Characterization of Synthetic Lung Surfactant Activity against Proinflammatory Cytokines in Human Monocytes

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Our previous study demonstrated that the smallest synthetic peptide with the sequence CPVHLKRLLLLL-LLLLLLLLLLL, SP-CL16(6—28), admixed with phospholipid (synthetic lung surfactant, SLS) showed strong surface activity. In this study, we attempted to develop a dual-type surfactant with both anti inflammatory and surface activities. SP-CL16(6—28) was first chemically synthesized and then purified for use by centrifugal partition chromatography. A mixture of SP-CL16(6—28) and phospholipid complex was tested for anti inflammatory activity using the human monocyte cell line THP-1. Whether the suppression of tumor necrosis factor-α (TNF-α), interleukin (IL)-8, IL-6, IL-1β, and macrophage migration inhibitory factor (MIF) was reduced by lipopolysaccharide (LPS) in monocytes was examined. Levels of these cytokines were measured by enzyme-linked immunosorbent assay. It was found that SLS significantly and dose dependently inhibited the secretion of TNF-α by THP-1 cells following stimulation with LPS. Dipalmitoylphosphatidylcoline did not inhibit the release of cytokines. These findings suggest that SLS has anti inflammatory activity. Therefore it should be possible to develop a SLS with both anti inflammatory activity and surface activity.

Key words synthetic lung surfactant; proinflammatory cytokines; suppression; human monocyte cell

Lung surfactant, synthesized by alveolar type II cells, is a mixture of a large amount of phospholipids and a small amount of specific proteins. Surfactant helps to keep the lungs expanded by lowering the surface tension at the air–water interface in the alveoli.[1—4] The lung surfactant is composed primarily of one phospholipid and four unique proteins that have been isolated.[5] Regarding the functions of lung surfactant proteins other than surface activity are still unknown.

Tumor necrosis factor-α (TNF-α), interleukin (IL)-8, IL-6, and IL-1β are major proinflammatory cytokines that induce the synthesis and release of many inflammatory mediators.[6,7] They are involved in immune regulation, autoimmune diseases, and inflammation. Macrophage migration inhibitory factor (MIF) also plays an important role in systemic as well as local inflammatory and immune responses.[8—10] MIF was first identified as a T cell-derived factor that inhibited the random migration of macrophages *in vitro*.[11,12]

Recently, it was found that the inflammatory reaction can be controlled by lipopolysaccharide (LPS) induction of lung surfactant.[13—15] Therefore the anti inflammatory activity of surfactant is of clinical interest and was investigated here.

We previously showed that SP-C was indispensable in the surface activity of a series of lung surfactants.[16] Thus we synthesized various forms of the SP-C analogue and found that the smallest synthetic protein unit with the sequence CPVHLKRLLLLLLLLLLLLLLL, SP-CL16(6—28), showed a high level of surface activity.[17] The poly leucine-substituted SP-CL analogues were comparable in surface activitiy with Surfacten® (Surfactant-TA), a modified surfactant preparation which has been used for the treatment of respiratory distress syndrome (RDS).

In this study, we undertook the chemical synthesis of SP-CL16(6—28) and then purified the peptide by centrifugal partition chromatography (CPC). The activity of the peptide improved the *in vitro* surface activity of a ternary lipid mixture composed of dipalmitoylphosphatidylcholine (DPPC), egg-phosphatidylglycerol (PG), and palmitic acid (PA) (DPPC/PG/PA, 75 : 25 : 10, w/w) in a Langmuir–Wilhelmy surface balance. Furthermore, we investigated the anti inflammatory activity of synthetic lung surfactant (SLS) and assayed the reaction of the proinflammatory cytokines TNF-α, IL-8, IL-6, IL-1β, and MIF, which they secretes in response to LPS, using THP-1 cells *in vitro*.

MATERIALS AND METHODS

9-Fluorenylmethyloxycarbonyl (Fmoc) esters of amino acids, a link amide polyethyleneglycol-polystyrene (PEG-PS) resin, and other reagents used for peptide synthesis were purchased from Kokusan Chemical Works (Tokyo, Japan) and Nihon PerSeptive (Tokyo, Japan). DPPC and PG (sodium salt prepared from egg yolk phosphatidylcholine) were obtained from Sigma Chemical (St. Louis, MO, U.S.A.). PA was from Tokyo Kasei Kogyo (Tokyo, Japan). Surfacten® (Surfactant-TA) was a modified bovine lung surfactant containing a small percentage of proteins and about 95% lipids, and was supplied by Mitsubishi-Tokyo Pharmaceuticals (Tokyo, Japan).

Human monocyte THP-1 cells were obtained from the American Type Culture Collection. RPMI-1640 medium was also purchased from Gibco (NY, U.S.A.). Fetal calf serum (FCS) was obtained from Gibco. The FCS used was heat inactivated. Phorbol myristate acetate (PMA), dexamethasone (Dex) and LPS (*E. coli* 055 : 55) were purchased from Sigma Chemical.

TNF-α, IL-1β, IL-6 and IL-8 enzyme-linked immunosorbent assay (ELISA) kits were purchased from Genzyme Techne (MA, U.S.A.). Recombinant human MIF, anti human MIF antibody, and anti human MIF biotinated antibody were purchased from R & D Research (MA, U.S.A.). All other chemicals were of reagent grade.

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Synthesis and Purification of SP-CL16(6—28) Peptide synthesis was carried out by the stepwise solid-phase method with a peptide synthesizer Pepsynthesizer® model 9050 plus (Nihon PerSeptive) as described previously.[17)]

The purification of cleaved peptide was described in detail previously.[18)] Briefly, the peptide was purified by CPC performed on a model LLN-B (Sanki Engineering, Tokyo, Japan) machine equipped with a 2056-cartridge column, and developed with the *n*-hexane/CH_3OH/H_2O/trifluoroacetic acid (TFA) solvent system by the descending method. Purified peptide was stored at −20 °C.

Analysis of Synthetic Peptide by Mass Spectrometry The peptide was identified using an electrospray-ionization mass spectrometry analyzer model Quatro LC (JASCO International, Tokyo, Japan).

Quantitative Amino Acid Analysis The composition of the purified peptide was determined as described previously.[18)]

Preparation of SLS SLS containing the peptide was prepared in the following manner[17)]: Synthetic peptide dissolved in a small volume of TFA was mixed with a ternary lipid mixture of DPPC/PG/PA (75 : 25 : 10, w/w) in a $CHCl_3$/CH_3OH (2 : 1, v/v) solution to produce a peptide-lipid mixture containing 2% peptide on a weight/weight basis relative to the phospholipids. The peptide-lipid mixture was suspended in 10% ethanol, incubated at 40—45 °C for 15 min and then lyophilized to obtain a white powder. This admixture was dispersed in saline by sonication for 1 min in ice-cold water and stored in nitrogen at −20 °C. The final concentration of phospholipids was 10 mg/ml. Surfactant-TA was also dispersed in saline at a phospholipid concentration of 25 mg/ml.

Measurement of Surface Properties with a Langmuir–Wilhelmy Surface Balance Surface spreading and dynamic surface activities were measured with a modified Wilhelmy surface balance (Kyowa, Tokyo, Japan) at 37±1 °C as described previously.[13)] Data of the fourth surface tension-area diagram is shown in the tables. The surface area at 10 mN/m obtained from the diagram was expressed as a percentage of the total area.

Cell Culture THP-1 cells were cultured in complete RPMI-1640 medium supplemented with 10% heat-inactivated FCS, 1 mM L-glutamine, 50 units/ml of penicillin, 50 μg/ml of streptomycin and 50 μM of 2-mercaptoethanol at 37 °C in a moist 5% CO_2 incubator. Assays were performed at a density of 10^7 cells/ml.

Cytokine Assays THP-1 cells were suspended in complete RPMI-1640 medium. *In vitro* cultivation was done in triplicate over 24 h at 37 °C in a humidified atmosphere containing 5% CO_2, under the following culture conditions: Unstimulated THP-1 cells were cultivated in complete RPMI-1640. In contrast, THP-1 cells were pretreated with PMA (10 nM) for 6 h. After the incubation, the culture medium was removed and cells were washed three times with RPMI-1640 without serum. PMA-pretreated cells were stimulated with either LPS at a concentration of 10 μg/ml, or by LPS combined with SLS, Surfactant-TA, and DPPC at 1—100 μg/ml. Cultures were incubated for 24 h at 37 °C. After incubation, supernatants were collected and assayed for TNF-α, IL-8, IL-6 and IL-1β using commercial ELISA kits. MIF was measured by ELISA as described previously.[19)]

RESULTS

Surface Activity of SLS in a Langmuir–Wilhelmy Surface Balance The surface activity of the synthetic peptide improved the *in vitro* surface activity of a ternary lipid mixture composed of DPPC/PG/PA (75 : 25 : 10, w/w) in a Langmuir–Wilhelmy surface balance. The levels of surface activity are shown in Table 1, with a summary of the spreading kinetics and dynamic compression-expression properties of SP-CL16(6—28) after association with the lipid mixture of DPPC/PG/PA.

SLS markedly increased the spreading rates, compared with the activity of the ternary lipid mixture, and the surface tension reached 27.7 mN/m at 30 s, which was obtained with the SP-CL16(6—28) peptide. After 3 min of spreading, the surface tension area of the surfactant film during dynamic cycling was measured with phospholipids at an internal concentration of 1.5 μg/cm^2. These films containing the peptide were compressed by 24.9% to obtain a surface tension of less than 10 mN/m and exhibited minimum and maximum surface tensions of 2.3 and 28.9 mN/m, respectively. In contrast, with the ternary lipid mixture of DPPC/PG/PA, after 3 min of spreading the film containing the peptide was compressed by 54.6% to reach a surface tension of less than 10 mN/m, and the minimum and maximum surface tensions were 2.7 and 44.5 mN/m, respectively. Moreover, after 3 min of spreading, the film containing DPPC had not reached a surface tension of less than 10 mN/m, and exhibited minimum and maximum surface tensions of 68.4 and 70.2 mN/m, respectively.

Effects of DPPC on Proinflammatory Cytokines from LPS-stimulated THP-1 Cells Concentrations of TNF-α, IL-8, IL-6, IL-1β, and MIF secreted by unstimulated THP-1 cells were 8.2±2.06 pg/ml, 633.7±26.01 pg/ml, 2.9±2.15 pg/ml, 27.4±1.09 pg/ml, and 10.5±0.79 ng/ml (mean±S.D.),

Table 1. Surface Activities of DPPC, a Ternary Lipid Mixture of DPPC/PG/PA, and SP-CL16(6—28) Plus a Ternary Lipid Mixture and Surfactant-TA

Sample[a)]	Surface tension (mN/m)		Surface area (%)	Surface spreading (mN/m)	
	γ_{min}[b)]	γ_{max}[c)]	at 10 mN/m	at 30 s	at 180 s
DPPC	68.4±2.5	70.2±4.8	N.D.[d)]	68.2±1.8	69.0±3.4
DPPC/PG/PA	2.7±1.5	44.5±6.8	45.4±2.2	48.2±1.3	45.8±2.3
DPPC/PG/PA plus SP-CL16(6—28)	2.3±1.4	28.9±0.8	75.1±1.3	27.7±11.4	26.8±0.7
Surfactant-TA	6.5±2.2	30.5±1.4	74.4±5.6	28.3±1.9	28.5±1.2

a) A ternary lipid mixture of DPPC/PG/PA (75 : 25 : 10, w/w), admixed with synthetic peptide at 2% of the weight of phospholipids was applied to the air-water interface with 1.5 μg of phospholipids/cm^2 surface area and allowed to spread spontaneously for 3 min. The surface area was then chosen from an area of 54.0 to 21.6 cm^2, and dynamic surface tension was recorded as described in Materials and Methods. *b*) Minimum surface tension. *c*) Maximum surface tension. Each value represents mean±S.D. (n=3). *d*) Not detected.

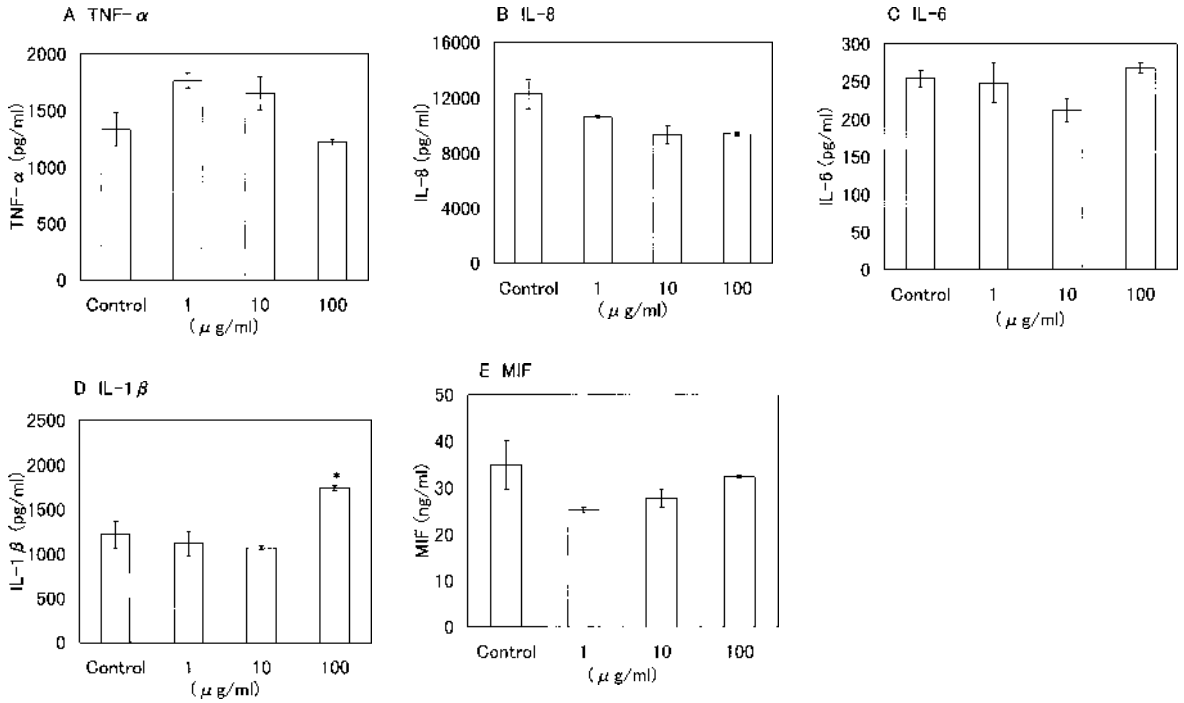

Fig. 1. Comparison of the Effect of DPPC on the Production of Various LPS-Stimulated Proinflammatory Cytokines by THP-1 Cells

A: TNF-α; B: IL-8; C: IL-6; D: IL-1β; E: MIF. DPPC was tested at concentrations of 1—100 μg/ml. Conditions for cultures are described under Materials and Methods. Data are given as mean±S.D. (n=2). *$p \leqq 0.05$, significantly different from control (Dunnett's multiple-comparison test).

respectively, after incubation for 24 h.

The findings are shown in Fig. 1. In this experiment, DPPC did not affect the release of TNF-α, IL-8, IL-6, and MIF from LPS-stimulated THP-1 cells (Fig. 1A—D). In contrast, IL-1β was increased by DPPC at a dosage of 100 μg/ml (Fig. 1D).

Effects of SLS, DPPC/PG/PA, and Surfactant-TA on Proinflammatory Cytokines From LPS-Stimulated THP-1 Cells Concentrations of TNF-α, IL-8, IL-6, IL-1β, and MIF secreted by unstimulated THP-1 cells were 7.8±4.70 pg/ml, 23.0±2.50 pg/ml, 2.9±0.18 pg/ml, 5.7±4.71 pg/ml, and 4.7±0.50 ng/ml (mean±S.D.), respectively, after incubation for 24 h. The SP-CL16(6—28) plus phospholipid mixture SLS and DPPC/PG/PA concentration-dependently (1—100 μg/ml) inhibited the secretion of TNF-α induced by LPS by human monocyte THP-1 cells (Fig. 2A). The IC_{50} values of SLS and DPPC/PG/PA were 48 and 85 μg/ml, respectively. Surfactant-TA inhibited the release of TNF-α from LPS-stimulated THP-1 cells in a dose-dependent manner (1—100 μg/ml). IL-8 and MIF were not affected by LPS (Figs. 2B, E).

SLS and Surfactant-TA inhibited IL-6 secretion at concentrations of 10—100 μg/ml (Fig. 2C). Surfactant-TA alone inhibited the secretion of IL-6 at a concentration of 1 μg/ml. DPPC/PG/PA inhibited IL-6 secretion at 100 μg/ml. Furthermore, SLS and DPPC/PG/PA inhibited the release of IL-1β at 1 μg/ml, but increased it at a concentration of 100 μg/ml (Fig. 2D).

Effects of Dex on Proinflammatory Cytokines Secreted by LPS-Stimulated THP-1 Cells Dex 0.1—10 μM inhibited the secretion of TNF-α from human monocyte THP-1 cells induced by LPS (Fig. 3A) concentration dependently. The IC_{50} value was 1 μM. IL-8 was similarly inhibited in a dose-dependent manner (Fig. 3B). IL-6 showed a similar response and the IC_{50} was more than 0.1 μM (Fig. 3C). In contrast, Dex had no effect on the secretion of cytokines (IL-1β and MIF) from THP-1 cells induced by LPS (Figs. 3D, E).

DISCUSSION

The ultimate goal of this research is to develop a synthetic lung surfactant with surface and anti-inflammatory activities. The purpose of the present study was to characterize further the biological activity of SLS, which is composed of DPPC, PG, PA, and SP-CL16(6—28). We previously demonstrated that the synthetic SP-C analogue SP-CL16(6—28) has surface activity equivalent to that of the acute RDS (ARDS) treatment agent Surfactant-TA.[17,18]

In this study, the surface activity of the lung surfactant was judged on the basis of the criteria of Tanaka *et al.*[20,21] The Langmuir–Wilhelmy surface balance method. The criteria were as follows: surface spreading is less than 35 mN/m after the sample is allowed to stand for 180 s. Maximum surface tension (γ_{max}) is less than 35 mN/m. Furthermore, more than 60% of the surface area (compression) has tension of 10 mN/m. As shown in Table 1, the only synthetic surfactant samples that satisfied the criteria were DPPC/PG/PA plus SP-CL16(6—28). Therefore to produce a SLS with surface activity, phospholipids plus SP-CL protein are needed.

In addition, we examined the anti inflammatory activity of SLS using a monocyte cell line. DPPC alone had no effect on the LPS-induced secretion of TNF-α from human monocyte THP-1 cells. Modified bovine surfactant (Surfactant-TA) contains the surfactant-associated proteins B and C and several different phospholipids.[22] The most active SLS is composed of DPPC, PG, PA, and SP-CL16(6—28). No anti in-

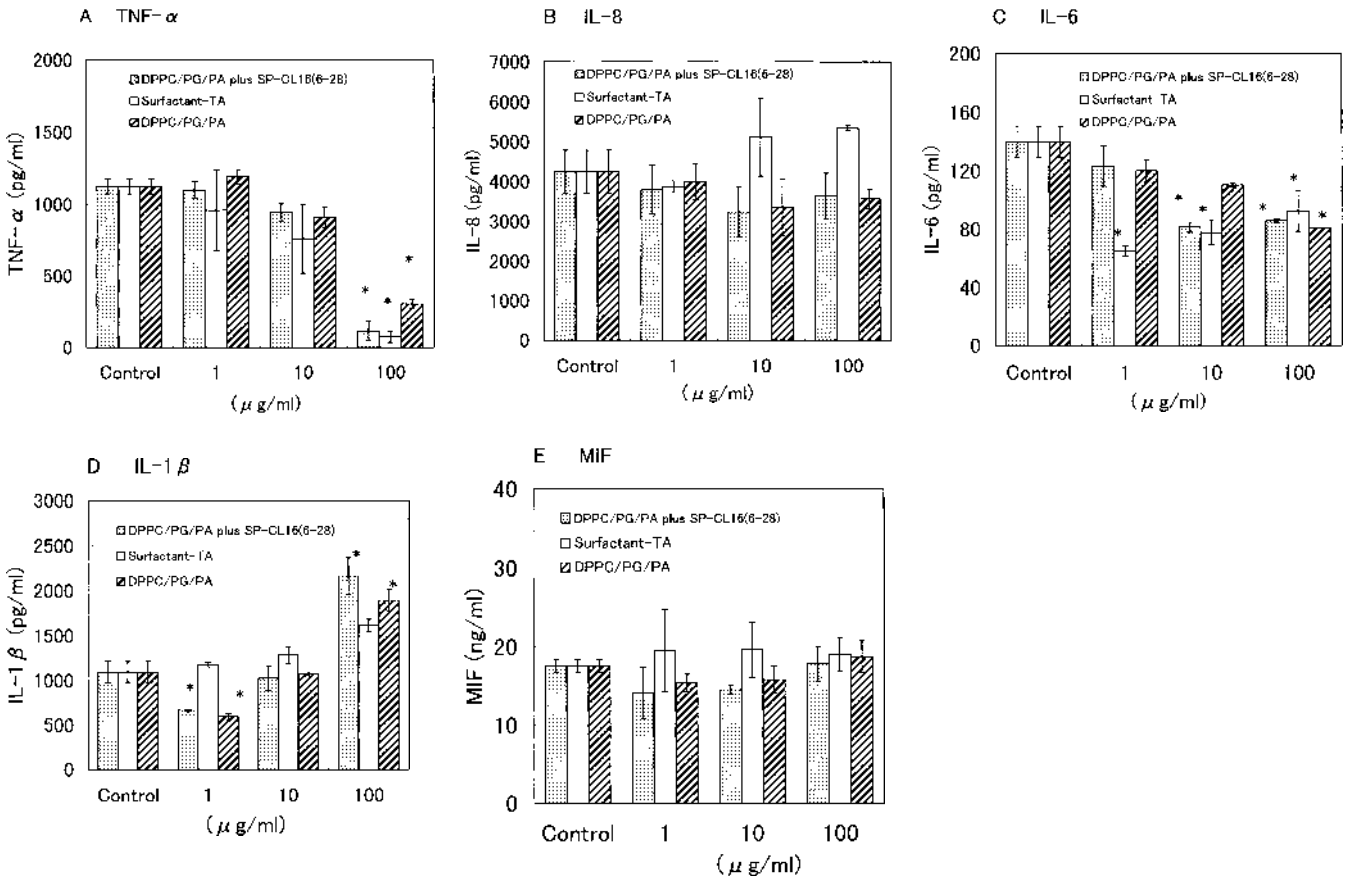

Fig. 2. Comparison of the Effect of Synthetic Surfactant and Surfactant-TA on the Prodution of LPS-Stimulated Proinflammatory Cytokines by THP-1 Cells

A: TNF-α; B: IL-8; C: IL-6; D: IL-1β; E: MIF. A ternary lipid mixture of DPPC/PG/PA (75 : 25 : 10, w/w) admixed with SP-CL16(6—28) at 2% of the weight of phospholipids. SLS and Surfactant-TA were tested at concentrations of 1—100 μg/ml. Conditions for cultures are described under Materials and Methods. Data are given as mean±S.D. (n=3). $*p \leqq 0.05$, significantly different from control (Dunnett's multiple-comparison test).

flammatory activity against LPS was exhibited by the phospholipid, at least when mixed with surfactant protein.

Human monocyte THP-1 cells differentiate into mature cells in response to various inducers. PMA induces the cells to commit to the macrophage lineage. We investigated changes in proinflammatory cytokines, especially TNF-α, IL-8, IL-6, IL-1β, and MIF when THP-1 cells were treated with PMA. SLS inhibited the release of TNF-α marked in a dose-dependent manner. Although the level of IL-6 was decreased by SLS at concentrations of 1—10 μg/ml, it was not inhibited at 100 μg/ml.

SLS incerased the level of IL-1β at a concentration of 100 μg/ml. However, in the control experiment, DPPC 100 μg/ml increased the level similarly. This suggests that the synthetic surfactant deos not necessarily act directly on IL-1β. Yisheng *et al.*[23] examined the reaction of Dex with PMA using THP-1 cells. According to their report, the signal transduction occurring *via* protein kinase C (PKC) when PMA was used to induce differentiation is closely related to the increase in secretion of IL-1β. In our assay, the main contributor to the increase in IL-1β was not LPS but PMA, an agent specific for THP-1 cells. Furthermore, the secretion of cytokines by LPS-stimulated macrophages should be inhibited by a non specific anti inflammatory agent such as Dex. However, IL-1β secretion was not well controlled by Dex in THP-1 cells, excepted at surprisingly high concentrations 0.1—10 μM. The mechanism of the secretion of IL-1β from THP-1 appears to depend on PMA rather than LPS. Previously, surfactant protein-A (SP-A) was reported to act on the LPS receptor and especially on CD14.[24] However, there is no evidence of a signaling pathway involving SP-C. We speculate that the CD14 receptor is a possible site of action of SLS. As a result, PKC is activated and the level of IL-1β increases in the presence of SLS. After the removal of PMA, activation of PKC would continue within the cell. SLS may act to suppress the secretion of IL-1β through CD14.

Recently, intervention with the use of proinflammatory cytokines was reported in cases of ADRS. TNF-α, IL-8, IL-6, IL-1β, and MIF were detected in high concentrations in the bronchoalveolar fluids of patients with ARDS, and their relationship to ARDS is of interest.[25,26] Furthermore, it was recently shown that MIF inhibits the anti inflammatory activity of endogenous steroids.[27] We consider that pharmacotherapy for ARDS targets all the internal organs and may serve to prevent the morbidity itself. Therefore treatment with a synthetic steroid and a surfactant would be effective.

A clinical trial of the surfactant was performed in ARDS.[28] This agent acts on a lesion directly since it is released in the respiratory tract. Therefore a dual-type synthetic surfactant with surface activity to prevent ARDS alveolus atrophy and anti inflammatory activity would be useful clinically.

To extend our understanding of the inhibitory effects of

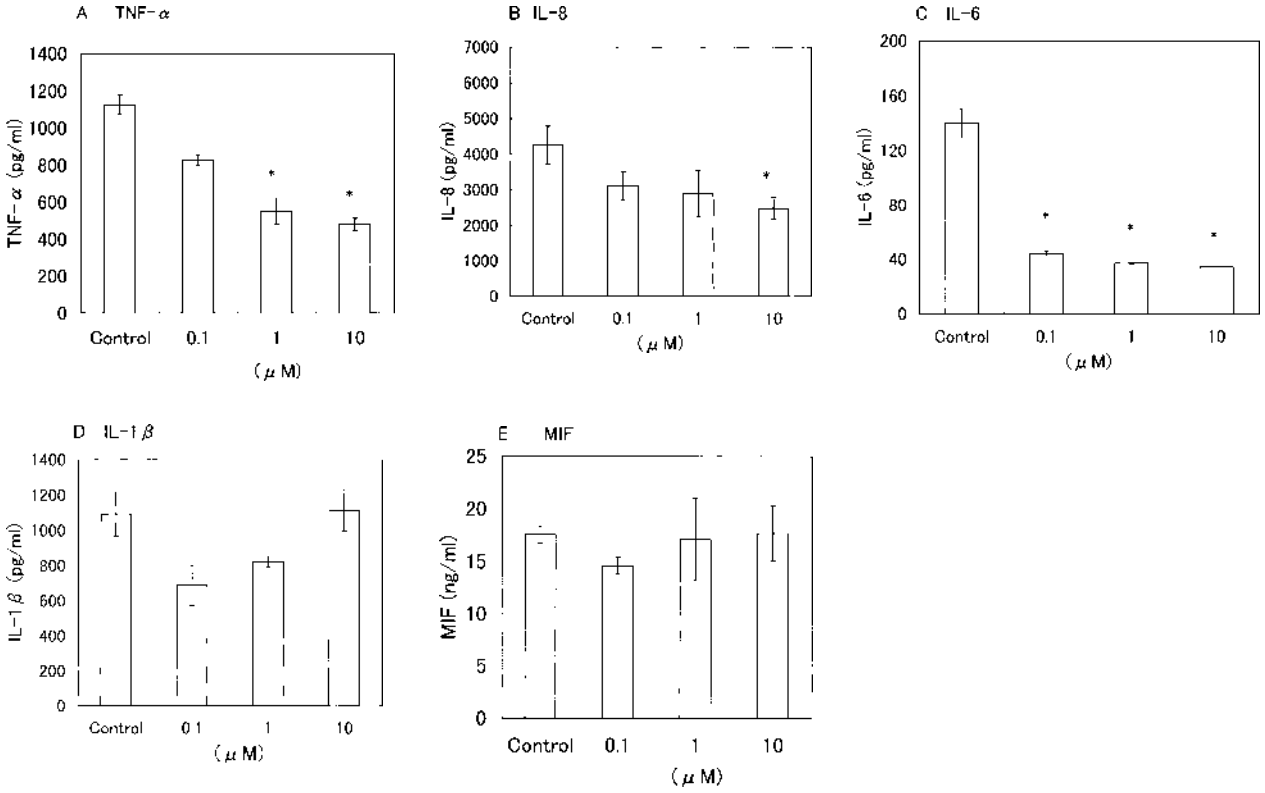

Fig. 3. Effect of Dex on the Production of LPS-Stimulated Proinflammatory Cytokines by THP-1 Cells

A: TNF-α; B: IL-8; C: IL-6; D: IL-1β; E: MIF. Dex was tested at concentrations of 0.1—10 μM. Conditions for cultures are described under Materials and Methods. Data are given as mean±S.D. (n=3). *p≦0.05, significantly different from control (Dunnett's multiple-comparison test).

SLS on the production of proinflammatory cytokines, exprIments were performed using Dex. Generally, Dex is used as non specific anti inflammatory steroid and prevent the activation of a transcriptional factor through glucocorticoid receptor. In a series of experiments, we showed the dual-type agent to have the surface activity of a SLS along with anti inflammatory activity. Neither activity was exhibited by DPPC. SLS contains various phospholipids and surfactant proteins like Surfactant-TA. We were able to characterize the surface activity of this synthetic surfactant, as well as its anti inflammatory activity, in this study.

Steroids inhibit the production of cytokines by controlling the translation of mRNA within the macrophage. They have strong anti inflammatory activity that blocks almost the entire cytokine cascade. It has been reported that the effectiveness of steroids is questionable in the treatment of ARDS.[29] On the other hand, the treatment of infection is improved by antibiotic medication, and one can expect good performance at low cost. However, the clinical value of steroids is not necessarily established.[30]

In this investigation, a synthetic surfactant in a lipid mixture of DPPC/PG/PA plus SP-CL16(6—28) exhibited both surface and anti inflammatory activity. As compared with Dex, the SLS suppressed cytokine production only weakly. However, we have characterized its anti inflammatory activity except for the surface activity. It appears that the combined use of this SLS and synthetic steroid may be a potential treatment for ARDS.

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