Biochimica et Biophysica Acta 1838 (2014) 2046–2052

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Improvement of pulmonary surfactant activity by introducing D-amino acids into highly hydrophobic amphiphilic α-peptide Hel 13-5

Yoshihiro Nakamura [a], Ko Yukitake [b], Hiromichi Nakahara [c], Sooyoung Lee [d], Osamu Shibata [c,*], Sannamu Lee [c,e]

[a] Muromachi Chemical Co. Ltd., Ohmuta, Fukuoka 836-0895, Japan
[b] Fukuoka University Hospital, Fukuoka 814-0180, Japan
[c] Department of Biophysical Chemistry, Faculty of Pharmaceutical Sciences, Nagasaki International University, Sasebo, Nagasaki 859-3298, Japan
[d] Department of Pediatrics, Graduate School of Medical Sciences, Kyushu University and Emergency & Critical Care Center, Kyushu University Hospital, Fukuoka 812-8582, Japan
[e] Department of Chemistry, Faculty of Science, Fukuoka University, Fukuoka 814-0180, Japan

ARTICLE INFO

Article history:
Received 5 February 2014
Received in revised form 3 April 2014
Accepted 23 April 2014
Available online 2 May 2014

Keywords:
Amphiphilic α-helical peptide
Artificial pulmonary surfactant
Recovery of lung compliance
Respiratory distress syndrome
Soy lecithin
Surface tension–area diagram

ABSTRACT

The high costs of artificial pulmonary surfactants, ranging in hundreds per kilogram of body weight, used for treating the respiratory distress syndrome (RDS) premature babies have limited their applications. We have extensively studied soy lecithins and higher alcohols as lipid alternatives to expensive phospholipids such as DPPC and PG. As a substitute for the proteins, we have synthesized the peptide Hel 13-5D3 by introducing D-amino acids into a highly lipid-soluble, basic amphiphilic peptide, Hel 13-5, composed of 18 amino acid residues. Analysis of the surfactant activities of lipid-amphiphilic artificial peptide mixtures using lung-irrigated rat models revealed that a mixture (Murosurf SLPD3) of dehydrogenated soy lecithin, fractionated soy lecithin, palmitic acid (PA), and peptide Hel 13-5D3 (40:40:17.5:2.5, by weight) superior pulmonary surfactant activity than a commercially available pulmonary surfactant (beractant, Surfacten®). Experiments using ovalbumin-sensitized model animals revealed that the lipid-amphiphilic artificial peptide mixtures provided significant control over an increase in the pulmonary resistance induced by premature allergy reaction and reduced the number of acidocytes and neutrophils in lung-irrigated solution. The newly developed low-cost pulmonary surfactant system may be used for treatment of a wide variety of respiratory diseases.

1. Introduction

The pulmonary surfactant is a lipid–protein complex secreted from the alveolar cells and plays an essential role in the pulmonary function by reducing the surface tension [1–3]. The pulmonary surfactant contains phospholipid and neutral fat such as cholesterol and triglycerol. Phospholipids such as dipalmitoylphosphatidylcholine (DPPC) and phosphatidylglycerol (PG) are considered to be essential to the surfactant activity [2]. In addition, surfactant proteins (SPs) SP-A, SP-B, SP-C and SP-D account for approximately 5% of the total pulmonary surfactants [1]. Among these surfactant proteins, SP-B and SP-C are important and induce surfactant activities for pulmonary respiration [4,5]. Since SP-B localized to the surface of the cell membrane and SP-C is membrane resident, their activities in the pulmonary surfactant differ from their action modes. Their roles in the pulmonary surfactant activities remain largely unknown. It is thought that the proteins catalyze the migration of lipids from the monolayer membrane to the bilayer membrane occurring at the air–liquid interface of the lung surface at the time of respiration [4,5]. A recent study found that the interaction between the basic properties of PG and SP is of significance [6].

We have considered that a mixture of an amphiphilic basic peptide having both of a membrane-surface staying type and a membrane spanning type with an appropriate phospholipid can provide pulmonary activities. We have reported the synthesis of a basic peptide, Hel 13-5, that was amphiphilic and highly lipid-soluble [7]. This peptide is configured in such a manner that the lipid-soluble and the water-soluble portions are separated from each other along the helical axis (Fig. 1). Similarly to SP-B, this peptide is considered to occupy the cell surface. Experiments using liposomes showed that lipid-soluble portion of Hel 13-5 was able to penetrate into the acidic and neutral phospholipid membranes [8]. Further, when the lipid was mixed with the peptide at a rate of 5-3 (lipid):1 (peptide), the peptide formed a nanotube together with the lipid [9,10]. Hel 13-5 is similar to SP-C in that the peptide is involved in the interaction with the lipid-soluble portion of the lipid. The surface tension–surface area curve (hysteresis curve) of the mixture of the peptide with the lipid constructed using the Wilhelmy surface

Abbreviations: ARDS, acute respiratory distress syndrome; DPPC, dipalmitoyl-L-α-phosphatidylcholine; egg PC, egg yolk phosphatidylcholine; egg PG, egg yolk phosphatidylglycerol; OD, *n*-1-octadecanol; PA, palmitic acid; RDS, respiratory distress syndrome; SP- B and C, surfactant proteins B and C

* Corresponding author at: Department of Biophysical Chemistry, Faculty of Pharmaceutical Sciences, Nagasaki International University, 2825-7 Huis Ten Bosch, Sasebo, Nagasaki 859-3298, Japan. Tel./fax: +81 956 20 5686.
E-mail address: wosamu@niu.ac.jp (O. Shibata).
URL: http://www.niu.ac.jp/~pharm1/lab/physchem/indexenglish.html (O. Shibata).

http://dx.doi.org/10.1016/j.bbamem.2014.04.024
0005-2736/

Hel13-5 : KLLKLLLKLWLKLLKLLL
Hel13-5D3 : KLLKLLLKLWLKLLKLLL
Hel13-5D5 : KLLKLLLKLWLKLLKLLL
KL_4 : KLLLLKLLLLKLLLLKLLLLK

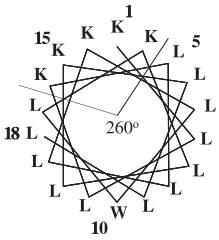

Fig. 1. Primary structure of peptides and α-helical wheel representation of Hel 13-5. Underlines represent D-amino acids.

tensiometer simulating variations in the lung respiration revealed that a DPPC/PG/PA/Hel 13-5 system had a high pulmonary surfactant activity [11]. The experiments on the system further revealed that the peptide and PG are squeezed out in the form of a bimolecular structure (or surface-associated reservoir) from the monomolecular membrane in the course of compression and DPPC is stayed on the monomolecular membrane [11–13]. Recently, PA was found to improve the ordering of DPPC at the surface while stabilizing the surface-associated reservoirs below the surface [14]. Further, it has been shown that these lipid components control the secondary structures of the peptide during the compression and expansion of the surface monolayers, demonstrating the importance of specific interaction between anionic lipids and cationic peptide in the pulmonary surfactant system [15–17]. Notably, a hydrogenated soy lecithin/soy lecithin/PA/Hel 13-5 (40/40/17.5/2.5, by weight) showed surfactant activity comparable to that of beractant (Surfacten®), a commercially available surfactant [18]. This indicated that the soy lipid can be used as an inexpensive substitute for DPPC and PG.

The basic amphiphilic peptide interacts with the biomembrane and exhibits various properties, including antibacterial activity, membrane fusion, and hemolysis [19]. The basic and amphiphilic surfactant protein SP-B not only has the surfactant activity, but also plays a role in controlling the lung inflammation [20] and exhibits antibacterial property [21]. One study found that a derivative of SP-B might be effective against pneumonia. We have synthesized the basic and amphiphilic peptide Hel 13-5 that possesses a membrane fusion activity, channel formation, and hemolytic activity [7]. However, its complex with the lipid loses its activities at the above rate.

It has been reported that the introduction of a D-amino acid into the basic and amphiphilic peptide with the hemolytic and antibacterial activities improves the membrane specificity and selectivity and decreases the hemolytic activity while maintaining its strong antibacterial activity [22,23]. There is also the recent report of an increased selectivity between tumor cells and normal cells [24]. Therefore, we prepared two compounds, {D-L ^{7,14}, K^8} Hel 13-5 (Hel 13-5D3) and {D-L ^{7,11,14,16}, K^8} Hel 13-5 (Hel 13-5D5) (Fig. 1), by replacing a portion of the amino acid sequence of the peptide Hel 13-5 with D-amino acids. The influence of the amino acid replacement on the in vitro and in vivo pulmonary surfactant activities of the hydrogenated soy lecithin/soy lecithin/PA/synthetic peptide (40/40/17.5/2.5, by weight) preparations was assessed. This assessment was made based on the hysteresis curve, the recovery of pulmonary compliance in lung-irrigated rats, and the asthma control effects in ovalbumin-sensitized model animals. The experimental results revealed that the 18-amino acid peptide containing the D-amino acids was more active than the commercially available pulmonary surfactant (Surfacten®). Experiments with ovalbumin-sensitized model animals further demonstrated that an induced elevation of a pulmonary resistance (early phase) can be suppressed.

In this study, we examined the possibility of applying the pulmonary surfactant system broadly to the pulmonary diseases caused by pulmonary surfactant deficiency.

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine (egg PC, purity > 99%) and egg phosphatidylglycerol (egg PG, >99%) were purchased from Avanti Polar Lipids, Inc. and Sigma, respectively. Hydrogenated soy lecithin (SLP White H), fractionated soy lecithin 70 (fractional lecithin SLP-PC70), and hydrogenated soy lecithin 70H (prepared by hydrogenating soy lecithin SLP-PC70) were purchased from True Lecithin Mfg. Co., Ltd. (Mie, Japan). Surfacten® was purchased from Mitsubishi Pharma Corp., (Tokyo, Japan). All other lipids were purchased from Wako Pure Chemical Industries (Osaka, Japan). The lipid–peptide mixtures corresponding to Exosurf® and Surfaxin were prepared in our laboratory to yield a dipalmitoylphosphatidylcholine (DPPC, 99%)/1-hexadecanol (HD, 98%)/tyloxapol (84/16/0.25, by weight) system and a DPPC/egg PG/palmitic acid (PA, >98%)/KL_4 (75/25/10/3, by weight) system, respectively. As model pulmonary surfactant preparations, five mixtures were used; Murosurf L: 1-octadecanol (OD, >98%)/egg PC/PA (35/40/25), Murosurf SL: SLP White H/SLP-PC70/PA (40/40/20, by weight), Murosurf SLP: SLP White H/SLP-PC70/PA/Hel 13-5 (40/40/17.5/2.5), Murosurf SLPD3: SLP White H/SLP-PC70/PA/Hel 13-5D3 (40/40/17.5/2.5), and Murosurf SLPD5: SLP White H/SLP-PC70/PA/Hel 13-5D5 (40/40/17.5/2.5).

2.2. Synthesis of peptides

The peptides were synthesized using an automated synthesizer (PerSeptive Biosystems) by Fmoc solid phase synthesis method of the continuous flow type using Fmoc-Leu-PEG resin (Fmoc-Leu-OH resin, 0.21 mmol/g; Watanabe Kagaku K.K.) as a starting material [7]. The crude peptide synthesized was then dissolved in 30% acetic acid and subjected to chromatography using a Sephadex® G-25 column. The peptide portions eluted were collected and further purified with reverse-phase high-throughput liquid chromatography (HPLC) (COSMOSIL 5C18-AR 20 × 250 mm) using 0.1% TFA/water and acetonitrile as solvents. The purity of the peptide product was confirmed by the presence of a single peak in its HPLC (COSMOSIL 5C18-AR 4.6 × 150 mm) profile and by the TOF-Mass analysis method (Voyager type; PerSeptive Biosystems, Tokyo). Water containing 0.1% TFA and acetonitrile were used as a solvent for elution.

2.3. Circular dichroism (CD) measurement

The circular dichroism (CD) spectrum in the wavelength region of 196–260 nm was recorded at 25 °C on a J-700 spectrometer (J-700; Nihon Bunkou, K.K., Hachiouji, Tokyo) using a quartz cell (with water jacket) of cell length 0.1 cm. The measurements were repeated four times.

A peptide solution was prepared by dissolving approximately 2 mg of the peptide in 2 mL of 20 mM TES Buffer containing 150 mM NaCl. The concentration of the peptide solution was estimated from the absorbance of the solution at 280 nm (molar ellipticity of Trp, 5000 cm/mol). The peptide–lipid solution was prepared as described in the following section. The suspensions were sonicated for 10 min (×3) by using a titanium tip sonicator under ice-cold conditions and nitrogen flowing.

2.4. Preparation of sample (peptide–lipid mixture)

An amount of each of the peptide, lipid, and aliphatic acid was weighed and dissolved in a mixture of chloroform/methanol. To the resulting solution, the peptide was added to the amount to 2.5% or 5% w/w. The peptide–lipid solution was purged with nitrogen (N_2) gas and dried under reduced pressure to yield a dry film coating on the surface of the vessel. A physiological saline was added to the vessel and the

dry film coating was stirred to yield a suspension that was in turn used as a sample.

2.5. Processes for formation experiments of surface tension–area diagram

The surface tension was measured with a Wilhelmy Balance (Acoma Medical Industry Co., Ltd., Japan). A Teflon®-coated water vessel (78 × 138 × 30 mm) was filled with physiological saline to form a closed liquid surface. The air–liquid interface of the liquid surface was developed using 100 μg of the peptide–lipid mixture and was allowed to stand for 3 min to permit the mixture to spread thereon spontaneously. Variation in the surface tension during this period was recorded as the surface spreading rate with a platinum plate hanging vertically in the vessel. The surface area of the monolayer formed in 3 min, which repeatedly spread and compressed alternately at a speed of 3 min per cycle (maximum area of 45 cm^2 to the minimum area of 9 cm^2) was recorded. The surface tension acting onto the platinum plate was converted into electrical signals with a force converter. The electrical signals, together with the variation in the surface area, were automatically recorded continually with an X-Y recorder. The recording was continued until variation could no longer be detected. The curves for all systems during the seventh cycle, which was close to the equilibrium state, are represented in this study.

2.6. Pulmonary surfactant-deficient animal models

A pulmonary surfactant-deficient rat model was generated by irrigating the lung of the Wistar rats with warm physiological saline and by ventilating the animals artificially under 100% oxygen. The model rats were administered with the artificial pulmonary surfactant preparation and its effects on the prolongation of life and pulmonary compliance were examined. Three pulmonary surfactants were used as controls. These are, Surfacten® that is derived from the bovine pulmonary surfactant and being currently under clinical trial, Surfaxin® that is composed of DPPC as a major component and synthetic peptides consisting of lysine (K) and leucine (L) (KL_{24}), and colfosceril palmitate (Exosurf®), which is composed solely of the lipid system and containing no peptide. Physiological saline with no pulmonary surfactant also served as a control. Surfacxin® and Exosurf® were synthesized in our laboratory according to reported methods. The rat lung was irrigated until its pre-irrigation pulmonary compliance of ca. 0.60 mL/cm H_2O dropped to ~0.2 mL/cm H_2O. After the formation of the pulmonary surfactant-deficient rat model was confirmed, the rats were subjected to experiments for the administration of various pulmonary surfactants. A group of six rats or more was used for testing the effects of each pulmonary surfactant. Experimental details have been described elsewhere [18]. The Tukey–Kremer method was used for statistical analysis, $P < 0.05$ was considered statistically significant.

2.7. Experimental conditions and method of asthma models

For generating rat models of asthma the Brown Norway rats were sensitized with egg white albumin (OVA), which elevated the pulmonary resistance upon inhalation. To measure pulmonary resistance (RL), the rats were anesthetized by intraperitoneal administration of urethane (1 g/kg, 25% wt/vol). The RL was monitored using a PULMOS-II system (M.I.P.S., Osaka, Japan) [25]. Sensitized rats were exposed three times to OVA according to the methods of Abe et al. [26]. Animals were pre-treated by administering 0.1 mL (20 mg/mL) of each of the Murosurf SL, Murosurf SLP, Murosurf SLPD3, and Surfacten® compositions into the respiratory tract. After 10 min, the animals were sensitized with inhaled OVA. The pulmonary resistance was measured periodically according to the method of Abe et al. [26]. The effects on asthma were assessed by examining the ability of the surfactants to suppress the enhancement in the pulmonary resistance.

After the final measurement of the pulmonary resistance, bronchoalveolar lavage fluid was collected via the tracheal cannula using 2 × 10 mL of saline containing 1 mM EDTA. The fluid collected was centrifuged at 300 ×g for 5 min at 4 °C and the cell pellet was resuspended in 1.0 mL sterile saline containing 0.2% rat serum. The total cell count was determined by adding an equal volume of trypan blue stain and then counting the cells under a light microscope. The differential cell count was carried out using a smear preparation stained with Diff-Quik (International Reagents Corp., Kobe, Japan). Two hundred cells were counted at random under ×200 magnification. The cells were identified by their morphologies.

The Tukey–Kremer method was used for statistical analysis. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Designing highly specific peptides related to Hel 13-5 containing D-amino acids

As described earlier [7], we prepared the amphiphilic peptide Hel 13-5 with the ideally separated hydrophobic and hydrophilic parts, when it was drown in a helical wheel diagram (Fig. 1). In the presence of a lipid membrane, the lipid-soluble portion of Hel 13-5 penetrates deeply into the bimolecular membrane [8]. This provides a high pulmonary surfactant activity that is comparable to the artificial pulmonary surfactant derived from the pig lung, but Hel 13-5 shows a strong hemolytic activity upon single application in the absence of lipids [18]. Previous studies have shown that the introduction of D-amino acids into the amphiphilic peptide forces a conformational change and causes a decrease in hydrophobicity, leading to reduced hemolytic activity [22,23]. Therefore, we introduced D-amino acids into Hel 13-5 to suppress its hemolytic activity. The D-amino acids were introduced at optional, but well-balanced positions, and the D-amino acid content of the peptide was set at 20% to 30% (Fig. 1).

3.2. Conformation and lipid-solubility of peptides

To understand the changes in the structure and properties of the peptide brought about by the introduction of the D-amino acids, we analyzed their CD spectra and reverse-phase HPLC profiles (Fig. 2). The CD spectra revealed that the peptide Hel 13-5 assumed an α-helical structure in aqueous solution, whereas the peptides Hel 13-5D3 and Hel 13-5D5 each assumed a random structure (Fig. 2A). In the presence of neutral and acidic liposomes, Hel 13-5D3 showed a β-structure-like CD spectrum containing partially α-helical structures, while Hel 13-5D5 did not show any specific structure. Measurements in the presence of soy lecithin indicated that Hel 13-5D3 was in a helix-like shape with a considerably weak molar ellipticity, whereas Hel 13-5D5 showed weak molar ellipticity in all wavelengths measured (Fig. 2). As opposed to Hel 13-5, Hel 13-5D3 and Hel 13-5D5 failed to assume an amphiphilic α-helical structure in buffer solution, likely because of the presence of D-amino acid moieties in their structures. However, in the presence of lipids, Hel 13-5D3 shows secondary structures such as α-helical and β-structure. In contrast, Hel 13-5D5 did not assume any specific structure in the media examined. Thus, it was found that the secondary structure is controlled both by the environmental lipids and by the D-amino acid content of the peptide sequences. The interaction of amphiphilic α-helical peptides with the biomembrane constituents has been previously evaluated for the hydrophobicity related to the composition of the α-helical structure [7,8].

The reverse phase (C18) HPLC analysis was carried out to assess the hydrophobicity of the peptide. In general, retention time increases with hydrophobicity [27]. The retention time decreased with increasing D-amino acid content, indicating that the synthetic peptide–lipid monolayer interaction became weaker with the introduction of D-amino acids, which altered the secondary structure of the peptide (Fig. 2B).

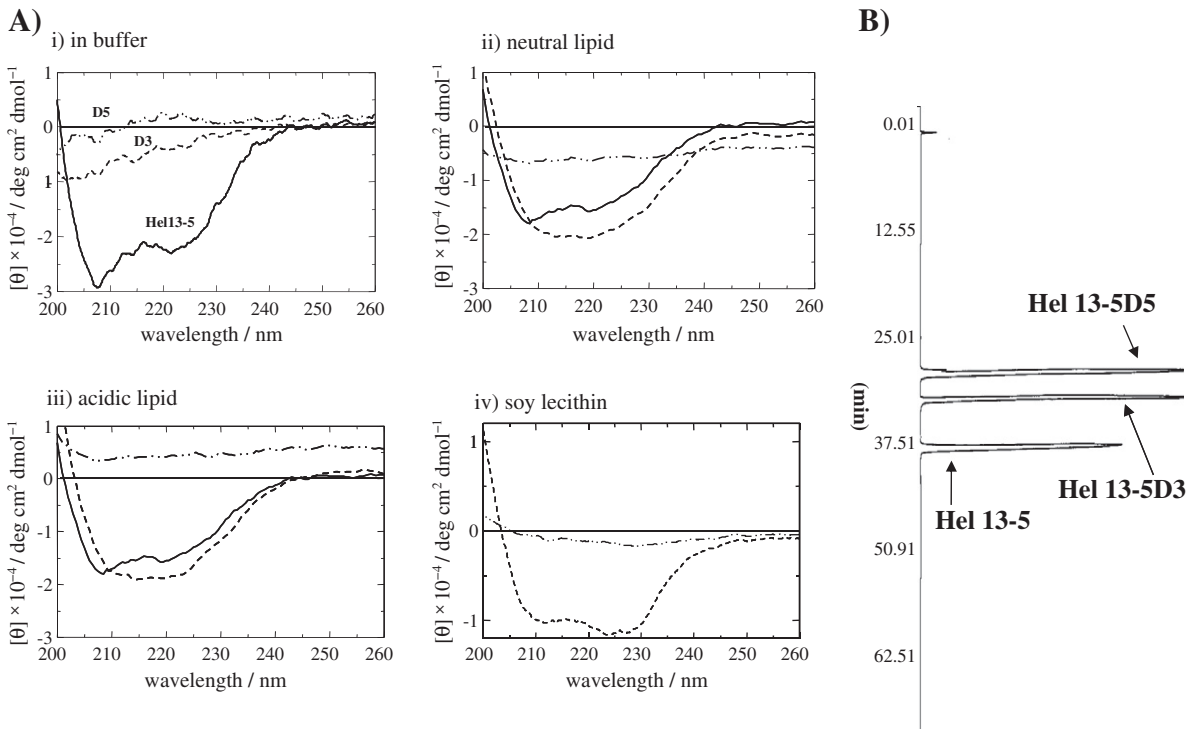

Fig. 2. CD (A) and HPLC (B) data of Hel 13-5 peptide containing the D-amino acid. (A) Hel 13-5 (solid line), Hel 13-5D3 (broken line), and Hel 13-5D5 (two-dot chain line). i) in buffer, ii) in the presence of neutral lipids (egg PC), and iii) in the presence of acidic lipids (egg PC/egg PG = 3/1), and hydrogenated soy lecithin/fractionated soy lecithin PC70/PA/peptides (9/9/1/1, by weight) in iv). Peptide and lipid concentrations were 10 μM and 100 μM, respectively.

3.3. Assessment of in vitro pulmonary surfactant activity

A stable mutual transfer from the monolayer membrane to the bilayer membrane is proposed as the basis of the expression of the pulmonary surfactant activity [28]. Phospholipids are essential for this transfer. The pulmonary surfactant is composed of the protein and the lipid. The protein is considered to smoothly catalyze the transfer between the monolayer membrane and the phospholipid bilayer on the air–liquid interfaces of the alveoli. This transfer can be readily observed by constructing the surface tension–surface area curve (hysteresis curve) with the Wilhelmy surface tensiometer representing a variation in the respiratory pressure of the lung [29]. In general, a faster drop in the surface tension or a faster expansion of the surface at the time of compression or indicates better activity in vivo. In other words, the greater the area to be formed by the hysteresis curve or the smaller the surface tension at the time of compression, the higher the activity.

The natural pulmonary surfactant contains a large amount of DPPC and PG. We have previously used soy lecithin as substitutes for such expensive lipids [18]. In this study, we have used lipid mixture systems. Fig. 3 shows a hysteresis diagram of the D-amino acid-containing peptide–lipid mixture systems. In the system containing soy-been lipids, Hel 13-5D3 hysteresis curves, compared with those of Surfacten® and the minimum surface tension (13 mN m^{-1}), did not amount to that of Surfacten®. Hel 13-5D5 showed a more deformed curve. These indicate that Hel 13-5D3 may have more favorable surfactant activity than Hel 13-5D5.

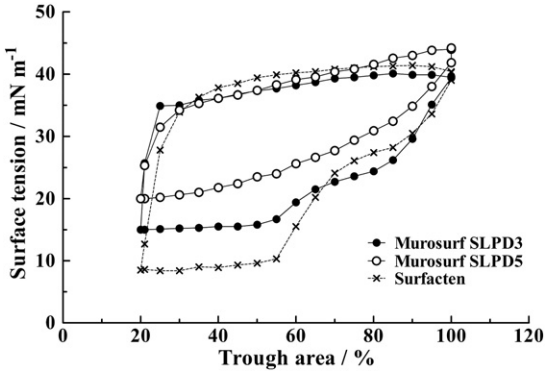

Fig. 3. The surface tension and the surface area curves in a soy lecithin lipid and peptide mixture system.

3.4. Assessment of pulmonary surfactant activity using lung-irrigated rat models

Assessments for the pulmonary surfactant activity of the peptide–lipid systems were made on the basis of the pulmonary compliance using lung-irrigated rabbit as an RDS model [30]. In this study, the rat models were generated by irrigating the lungs of Wistar rats with physiological saline. Pulmonary surfactant was administered to measure the pulmonary function (compliance). The results are shown in Fig. 4. Pulmonary surfactants are considered better when the values of the compliance rose faster and higher immediately after the administration of the pulmonary surfactant. Surfacten® raised its compliance steadily immediately after administration and reached a constant compliance in approximately 2 h.

The mixture system composed of Hel 13-5D3 and the lipid Murosurf SLPD3 showed similar trends as that of Surfacten®, but reached rather higher values at 0.5–1 h. This suggested that the recovery of the pulmonary functions was better than that with Surfacten®. The mixture system composed of Hel 13-5D5 and the lipid Murosurf SLPD5 produced a weaker recovery of the pulmonary functions.

The peptide Hel 13-5D3 we have developed performed better than the mixture system composed of the peptide Hel 13-5 containing no D-amino acids and the less expensive lipid Murosurf SLP. An artificial pulmonary surfactant (Surfaxin) developed as a peptide-lipid mixture system contains KL_4 as a peptide. However, its surfactant

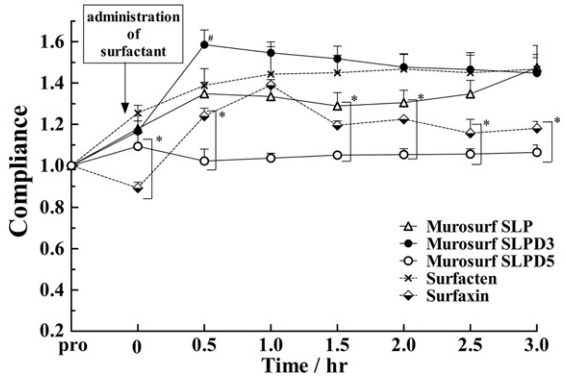

Fig. 4. Recovery of the pulmonary function by various pulmonary surfactants. Serial measurements of recovery ratio of compliance (Crs) of respiratory system are shown. Recovery ratio of Crs was calculated using the formula: (recovery ratio of Crs) = (Crs after surfactant treatment) / (Crs after lung lavage but before surfactant treatment). The data represents the mean ± S.D. $^{*}P < 0.05$ vs. surfactant treatment group.

activity was lower than that of the mixture system containing the peptide Hel 13-5D3.

3.5. Results of experiments with asthma models

It has been reported that the exogenous surfactant administration significantly improved lung function in the patients during an asthmatic attack [31]. Inhaled synthetic surfactant (Pumactant, Britannia Pharmaceuticals Ltd. Redhill, UK) abolished the early allergen-induced response in asthma [32]. Thus, we examined whether D-amino acid-containing peptide-lipid mixture abolished the symptoms in rats sensitized with egg white albumin (OVA). When compared with controls, Murosurf SL, Murosurf SLP, Murosurf SLPD3, and Surfacten® significantly suppressed the enhancement of resistance to the respiratory tract induced by OVA in 45 min after the administration of OVA (Fig. 5).

After the experiments were terminated, the lung-irrigated solution was subjected to cytodiagnosis of leukocytes and the cell numbers were estimated (Fig. 6A). When compared to that in non-sensitized rats, the number of leukocytes increased in rats sensitized with OVA. Notably, in rats administered with Murosurf SLPD3 before the administration of OVA, the number of leukocytes was lower. Murosurf SL also decreased the number of leukocytes, while Murosurf L had no effects on the number of leukocytes (data not shown).

Cellular differentiation in the lung-irrigated solution was examined (Fig. 6B). When compared to non-sensitized rats, the rats administered with each of the surfactants, Murosurf SL, Murosurf SLP, Murosurf SLPD3, and Surfacten®, caused an increase in the number of acidocytes and neutrophils, and a decreased in the number of macrophages. Administration of Murosurf SLPD3 before the sensitizing with OVA decreased the number of acidocytes while increasing the number of macrophages. Murosurf SL also decreased the number of acidocytes and increased the number of macrophages. Murosurf L had no effects

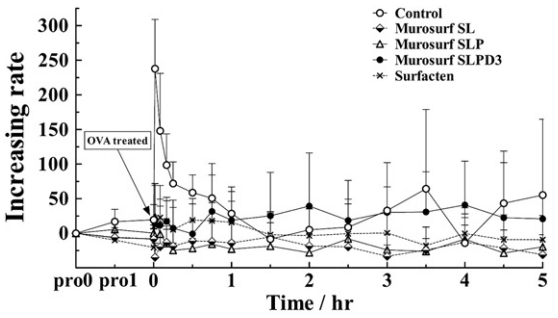

Fig. 5. The time courses of pulmonary resistance (RL) changes on inhaled D-amino acid-containing peptide–lipid mixture with asthma model rats.

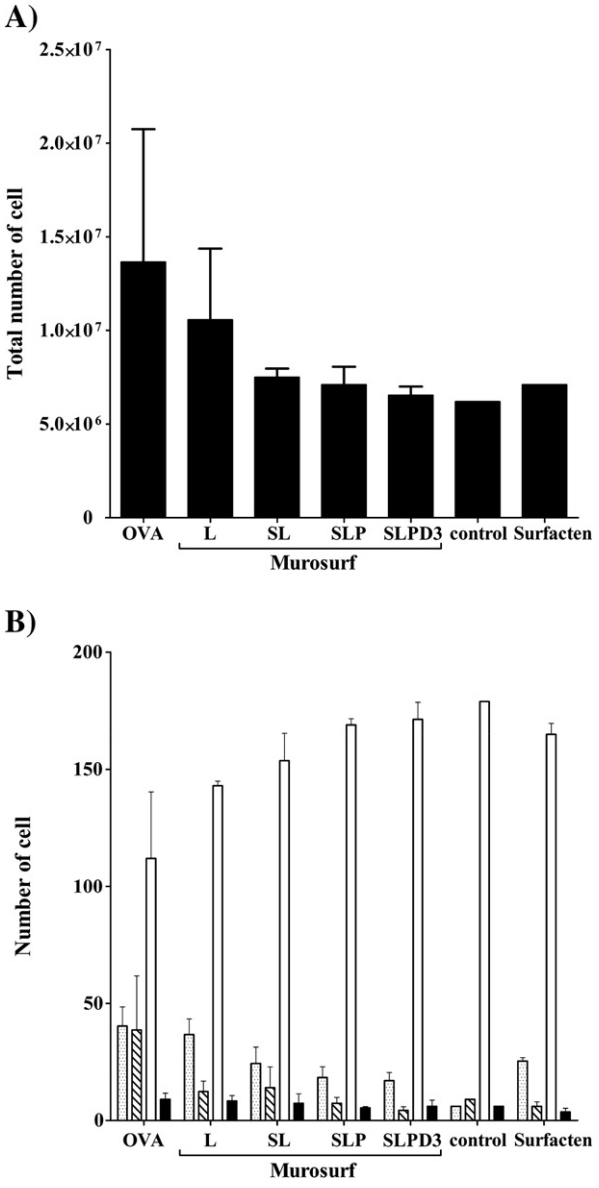

Fig. 6. A cytological analysis of inflammatory cells in bronchoalveolar fluid on inhaled D-amino acid-containing peptide/lipid mixture. (A) The total cell number of leukocyte after double OVA challenges. (B) The number of differential cells in leukocyte. Bar graves are marked as follows: eosinophil (dots), neutrophil (slanting lines), macrophage (white), and lymphocyte (black).

on the number of the acidocytes, indicating that peptides may be important for decreasing the number of acidocytes and increasing macrophage counts.

3.6. Hemolytic activity

The hemolytic activity of Hel 13-5 and Hel 13-5D3 increased in a concentration-dependent fashion and the hemolytic level reached nearby 100% at approximately 20-μM concentration of the peptide. The peptide Hel 13-5D5 increased the hemolytic activity to its maximum level of 35% at 30–50 μM concentrations and in a concentration-dependent fashion within the range of 50–70 μM (figure not shown).

4. Discussion

The amphiphilic structure with the ideally separated hydrophobic and hydrophilic parts has the specific hydrophobic–hydrophilic balance

(HHB) depending on amino acid species. The HHB determines the three-dimensional structure, stability, and physiological functions of proteins and peptides [7,33]. It is shown that the structure of the water-soluble residues scatter (e.g. KL_4) is more important in the high spreading effect than the ideal amphipathic structure of Hel 13-5, in which the helical structure is built up.

Introduction of D-amino acids into a portion of these amphiphilic peptides caused changes in the secondary structure of the peptide, leading to significant changes in their physiological activity. Shai et al. reported that introduction of D-amino acids into an amphiphilic peptide with antibacterial and hemolytic activities caused no change in the antibacterial activity while losing only the hemolytic activity [22]. Further, these peptides were less toxic to normal cells and selectively acted on the tumor cells alone, leading to the possibility of developing new anti-tumor chemotherapeutic agents [24]. This was likely due to the differences in the mechanism of action the peptides toward cell membranes.

In this study, we synthesized the D-amino acid-containing peptides with the goal of achieving membrane specificity. The D-amino acids influenced the structure and lipid solubility. These influences were considered to have originated from the three-dimensional restrictions on the freedom of movement of amino acid residues in the peptide structure. The results of the CD spectral measurements led to the assumption that the original α-helical structure in a buffer solution would be broken into a random-like structure resulting in a partial breakdown of the amphiphilic configuration and causing a decrease of lipid solubility. In the presence of the lipid, the peptide is considered to assume a new structure. Although the peptide produced a curve indicative of β-structure, the structure is yet to be determined. A double minimum indicative of a helix shape was shown in the presence of the soy lipid. However, the intensity was lower than that expected for a usual helical structure, suggesting that structure changed considerably in the presence of the lipid.

Among the compounds synthesized in this study, Hel 13-5D3 and Hel 13-5D5 showed similar hysteresis curves in a mixture with the soy lipid to Surfacten (Fig. 3). In particular, the surface activity of the Hel 13-5D3 preparation was better in that the curve for Murosurf LPD3 exhibited lower (minimum) surface tension during the compression cycle than that for Murosurf LPD5. The Hel 13-5D3 preparation also demonstrated a high pulmonary surfactant activity as indicated by the results of the pulmonary compliance experiments using the lung-irrigated rats. The pulmonary surfactant activity of the above peptide was superior to that of Hel 13-5 and surpassed that of Surfacten®.

It has been reported that the pulmonary surfactant activity of a 21-amino acid peptide showing an amphiphilic property along an α-helical axis was lower than that of the non-amphipathic KL_4 [34]. Therefore, it can be considered that the reduced lipid solubility caused by the structural changes that occurred following the introduction of the D-amino acids into the peptide backbone would contribute to the enhanced surfactant activity of Hel 13-5D3. Results of our fluorescence experiments suggested that Hel 13-5D3 inserts itself deep into the lipid membrane (data not shown).

In this study, we used the inexpensive soy lecithin for preparing the lipid/Hel 13-5D3system as a lipid substitute for expensive DPPC and PG. Soy lecithin is used for treating hyperlipidemia and is not toxic to the tissues. Artificial animal lung-derived surfactants show antigenicity and have potential risk of infection such as BSE. In contrast, the artificial pulmonary surfactant we have developed can be safely used as an agent for treating respiratory distress syndromes (RDS) of newborn babies. Therefore, the newly synthesized peptides are candidate compounds for use as artificial pulmonary surfactants.

As the D-amino acid-containing peptides are synthesized by solid phase automated synthesis method, large-scale production is difficult and costs of production are somewhat high. A large-scale production of the peptides is also feasible by liquid phase peptide synthesis methods. We have developed methods for the liquid phase synthesis of Hel13-5D3.

If the cost of pulmonary surfactants is low, they can be used to treat adults with acute respiratory distress syndrome (ARDS) as well as to newborn babies with RDS [35]. Studies have shown that the pulmonary surfactant can be used to temporarily treat ARDS with no effect on the prolongation of life [34]. The reports of phase II experiments on some artificial pulmonary surfactants showed that their application in treating ARDS could provide temporary improvements, yet no improvement in the progress [36]. It has been reported that the enhancement of surfactant functions using the surfactant protein SP-B in endotoxin lung injury animal models could protect the lung from injuries and that the surfactant with KL_4 mimic of SP-B could prevent hyperoxide and LPS-induced lung injury [37]. These problems are debatable. Furthermore, it was temporarily recognized that ARDS accompanied injury of functions of the pulmonary surfactant and the pulmonary surfactant supplement therapy had the effect on recovery of the respiratory functions. The strategy of treating the cause of ARDS is considered to lead to the prolongation of life by the use of pulmonary surfactant to improve the respiratory functions and consequently protect the lung from barotrauma accompanied by a respirator and from oxygen toxicity. Considering such observations, the pulmonary surfactant can be applied easily to reduce an occurrence of respiratory distress only if it would be inexpensive in terms of production and expenses for administration.

Results of our experiments using asthma models suggested that the Hel 13-5D3/soy lipid mixture system could be used for treating asthma. Reduced number of acidocytes and neutrophils in the lung-irrigated solution of model animals treated with the Hel 13-5D3/soy lipid mixture suggested that the system could be used for controlling the allergic inflammation. Similar effects were also obtained with SP-B and KL_4/surfactant mixture [20]. It was reported that the administration of the pulmonary surfactant was effective in allergy-sensitized model animals and that it provided relief from introsusception attack of advanced bronchial asthma.

In this study, we showed that the new pulmonary surfactant systems we developed are inexpensive and nontoxic, and they can be used to treat asthma in addition to ARDS. Furthermore, the new pulmonary surfactants posed no risk of antigenicity or infection, as opposed to the pulmonary surfactants derived from animals. Our pulmonary surfactants are entirely synthetic, which is why that they can be prepared with high integrity on a large scale. Our development can be used to greatly improve present medicinal technology, along with RDS and ARDS treatment strategies.

Acknowledgment

We thank Prof. Masayoshi Abe for the asthma model experiment and its helpful discussion. This work was supported by the grant of Research and Development, Fukuoka Industry Technology Foundation (2004~2005).

References

[1] J. Goerke, Pulmonary surfactant: functions and molecular composition, Biochim. Biophys. Acta 1408 (1998) 79–89.
[2] R. Veldhuizen, K. Nag, S. Orgeig, F. Possmayer, The role of lipids in pulmonary surfactant, Biochim. Biophys. Acta 1408 (1998) 90–108.
[3] J.A. Zasadzinski, J. Ding, H.E. Warriner, F. Bringezu, A.J. Waring, The physics and physiology of lung surfactants, Curr. Opin. Colloid Interface Sci. 6 (2001) 506–513.
[4] S. Hawgood, M. Derrick, F. Poulain, Structure and properties of surfactant protein B, Biochim. Biophys. Acta 1408 (1998) 150–160.
[5] J. Johansson, Structure and properties of surfactant protein C, Biochim. Biophys. Acta 1408 (1998) 161–172.
[6] C.G. Cochrane, Pulmonary surfactant in allergic inflammation: new insights into the molecular mechanisms of surfactant function, Am. J. Physiol. Lung Cell. Mol. Physiol. 288 (2005) L608–L609.
[7] T. Kiyota, S. Lee, G. Sugihara, Design and synthesis of amphiphilic α-helical model peptides with systematically varied hydrophobic–hydrophilic balance and their interaction with lipid- and bio-membranes, Biochemistry 35 (1996) 13196–13204.

[8] A. Kitamura, T. Kiyota, M. Tomohiro, A. Umeda, S. Lee, T. Inoue, G. Sugihara, Morphological behavior of acidic and neutral liposomes induced by basic amphiphilic α-helical peptides with systematically varied hydrophobic–hydrophilic balance, Biophys. J. 76 (1999) 1457–1468.
[9] S. Lee, T. Furuya, T. Kiyota, N. Takami, K. Murata, Y. Niidome, D.E. Bredesen, H.M. Ellerby, G. Sugihara, De novo-designed peptide transforms Golgi-specific lipids into Golgi-like nanotubules, J. Biol. Chem. 276 (2001) 41224–41228.
[10] T. Furuya, T. Kiyota, S. Lee, T. Inoue, G. Sugihara, A. Logvinova, P. Goldsmith, H.M. Ellerby, Nanotubules formed by highly hydrophobic amphiphilic α-helical peptides and natural phospholipids, Biophys. J. 84 (2003) 1950–1959.
[11] H. Nakahara, S. Lee, G. Sugihara, C.-H. Chang, O. Shibata, Langmuir monolayer of artificial pulmonary surfactant mixtures with an amphiphilic peptide at the air/water interface: comparison of new preparations with Surfacten (Surfactant TA), Langmuir 24 (2008) 3370–3379.
[12] H. Nakahara, S. Lee, G. Sugihara, O. Shibata, Mode of interaction of hydrophobic amphiphilic α-helical peptide/dipalmitoylphosphatidylcholine with phosphatidylglycerol or palmitic acid at the air–water interface, Langmuir 22 (2006) 5792–5803.
[13] H. Nakahara, S. Lee, O. Shibata, Pulmonary surfactant model systems catch the specific interaction of an amphiphilic peptide with anionic phospholipid, Biophys. J. 96 (2009) 1415–1429.
[14] H. Nakahara, S. Lee, Y. Shoyama, O. Shibata, The role of palmitic acid in pulmonary surfactant systems by Langmuir monolayer study: lipid–peptide interactions, Soft Matter 7 (2012) 11351–11359.
[15] H. Nakahara, A. Dudek, Y. Nakamura, S. Lee, C.H. Chang, O. Shibata, Hysteresis behavior of amphiphilic model peptide in lung lipid monolayers at the air–water interface by an IRRAS measurement, Colloids Surf. B 68 (2009) 61–67.
[16] H. Nakahara, S. Lee, O. Shibata, Specific interaction restrains structural transitions of an amphiphilic peptide in pulmonary surfactant model systems: an in situ PM-IRRAS investigation, Biochim. Biophys. Acta 1798 (2010) 1263–1271.
[17] H. Nakahara, S. Lee, O. Shibata, Surface pressure induced structural transitions of an amphiphilic peptide in pulmonary surfactant systems by an in situ PM-IRRAS study, Biochim. Biophys. Acta 1828 (2013) 1205–1213.
[18] K. Yukitake, Y. Nakamura, M. Kawahara, H. Nakahara, O. Shibata, S. Lee, Development of low cost pulmonary surfactants composed of a mixture of lipids or lipids–peptides using higher aliphatic alcohol or soy lecithin, Colloids Surf. B 66 (2008) 281–286.
[19] G. Saberwal, R. Nagaraj, Cell-lytic and antibacterial peptides that act by perturbing the barrier function of membranes: facets of their conformational features, structure–function correlations and membrane-perturbing abilities, Biochim. Biophys. Acta 1197 (1994) 109–131.
[20] R. Epaud, M. Ikegami, J.A. Whitsett, A.H. Jobe, T.E. Weaver, H.T. Akinbi, Surfactant protein B inhibits endotoxin-induced lung inflammation, Am. J. Respir. Cell Mol. Biol. 28 (2003) 373–378.
[21] M.A. Ryan, H.T. Akinbi, A.G. Serrano, J. Pérez-Gil, H. Wu, F.X. McCormack, T.E. Weaver, Antimicrobial activity of native and synthetic surfactant protein B peptides, J. Immunol. 176 (2006) 416–425.
[22] Z. Oren, J. Hong, Y. Shai, A repertoire of novel antibacterial diastereomeric peptides with selective cytolytic activity, J. Biol. Chem. 272 (1997) 14643–14649.
[23] T. Kiyota, R. Yanagida, M. Oka, M. Miyoshi, S. Lee, G. Sugihara, The effect of D-amino acid-containing basic peptides with different hydrophobicity on the antimicrobial and cytotoxic activity, Bull. Chem. Soc. Jpn. 73 (2000) 2363–2370.
[24] N. Papo, M. Shahar, L. Eisenbach, Y. Shai, A novel lytic peptide composed of DL-amino acids selectively kills cancer cells in culture and in mice, J. Biol. Chem. 278 (2003) 21018–21023.
[25] M. Abe, K. Shibata, H. Akatsu, N. Shimizu, N. Sakata, T. Katsuragi, H. Okada, Contribution of anaphylatoxin C5a to late airway responses after repeated exposure of antigen to allergic rats, J. Immunol. 167 (2001) 4651–4660.
[26] M. Abe, Y. Hayashi, A. Murai, K. Shibata, N. Sakata, R. Igarashi, T. Katsuragi, K. Tanaka, Effects of inducible nitric oxide synthase inhibitors on asthma depending on administration schedule, Free Radic. Biol. Med. 40 (2006) 1083–1095.
[27] R.A. Houghten, S.T. DeGraw, Effect of positional environmental domains on the variation of high-performance liquid chromatographic peptide retention coefficients, J. Chromatogr. A 386 (1987) 223–228.
[28] J. Pérez-Gil, K.M.W. Keough, Interfacial properties of surfactant proteins, Biochim. Biophys. Acta 1408 (1998) 203–217.
[29] R.H. Notter, J.N. Finkelstein, Pulmonary surfactant: an interdisciplinary approach, J. Appl. Physiol. 57 (1984) 1613–1624.
[30] T. Kobayashi, H. Kataoka, T. Ueda, S. Murakami, Y. Takada, M. Kokubo, Effects of surfactant supplement and end-expiratory pressure in lung-lavaged rabbits, J. Appl. Physiol. 57 (1984) 995–1001.
[31] K. Kurashima, H. Ogawa, T. Ohka, M. Fujimura, T. Matsuda, T. Kobayashi, A pilot study of surfactant inhalation in the treatment of asthmatic attack, Arerugi 40 (1991) 160–163.
[32] K.S. Babu, D.A. Woodcock, S.E. Smith, J.N. Staniforth, S.T. Holgate, J.H. Conway, Inhaled synthetic surfactant abolishes the early allergen-induced response in asthma, Eur. Respir. J. 21 (2003) 1046–1049.
[33] A. Zemel, A. Ben-Shaul, S. May, Perturbation of a lipid membrane by amphipathic peptides and its role in pore formation, Eur. Biophys. J. 34 (2005) 230–242.
[34] G. Nilsson, M. Gustafsson, G. Vandenbussche, E. Veldhuizen, W.J. Griffiths, J. Sjövall, H.P. Haagsman, J.-M. Ruysschaert, B. Robertson, T. Curstedt, J. Johansson, Synthetic peptide-containing surfactants, Eur. J. Biochem. 255 (1998) 116–124.
[35] K. Raghavendran, D. Willson, R.H. Notter, Surfactant therapy for acute lung injury and acute respiratory distress syndrome, Crit. Care Clin. 27 (2011) 525–559.
[36] T.J. Gregory, W.J. Longmore, M.A. Moxley, J.A. Whitsett, C.R. Reed, A.A. Fowler III, L.D. Hudson, R.J. Maunder, C. Crim, T.M. Hyers, Surfactant chemical composition and biophysical activity in acute respiratory distress syndrome, J. Clin. Invest. 88 (1991) 1976–1981.
[37] P. Kinniry, J. Pick, S. Stephens, D. Jain, C.C. Solomides, R. Niven, R. Segal, M. Christofidou-Solomidou, KL_4-surfactant prevents hyperoxic and LPS-induced lung injury in mice, Pediatr. Pulmonol. 41 (2006) 916–928.