. 100 ng of SP-A (*a*), SP-D (*b*), chimera (*c*), and CRF (*d*) were subjected to 13% SDS-PAGE under reducing and denaturing conditions and transferred onto polyvinylidene difluoride membranes. The membranes were immunoprobed with anti-human SP-D or anti-human SP-A polyclonal antibody (*pAb*) (5 μg/ml), or with anti-human SP-D monoclonal antibody (*mAb*) 7C6 or 7A10 (2 μg/ml), followed by incubation with HRP-conjugated anti-rabbit IgG or HRP-conjugated anti-mouse IgG. The proteins that reacted with the antibodies were visualized as described under "Experimental Procedures."

stimulated by both smooth (Fig. 4*A*) and rough serotypes (Fig. 4, *B* and *C*) of LPS. The NF-κB activities in the presence of SP-A (10 μg/ml) were 30% (the mean of two experiments) and 119% of those in the absence of SP-A when the cells were stimulated with O26:B6 LPS and Re595 LPS, respectively. These results are consistent with those obtained by examining TNF-α secretion.

SP-D Inhibits Cell Surface Binding of LPS to TLR4/MD-2-expressing Cells and Alters MD-2-LPS Interaction—We next examined whether SP-D alters the binding of LPS to TLR4/MD-2-expressing HEK293 cells by using Alexa488-labeled *E. coli* O55:B5 LPS (smooth LPS) and Alexa488-labeled *S. minnesota* LPS (rough LPS). Flow cytometric analysis revealed significant LPS binding to the cell surface (Fig. 5, *A* and *B*, *upper*, *gray shadow*) when the cells were incubated with the fluorescent LPS at 4 °C. The binding of smooth and rough serotypes of Alexa488-conjugated LPS was inhibited by the presence of SP-D (Fig. 5, *A* and *B*, *upper*, *dotted line*). SP-D significantly

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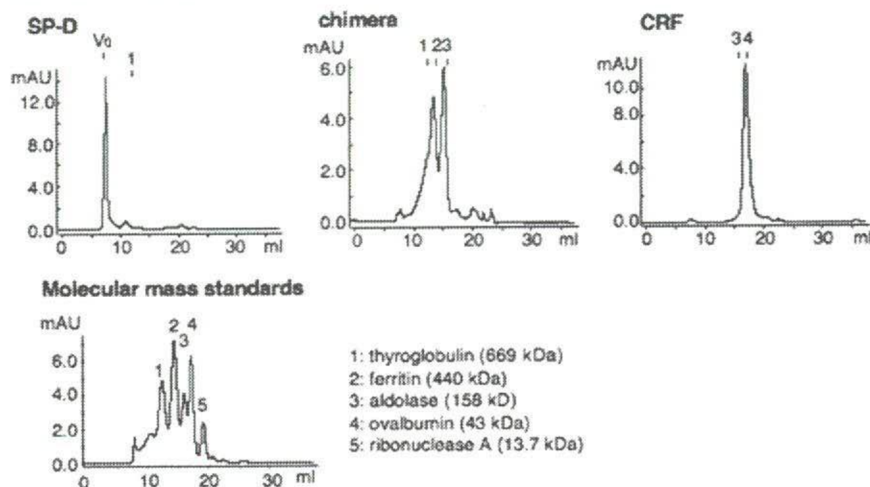

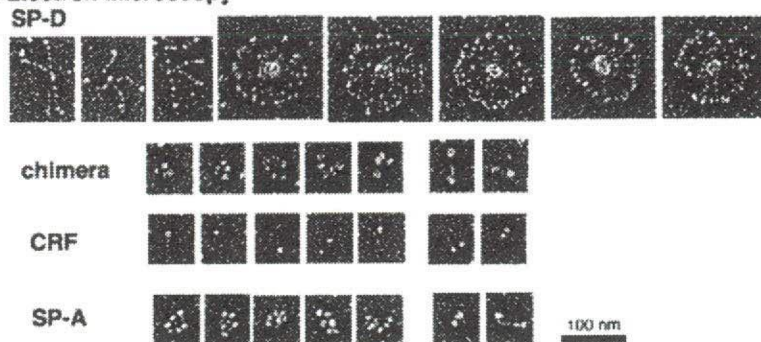

FIGURE 7. **Analysis of recombinant SP-D proteins by gel filtration and electron microscopy.** *A*, SP-D, A/D chimera, and CRF were analyzed in PBS by gel-filtration chromatography using a Superose 610/300 GL column. The molecular mass standards are; *1*, thyroglobulin (669 kDa); *2*, ferritin (440 kDa); *3*, aldolase (158 kDa); *4*, ovalbumin (43 kDa); *5*, ribonuclease A (13.7 kDa). *B*, electron micrographs of rotary-shadowed SP-D, A/D chimera, and CRF. SP-D shows the cruciform structures produced by dodecamers. Another SP-D subpopulation includes higher order multimers. The A/D chimera shows the bouquet-like structure with either four or six globular heads. The CRF preparation is composed of one or two globular heads with a short stalk. SP-A shows the bouquet-like structure mainly with six globular heads.

attenuated the mean fluorescence intensity of cell surface binding of both O55:B5 LPS and *S. minnesota* LPS (Fig. 5, *A* and *B*, *lower*).

Because MD-2 is critical for TLR4-mediated LPS signaling (13) and MD-2 directly interacts with LPS (14) and its principal core structural constituent, lipid A (29), we examined whether SP-D affects the binding of sMD-2 to LPS coated onto microtiter wells. SP-D significantly inhibited the binding of sMD-2 to different serotypes of LPS (Fig. 5*C*). The binding of sMD-2 to Re595 LPS and Rc LPS was reduced to near background levels by SP-D. The binding of sMD-2 to O26:B6 LPS and O111:B4 LPS was reduced to ~50% by SP-D. The results clearly demonstrate that SP-D alters MD-2-LPS interaction.

Analysis of Chimeric Protein and CRF—SP-A binds to Re595 LPS and Rc LPS and does not bind to O26:B6 LPS or O111:B4 LPS (20). In this study SP-D also binds to Re595 LPS and Rc LPS but not to O26:B6 LPS, and SP-D suppresses inflammatory responses elicited by both serotypes of LPS, unlike SP-A. We thus constructed a chimera composed of the N terminus plus the collagenous domain of SP-A and the neck plus the CRD of SP-D (A/D chimera) to determine whether the different responses between SP-A and SP-D are consequences of different oligomeric structures. Recombinant SP-D and the A/D chimera (Fig. 6*A*) were expressed in CHO-K1 cells and purified by affinity chromatography using mannose-Sepharose. CRF consisting of the neck region plus the CRD of SP-D was also purified by gel filtration after collagenase digestion. The main protein bands of SP-D and SP-A migrated at the positions of apparent molecular masses of 45 and 36 kDa, respectively, under reducing conditions when analyzed by electrophoresis (Fig. 6*B*, *lanes a* and *b*). The A/D chimera and CRF exhibited the expected sizes of apparent molecular masses of 32 and 18 kDa, respectively (Fig. 6*B*, *lanes c* and *d*). We also analyzed these proteins by Western blotting using anti-SP-A polyclonal antibody, anti-SP-D polyclonal antibody, and anti-SP-D monoclonal antibodies 7C6 and 7A10 whose epitopes are located at the neck region and the CRD of SP-D, respectively. SP-D or SP-A was recognized only by anti-SP-D antibody or anti-SP-A antibody, respectively (Fig. 6*C*, *lanes a* and *b*). The chimera was recognized by all four of the antibodies used (Fig. 6*C*, *lane c*) and CRF was detected by anti-SP-D polyclonal antibody and antibodies 7C6 and 7A10 (Fig. 6*C*, *lane d*). These results indicate that the chimera and the CRF are correctly expressed and secreted.

We further analyzed these proteins by gel-filtration chromatography and by electron microscopy to examine their oligomeric structure in solution. Gel-filtration analysis with Superose revealed that SP-D eluted at void fraction (Fig. 7*A*), indicating that SP-D is highly oligomeric (molecular mass of ~5 MDa). The elution profile of the A/D chimera showed two main peaks with apparent molecular masses of 220 and 620 kDa. CRF eluted at a fraction corresponding to the size of 68 kDa. The ultrastructures of the proteins were observed by electron microscopy using the rotary shadowing technique (Fig. 7*B*). In the preparation of SP-D, heterogenous particles, presumably consisting of two, or four trimers, or multimers were observed. One subpopulation of SP-D was a cruciform dodecamer. Another subpopulation includes multimerized oligomer con-

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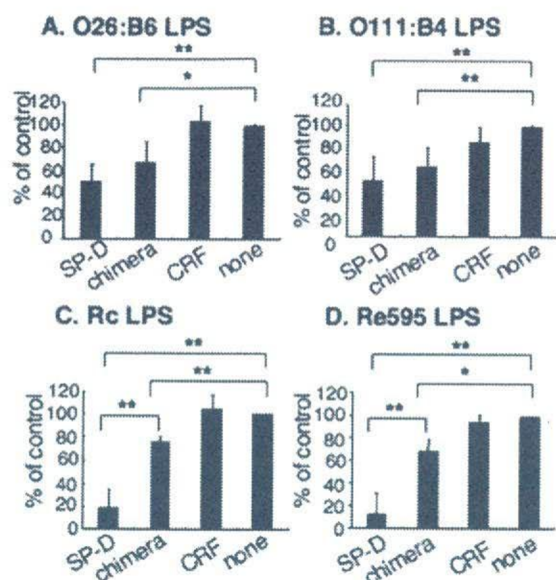

FIGURE 8. **The inhibitory effect of A/D chimera and CRF on the sMD-2 binding to LPS.** sMD-2 (1 μg/ml) was preincubated alone (none) or with 20 μg/ml SP-D, chimera, or CRF for 1 h at 37 °C, and the protein mixture was further incubated for 3 h at 37 °C with O26:B6 LPS (*A*) or O111:B4 LPS (*B*) coated onto microtiter wells. When Rc LPS (*C*) or Re595 LPS (*D*) was coated onto microtiter wells, 2 μg/ml sMD-2 was preincubated alone (none) or with 40 μg/ml SP-D, chimera, or CRF for 1 h at 37 °C. After the incubation, the wells were washed and further incubated with anti-His tag polyclonal IgG for 1 h, followed by incubation with HRP-conjugated anti-rabbit IgG for 1 h. The binding of sMD-2 to LPS was detected by measuring the absorbance at 492 nm, as described under "Experimental Procedures." The results are expressed as relative absorbance (% of control) compared with that obtained from the binding of sMD-2 to LPS in the absence of collectin (100%). The data shown are the means ± S.D. from five separate experiments (*A* and *B*) or three separate experiments (*C* and *D*). The mean absorbance at 492 nm obtained from the binding of sMD-2 to O26:B6 LPS, O111:B4 LPS, Rc LPS or Re595 LPS was 0.042, 0.037, 0.037, or 0.051, respectively. *, $p < 0.05$ and **, $p < 0.01$.

sisting of SP-D molecules associated at their N terminus. These observations are consistent with those of previous studies (8, 30). The preparation of the A/D chimera contained two, or four, or six trimers. Structures of four or six globular heads were seen and appeared to form a bouquet-like arrangement, which is typically observed for SP-A (7). The collagenous domain of SP-A consists of 23 Gly-*X*-*Y* repeats with an interruption near the midpoint that imparts a kink to the stem-like regions of the bouquet structure (31). The collagenous domain of SP-D contains 59 Gly-*X*-*Y* triplets without interruption (6).

The A/D Chimera and CRF Are Less Potent as Inhibitors for sMD-2 Binding to LPS, and Antagonists for LPS Signaling and TNF-α Secretion, by TLR4/MD-2-expressing Cells—The effects of the A/D chimera and CRF on the binding of sMD-2 to LPS coated onto microtiter wells were examined. SP-D and the chimera significantly attenuated the sMD-2 binding to various serotypes of LPS tested (Fig. 8), but the inhibitory effect of CRF on the sMD-2 binding to LPS was insignificant. Although active, the effect of the chimera as an inhibitor was generally less potent than that of SP-D. The A/D chimera was most different from SP-D at inhibiting sMD-2 binding to Re595 LPS and Rc LPS (Fig. 8, *C* and *D*). The activity of the A/D chimera was comparable to SP-D for inhibiting sMD-2 binding to O26:B6 LPS and O111:B4 LPS (Fig. 8, *A* and *B*).

LPS-elicited TNF-α secretion and NF-κB activity were also determined in the presence of SP-D, the chimera, and CRF. Because the oligomerization states are different, the findings are expressed as a function of monomer concentrations for the various recombinant proteins. The A/D chimera was less potent as an inhibitor of LPS-induced TNF-α secretion from alveolar macrophages than SP-D (Fig. 9, *A*–*C*). CRF was very weak at inhibiting TNF-α secretion. When normalized per mole of monomer, the IC_{50} was 181.3 ± 50.5 nM (mean ± S.D., $n = 3$) for SP-D, 239.9 ± 184.4 nM for the chimera, and 347.0 ± 87.4 nM for CRF in O26:B6 LPS-stimulation; 126.8 ± 45.8 nM for SP-D and 341.6 ± 122.9 nM for the chimera in Rc LPS-stimulation ($p < 0.05$, SP-D *versus* the chimera); 436.8 ± 226.2 nM for SP-D and 1201.6 ± 75.2 nM for the chimera in Re595 LPS stimulation ($p < 0.01$, SP-D *versus* the chimera). The luciferase assay for LPS-elicited NF-κB activation (Fig. 9, *D*–*F*) showed that SP-D was the most potent in attenuating LPS signaling and that the chimera and CRF were weak inhibitors in rank order of the chimera > CRF. These results obtained from cellular experiments are consistent with those obtained from the *in vitro* sMD-2 binding to LPS, indicating that the inhibitory effect of SP-D is dependent upon the formation of cruciform structure and its multimer.

DISCUSSION

Previous *in vivo* studies (17–19, 32) have provided compelling evidence that pulmonary collectins modulate pulmonary inflammation caused by microbes and their components. Several mechanisms of collectin-mediated modulation of inflammation have been proposed. Gardai *et al.* (33) have proposed that, in the absence of microbes, the direct binding of CRD of pulmonary collectins to signal inhibitory regulatory protein α induces the activation of tyrosine phosphatase SHP-1 and blocks the downstream signaling, resulting in inhibiting pulmonary inflammation. However, in the presence of microbes, the binding of the aggregated collagenous tail of SP-A to calreticulin/CD91 stimulates p38 phosphorylation, NF-κB activation, and cytokine production. They concluded that SP-A plays dual inflammatory roles by its interaction with signal inhibitory regulatory protein α and calreticulin/CD91. We have previously shown that SP-A inhibits inflammatory responses stimulated with O26:B6 LPS, peptidoglycan, and zymosan by direct interactions with TLR2 and TLR4 (21, 34, 35) and that SP-A does not attenuate but rather enhances Re595 LPS-induced signaling and TNF-α secretion (20). Because Re595 LPS but not O26:B6 LPS, peptidoglycan, or zymosan is a ligand for SP-A, this collectin exhibits dual functions in a ligand-specific manner. However, SP-D appears to exhibit anti-inflammatory effects regardless of ligand or non-ligand, because SP-D binds to Re595 LPS and Rc LPS but not to O26:B6 LPS and inhibits signaling and cytokine secretion stimulated with these different serotypes of LPS by altering LPS binding to its receptor (see Figs. 2–5). Gardai *et al.* (33) did not examine whether SP-D binds to calreticulin/CD91 via its collagenous tail. Because the collagenous tails and the globular domains of SP-A and C1q bind to the calreticulin/CD91 and the microbes, respectively, the orientation of the bouquet-like structure could be important. In contrast, the oligomeric structure of SP-D showing a cruciform is quite dif-

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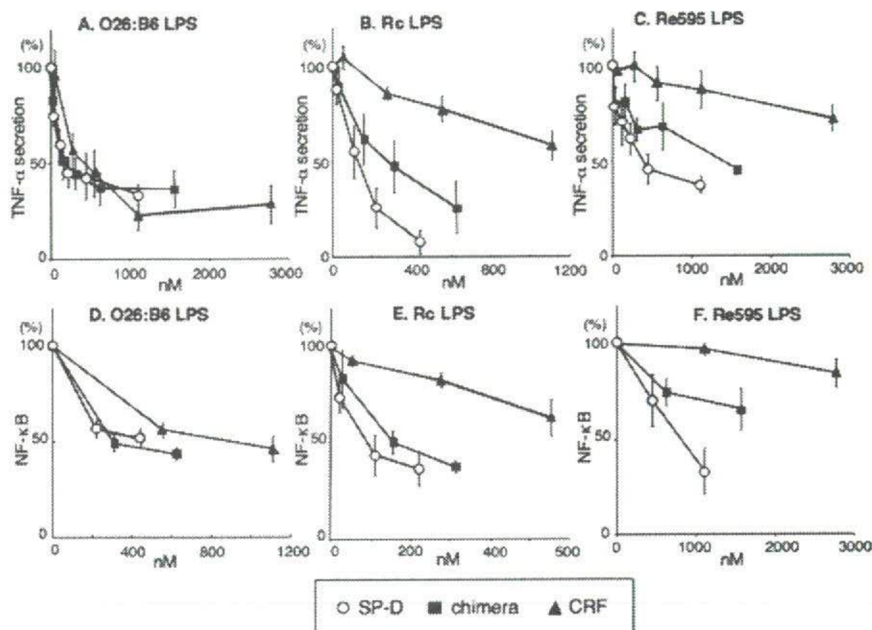

FIGURE 9. **The inhibitory effects of A/D chimera and CRF on the LPS-induced cell responses.** *A–C*, rat alveolar macrophages (10^5 cells) were preincubated with the indicated concentrations of SP-D (○), A/D chimera (■), or CRF (▲) for 30 min at 37 °C with 5% CO_2 and then stimulated with 100 ng/ml O26:B6 LPS (*A*), Rc LPS (*B*), or Re595 LPS (*C*) for 5 h. TNF-α secreted into the media was measured, and the results are expressed as percentages of LPS-induced TNF-α secretion in the absence of the protein. *D–F*, HEK293 cells transfected with TLR4 and MD-2 were incubated with the indicated concentration of SP-D (○), chimera (squlf]), or CRF (▲) for 1 h at 37 °C in an atmosphere of 5% CO_2 before adding LPS. The cells were stimulated with 100 ng/ml O26:B6 LPS (*D*), Rc LPS (*E*), or Re595 LPS (*F*) and further incubated for 5 h at 37 °C. LPS-induced NF-κB activation was determined as described under "Experimental Procedures." The results are expressed as percentages of luciferase activity of LPS-induced NF-κB activation in the absence of the protein. The *abscissa* values are expressed as the concentration of monomeric collectin present in the reactions to normalize the differences in oligomerization among the recombinant proteins. The data shown are the means ± S.D. from three separate experiments.

ferent from those of SP-A and C1q (see Fig. 7). Thus, it is possible to assume that SP-D might not bind to calreticulin/CD91, which cause pro-inflammatory response. SP-D may contribute to keeping the lung in a relatively uninflamed state by its anti-inflammatory functions. These studies support the idea that interactions of pulmonary collectins with cell surface receptors and/or their ligands result in the modulation of pulmonary inflammation.

It is difficult to determine the actual concentrations of pulmonary collectins *in vivo*, because alveolar hypophase (the epithelial lining fluid of the alveolus) cannot be directly measured. Nevertheless, their concentrations can be estimated based on the recovery of the proteins in the bronchoalveolar lavage fluids and the extrapolated hypophase volume (100–1000 μl/lung) (36, 37). The SP-D concentration in the alveolar hypophase can be calculated as ~63 μg/ml (38) when estimated from the average concentration (0.88 μg/ml) in the bronchoalveolar lavage fluids of human healthy volunteers (39). The calculated SP-A concentrations can be in the alveolar hypophase range from 180 μg/ml to 1.8 mg/ml (38, 40–42). Although the concentrations of pulmonary collectins in the hypophase under healthy and diseased states cannot be directly determined, the SP-D concentrations used in this study are within the best estimates of the physiological ranges.

We have previously shown that SP-D directly binds to the extracellular TLR4 domain (22). In this study sMD-2 was immunoprecipitated together with sTLR4 and SP-D, and biotinylated SP-D also co-precipitated with sMD-2 and sTLR4; demonstrating that SP-D can bind an sTLR4-sMD-2 complex. Because the formation of a complex with TLR4 and MD-2 is critical for initiating LPS signaling, it is reasonable to assume that interaction of SP-D with a receptor complex may affect LPS signaling. Consistently, SP-D attenuates cell surface binding of Alexa-labeled LPS to TLR4/MD-2-expressing cells. We also examined the effect of SP-D on the binding of sMD-2 to LPS, because lipid A avidly binds to sMD-2 but not to sTLR4 (29). The results indicate that SP-D significantly decreases the sMD-2 binding to LPS. Taken together, these results support the conclusion that SP-D dampens LPS-induced inflammation by altering LPS-receptor interaction.

This study shows that SP-D binds *S. minnesota* Re595 LPS and *E. coli* Rc LPS, but not *E. coli* O26:B6 LPS. The experiments with anti-SP-D monoclonal antibodies indicate that the neck domain and the CRD are involved in the binding of SP-D to Re595 LPS and Rc LPS, respectively. A previous study (28) has shown by lectin blot analysis that SP-D interacts with Rc and Rd LPS, but not with Re LPS or smooth LPS. Although the differences between studies with Re LPS binding may be due to the methods used, this and our previous studies (20, 43) using microtiter well assays indicate that SP-A and SP-D exhibit significant binding to Re595 LPS, but not to O26:B6 LPS. Because SP-A inhibits inflammatory responses elicited by O26:B6 LPS but not by Re595 LPS (20), we tested whether SP-D modulates LPS signaling in a ligand-specific manner, as observed in SP-A. Unlike SP-A, SP-D inhibits inflammatory cell responses induced by rough LPS, an SP-D ligand, as well as smooth LPS, which is not a ligand for SP-D. The precise mechanism by which SP-D inhibits rough LPS-elicited signaling remains to be determined. The profiles of concentration-dependent inhibition by SP-D and the SP-A/SP-D chimera indicate that the difference between these molecules in the inhibitor activities on smooth LPS-induced responses appears small (see Fig. 9, *A* and *D*). In contrast, the difference of the inhibition of rough LPS-induced responses is more pronounced (see Fig. 9, *B*, *C*, *E*, and *F*). Because the chimera also binds to Re595 LPS and Rc LPS (data not shown) as well as SP-A and SP-D, the finding that

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the inhibitory activity of the chimera on rough LPS-induced responses is less potent than that of SP-D may be a consequence of the orientation of the CRD and/or the length of the collagenous tail, when the proteins interact with rough LPS and an LPS receptor.

The recombinant SP-D prepared in this study is found to be highly oligomeric in solution by gel-filtration analysis and is organized as a cruciform dodecamer, or higher order multimers in electron micrographs. Disruption of interchain disulfide bond formation at the N terminus by Cys → Ser mutations at Cys^{15} and Cys^{20} prevents the covalent oligomerization of trimeric subunits, resulting in loss of functions, such as inducing viral aggregation and enhancing interactions of influenza A virus with neutrophils (44). Expression of wild-type SP-D but not of $SP\text{-}D^{C15S, C20S}$ in SP-D-null mice corrects the pulmonary phospholipid accumulation and emphysema phenotype of the $SP\text{-}D^{-/-}$ mice (45). These studies indicate that the supratrimeric oligomerization of SP-D is required for native protein functions. In this study CRF is ineffective in modulating LPS signaling, although CRF possesses the neck plus the CRD, which are the functional domains for interactions with LPS and TLR4 (22). This clearly demonstrates the importance of the oligomerization of SP-D in immunomodulatory functions of the protein. In addition, the SP-A/SP-D chimera that is assembled in a bouquet-like octadecamer characteristic of SP-A is less potent at inhibiting the LPS-induced inflammatory responses. This suggests that the integrity of the cruciform formation is critical for the expression of the full activities of SP-D in modulating LPS-stimulated inflammatory responses.

In conclusion, SP-D can bind a complex of TLR4 and MD-2 and inhibits TLR4-mediated inflammatory responses caused by both smooth and rough serotypes of LPS. SP-D inhibits the cell surface binding of smooth and rough LPS to TLR4/MD-2-expressing cells and attenuates the MD-2 binding to LPS. SP-D is a more potent inhibitor of LPS-elicited inflammation than SP-A/SP-D chimera and CRF. These results clearly demonstrate that SP-D inhibits LPS-induced inflammatory cell responses by altering LPS-receptor interaction and that the organization of the cruciform dodecamer and its multimer is a critical feature of its function.

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N-アセチルシステインによる肺線維化の抑制

N-acetylcysteine as a new therapy for IPF

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◎オキシダントは直接的あるいは間接的に肺傷害の中心的なメディエーターとして間質性肺炎の病態に関与している．N-アセチルシステイン(NAC)はグルタチオン(GSH)の前駆体で細胞内GSH増加作用を有するうえ，それ自体にもantioxidant作用を有するため，IPF治療に有用である可能性がある．ヨーロッパではIPF症例を対象にプレドニン＋アザチオプリン併用のうえにNAC(1,800 mg/day)を1年間経口投与する臨床試験が行われ，プラセボ群と比較して，D_{LCO}およびVCの悪化の有意な減少が認められた．著者らはマウスブレオマイシン肺臓炎モデルを用いてNAC吸入治療の有用性を証明し，さらにNAC吸入療法のオープン臨床試験を行い，良好な結果を得た．現在，わが国ではNAC吸入療法の多施設臨床試験が施行中である．NACは副作用もほとんどなくIPFの治療薬として有用性が期待できる薬剤である．

Key word N-アセチルシステイン，グルタチオン，オキシダント，特発性肺線維症

肺線維症とオキシダント

間質性肺炎の病態においては，未知の刺激により肺胞マクロファージや集積した好中球などからオキシダントが放出され，組織傷害を惹起している．このような病態における炎症細胞の集積や活性化にはサイトカインや接着分子が重要な役割を演じているが，細胞内情報伝達や遺伝子の転写因子の活性化などにもオキシダントが関与し，サイトカイン産生や接着分子発現といった炎症の病態を調節していることも示されている．このようにオキシダントは直接的あるいは間接的に肺傷害の中心的なメディエーターとして間質性肺炎の病態に関与している．

一方，正常の気道粘膜には種々のオキシダント刺激を防御するためのanti-oxidant defense mechanismが存在する．そのひとつであるグルタチオン(GSH)はglutathione redox cycleのなかで働き，過酸化水素を水に変換する役割を果たしている．気道上皮被覆液(epithelial lining fluid：ELF)にはこのGSHが豊富に存在し，肺胞・気道上皮をoxidative stressから保護していると考えられる[1]．しかし，特発性肺線維症(IPF)をはじめとする間質性肺疾患の病態においては，肺胞マクロファージなど気道炎症細胞からの活性酸素産生が亢進し，antioxidantである気道上皮被覆液中のGSH濃度が低下していることが知られている[2,3]．したがって，これらの症例において，減少したGSHを補充するなど抗酸化療法が治療として有効である可能性が考えられる．

抗酸化薬として，SODやチオレドキシンなどのアンチオキシダントの治療への可能性が肺線維化動物モデルで報告されている．著者らも，N-アセチルシステイン(NAC)の吸入がマウスにおけるブレオマイシンによる肺線維化を抑制することを示した[4]．

NACとは

NACは古くから去痰剤として用いられてきた