In Vitro Inhibition of Biophysical Surface Properties and Change in Ultrastructures of Exogenous Pulmonary Surfactant by Albumin or Fibrinogen

In order to observe the effects of serum albumin and fibrinogen on biophysical surface properties and the morphology of pulmonary surfactant in vitro, we measured the surface adsorption rate, dynamic minimum and maximum surface tension (min-, max-ST) by Pulsating Bubble Surfactometer, and demonstrated ultrastructures on a series of mixtures with varying concentrations of albumin or fibrinogen and Surfactant-TA. The albumin and fibrinogen significantly inhibited the adsorption rate and ST-lowering properties of surfactant through increasing STs of adsorption rate, min-ST, and max-ST. The characteristic morphology of the Surfactant-TA changed from lamellar rod-like structure with open ends into spherical structures with loss of their open ends by mixing with albumin or fibrinogen. These inhibitory effects of albumin and fibrinogen on surface properties of surfactant were dependent upon the increasing concentration of albumin or fibrinogen. We concluded that albumin and fibrinogen significantly altered surfactant function and its ultrastructural morphology in vitro. These findings support the concept that albumin and fibrinogen-induced surfactant dysfunction may play an important role in the pathophysiology of adult respiratory distress syndrome, and this adverse effect of albumin and fibrinogen on surfactant might be overcome by administration of large doses of exogenous surfactant.

Key Words : *Respiratory distress syndrome; Respiratory distress syndrome adult; Pulmonary surfactants; Surface tension; Albumins; Fibrinogen*

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INTRODUCTION

Pulmonary surfactant is essential to normal lung function. Its primary function is to lower surface tension (ST) at the air-water interface of the lung alveoli, which prevents the lung from collapsing at the end of expiration, thereby stabilizing lung volume. Pulmonary surfactant is also related to the pathophysiology of neonatal respiratory distress syndrome (RDS) and other types of respiratory failures (1).

Adult respiratory distress syndrome (ARDS) is a syndrome of acute parenchymal lung dysfunction characterized by the following clinical presentation: a) a major antecedent precipitating event, b) the sudden onset of tachypnea and hypoxemia refractory to high concentrations of inspired oxygen, c) the presence of diffuse patchy infiltrates on chest radiograph, d) a decrease in static lung compliance, and e) the exclusion of cardio-

genic pulmonary edema or chronic pulmonary diseases as the sole causes of pulmonary dysfunction (2).

The pathophysiology of ARDS is complex and related to several factors such as the formation of pulmonary edema, the roles of chemical mediators, alterations in surfactant activity, mechanics related to lung volumes, and cardiovascular alterations (3). Among these factors, alterations in alveolar surfactant function may well contribute to a variety of pathophysiological key events encountered in ARDS (4). In recent studies, ARDS has been found to lead to injury of the alveolar-capillary membrane, which ultimately results in pulmonary edema. Leakage of blood components due to alteration of permeability and pulmonary hemorrhage disturb the pulmonary surfactant system. Secondary functional deficiency of pulmonary surfactant due to blood component-induced surfactant dysfunction may thus play a role in the pathophysiology of ARDS.

The relationship between blood component-induced surfactant dysfunction and its morphological changes has not yet been fully explained, however. This study was carried out a) to observe the changes of the surface biophysical properties such as surface adsorption rate, dynamic minimum and maximum ST (min-ST, max-ST), and of electron microscopic ultrastructure of surfactant by the major components of blood, albumin and fibrinogen. And also b) to observe dose-related effects in their action on the surfactant in vitro.

MATERIALS AND METHODS

Preparation of surfactant, surfactant-albumin, and surfactant-fibrinogen mixtures

The surfactant used in this study was Surfactant-TA (Surfacten™, Tokyo Tanabe Co, Japan), a reconstituted bovine surfactant containing 100 mg phospholipid (PL) per vial (5). Surfactant preparation was done by adding 4 ml of saline for 1 vial of Surfactant-TA making 25 mg/ml concentration, and then serially diluted with saline to make surfactant suspension at a concentration of 6.25 mg/ml and 1.56 mg/ml.

Bovine serum albumin (Sigma, USA), and human fibrinogen (Baxter Diagnosis Inc, USA) were used and each was dispersed in saline to make of 16 mg/ml concentration solution, and then serially diluted with saline.

Surfactant-albumin, and surfactant-fibrinogen mixtures was made by mixing equal volume of surfactant suspension (6.25 or 1.56 mg PL/ml) with albumin or fibrinogen solution serially, resulting in the following concentrations: albumin 8.0, 4.0, 2.0, 1.0 mg/ml; fibrinogen 8.0, 4.0, 2.0, 1.0, 0.5, 0.25 mg/ml.

Surface adsorption rate and dynamic surface tension measurement

Surface properties were tested by measuring the surface adsorption rate, dynamic min-ST, and max-ST with Pulsating Bubble Surfactometer™ (6) (Electronetic Corporation, Tonawanda, NY) of surfactant at PL concentration of 1.56 and of 6.25 mg/ml and surfactant-albumin or surfactant-fibrinogen mixtures. A bubble was created in 25 μ l of sample, which was then pulsated at a rate of 20 cycles per minute at 37°C, treatment that compressed the surface area of the bubble by 50%. First, adsorption rate of the bubble was measured, and then min-ST and max-ST were calculated at minimum and maximum bubble sizes after pulsation at the fifth cycle and at 1, 3, and 5 min, according to the formula of Laplace (6). Each sample was studied five times in a blind

fashion.

Electron microscopic examination

Ultrastructures were studied using Notter's method (7), with some modification. Surfactant concentration was 1.56 mg/ml; surfactant-albumin and surfactant-fibrinogen mixtures were at a concentration of 1.56 mg surfactant/ml and 4 mg albumin or fibrinogen/ml. To each of 1.5 ml volume of dispersed surfactant alone or surfactant-albumin or surfactant-fibrinogen mixture, an equal volume of 2% tannic acid in 0.1 M sodium phosphate buffer, pH 7.2, was added. After 30 minutes fixation, the mixtures were washed five times in buffer and pelleted at 3,000 rpm for 10 minutes after each wash to remove excess tannic acid, the supernatant being discarded. Each pellet was then fixed for 1 hr in 1% OsO₄ in 0.1 M sodium phosphate buffer, pH 7.2, and dehydrated in increasing concentrations of ethanol. Embedding was in polybed 812, nadic methyl anhydride, dodecenyl succinic anhydride and DMP-30. Thin sections were stained with uranyl acetate and lead citrate for observation and photography.

Statistical analysis

The STs of adsorption rate, min-ST and max-ST were measured at each concentration after the addition of albumin or fibrinogen and multiple comparisons were made with surfactant alone at a concentration of 1.56 and 6.25 mg/ml of surfactant, employing the Dunnett method for analysis of variance (ANOVA) and using Statview II Software (Abacus Concepts, Berkeley, CA, USA). Statistical significance was assigned to p values < 0.01.

RESULTS

Surface adsorption rate

The STs of surface adsorption rates of surfactant-alone were 27.4 ± 0.8 and 27.2 ± 0.5 mN/m at a concentration of 1.56 mg/ml and 6.25 mg/ml.

In surfactant-albumin mixtures, the STs of adsorption rate were significantly higher than those of surfactant-alone (32.8 ± 1.1 and 34.0 ± 1.4 vs 27.4 ± 0.8 mN/m) at an albumin concentration of 4.0 and 8.0 mg/ml in surfactant suspension of 1.56 mg/ml ($p < 0.01$) and increases of STs of adsorption rates by albumin were dependent to the albumin concentration. But these were no significant increases in the STs of adsorption rate at varying concentrations of albumin in mixtures with surfactant

Table 1. Adsorption rates of surfactant alone, surfactant-albumin, and surfactant-fibrinogen mixtures

Surfactant (mg/ml)	Albumin (mg/ml)	Adsorption Rate (ST: mN/m)	Surfactant (mg/ml)	Fibrinogen (mg/ml)	Adsorption Rate (ST: mN/m)
1.56	0	27.4 ± 0.8	1.56	0	27.2 ± 0.8
			1.56	0.25	28.8 ± 1.1
			1.56	0.5	30.0 ± 2.0*
1.56	1.0	27.6 ± 0.6	1.56	1.0	30.2 ± 2.3*
1.56	2.0	27.8 ± 0.8	1.56	2.0	34.8 ± 1.8*
1.56	4.0	32.8 ± 1.1*	1.56	4.0	43.6 ± 1.7*
1.56	8.0	34.0 ± 1.4*	1.56	8.0	47.0 ± 2.0*
6.25	0	27.2 ± 0.5	6.25	0	27.2 ± 0.5
			6.25	0.25	27.0 ± 1.0
			6.25	0.5	27.2 ± 1.1
6.25	1.0	28.2 ± 1.1	6.25	1.0	27.6 ± 0.9
6.25	2.0	28.8 ± 1.1	6.25	2.0	27.6 ± 0.9
6.25	4.0	29.2 ± 1.8	6.25	4.0	28.4 ± 0.9
6.25	8.0	29.2 ± 1.8	6.25	8.0	37.6 ± 1.7*

Adsorption rates (unit: ST, mN/m) were measured by Pulsating Bubble Surfactometer.

Values are mean ± S.D.: n=5.

* P<0.01 vs surfactant alone (albumin or fibrinogen: 0 mg/ml) (ANOVA).

ST, surface tension; Surfactant, Surfactant-TA.

suspension of 6.25 mg/ml (Table 1).

In the surfactant-fibrinogen mixtures, the STs of adsorption rate were significantly higher than those of surfactant-alone at an fibrinogen concentration of 0.5 mg/ml or above in surfactant suspension of 1.56 mg/ml ($p<0.01$), and the increases of STs were dependent to the concentration of fibrinogen. But there were no significant increases in the STs of adsorption rate at varying concentrations of fibrinogen in mixtures with surfactant suspension of 6.25 mg/ml except for very high concentration of fibrinogen (8.0 mg/ml) (Table 1).

Dynamic minimum and maximum surface tension (min-ST and max-ST)

Dynamic min-ST

The dynamic min-STs at 5 min of surfactant-alone were 4.8 ± 0.5 and 5.1 ± 0.2 mN/m at a concentration of 1.56 mg/ml and 6.25 mg/ml.

In the surfactant-albumin mixtures, the dynamic min-STs were significantly higher than those of surfactant-alone (6.5 ± 0.4 vs 4.8 ± 0.5 mN/m) at an albumin concentration of 8 mg/ml in surfactant suspension of 1.56

Table 2. Minimum and maximum surface tensions of surfactant alone, surfactant-albumin, and surfactant-fibrinogen mixtures at 5 minutes

Surfactant (mg/ml)	Albumin (mg/ml)	5 minutes		Surfactant (mg/ml)	Fibrinogen (mg/ml)	5 minutes	
		min-ST (mN/m)	max-ST (mN/m)			min-ST (mN/m)	max-ST (mN/m)
1.56	0	4.8 ± 0.5	33.8 ± 0.8	1.56	0	4.8 ± 0.5	33.8 ± 0.8
				1.56	0.25	7.4 ± 0.2*	36.9 ± 0.5*
				1.56	0.5	7.5 ± 0.2*	37.9 ± 0.3*
1.56	1.0	5.8 ± 0.2	36.2 ± 0.6	1.56	1.0	7.6 ± 0.4*	38.3 ± 0.5*
1.56	2.0	6.3 ± 0.4	36.9 ± 0.5*	1.56	2.0	9.8 ± 0.4*	46.8 ± 1.2*
1.56	4.0	6.2 ± 0.3	37.1 ± 0.3*	1.56	4.0	11.7 ± 0.6*	52.0 ± 1.2*
1.56	8.0	6.5 ± 0.4*	37.7 ± 1.3*	1.56	8.0	17.2 ± 3.8*	55.6 ± 0.7*
6.25	0	5.1 ± 0.2	32.5 ± 0.4	6.25	0	5.1 ± 0.2	32.5 ± 0.4
				6.25	0.25	6.4 ± 0.2*	35.4 ± 0.3*
				6.25	0.5	6.9 ± 0.7*	35.9 ± 1.3*
6.25	1.0	6.2 ± 0.4*	33.0 ± 0.5	6.25	1.0	7.4 ± 0.4*	36.8 ± 0.9*
6.25	2.0	6.3 ± 0.2*	33.4 ± 0.8	6.25	2.0	8.0 ± 0.7*	38.6 ± 0.3*
6.25	4.0	6.4 ± 0.1*	33.4 ± 1.4	6.25	4.0	8.5 ± 0.3*	40.2 ± 0.8*
6.25	8.0	6.4 ± 0.5*	34.8 ± 1.2*	6.25	8.0	12.8 ± 1.3*	50.4 ± 1.4*

Minimum and maximum surface tensions (mN/m) were measured by Pulsating Bubble Surfactometer.

Values are mean ± S.D.: n=5.

* P<0.01 vs surfactant alone (albumin or fibrinogen: 0 mg/ml) (ANOVA).

min-ST, minimum surface tension; max-ST, maximum surface tension; Surfactant, Surfactant-TA.

mg/ml ($p < 0.01$), and (6.2 ± 0.4 , 6.3 ± 0.2 , 6.4 ± 0.1 , and 6.4 ± 0.5 vs 5.1 ± 0.2 mN/m) at an albumin concentration of 1.0 mg/ml or above in surfactant suspension of 6.25 mg/ml ($p < 0.01$) (Table 2).

In the surfactant-fibrinogen mixtures, the dynamic min-ST were significantly higher than those of surfactant-alone at a fibrinogen concentration of 0.25 mg/ml or above in surfactant suspension of 1.56 mg/ml ($p < 0.01$), and the increases of min-STs were dependent to the concentration of fibrinogen. Also, there were significant increases in the min-STs at a fibrinogen concentration of 0.25 mg/ml or above in mixtures with surfactant suspension of 6.25 mg/ml ($p < 0.01$) (Table 2).

Dynamic max-ST

The dynamic max-ST at 5 min of surfactant-alone were 33.8 ± 0.8 and 32.5 ± 0.4 mN/m at a concentration of 1.56 mg/ml and 6.25 mg/ml.

In the surfactant-albumin and surfactant-fibrinogen mixtures, the dynamic max-STs were significantly higher than those of surfactant-alone ($p < 0.01$), in a similar manner as the changes of dynamic min-ST. The increases of max-STs by albumin or fibrinogen were dependent to the concentrations of albumin or fibrinogen (Table 2).

Electron microscopic changes

The characteristic morphology of Surfactant-TA (concentration; 1.56 mg/ml) was observed and revealed lamellar rod-like micelles and multiple open-ends between the ends of rod-like micelles at both sides (Fig. 1). However, after mixing the surfactant with albumin or fibrinogen (concentration: surfactant 1.56 mg/ml, albumin or fibrinogen 4 mg/ml), the characteristic morphology of the surfactant changed from rod-like structures into a structure composed of deformed rods and spherical lamellae; the open-ends at both sides of the surfactant rods were thereby lost (Fig. 2, Fig. 3). In addition, the attachment of albumin or fibrinogen particles to the lamellar rod-like structures of the surfactant was observed in ultrastructures of surfactant-albumin (Fig. 2) and surfactant-fibrinogen mixture (Fig. 3).

DISCUSSION

This study revealed that albumin and fibrinogen may cause significant inhibition of the surface adsorption rates and ST-lowering properties of the surfactant due to the

Fig. 1. The electron microscopic ultrastructure of the surfactant-alone (Surfactant-TA, concentration: 1.56 mg/ml) shows the characteristic rod-like structure with multiple open ends (arrow) (A, $\times 10,000$; B, $\times 40,000$).

Fig. 2. After mixing the surfactant with albumin (Surfactant: 1.56 mg/ml, albumin: 4 mg/ml), the characteristic morphology of the surfactant changed to a spherical structure with losing at the open ends (A, $\times 10,000$) and attachment of albumin particles to the rod-like structure of the surfactant were also observed (B, $\times 40,000$).

Fig. 3. After mixing surfactant with fibrinogen (Surfactant: 1.56 mg/ml, fibrinogen: 4 mg/ml), the characteristic morphology of the surfactant changed to a spherical structure with losing at the open ends (A, $\times 10,000$); attachment of fibrinogen particles to the rod-like structure of the surfactant were observed (B, $\times 40,000$).

increased STs of adsorption rates, min-ST and max-ST, as measured by a Pulsating Bubble Surfactometer. Furthermore, inhibitory activity after mixing albumin or fibrinogen was dependent on the increasing concentration of albumin or fibrinogen. The inhibitory potency of fibrinogen to surfactant was stronger than that of albumin.

Surfactant-TA itself has good ST-lowering property, because the ST was below 11 mN/m, a value that was considered to be compatible with normal physiologic function (8). Fujiwara (9) and Tanaka et al. (10) have suggested surface physical criteria for good artificial surfactant. According to their suggestions, Surfactant-TA showed the following surface activities: rapid spreading (less than 10 seconds to reach an equilibrium ST of 24-27 mN/m); rapid adsorption (less than 1 minute to reach a ST of 27-30 mN/m; this means a SP of 42-45 mN/m) with a hypophase concentration of 50 μ g PL/ml; a min-ST less than 10 mN/m with only 20-30% surface compression, and a reproducible ST-area diagram with max-ST of 27-30 mN/m, using a modified Wilhelmy surface balance.

The results of the adsorption rate, min-ST and max-ST of Surfactant-TA alone were very similar to Fujiwara's criteria for good surfactant (9). After mixing with albumin or fibrinogen and surfactant, the STs of adsorption rate, min-ST, and max-ST of the mixtures were significantly higher than surfactant alone. These alterations revealed that an albumin or fibrinogen-contaminated surfactant does not have a good enough exogenous surfactant activity compared with the *in vitro* criteria for a good exogenous surfactant. These inhibitory activities were dependent on their relative concentrations of albumin or fibrinogen. At high albumin or fibrinogen concentration, albumin or fibrinogen-induced inactivation may be severe, while at low concentrations inactivation may be minimal. Albumin or fibrinogen, in itself, might therefore interfere with the adsorptive action of surfactant from the hypophase of solution to the surface of air-fluid interface and interfere with the ST-lowering activity of exogenous surfactant. These findings support suggestions that albumin or fibrinogen-induced dysfunction of surface physical properties of a surfactant may contribute, in part, to the pathophysiology of ARDS.

The pathophysiology of ARDS is complex. There are several factors relating to a loss of surfactant activity in ARDS. Seeger et al. (4) emphasize that alteration in alveolar surfactant function may well contribute to a variety of pathophysiological key events encountered in ARDS and suggest that there are mechanisms of surfactant alteration in ARDS. The mechanisms were as follows; a) a lack of surfactant due to its reduced generation/release by diseased pneumocytes or to an increased loss of surfactant, b) inhibition of surfactant function by

plasma protein leakage (inhibitory potencies of different plasma proteins). Leakage of plasma proteins into the alveolar space may substantially contribute to surfactant alterations in ARDS. c) incorporation of surfactant phospholipids and surfactant-proteins into polymerizing fibrin upon hyaline membrane formation. d) damage/inhibition of surfactant compounds by inflammatory mediators.

In 1965, Tierney and Johnson (11) reported that surfactant function was inhibited *in vitro* by plasma or serum. In 1966, Avery and Said (12) correlated pulmonary edema formation with surfactant functional abnormalities and postulated that the interaction of surfactant and plasma proteins results in hyaline membrane formation and sequestration of alveolar surfactant within the alveolar spaces. Several experimental studies *in vitro* and *in vivo* have demonstrated that contamination by leakage of blood components, e.g., blood itself, plasma or serum, compromise biophysical surfactant function (13-17). Among different proteins involved, albumin (15, 18-20), hemoglobin (21), and fibrinogen or fibrin monomer (15, 18, 20, 21-25) possess strong surfactant-inhibitory properties.

Through *in vitro* studies, an increase in surfactant ST was reported as a result of mixing albumin (26, 27), fibrinogen (27), and alpha-globulin (27), as measured by a Wilhelmy balance, and increased ST and inhibition of adsorption rate resulted from mixing albumin (15, 18), fibrinogen (15, 18), human serum (15), 55,000-dalton serum protein (15), bilirubin (15), hemoglobin (21), erythrocyte membrane lipids (21), and fibrinogen degradation product, as measured by a Pulsating Bubble Surfactometer.

In *in vivo* studies, Kobayashi et al. (17) have concluded that lung edema fluid impairs ventilation through surfactant inactivation when the protein-to-surfactant ratio increases, and that albumin and fibrinogen are the main causes of this impairment in animal models of ARDS. In addition, Seeger et al. (20) have demonstrated that IgG, IgM, elastin, albumin, fibrin, and fibrinogen monomer interfered with the pressure-volume diagram of rabbit models of ARDS. Among different proteins, fibrin monomers are especially effective in interfering with surfactant function.

Generally, fibrin degradation products and fibrinogen have the most potent inhibitory potentials (15, 20, 23, 27). Their rank order of potency in interfering with surfactant function is: IgG, IgM and elastin < albumin < fibrinogen < fibrin monomer (20). Proteins compete with the surfactant for the surface and thus interfere with monolayer formation (28). The relationship between surfactant and inhibitory proteins depends on their relative concentrations (16, 19). At high surfactant concentration, protein inactivation may be minimal, while at low con-

centrations inactivation may be severe, even with albumin predominantly present in the airspaces. Thus, a decreased alveolar pool size of functionally active surfactant should increase the risk of inactivation.

In addition, our study demonstrated that morphological changes occurred after surfactant activities were inhibited by albumin or fibrinogen. Sasaki (29) compared the in vitro surface properties and ultrastructures of five surfactant preparations, namely purified natural surfactant, natural surfactant lipids, PL fraction of pig surfactant, dipalmitoylphosphatidyl-choline (DPPC) + phosphatidyl-glycerol (PG) (7:3), and Surfactant-TA. The latter had the best in vitro surface active properties and showed the characteristic ultrastructure of large open-ended, thin-walled, lamellar rod-like structures. Other surfactant preparations, however, had spherical multilamellar bodies but few open-ended structures. Sasaki concluded that these structural differences between Surfactant-TA and other surfactants correlated well with the physicochemical differences of these surfactants. It was deduced that this morphology of a surfactant with an open-ended structure can more easily adsorb to the air-liquid interface than a spherical multilamellar structure. Our data revealed that the ultrastructure of Surfactant-TA changed from rod-like structures with open-ends to a deformed rod and spherical structure with loss of open-ends. The deposition of albumin or fibrinogen particles on rod-like micelles occurred after mixing the surfactant with albumin or fibrinogen. These findings suggest that inhibition of the physical properties of pulmonary surfactant by albumin and fibrinogen occurred through the mechanism whereby albumin and fibrinogen alter the morphology of the surfactant.

Finally, with regard to the clinical aspect of the consequences of ARDS, a loss of alveolar surfactant activity due to severe impairment of surfactant and other systems in ARDS conditions increases ST. This causes changes in lung mechanics, the impairment of gas-exchange (ventilation-perfusion mismatch), lung edema, reduction of host defence mechanism, enhanced inflammatory process and fibrosis. As can be expected, there have been several reports of exogenous surfactant replacement therapy in cases of ARDS in children and adults, and in ARDS cases due to secondary surfactant dysfunction in neonates with meconium aspiration syndrome, pulmonary hemorrhage, lung edema, etc. They also demonstrated greater clinical improvement of oxygenation in the acute stage and in long term outcome in cases in exogenous surfactant-treated group than in the control group. Some reports have therefore recommended the use of exogenous surfactant replacement therapy in cases of ARDS.

In conclusion, albumin and fibrinogen significantly

increased the STs of adsorption rates and the min-ST and max-ST of pulmonary surfactant, and changed electron microscopic findings of the surfactant. These inhibitory effects of albumin and fibrinogen on surface properties of surfactant were dependent upon the increasing concentration of albumin or fibrinogen. These findings support the hypothesis that albumin and fibrinogen-induced surfactant dysfunction may play a role in the pathophysiology of ARDS. According to the results of this study, adverse effects of albumin and fibrinogen on the surfactant might be overcome by administration of large doses of exogenous surfactant, and exogenous surfactant therapy or surfactant lavage may be beneficial in cases of a respiratory distress due to ARDS.

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