Table 1. *Demographic data on study subjects who underwent WLL*

Case	Age, yr	Sex	Symptoms*	Onset to WLL†, mo	DSS‡	Smoking Status	Occupational Dust Exposure	Complications
1	45	M	DOE, Occasional Cough	66	2	Ex-Smoker	No	Psoriasis, HT, DL
2	54	F	DOE	43	2	Never	No	HT, DL
3	67	M	Dyspnea, Cough, Hemosputum	10	5	Ex-Smoker	No	Postcerebral Infarction
4	34	M	Dyspnea	42	5	Current Smoker	No	None
5	53	F	Dyspnea, Cough	96	5	Never	No	SSS
6	46	M	None	30	1	Current Smoker	Yes	None
7	66	M	DOE	25	4	Never	No	DM
8	67	F	Dyspnea	36	5	Never	No	None
9	57	M	DOE	28	2	Never	No	None

*Symptoms were recognized as respiratory symptoms. †Onset: time when the 1st respiratory symptom emerged or time of finding an abnormal image that was compatible with pulmonary alveolar proteinosis (PAP). ‡Disease severity score (DSS): defined based on respiratory symptoms and arterial oxygen tension (PaO_2; see Ref. 3). DSS 1: no symptoms and $PaO_2 \geq 70$ mmHg. DSS 2: symptomatic and $PaO_2 \geq 70$ mmHg. DSS 3: 60 mmHg $\leq PaO_2 < 70$ mmHg. DSS 4: 50 mmHg $\leq PaO_2 < 60$ mmHg. DSS 5: $PaO_2 < 50$ mmHg. WLL, whole-lung lavage; DOE, dyspnea on exertion; HT, hypertension; DL, dyslipidemia; SSS, sick sinus syndrome; DM, diabetes mellitus.

ranged 10–225 s, 120–425 s, 52–225 s, and 0–96 s, respectively. In 11 of 17 lungs, the retaining time was designed to be 120 s, but it was variable with 132–425 s in 6 lungs. Time required for other stages varied remarkably as shown in Table 3.

The initial volume of instilled saline ranged within 600–2,300 ml (1,359 ± 435 ml). The initial discharged volume ranged from 150 to 1,282 ml (697 ± 341 ml), with percentage of recovery ranging from 24.3 to 71.4%. The mean volume of instilled saline from the second to the last lavage in each patient varied from 489 to 1,938 ml (893 ± 374 ml). In each cycle, 461–1,896 ml (859 ± 364 ml) of discharged fluid was recovered; the recovery percentage was relatively constant (89.7–100.3%). As a whole, the total volume of saline instilled into each lung was 9,900–28,200 ml, and the total volume of discharged lavage fluid was 8,910–27,000 ml, with total percentage of recovery of 93.4 ± 3.3 (82.1–95.9%).

Simulation of Protein Concentrations in the Drained Lavage Fluid

The theoretical concentrations of IgG, transferrin, albumin, and β_2- microglobulin in the drained lavage fluid were calculated at the end of the draining stage according to the equation described above and by using the procedures detailed in MATERIALS AND METHODS. The theoretical concentrations were plotted on a log scale against time after the beginning of WLL (Fig. 2). The plot for each patient was manually fitted with the protein concentration measured in the drained lavage fluid of each cycle by changing K_s and K_b. Plots for the theoretical concentrations of IgG, transferrin, albumin, and β_2-microglobulin coincide with the measurements (Fig. 2, *A–D*). Data for K_s and K_b are shown in Fig. 3. K_s values for IgG, transferrin, albumin, and β_2-microglobulin ($2.03 \times 10^{-7} \pm 0.902$, $1.95 \times 10^{-7} \pm 0.589$, $1.84 \times 10^{-7} \pm 0.564$, and $1.85 \times 10^{-7} \pm 0.658$, respectively) did not vary among patients (Fig. 3*A*). Importantly, there was no significant difference in K_s values among these four proteins, suggesting that transfer from the surfactant to lavage fluid was independent of molecular weight. However, there was relative variability in K_b among proteins, especially with β_2-microglobulin, which had a K_b that was two orders of magnitude higher than that of the other proteins. K_b values for IgG, transferrin, albumin, and β_2-microglobulin were $4.97 \times 10^{-10} \pm 4.166$, $5.61 \times 10^{-10} \pm 1.990$, $3.82 \times 10^{-10} \pm 1.661$, and $2.28 \times 10^{-8} \pm 0.773$, respectively (Fig. 3*B*). No differences in K_s or K_b values of each protein were found between left and right lungs (data not

Table 2. *Clinical parameters of patients*

	Arterial Blood Gas Analysis			Serum Biomarkers				Pulmonary Function Test			
Case	PaO_2, mmHg	$PaCO_2$, mmHg	A-aDO_2, mmHg	KL-6, JU/ml	SP-D, ng/ml	CEA, ng/ml	GM-Ab, μg/ml	%VC, %	FEV_1/FVC, %	FRC, liters	$\%D_{LCO}$, %
1	81.3	35.8	24.0	7611	963	6.9	6.8	85.8	82.7	2.51	60.5
2	75.6	39.4	25.1	32070	187	12.5	116.0	81.9	79.43	1.77	38.2
3	67.4*	35.5*	269.0*	46700	518	41.4	25.2	51.4	84.5	2.36	19.2
4	55.2*	38.4*	167.7*	26300	799	49.1	25.0	47.7	78.98	1.52	30.0
5	46.4	34.8	60.1	31093	422	19.8	35.7	47.4	98.1	1.41	ND†
6	72.3	38.8	29.2	8356	236	12.8	35.5	85.9	82.9	2.49	70.7
7	51.2	36.7	52.9	10969	407	73.3	6.7	87.5	109.4	1.71	70.8
8	46.2	38.2	55.1	15700	531	16.5	10.4	77.5	80.3	1.53	ND
9	75.3	39.6	24.9	7684	172	7.9	150.9	83.9	75.8	2.50	67.8

Normal Krebs von den Lungen-6 (KL-6), surfactant protein D (SP-D), carcinoembryonic antigen (CEA), and granulocyte/macrophage colony-stimulating factor autoantibody (GM-Ab) levels were within 500 IU/ml, 110 ng/ml, 5.0 ng/ml, 1.0 μg/ml, respectively. *Nasal oxygen supply. †Diffusing capacity of the lung for carbon monoxide (D_{LCO}) of case 5 was not detected because of low vital capacity (VC). ND, not done. A-aDO_2, alveolar-arterial oxygen difference that was measured; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; FRC, functional residual capacity.

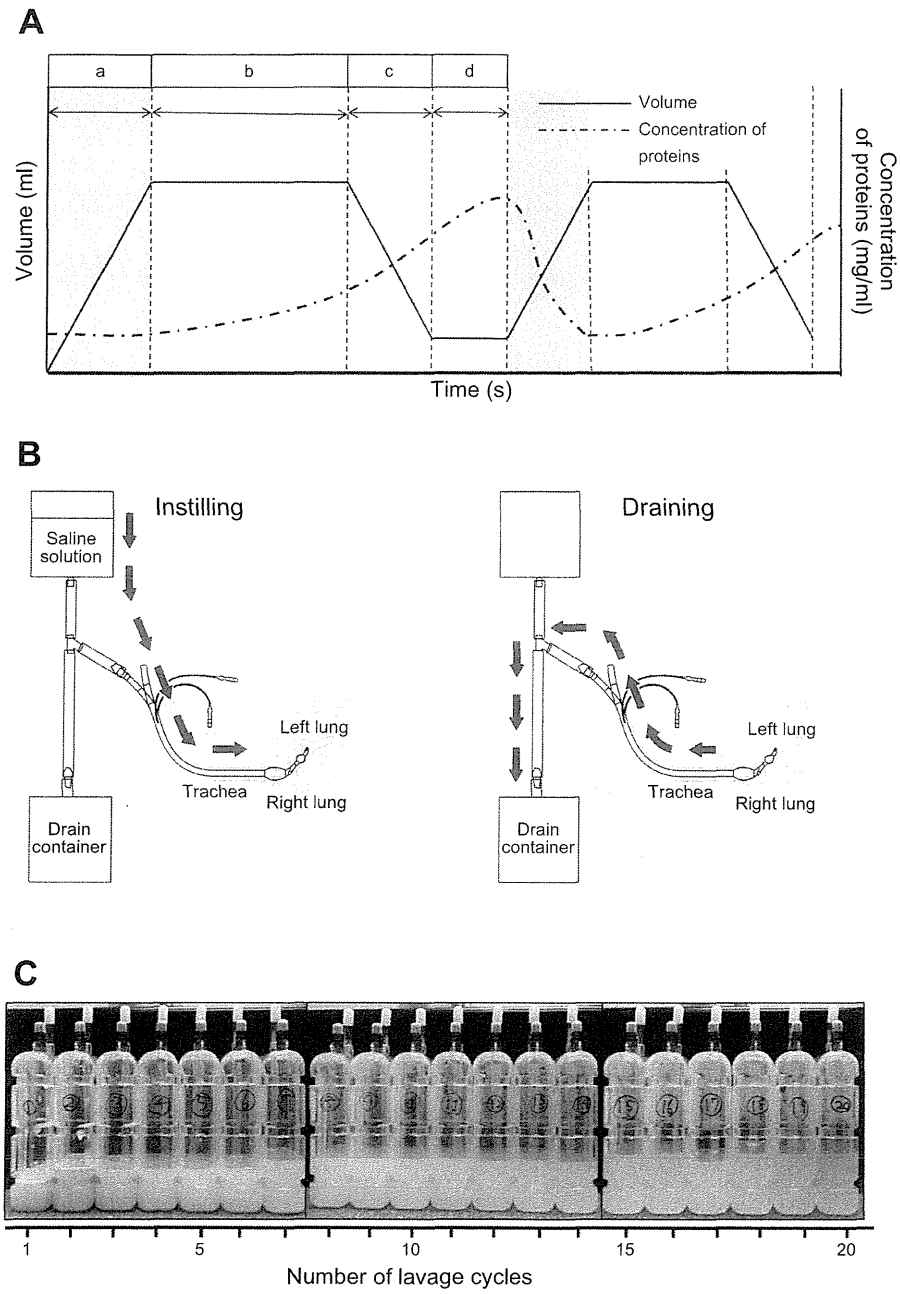

Fig. 1. *A*: conceptual schematic of the time course of the lavage fluid volume in the lung and the concentration of a protein during whole-lung lavage (WLL). Each lavage cycle involved 4 stages: instilling (*a*, from the beginning to the end of saline instillation), retaining (*b*, from the end of saline instillation until the beginning of drainage), draining (*c*, from the beginning to the end of drainage), and preparing (*d*, from the end of drainage to the beginning of the next saline instillation). *B*: schematic of the procedures for instilling (*left*) and draining (*right*) stages. ~0.5 to 2.0 l of saline solution from a bottle was instilled through an endotracheal tube into the lavage lung, retained for a few minutes, and then drained into a draining container. *C*: appearance of the drained fluid obtained from the first to the 20th lavage cycle.

shown). Thus the simulation data confirm the appropriateness of our mathematical model and indicate that the transfer kinetics of proteins into the drained fluid was time dependent.

Durable Effects of the Time on the Lavage Efficiency

To determine the durable effect of each lavage cycle on the slope for the decreasing concentration of each protein in the drained lavage fluid, we evaluated the change in slope of the theoretical curves by varying the duration of the retaining stage in silico. For this purpose, we used the initial data settings in case 4, i.e., the instilling volume of saline; the durations (s) of instillation, retaining, draining, and preparing; and the volume of drained lavage fluid in the first lavage cycle. We found that decreasing curves for the albumin concentration became steeper upon substitution of the shorter time (Fig. 4*A*).

Next, we proceeded to confirm the effects observed in the simulation by using measurements in case 1. We evaluated the rate of declining albumin concentration in the lavage aliquots from a patient who occasionally underwent WLL for the left lung with short-term cycles (120 s, 1–20 cycles) and for the right lung with long-term cycles (540 s, 4–11 cycles). As shown in Fig. 4*B*, the slope of decline for the left lung appeared

Table 3. *Timetable of the stages in each cycle of WLL*

Case	Left/Right	No. of Cycles	Total Time, s	Mean Time ± SD, s*				
				Each Cycle, s†	Stage a, Instilling, s	Stage b, Retaining, s	Stage c, Draining, s	Stage d, Preparing, s
1	L	20	6260	258 ± 15.0	42 ± 3.0	120 ± 1.6	83 ± 42.2	13 ± 18.6
	R‡	11	6895	561 ± 161.7	41 ± 8.1	425 ± 196.2	59 ± 16.4	8 ± 2.6
2	L	20	5200	210 ± 21.9	28 ± 3.0	120 ± 0	52 ± 23.2	13 ± 5.1
	R	20	5450	220 ± 8.3	45 ± 5.9	120 ± 0	52 ± 16.1	8 ± 8.1
3	L	20	5583	237 ± 15.1	36 ± 7.0	120 ± 0	73 ± 18.4	10 ± 2.6
	R	20	6060	236 ± 38.8	35 ± 6.0	120 ± 0	64 ± 26.8	20 ± 16.8
4	L	24	5926	215 ± 19.4	35 ± 4.1	120 ± 0	54 ± 24.7	10 ± 3.3
	R	29	9018	285 ± 17.5	57 ± 3.9	120 ± 0	102 ± 16.8	8 ± 3.3
5	L	20	5230	227 ± 30.6	31 ± 11.9	120 ± 0	76 ± 31.9	11 ± 4.7
	R	20	6480	284 ± 25.3	31 ± 6.2	120 ± 0	121 ± 20.2	19 ± 24.8
6	L	20	5395	266 ± 32.3	10 ± 0	120 ± 0	133 ± 32.0	5 ± 0
	R	20	5680	278 ± 42.2	28 ± 11.2	132 ± 24.6	112 ± 24.8	5 ± 0
7	R	20	6180	283 ± 31.6	52 ± 10.1	120 ± 0	109 ± 41.4	13 ± 11.5
8	L	16	10380	634 ± 264.9	180 ± 32.1	199 ± 28.7	148 ± 50.0	96 ± 250.3
	R	20	11796	550 ± 49.5	188 ± 41.3	200 ± 6.2	153 ± 20.5	16 ± 20.6
9	L	11	6180	553 ± 185.8	146 ± 42.8	193 ± 24.2	174 ± 82.2	40 ± 112.3
	R	13	8280	627 ± 98.1	225 ± 45.2	231 ± 64.1	225 ± 63.3	0 ± 0

*Data are presented as a mean ± SD of time (s) required for 1 lavage cycle. Time for total on each stage of lavage cycle is expressed as a mean ± SD. Instilling time (stage a) is mean time (s) required for instilling saline into the lung. Retaining time (stage b) is mean time (s) applied for retaining saline in the lung. Draining time (stage c) is mean time (s) required for draining lavage fluid to the container. Preparing time (stage d) is mean time (s) required for preparation for the next saline instillation. †Each cycle time is the mean of stage a to d from 2nd to the last lavage. The 1st cycle required 120–1080 s. ‡Time (s) for the 1st 3 cycles ranged within 230–270 s, and that for the 4th to 11th cycles ranged within 625–680 s.

steeper than that for the right lung. The time required to reach 10% of the initial concentration of albumin in the first lavage was 2,730 s for the left lung, whereas it was 4,390 s for the right lung. Notably, both K_S and K_b of the left and right lungs were comparable (1.77×10^{-7} and 4.97×10^{-10} cm/s, respectively, for the left lung; 1.60×10^{-7} and 3.20×10^{-10} cm/s, respectively, for the right lung).

When 1,000 ml of saline was assumed to be instilled into the lung in each cycle, the cumulative amount of albumin drained into the lavage fluid did not differ remarkably within retaining time of 90–570 s (Fig. 4*C*). The curve in short retaining time (90 s) slightly exceeded those in long retaining time (450–570 s) but reversed after 4,000 s. In this setting, the simulation impressed that ~3,200 s (53.3 min) would be required for enough elimination of albumin but that the efficiency of elimination would not significantly change after 5,400 s (90 min).

Effect of Instilled Saline Volume on the Efficiency of WLL

Eqs. 1–3 described in MATERIALS AND METHODS meant that the effect of WLL on elimination of proteins was affected by the instilled saline volume into the lung. As shown in Fig. 4*D*,

Table 4. *Volume balance during WLL*

Case	Left/Right	First Cycle		Second to the Last Cycle			
		Instilled Volume, liters	Drained Volume, liters (recovery %)	Average Instilled Volume, liters	Average Drained Volume, liters (recovery %)	Total Instilled Volume, liters	Total Drained Volume, liters (recovery %)
1	L	1.70	1.05 (61.8)	0.96	0.93 (96.6)	20.0	18.7 (93.7)
	R	1.90	1.10 (57.9)	1.04	1.01 (97.1)	12.3	10.1 (82.1)
2	L	1.40	0.50 (35.7)	0.55	0.51 (91.4)	11.9	10.1 (84.9)
	R	1.50	0.90 (60.0)	0.84	0.81 (96.6)	17.5	16.4 (93.5)
3	L	1.50	1.00 (66.7)	0.85	0.82 (96.3)	16.9	16.6 (93.8)
	R	1.70	0.90 (52.9)	0.88	0.89 (100.3)	18.5	17.8 (95.9)
4	L	0.90	0.37 (41.1)	0.64	0.63 (97.1)	16.5	14.7 (89.3)
	R	1.40	1.00 (71.4)	0.96	0.93 (97.0)	28.2	27.0 (95.7)
5	L	0.60	0.15 (25.0)	0.49	0.46 (94.2)	9.90	8.91 (90.0)
	R	1.00	0.32 (32.0)	0.59	0.56 (95.4)	12.2	11.0 (90.2)
6	L	1.00	0.60 (60.0)	0.55	0.53 (96.6)	11.4	10.7 (93.4)
	R	1.00	0.50 (50.0)	0.63	0.61 (95.8)	13.0	12.0 (92.3)
7	R	1.00	0.50 (50.0)	0.76	0.75 (98.6)	15.5	14.8 (95.5)
8	L	1.10	0.27 (24.3)	0.94	0.85 (89.7)	15.2	13.0 (85.0)
	R	1.30	0.47 (36.0)	0.97	0.95 (98.5)	19.8	18.5 (94.4)
9	L	1.80	0.93 (51.9)	1.58	1.48 (93.8)	17.6	15.7 (89.5)
	R	2.30	1.28 (55.7)	1.94	1.90 (97.8)	25.6	24.0 (94.1)

The instilling volume of the 1st cycle in case 1–7 was determined by the following equations: functional residual capacity (ml) × 0.45 or 0.55 + tidal volume for the left and right lung, respectively. In case 8 and 9, saline was allowed to be instilled into the lung as much as possible from a bottle at 30 cm height from the tracheal tube.

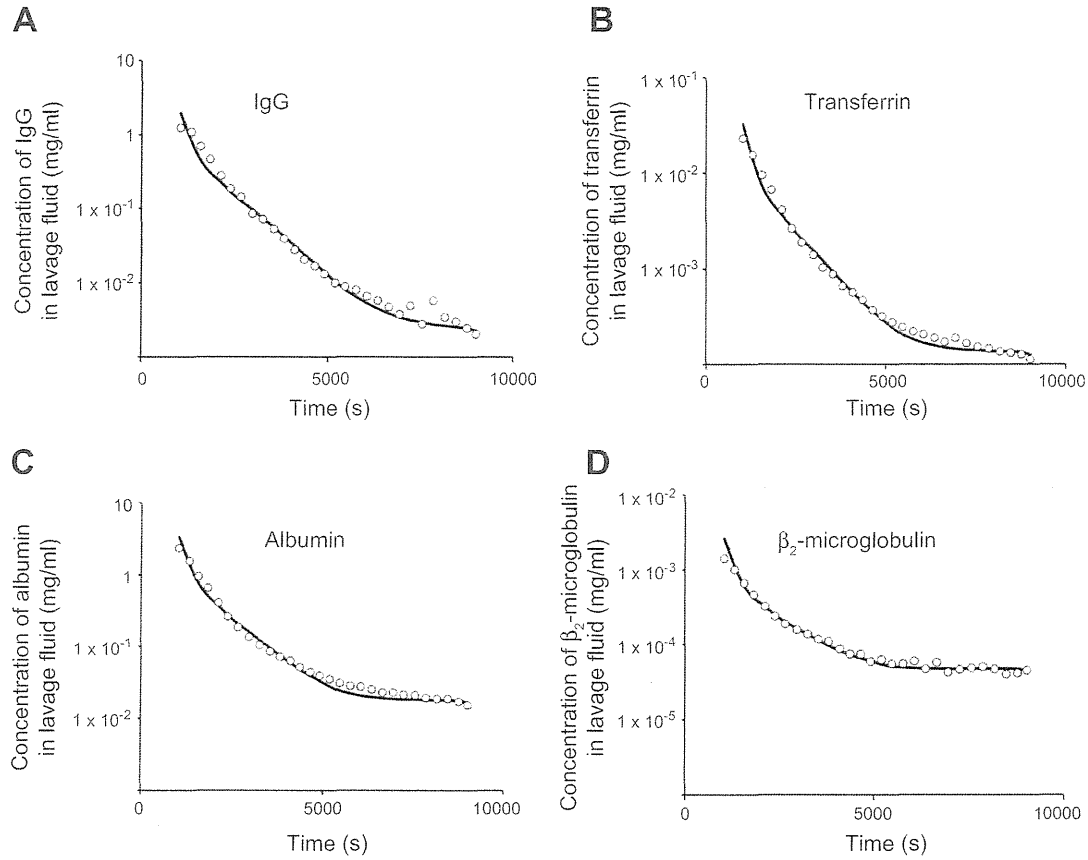

Fig. 2. Theoretical concentrations (lines) and measured concentration (plots) of IgG (*A*), transferrin (*B*), albumin (*C*), and β_2-microglobulin (*D*) in the drained aliquot of lavage fluid for each cycle. The vertical axis is the concentration of the protein on a log scale, and the horizontal axis indicates the time after the beginning of WLL.

when the cumulative eliminated albumin in case 1 was estimated in silico with fixed lavage cycle time at 240 s, the eliminated albumin appeared to increase as the instilled volume increased during 0 to ~3,200 s. After 3,200 s, the eliminated albumin gradually increased, but the volume effect seemed to be diminished.

Exceptional Substances That Fail to Follow the Mathematical Model

Although we applied our mathematical model to the transfer of various substances during WLL, we found that the following substances did not follow the model.

Gastrin and urea. Measured levels of gastrin and urea did not exhibit an exponential decreasing phase but instead reached a plateau in the early stage of WLL (Fig. 5, *A* and *B*). Thus calculation of K_s was difficult. Permeation of gastrin and urea from the blood to the lavage fluid occurred so quickly that the theoretical curves were hardly matched with the actual measurements, which themselves fluctuated markedly during the plateau phase.

SP-D. The SP-D concentration in the drained lavage fluid decreased consistently to a minor extent in the four lungs in the absence of an exponential phase and quickly reached a plateau in the early phase (Fig. 5*C*). As alveolar type II cells and nonciliated Clara cells abundantly release SP-D into the lower respiratory tract, this early plateau phase reflects its active release in situ.

GM-CSF autoantibody. Although the quantified GM-CSF autoantibody belongs to an IgG isotype, theoretical curves of the concentration in the drained lavage fluid did not fit with the measured autoantibody concentration even upon substitution of various sets of coefficients with K_s and K_b in all 17 lungs (Fig. 5*D*).

DISCUSSION

By using a mathematical model based on measured concentrations of proteins, this study investigated the transfer of proteins from the surfactant and blood into the lavage fluid during WLL. We confirmed that the transfer followed a time-dependent differential equation, which assumes that the rate of transfer is proportional to the transmission coefficient, the effective surface area, and the protein gradient between the body compartment and lavage fluid (44).

By using various methods (e.g., comparisons of the protein concentrations between the plasma, sputum, and BALF) and by proving that the IgG1/IgG2 ratio between the BALF and serum are comparable, previous studies demonstrated the transfer of circulating proteins into the alveolar spaces (2, 14, 18, 28, 39).

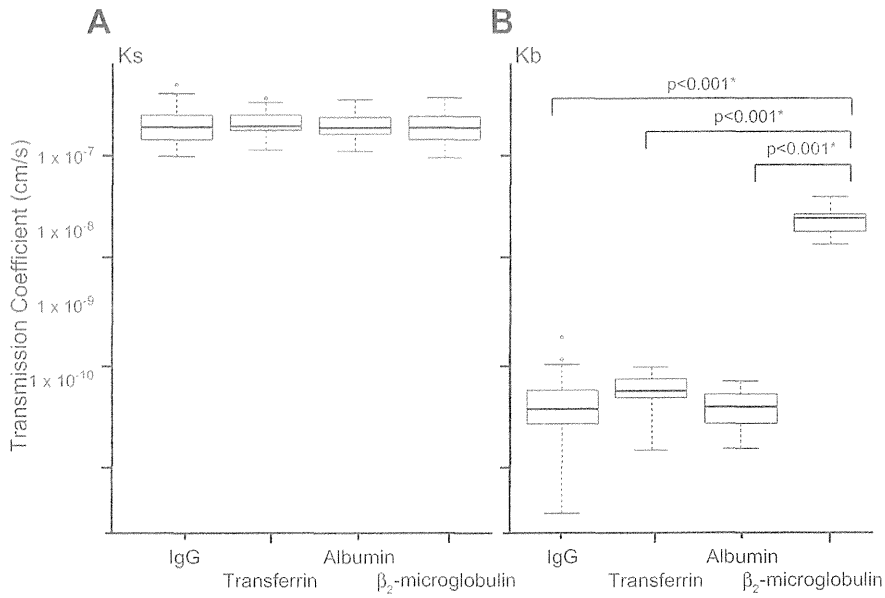

Fig. 3. Coefficients of transfer of IgG, transferrin, albumin, and β_2-microglobulin from surfactant (K_s) (*A*) and blood (K_b) (*B*) to the lavage fluid. The vertical axis indicates the transmission coefficients (cm/s) on a log scale. Statistical significance of coefficients between 2 proteins are shown in the figure.

More recently, intravenously injected GM-CSF autoantibodies were detected in the BALF of nonhuman primates and were observed to reproduce PAP (35). These results indicate that the antibody can cross the air-blood barrier (35). The kinetics of transfer from the blood to the air space and vice versa was studied both in vitro and in vivo (3, 23, 26, 27, 34). In one study, the transmission coefficient (10^{-7}-10^{-5} cm/s) of various proteins across a monolayer of A549 cells was shown to indicate bidirectional transfer. These coefficients appear to be inversely correlated with the molecular weight of proteins (22). In another study, the transmission coefficient for proteins in a monolayer of rat alveolar epithelial cells in vitro was within 10^{-9}-10^{-7} cm/s, whereas that for albumin in sheep lung in vivo was 5×10^{-10} cm/s (11, 17). Thus mass transfer from the blood to the air spaces may be continuously taking place even at steady state.

In previous studies by Ikegami et al. (15), surface tension maintained by surfactant materials covering the alveolar surface was found to have a probable role in interfering with massive transfer and subsequent accumulation of circulating proteins in the air spaces. Interference with the transfer is known to be disrupted by the elimination or deficiency of SP-B (15, 16). Lung lavage may remove surface-active materials in the alveoli and thus temporally disrupt the mechanisms that interfere with the influx of circulating proteins. It is for this reason that we focused on WLL to clarify the mechanism of protein transfer from the blood or surfactant to the lavage fluid. We found that the protein transfer followed a time-dependent mathematical model that was made analogous to the heat transmission model. To our knowledge, this is the first study that has clarified the mechanism of protein transfer in the lung during WLL.

To postulate a mathematical model, we assumed that the transfer of proteins from each body compartment to the lavage fluid consists of two pathways, namely transfer from the accumulated surfactant to the lavage fluid and transfer from the blood to the lavage fluid. The latter may be further divided into two pathways, namely transfer from the blood through the surfactant and direct transfer to the lavage fluid. However, we did not distinguish between these two latter pathways in this study because the transfer of a protein across the air-blood barrier seemed to be rate limiting. We found that protein transfer from the surfactant to the lavage fluid appeared to have K_s values independent of the molecular weight and other properties. It is notable that the K_s values did not differ among patients, indicating the reproducibility of the model. However, mass transfer from the blood to the lavage fluid with variable K_b values did appear to be affected by the molecular weight of the protein because the protein was transferred through a semipermeable membrane consisting of endothelial cells, basement membrane, and type I pneumocytes. Transcytosis was proposed as the primary mechanism of protein transfer for large molecules and of partial paracellular diffusion of small molecules (7, 23). However, the true mechanism remains controversial. As indicated in this study, transfer of β_2-microglobulin (molecular weight of 11 kDa) from the blood to the lavage fluid had K_b values that were two orders of magnitude higher than those of albumin, transferrin, and IgG, which had molecular weights of 66, 80, and 150 kDa, respectively. This difference suggests that β_2-microglobulin diffusion possesses a mechanism that is different from that of other proteins, i.e., it is supposed to be mainly transcytosis for albumin, transferrin, and IgG but mainly paracellular diffusion for β_2-microglobulin. Further analyses will be required to clarify the mechanisms by measuring the permeability of various substances with molecular weight of 10–60 kDa to confirm a "gap" in permeability coefficient K_b among substances with molecular weights in this range.

It is notable that the decrease in concentrations of low-molecular-weight substances in the lavage fluid, namely urea (molecular weight of 60 kDa) and gastrin (molecular weight of 2.1 kDa), was inconsistent with our mathematical model. The measured concentrations appeared to fluctuate and appeared to be independent of time. Moreover, the phase of exponential

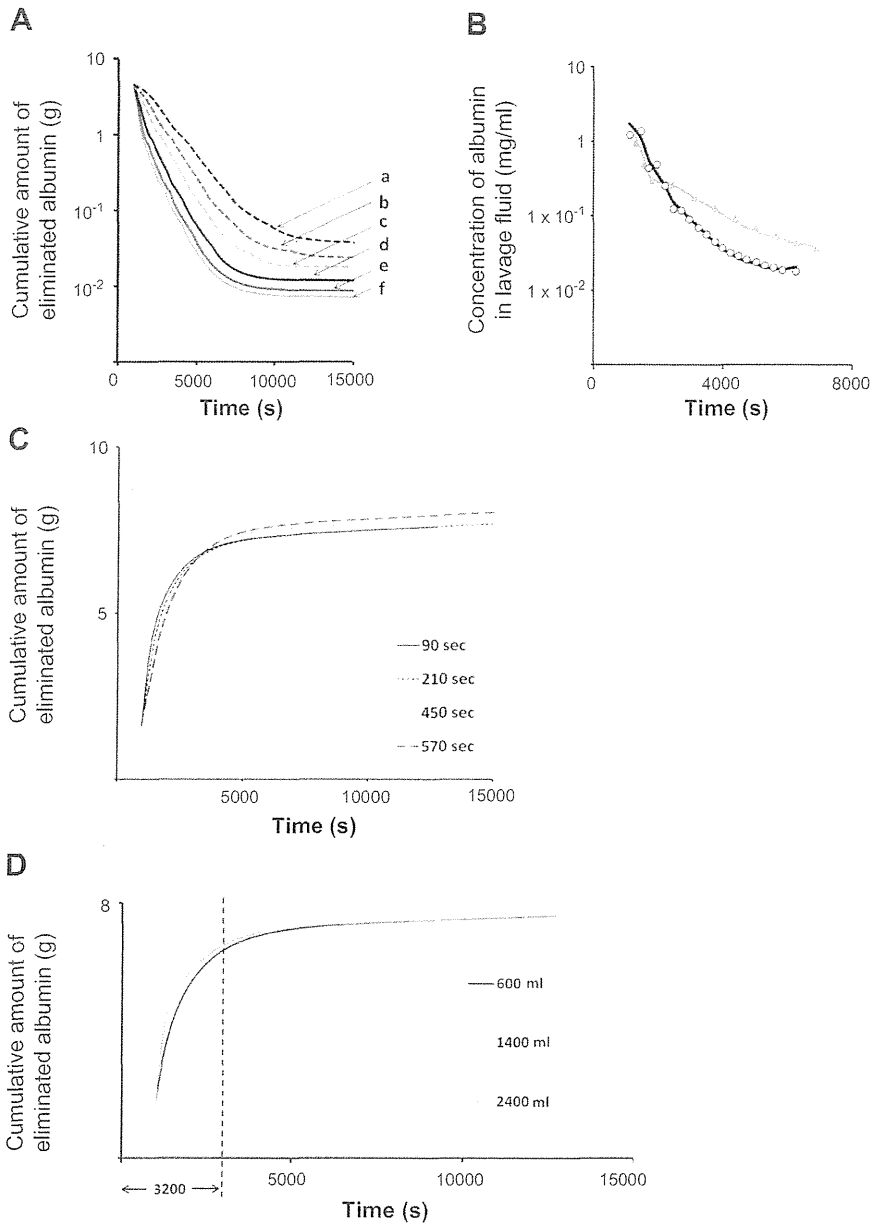

Fig. 4. *A*: durable effect of the retaining stage in each lavage cycle on the theoretical decreasing curve of albumin concentration in the drained lavage fluid. The time assumed for the retaining stage was variable: *a*, 540 s; b, 360 s; *c*, 240 s; *d*, 120 s; *e*, 60 s; and *f*, 30 s. The vertical axis indicates the albumin concentration in the lavage fluid (mg/ml). The horizontal axis indicates the time after the beginning of WLL. *B*: theoretical (lines; black, left; gray, right) and measured (plots; ○, left, ▲, right) concentrations of albumin in the drained lavage fluid in each cycle. The vertical axis indicates the albumin concentration in the lavage fluid (mg/ml). The horizontal axis indicates the time after the beginning of WLL. *C*: simulation curves of cumulative amount of albumin drained in the drained lavage fluid when the retaining time varied with 90 (solid line), 210(small dashed line), 450(dotted line), or 570(large dashed line) s. *D*: cumulative amount of eliminated albumin in the drained lavage fluid. An in silico evaluation by changing instilled saline volume varied with 600 (black solid line), 1,400 (dotted line), or 2,400 (gray solid line) ml.

decrease was hardly defined in six out of ten lungs examined: when there was any decrease, the phase lasted within 1,000 s after the start of WLL (data not shown). This characteristic was likely due to the high permeability of the air-blood barrier to the molecules. Similarly, Rennard et al. (32) reported that urea was more able than glucose and albumin to permeate into the lavage fluid, as observed in normal volunteers with saline instilled into their lung segments.

SP-D is produced by alveolar type II cells and nonciliated Clara cells in the lower respiratory tracts and is secreted into the air space (43). Although SP-D is detectable in the sera of patients with aPAP, its levels are much lower than those of BAL (12). Thus SP-D transfer from the blood to the air space is negligible. The high concentration of SP-D in the lavage fluid was likely due to its continuous production in the lung. The rate of its production was estimated to be 6–13 mg/h on the basis of evaluation of four lungs (data not shown).

The lung is the organ that most abundantly produces GM-CSF, a factor that is critical for terminal differentiation of alveolar macrophages, as it promotes the expression of the transcription factor, PU.1 (38). It is suggested that IgG-type GM-CSF autoantibody is pathogenic and is known to be transferred from the lung capillaries into the air spaces immediately formed by GM-CSF autoantibody complex to become undetectable by our GM-CSF autoantibody ELISA system (30).

Furthermore, we had better to reconsider the adequacy of the present mathematical model when it was applied to substances

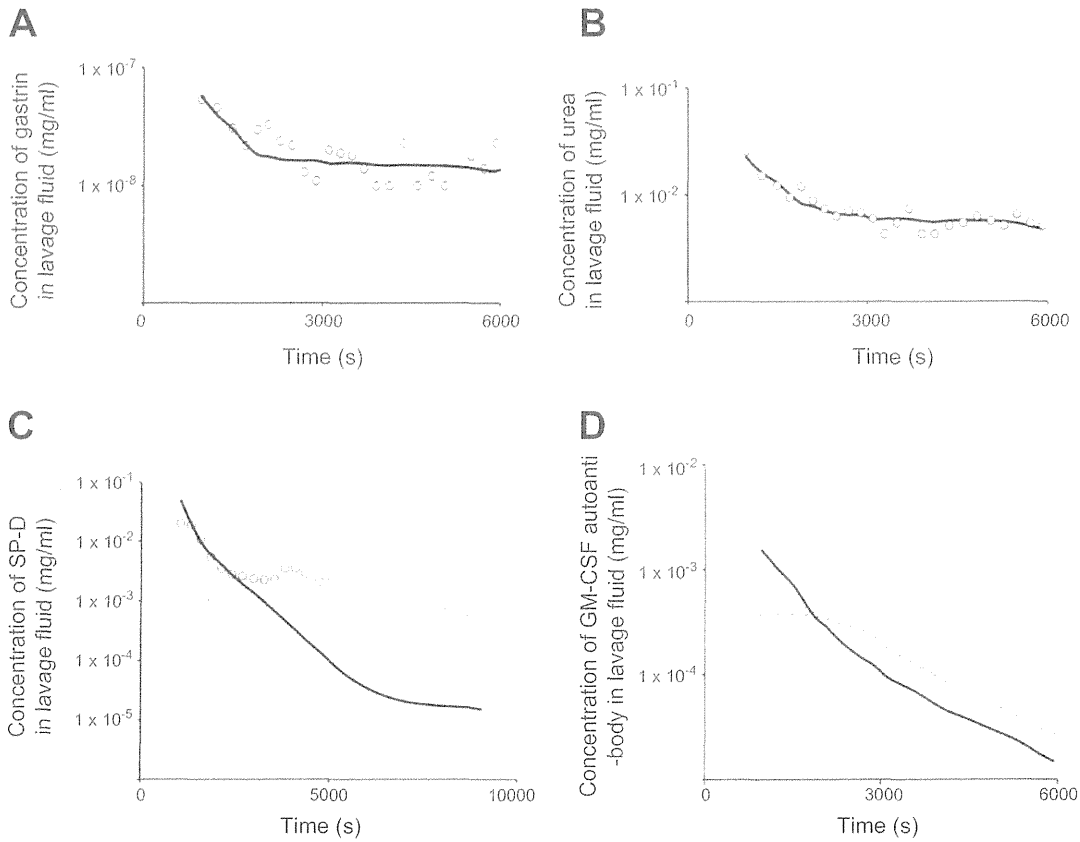

Fig. 5. *A* and *B*: actual measurements (plots) of gastrin or urea concentration in the drained lavage fluid did not exhibit the exponential decreasing phase but reached a plateau fluctuating in the early term. These seemed to migrate immediately from the blood to the lavage fluid. Thus the theoretical curves (lines) were hardly fitted with the actual measured concentration. *C*: concentration of surfactant protein D (SP-D) in the drained lavage fluid revealed slight decrease without exponential phase and soon reached a plateau phase in the early term. As SP-D is abundantly released from alveolar type II cells into the lower respiratory tracts, this early plateau phase probably reflected the active release in situ. *D*: actually measured granulocyte/macrophage colony-stimulating factor (GM-CSF) autoantibody concentrations were consistently under the theoretical curve especially in the early stage.

with lower molecular weights by assuming two permeation coefficients, such as K_{b1} (coefficients from the blood to the lavage fluid through surfactant) and K_{b2} (from the blood directly to the lavage fluid).

In the present study, the recovery rate in the first draining lavage fluid was lower than those after the second lavage. Although the first instilled saline remained in the lower respiratory tracts, we did not mind the remaining volume at the first draining because we thought that the remaining lavage fluid could be recovered after the second draining. Therefore, we did not intentionally extend the first draining time longer than those of other cycles. Although we usually perform percussion or vibration on the patient's chest, the recovery rate at the first draining was not improved by these procedures. It is likely that the low recovery rate and its variability of the first lavage shown in Table 4 were due to the early cessation of the first draining.

To date, methods of WLL for the treatment of PAP have not been standardized (25). Michaud et al. (29) recommended instilling 1 l of saline into the lavage lung and then to clamp the draining tube for 4–5 min (29). Bonella et al. (4) and Paschen et al. (31) determined the number of lavage cycles by measuring the optical density of each lavage fluid. They applied statistical evaluation to data from a number of WLLs to find the relationship between instilled saline volume and eliminated proteins. Although their approach is fundamentally different from ours, their finding that instilling volume is an important element for determining the amount of eliminated protein was confirmed in this study (Fig. 4*D*). The protocol for WLL used in this study were variable among participating hospitals, and thus time of each cycle varied between 213–630 s, including 120–540 s for the retaining time. As for our mathematical model, the number of cycles and the retaining times did not influence the efficiency of WLL. Based on *Eq. 1*, the amount of proteins eliminated by WLL was dependent on time after the beginning. According to the volume effect demonstrated by in silico simulation in this study (Fig. 4*D*), larger instilled volume appeared to improve the efficiency of lavage. However, the simulation also suggested that the effect is limited within some range of time. Previous studies, however, demonstrated the volume effect (4). In this regard, total eliminated albumin concentration significantly correlated with instilling saline volume in actually measured values in 17 WLLs of the present study with Rho value at 0.69. However, we have to consider the possibility that it also prolonged the duration of instilling and draining time, and thus longer time for each lavage cycle increases the elimi-

nated protein(s). Thus our mathematical model may be useful to predict the amount of eliminated proteins at a certain time point after the beginning of WLL.

In conclusion, we demonstrated that protein transfer in the lung during WLL followed a relatively simple, mathematical model based on diffusion and that this model could be expressed in terms of a number of differential equations. As an exception of the present mathematical model, substances with low molecular weight do not follow the theory. Our study, not only contributes to the design of an efficient regimen for WLL, but also reveals the mechanism of delivery of specific large drug molecules across the air-blood barrier, such as antibody drugs.

APPENDIX

The Effective Alveolar Surface Area

The effective alveolar surface area was calculated from the data for the alveolar volume, V_A according to the following equations: $A_s = 6.4 \cdot 10^3 \cdot V_A^{2/3}$. For a person with 74 kg body wt, both A_s and V_A were reported to be 143 m^2 and 3,338 ml, respectively (10). The effective surface area of the pulmonary capillaries, A_b, was estimated from the following formula (10): $A_b = 0.89 \cdot A_s$. The relationship between alveolar surface area, S_A, and alveolar volume, V_A, depends on the number of alveoli. S_A increases as the number of alveoli increases at a fixed value of V_A. According to Ref. 10, the average lung volume is 4,300 ml, and the average alveolar surface is $(143 \pm 12) \times 10^4$ cm^2 in normal subjects with an average body weight of 74 kg at 19–40 yr of age. Under these conditions, air-space volume density is 0.865 ± 0.013 cm^2/cm^3, and alveolar surface density is 370.6 ± 28.9 cm^2/cm^3.

We set

$$\beta = \frac{S_A^{\frac{1}{2}}}{V_A^{\frac{1}{3}}} \qquad (A1)$$

where, the right side of the equation is an expression for the constant shape parameter, β.

According to the report described above (V_A and S_A in the space V)

$$\frac{S_A}{V} = 370.6 \text{ cm}^2/\text{cm}^3 \qquad (A2)$$

$$S_A = 143 \times 10^4 \text{ cm}^2 \qquad (A3)$$

$$\frac{V_A}{V} = 0.865 \text{ cm}^3/\text{cm}^3 \qquad (A4)$$

From *Eqs. A2* and *A3*,

$$V = 3859 \text{ ml} \qquad (A5)$$

and from *Eqs. A4* and *A5*

$$V_A = 3338 \text{ ml} \qquad (A6)$$

where the anatomical dead space is 4,300 − 3,338 = 962 ml. Introducing *Eqs. A3* and *A6* into *Eq. A1*,

$$\beta = \frac{\sqrt{143 \times 10^4}}{\sqrt[3]{3338}} = 80.02 \qquad (A7)$$

On the basis of *Eq. A1* (note that S_A is in m^2 and V_A is in ml),

$$S_A = \beta^2 \cdot V_A^{\frac{2}{3}} = 6.403 \times 10^3 \cdot V_A^{\frac{2}{3}} \qquad (A8)$$

The value of β may be considered as constant even with a change in V_A in the same subject, as the number of alveoli and the shape do not change, particularly in the supine position.

Method for Optimizing the Transmission Coefficients

A program was written in Visual Basic Application using various coefficients to calculate the theoretical substance concentrations in the lavage aliquots. For explanation, we show an example of simulation used to obtain the best fitting curve shown in Fig. 2*C*. As shown in Appendix Fig. A1*A*, the value for K_s could be determined to be 1.8×10^{-7} cm/s by the least-square method until 3,000 s when K_b was assumed to be 0 cm/s. Next, K_b value was determined to be 5.2×10^{-10} cm/s again by the least-square method by 9,018 s. As shown in Appendix Fig. A1*B*, the theoretical curve appeared closer to the dotted actual measurements. Then K_b was changed to 6.1×10^{-10} cm/s manually, as shown in Appendix Fig. A1*C*: the theoretical curve

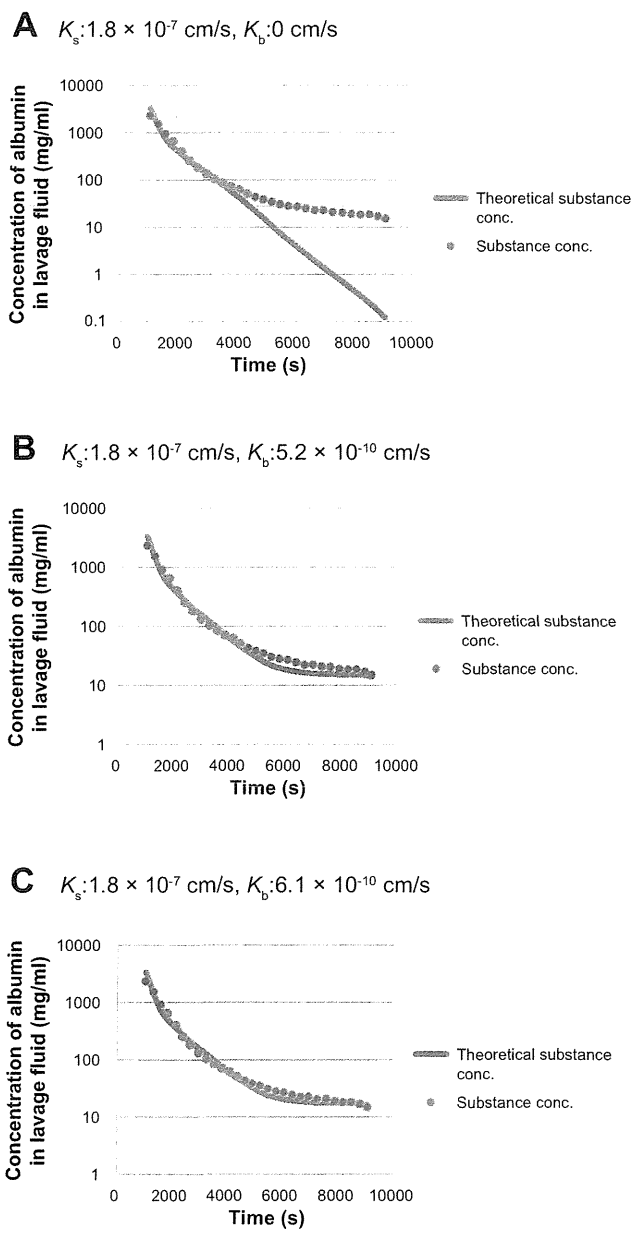

Fig. A1. Example of simulation used to obtain the best fitting curve shown in Fig. 2*C*.

completely coincides with the dotted actual measurements. Therefore, K_b was determined to be 6.1×10^{-10} cm/s.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

K.A., T.M., K.U., R.T., T.I., Y. Inoue, and K.N. conception and design of research; K.A., A.H., Y. Ito, H.W., T.W., Takero Arai, H.M., S.O., R.T., T. Takada, E.Y., T.I., M. Hirose, and Toru Arai performed experiments; K.A., T. Tanaka, T.M., N.K., M. Hayashi, M. Hirose, and K.N. analyzed data; K.A., T. Tanaka, T.M., N.K., R.T., E.Y., H.K., and K.N. interpreted results of experiments; K.A., T. Tanaka, R.T., and K.N. prepared figures; K.A., T. Tanaka, T.M., K.U., T.I., and K.N. drafted manuscript; K.A., T.M., K.U., R.T., Toru Arai, H.K., and K.N. edited and revised manuscript; K.A., T. Tanaka, T.M., N.K., A.H., H.W., Takero Arai, M. Hayashi, H.M., K.U., S.O., R.T., T. Takada, E.Y., T.I., M. Hirose, Toru Arai, Y. Inoue, H.K., and K.N. approved final version of manuscript.

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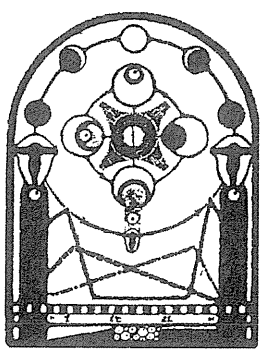

CHEST

Original Research

DIFFUSE LUNG DISEASE

Duration of Benefit in Patients With Autoimmune Pulmonary Alveolar Proteinosis After Inhaled Granulocyte-Macrophage Colony-Stimulating Factor Therapy

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Background: **Treatment of autoimmune pulmonary alveolar proteinosis (aPAP) by subcutaneous injection or inhaled therapy of granulocyte-macrophage colony-stimulating factor (GM-CSF) has been demonstrated to be safe and efficacious in several reports. However, some reports of subcutaneous injection described transient benefit in most instances. The durability of response to inhaled GM-CSF therapy is not well characterized.**

Methods: **To elucidate the risk factors for recurrence of aPAP after GM-CSF inhalation, 35 patients were followed up, monitoring for the use of any additional PAP therapies and disease severity score every 6 months. Physiologic, serologic, and radiologic features of the patients were analyzed for the findings of 30-month observation after the end of inhalation therapy.**

Results: **During the observation, 23 patients remained free from additional treatments, and twelve patients required additional treatments. There were no significant differences in age, sex, symptoms, oxygenation indexes, or anti-GM-CSF antibody levels at the beginning of treatment between the two groups. Baseline vital capacity (% predicted, %VC) were higher among those who required additional treatment ($P < .01$). Those patients not requiring additional treatment maintained the improved disease severity score initially achieved. A significant difference in the time to additional treatment between the high %VC group (%VC ≥ 80.5) and the low %VC group was seen by a Kaplan-Meier analysis and a log-rank test ($P < .0005$).**

Conclusions: **These results demonstrate that inhaled GM-CSF therapy sustained remission of aPAP in more than one-half of cases, and baseline %VC might be a prognostic factor for disease recurrence.**

Trial registry: **ISRCTN Register and JMACCT Clinical Trial Registry; No.: ISRCTN18931678 and JMAIIA00013; URL: http://www.isrctn.org and http://www.jmacct.med.or.jp**

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Abbreviations: A-aDO_2 = alveolar-arterial oxygen difference; Ab = antibody; aPAP = autoimmune pulmonary alveolar proteinosis; AT = additional treatment; BALF = BAL fluid; CEA = carcinoembryonic antigen; DLCO = diffusing capacity of the lung for carbon monoxide; DSS = disease severity score; FR = free from additional treatment; GM-CSF = granulocyte-macrophage colony-stimulating factor; IQR = interquartile range; KL-6 = Krebs von den Lungen-6; LDH = lactate dehydrogenase; PAP = pulmonary alveolar proteinosis; ROC = receiver operating characteristics curve; SP = surfactant protein; VC = vital capacity; WLL = whole-lung lavage

Autoimmune pulmonary alveolar proteinosis (aPAP) is a rare lung disease characterized by the accumulation of surfactant protein (SP), which causes progressive respiratory insufficiency.[1-3] The pathogenesis has been attributed to the excessive production of a neutralizing autoantibody against granulocyte-macrophage colony-stimulating factor (GM-CSF) that impairs GM-CSF-dependent surfactant clearance mediated by

alveolar macrophages.[4-8] On pulmonary function testing, the most common pattern seen is that of a restrictive defect, with a disproportionate reduction in diffusing capacity of the lung for carbon monoxide (DLCO) relative to a modest impairment of vital capacity (VC).[2] The disease is usually treated by whole-lung lavage (WLL), which remains the standard therapy to date.

The first patient successfully treated with subcutaneously administered GM-CSF was reported in 1996.[9] In an international multicenter phase 2 trial study, 14 patients were treated with GM-CSF by subcutaneous injection in escalating doses over a 3-month period, with an overall response rate of 43%.[10,11] A subsequent single-center study of 21 patients with aPAP treated with GM-CSF by subcutaneous administration in escalating doses for 6 to 12 months reported an overall response rate of 48%.[12] Several single cases of subcutaneous GM-CSF therapy have reported similar outcomes.[13,14] However, local reaction at sites of injection and other minor toxicities occurred in 85% of patients receiving subcutaneous GM-CSF.[2]

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GM-CSF inhalation is a promising alternative therapy for aPAP that has been demonstrated to lead to functional, biologic, and radiologic improvement.[15-18] Our national, multicenter phase 2 study revealed that the therapy reduced alveolar-arterial oxygen difference ($A\text{-}aDO_2$) by 12.3 mm Hg in 35 patients who completed the therapy, resulting in 24 responders. No treatment-related side effects were noted. Of importance, our previous phase 2 study showed that there was no significant difference in serologic, physiologic, and CT scan testing, except for serum Krebs von den Lungen-6 (KL-6) levels, between the responders and the nonresponders.[18]

There is limited information regarding the duration of benefit after various treatments of aPAP. In the literature analysis of 55 cases with a therapeutic response to WLL, the median duration of clinical benefit from lavage was 15 months.[2] A phase 2 study of subcutaneous GM-CSF administration demonstrated that 45% of patients required WLL during follow-up observation of 39 ± 17.3 months.[12] In a retrospective analysis of inhaled GM-CSF therapy (250 μg bid), five of 12 patients manifest progressive disease during observation.[17] As the disease progresses very slowly and can fluctuate in some cases, it is necessary to evaluate the prognosis by monitoring prospectively at the same time points after the treatment and by disease severity score as well as the need for additional treatment. The aim of this study was to define the duration of benefit among patients who underwent GM-CSF inhalation therapy.

Materials and Methods

Patients and Protocols

The present study prospectively observed patients who participated in a multicenter phase 2 trial (35 patients, registered as ISRCTN18931678 and JMAIIA00013) of GM-CSF inhalation therapy described previously. In brief, patients who had lung biopsy or cytologic findings diagnostic for pulmonary alveolar proteinosis (PAP), including elevated serum anti-GM-CSF antibody (Ab) levels and no improvement during a 12-week observation period, entered the treatment phase. Recombinant human GM-CSF dissolved in 2 mL of sterile saline was inhaled using an LC-PLUS nebulizer (PARI International). The treatment consisted of high-dose GM-CSF administration (125 μg bid on days 1-8, none on days 9-14; sargramostim) for six repetitions of 2-week cycles, then low-dose administration (125 μg once daily on days 1-4, none on days 5-14) for six repetitions of 2-week cycles (for a total dose of 15 mg). The clinical information including physiologic, serologic, and radiologic features obtained[18] was compared with the results of the following 30-month observation.

Patients were regularly evaluated by their physicians at the network hospitals after the GM-CSF inhalation therapy. The worsening dyspnea was evaluated with pulse oximetry, arterial blood gas analysis, or both in outpatient settings. Disease severity in patients was evaluated using PAP disease severity score (DSS) described previously.[19] Patients underwent additional treatments based on

either of the following criteria: (1) DSS is 3 or 4 and symptoms are worsening or (2) DSS 5, as shown in Figure 1. The consortium office of Niigata University contacted the network hospitals every 6 months with a questionnaire regarding additional treatment and disease severity score of the patient. The follow-up clinical information obtained at each network hospital was entered into a database to be compared with the results of the baseline clinical evaluation of each patient. The data were collected from nine clinical research centers in Japan (Hokkaido University, Tohoku University, Chiba University, Kitasato University, Niigata University, Aichi Medical University, National Hospital Organization Kinki-Chuo Chest Medical Center, National Hospital Organization Yamaguchi-Ube Medical Center, and Nagasaki University Institute of Tropical Medicine).

The study was approved by institutional review board of Niigata University (approval No. NH17-006) and the institutional review boards at all participating centers. Informed consent was obtained from all control subjects. The clinical information obtained by the clinical studies was entered into a database to be compared with the results of the 30-month observation. The study was designed and monitored for data quality and safety by a steering committee composed of the principal investigator at each participating site. The steering committee held a conference twice a year, where the findings of the observation were monitored.

BAL Procedures and GM-CSF Autoantibodies

The steering committee edited a standard operational procedure for BAL, which was followed by all participating institutes and described previously.[18,20] The concentration of GM-CSF autoantibodies in BAL fluid (BALF) or in serum were measured using a sandwich enzyme-linked immunosorbent assay as described previously.[4,21]

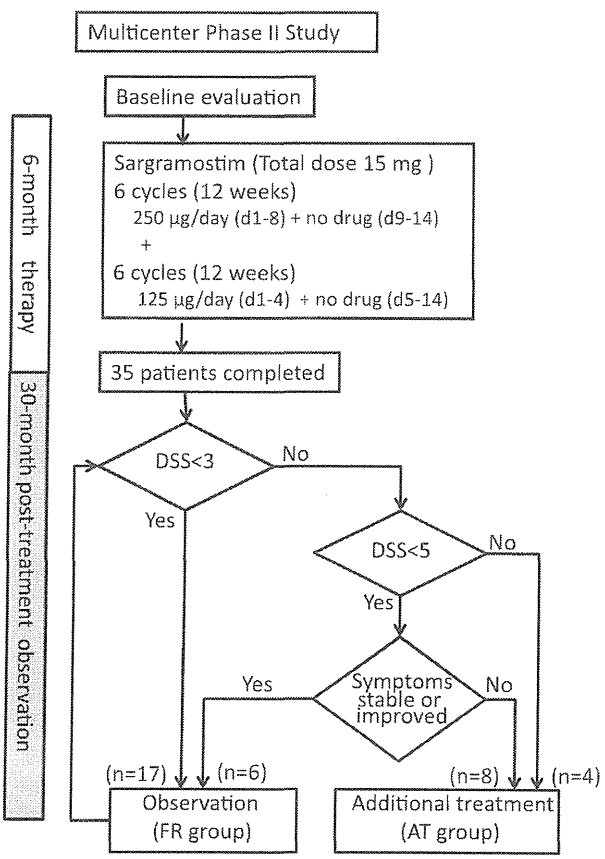

FIGURE 1. Profile of the study cohort. AT = additional treatment; DSS = disease severity score; FR = free from additional treatment.

Statistical Analysis

Numerical results are presented as the mean ± SE or the median and interquartile range (IQR). The χ^2 test was used to evaluate proportions for variables between high and low responders. The paired *t* test was used for comparisons between normally distributed data and the treatment periods. Comparisons of nonparametric data were made using the Wilcoxon signed-rank test. For group comparisons, unpaired *t* tests and Wilcoxon rank-sum tests were used. All *P* values were reported as two-sided. Analysis was performed using JMP software, version 8.0.2 (SAS Institute Inc).

RESULTS

Patient Characteristics and Requirements for Additional Treatments as an Indicator of Recurrence

Demographic data of patients are shown in Table 1. During the 30 months of observation after the end of GM-CSF inhalation, the need for treatments was monitored as an indicator of disease recurrence in each patient. Twenty-three patients were free from additional treatments during 30 months of observation and were designated as FR (free from additional treatment). Twelve patients who required additional treatments, including six patients with recurrence described in our previous study,[18] were designated as AT (additional treatment). Of those, two patients maintained most severe disease (DSS 5) even after the GM-CSF treatment and underwent subsequent WLL. One patient who had dyspnea, cough, and sputum production did not respond to the GM-CSF treatment and underwent subsequent WLL. One patient with cough and dyspnea showed worsening in PaO_2 and cough and had WLL 12 months after the GM-CSF inhalation. The other eight patients with dyspnea showed worsening in PaO_2/oxygen saturation by pulse oximetry (two patients worsened to DSS 5) and underwent additional therapy (e-Fig 1); five underwent additional GM-CSF inhalation treatments, two had WLL, and one patient, a nonresponder, declined WLL and underwent acetylcysteine inhalation, showing much improvement in hypoxia. Median time to additional treatment of the 12 patients was 50.5 weeks, with a range of 8.5 to 117.5 weeks. There was no significant difference in age, sex, symptoms, smoking status, history of dust exposure, arterial blood gas analysis, numbers of responders to GM-CSF inhalation, history of previous lung lavage, and anti-GM-CSF-Ab titer between the FR and AT groups (Table 1). There was no significant difference in disease markers, including baseline levels of PaO_2, $A\text{-}aDO_2$, %VC, %DLCO, CT scan scores, lactate dehydrogenase (LDH), and KL-6 between the patients who underwent WLL (n = 6, AT-WLL group) and those treated with GM-CSF inhalation (n = 5, AT-GM group)

Table 1—*Baseline Clinical Characteristics of Patients Free From Additional Treatment and Those Who Required Additional Treatment After GM-CSF Inhalation*

Characteristic	FR (n = 23)			AT (n = 12)			*P* Value
	No.	%	Median (IQR) or Mean ± SE	No.	%	Median (IQR) or Mean ± SE	
Age, y	23	...	52.5 (48-61)	12	...	52.5 (41.75-58)	.33[a]
Sex			...			...	.54[b]
Female	9	39		6	50		
Male	14	61		6	50		
Responders	17	74	...	7	58	...	.35[b]
Duration of symptoms, mo	23	...	20 (11-61)	12	...	18 (7.75-72)	.78[a]
Symptoms			...			...	
Dyspnea	22	96		12	100		.36[b]
Cough	10	43		7	58		.65[b]
Sputum	8	35		4	33		.71[b]
Smoking status			...			...	.39[b]
Current smoker	8	35		2	17		
Ex-smoker	5	22		2	17		
Never smoked	10	43		8	67		
Dust exposure	22	...	...	11	...	...	.27[b]
Yes	8	36		3	18		
No	14	64		8	82		
Arterial blood gas analysis							
$Paco_2$, Torr[c]	23	...	38.0 ± 0.7	12	...	39.0 ± 0.9	.40[d]
Pao_2, Torr[c]	23	...	60.6 ± 2.1	12	...	56.3 ± 3.0	.25[d]
A-aDO_2, Torr[e]	23	...	43.5 ± 2.4	12	...	46.2 ± 3.3	.51[d]
Disease severity score	23	...	3 (3-4)	12	...	3.5 (3-5)	.58[a]
GM-CSF autoantibody, μg/mL	23	...	22.8 (8.5-33.2)	12	...	23.1 (16.9-34.2)	.94[a]
Previous lung lavage (> 6 mo prior to study)			...			...	.22[b]
Yes	5	22		5	42		
No	18	78		7	58		

Thirty-five patients completed both the high-dose and low-dose period of GM-CSF inhalation therapy. A-aDO_2 = alveolar-arterial oxygen difference; AT = additional treatment; FR = free from additional treatment; GM-CSF = granulocyte-macrophage colony-stimulating factor; IQR = interquartile range (range from the 25th to the 75th percentiles of the distribution).

[a]Calculated using the Wilcoxon rank sum test.

[b]Calculated using the χ^2 test.

[c]Measured with patient in a supine position and breathing room air.

[d]Calculated using Student *t* test.

[e]Calculated using the following equation: A-aDO_2 = (PB − P_{H_2O}) × F_{IO_2} − $Paco_2$/R + {$Paco_2$ × F_{IO_2} × (1 − R)/R} − Pao_2, where PB = barometric pressure measured by local observatories; P_{H2O} = partial pressure of water vapor in inspired air (assumed to be 47 mm Hg); F_{IO_2} = fractional concentration of oxygen in dry gas (assumed to be 0.21); and R = respiratory quotient (assumed to be 0.8).

(e-Table 1). However, changes in A-aDO_2 during the GM-CSF treatment were significantly higher in the AT-GM group.

Association of Clinical Parameters With Requirement for Additional Treatment

There was no significant difference in baseline findings in terms of Pao_2, $Paco_2$, FEV_1, and DLCO between AT and FR groups. Both %VC (% predicted value) and %FVC were higher in the FR group ($P < .01$) (Fig 2A, Table 2, e-Fig 2). There was no correlation between baseline %VC and age ($P = .97$), sex ($P = .41$), baseline Pao_2 ($P = .18$), or baseline %DLCO ($P = .34$). There was no significant difference in high-resolution CT scan scores and serum markers, including LDH, KL-6, carcinoembryonic antigen (CEA), SP-A, and SP-D (Table 2).

As for differential blood cell counts, no significant difference was observed between FR and AT groups, except for numbers of basophils and platelets. The cell density of macrophages in BALF was lower in the FR group than those in the AT group ($P < .05$), whereas lymphocytes were lower in the AT group as compared with the FR group.

Next, clinical parameters at the end of treatment were evaluated. The %DLCO was lower in the AT group than that in the FR group, and serum markers (eg, LDH, KL-6, CEA, SP-D, SP-A) and CT scan scores were higher in the AT group than those in the FR group at the end of treatment ($P < .05$). However, there was no significant difference in A-aDO_2, blood cell counts, and cell differentials in BALF (Table 3). The patients free from additional treatment maintained the improved disease severity score initially achieved (e-Fig 3).

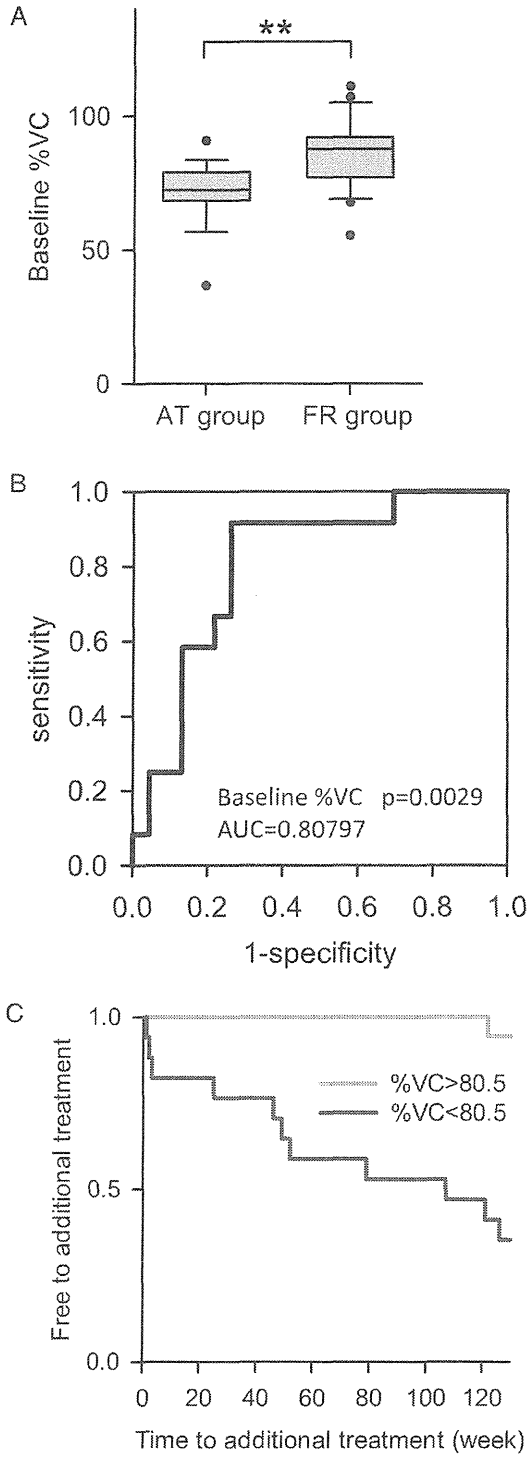

FIGURE 2. The association between VC (% predicted, %VC) and additional treatments during the 30-mo observation period (**$P < .01$). A, Baseline levels of %VC in FR and AT patient groups. B, Receiver operating curve of %VC. C, Kaplan-Meier plot showing patients of the high %VC group (%VC ≥ 80.5) and those of the low %VC group (%VC < 80.5). AUC = area under the receiver operating curve; VC = vital capacity. See Figure 1 legend for expansion of other abbreviations.

Predictive Value of VC for Prognosis After GM-CSF Inhalation

Because only %VC and %FVC differed between FR and AT groups among treatment-related pretreatment factors, the predictive value of parameters for recurrence after GM-CSF inhalation was evaluated using receiver operating characteristics curve (ROC) analysis and Kaplan-Meier analysis of time to additional treatment.

For ROC analysis, the area under the ROC curve was calculated nonparametrically, as proposed by Hanley and McNeil.[22] An additional therapy was defined as a positive indicator for disease recurrence. When the cutoff level of 80.5% was set for %VC, the baseline %VC predicted the additional therapy with a sensitivity of 92% and a specificity of 74% (Fig 2B).

For Kaplan-Meier analysis of time to additional treatment, we divided the patients into two groups, namely the high %VC group (%VC ≥ 80.5) and the low %VC group (%VC < 80.5). A significant difference in the time to additional treatment between the two groups was seen when the whole period of follow-up was compared ($P = .0001$) (Fig 2C). In the univariate Cox proportional analysis of baseline markers, %VC < 80.5% (hazard ratio, 18.42; 95% CI, 3.55-337.68; $P < .0001$) was associated with additional treatment, whereas no correlations were found between additional treatment and age, sex, baseline PaO_2, changes in A-aDO_2, and baseline levels of LDH, KL-6, SP-A, CEA, and anti-GM-CSF-Ab.

Subgroup Analysis: To test whether VC is an independent predictive factor for the time to additional therapy, we did subgroup analyses because of the small number of the AT patients. The patients were divided into two groups of an upper one-half and a lower one-half regarding age; sex; baseline PaO_2; change in A-aDO_2; baseline levels of LDH, KL-6, SP-A, CEA; and anti-GM-CSF-Ab. In these subgroups, a significant difference in the time to additional treatment between the high %VC group (%VC ≥ 80.5) and the low %VC group (%VC < 80.5) was still evident, suggesting that VC might be an independent factor predicting the time to additional therapy (e-Fig 4).

Time Course of Autoantibody Levels: In our previous reports, serum levels of anti-GM-CSF-Ab levels did not change during treatment.[16] To study longitudinal changes of serum levels of anti-GM-CSF-Ab after the inhaled GM-CSF therapy, serum samples were collected for anti-GM-CSF-Ab testing as an optional evaluation after the 30-month observation period. The serum levels were unchanged during the observation period except for three cases (e-Fig 5). In two cases, the serum levels increased by > 100 μg/mL, and one case required an additional treatment, whereas

Table 2—*Baseline Pulmonary Function, Radiologic Appearance, Serum Biomarkers, Hematologic Indexes, and BALF Cell Findings in Patients With PAP in FR and AT Groups Before GM-CSF Inhalation Treatment*

Measure	FR		AT		*P* Value
	No.	Mean ± SE or Median (IQR)	No.	Mean ± SE or Median (IQR)	
Pulmonary function					
VC, % predicted	23	85.9 ± 2.7	12	71.6 ± 3.8	.0045[a]
FVC, % predicted	23	85.3 ± 2.8	12	71.4 ± 3.9	.0064[a]
FEV_1/FVC	23	87.1 ± 2.0	12	84.9 ± 2.7	.51[a]
DLCO, % predicted	23	57.0 ± 3.4	10	46.0 ± 5.1	.082[a]
HRCT scan scores[b]					
Upper lung region	23	3 (2-5)	12	4.5 (2-5)	.12[c]
Middle lung region	23	4 (3-5)	11	4 (3-5)	.38[c]
Lower lung region	23	4 (3-5)	12	5 (4-5)	.36[c]
Serum biomarkers of PAP					
LDH, IU/L	23	287 ± 19	12	325 ± 26	.26[a]
CEA, ng/mL	23	6.2 ± 1.0	12	8.0 ± 1.4	.30[a]
KL-6, U/L	23	10,038 ± 1,531	12	9,434 ± 2,120	.81[a]
SP-A, ng/mL	23	127 ± 15	12	153 ± 20	.29[a]
SP-D, ng/mL	23	227 ± 25	12	290 ± 34	.14[a]
Hematologic indexes					
WBC count, cells/μL	23	5,608 ± 267	12	6,358 ± 370	.11[a]
Neutrophils, cells/μL	22	3,428 ± 200	12	3,596 ± 271	.62[a]
Monocytes, cells/μL	22	344 ± 21	12	396 ± 28	.15[a]
Lymphocytes, cells/μL	22	1,730 ± 147	12	2,122 ± 198	.12[a]
Eosinophils, cells/μL	22	107 ± 28	12	199 ± 38	.058[a]
Basophils, cells/μL	22	18.3 ± 4.3	12	45.3 ± 5.9	.0008[a]
Hemoglobin, g/dL	23	15.4 ± 0.3	12	14.4 ± 0.4	.058[a]
Platelets, $\times 10^3$ cells/μL	23	224 ± 9.1	11	271 ± 13	.0046[a]
BALF cell classification, %					
Alveolar macrophages	17	63 ± 3.6	5	38 ± 6.7	.0036[a]
Neutrophils	17	5.2 ± 1.5	5	10.8 ± 2.7	.082[a]
Eosinophils	17	0.84 ± 0.32	5	0.40 ± 0.60	.52[a]
Lymphocytes	17	31.2 ± 3.8	5	50.4 ± 7.1	.027[a]

BALF = BAL fluid; CEA = carcinoembryonic antigen; DLCO = diffusing capacity of the lung for carbon monoxide; HRCT = high-resolution CT; KL-6 = Krebs von den Lungen-6; LDH = lactate dehydrogenase; PAP = pulmonary alveolar proteinosis; SP = surfactant protein; VC = vital capacity. See Table 1 legend for expansion of other abbreviations.

[a]Calculated using Student *t* test.

[b]Described previously.[18] left lung.

[c]Calculated using the Wilcoxon rank sum test.

the others did not. In the third case, the serum levels decreased to 0.47 μg/mL, and additional treatments were not required.

Discussion

In the present study we have prospectively analyzed, for the time to our knowledge, the requirements of additional therapy and disease severity scores in 35 patients who completed GM-CSF inhalation therapy. The results demonstrate that 23 patients were free from administration of additional treatment during the 30-month observation period, indicating the enduring nature of the therapy. VC could be a useful predictive parameter for the recurrence of disease after GM-CSF therapy. This study contributes to the promotion of GM-CSF inhalation for initial therapy of aPAP.

WLL remains the standard of care today. A retrospective analysis of 231 cases found clinically significant improvement in Pao_2, FEV_1, VC, and DLCO and reported that the median duration of clinical benefit from lavage was 15 months.[2] In a report of 21 patients with PAP who underwent WLL in an experienced center, >70% of patients remained free from recurrent PAP during 7-year observation.[23] In our study, the median time to application of additional therapy was 30 months after GM-CSF therapy, suggesting the effects of GM-CSF inhalation may be comparable to those of WLL. Notably, the difference in changes in $A\text{-}aDO_2$ during the GM-CSF treatment between the AT-WLL group patients and the AT-GM group patients suggests that nonresponders to the first GM-CSF treatment might be likely to undergo WLL when disease recurred.

In a single-center, phase 2 study for subcutaneous administration of GM-CSF for PAP, Venkateshiah et al[12]

Table 3—*Pulmonary Function, Radiologic Appearance, Serum Biomarkers, Hematologic Indexes, and BALF Cell Findings in Patients With PAP in FR and AT Groups at the End of GM-CSF Inhalation Treatment and Before the 30-Mo Observation*

Measure	FR		AT		*P* Value
	No.	Mean ± SE or Median (IQR)	No.	Mean ± SE or Median (IQR)	
Pulmonary function					
VC, % predicted	23	93.4 ± 3.0	12	74.2 ± 4.2	.0007[a]
FVC, % predicted	23	80.5 ± 3.3	12	72.2 ± 4.5	.0025[a]
FEV_1/FVC	23	85.6 ± 1.6	12	84.7 ± 2.2	.73[a]
DLCO, % predicted	23	68.4 ± 3.4	11	46.8 ± 4.7	.0006[a]
HRCT scan scores[b]					
Upper lung region	23	2 (2-3)	12	3.5 (2-4)	.036[c]
Middle lung region	23	3 (2-3)	12	4 (2.25-4.75)	.023[c]
Lower lung region	23	2 (2-3)	12	4 (2.25-5)	.0039[c]
Serum biomarkers of PAP					
LDH, IU/L	23	242 ± 13	12	308 ± 18	.0064[a]
CEA, ng/mL	23	2.7 ± 0.6	12	5.7 ± 0.8	.0075[a]
KL-6, U/L	23	3,675 ± 735	12	6,565 ± 1,017	.028[a]
SP-A, ng/mL	23	80 ± 12	12	131 ± 16	.015[a]
SP-D, ng/mL	23	170 ± 34	12	304 ± 47	.027[a]
Hematologic indexes					
WBC count, cells/μL	23	5,213 ± 306	12	5,797 ± 424	.27[a]
Neutrophils, cells/μL	22	2,961 ± 205	12	3,026 ± 277	.85[a]
Monocytes, cells/μL	22	320 ± 30	12	338 ± 41	.74[a]
Lymphocytes, cells/μL	22	1,755 ± 131	12	2,153 ± 177	.080[a]
Eosinophils, cells/μL	22	145 ± 40	12	233 ± 55	.20[a]
Basophils, cells/μL	22	27.4 ± 5.9	12	43.7 ± 8.4	.12[a]
Hemoglobin, g/dL	23	14.8 ± 1.3	12	14.4 ± 1.4	.52[a]
Platelets, $\times 10^3$ cells/μL	23	214 ± 9.0	12	235 ± 12	.17[a]
BALF cell classification, %					
Alveolar macrophages	13	67 ± 4.1	5	58 ± 6.7	.28[a]
Neutrophils	13	6.6 ± 2.2	5	7.4 ± 3.5	.86[a]
Eosinophils	13	0.90 ± 0.46	5	0.82 ± 0.75	.93[a]
Lymphocytes	13	25.6 ± 4.8	5	33.2 ± 7.7	.41[a]

See Table 1 and 2 legends for expansion of abbreviations.
[a]Calculated using Student *t* test.
[b]Described previously.[15] left lung.
[c]Calculated using the Wilcoxon's rank sum test.

reported that nine of 21 patients (43%) required WLL. In a retrospective study of 12 patients who underwent aerosolized GM-CSF therapy, Wylam et al[17] reported that five of 11 responders had recurrence of disease. In four of five patients, the mean time to relapse was 6.3 months and ranged from 5.5 to 12 months.[15] It is notable that the dose of GM-CSF used in their study was twice that used in our study, although the prognosis of our cases was comparable to that of their study.

PAP is often described as a lung disorder with restrictive physiology. In the present study, 18 of 35 patients were in the normal range (≤ 80) in %FVC, whereas the other 17 patients were mildly to moderately restricted, which was comparable to previous studies.[24] Seymour et al[25] investigated 14 patients who underwent subcutaneous GM-CSF administration and suggested that higher VC before treatment was one marker to define responsiveness to GM-CSF therapy. In the present study, VC did not correlate with responsiveness to GM-CSF therapy, but it showed significant association with the requirement for additional treatment. Although limited by the small number of cases, the subgroup analyses suggested that VC is an independent factor from age, sex, baseline Pao_2, change in $A\text{-}aDO_2$, and baseline levels of serum markers, including anti-GM-CSF-Ab. However, there is a possibility that some clinical variables might be intrinsically related to VC. The physicians' decision for retreatment might be influenced by such clinical markers. Notably, a recent study of a series of patients with PAP followed in a reference center reported that the need for lavage was significantly associated with FVC.[26]

Reduction of VC might be due to two different factors: accumulation of surfactant-derived materials in the alveolar space and fibrotic changes of lung tissue. In a study of a quantitative CT scan analysis of patients with PAP who underwent WLL and showed improvements in %DLCO and %FVC, Perez et al[27] demonstrated that there was a reduction in lung weight

following lavage, which correlated with the dry weight of the lavage effluent. The study demonstrated a shift in the regional lung inflation toward more inflated lung with a corresponding increase in the mean lung inflation. Surfactant accumulation might be associated with an elevated ventilation-perfusion mismatch and disproportionately impaired DLCO in patients with aPAP.[2] Seymour et al[25] demonstrated serum levels of SP-A correlated with VC in 14 patients at baseline. The present study also showed that serum levels of SP-A correlated with VC at baseline as well as after treatment. However, requirement of additional therapy was not significantly associated with SP-A at baseline. Surfactant materials might be easily redistributed in alveolar spaces and may not be related to the impairment of lung tissue that might lead to additional treatment.

The other factor, fibrotic changes of lung tissue, might be maintained even after GM-CSF therapy or WLL. Pulmonary fibrosis has been reported to be associated with PAP, and exposure to oxygen or repeated WLL have been suggested as potential contributors to fibrosis. Although irreversible scarring of the lung is rarely associated with PAP, a small fraction of patients with PAP demonstrated substantially impaired %VC and rather poor prognosis. To investigate this possibility, two radiologists reevaluated baseline CT scans of 32 of the 35 participants for findings other than PAP without knowing the study results regarding responsiveness and prognosis of the GM-CSF inhalation. They only pointed out traction bronchiectasis in one patient (responder, FR), bronchiectasis in one patient (responder, FR), and multiple bullae in one patient (responder, AT). Thus, we failed to find any significant association between fibrotic change in CT scan and requirement of additional treatments. In the present study, the mean %VC levels of patients in the FR group improved from 85.9% to 93.4%, whereas those of patients in the AT group changed from 71.6% to 74.2%. The difference in improvement between the groups might be associated with the balance of surfactant accumulation and lung fibrosis in the lungs of patients.

For future studies, it would be useful to explore novel treatment regimens for patients with moderately impaired VC. As shown in this study, inhaled GM-CSF therapy did not change serum levels of anti-GM-CSF-Ab. However, the BALF titers of anti-GM-CSF-Ab were reduced in responders, which was likely due to the improved clearance in alveolar spaces. The future treatments might include a combination of GM-CSF inhalation with WLL to improve the environment of airway/alveolar spaces or with administration of rituximab to reduce the systemic production of anti-GM-CSF-Ab.

In conclusion, this study demonstrated that VC might be clinically useful in predicting the need for additional therapy in patients with aPAP who were treated with inhaled GM-CSF therapy. We believe this study contributes to improving the quality of life and treatments for patients with aPAP.

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Dr Tazawa: contributed to study conception and design, collection and analysis of data, and writing of the manuscript.
Dr Inoue: contributed to study design and assistance with the writing of the manuscript.
Dr Arai: contributed to data collection, manuscript preparation, and revision of the manuscript.
Dr Takada: contributed to data collection, manuscript preparation, and revision of the manuscript.
Dr Kasahara: contributed to manuscript preparation, critical patient samples and data, and revision of the manuscript.
Dr Hojo: contributed to data collection, manuscript preparation, and revision of the manuscript.
Dr Ohkouchi: contributed to data collection and analysis, manuscript preparation, and revision of the manuscript.
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Dr Nei: contributed to manuscript preparation, performance of research assays, and revision of the manuscript.
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Additional information: The e-Figures and e-Table can be found in the "Supplemental Materials" area of the online article.

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RESEARCH ARTICLE Open Access

Secondary pulmonary alveolar proteinosis complicating myelodysplastic syndrome results in worsening of prognosis: a retrospective cohort study in Japan

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Abstract

Background: Secondary pulmonary alveolar proteinosis (sPAP) is a very rare lung disorder comprising approximately 10% of cases of acquired PAP. Hematological disorders are the most common underlying conditions of sPAP, of which 74% of cases demonstrate myelodysplastic syndrome (MDS). However, the impact of sPAP on the prognosis of underlying MDS remains unknown. The purpose of this study was to evaluate whether development of sPAP worsens the prognosis of MDS.

Methods: Thirty-one cases of sPAP and underlying MDS were retrospectively classified into mild and severe cases consisting of very low-/low-risk groups and intermediate-/high-/very high-risk groups at the time of diagnosis of MDS, according to the prognostic scoring system based on the World Health Organization classification. Next, we compared the characteristics, disease duration, cumulative survival, and prognostic factors of the groups.

Results: In contrast to previous reports on the prognosis of MDS, we found that the cumulative survival probability for mild MDS patients was similar to that in severe MDS patients. This is likely due to the poor prognosis of patients with mild MDS, whose 2-year survival rate was 46.2%. Notably, 75% and 62.5% of patients who died developed fatal infectious diseases and exacerbation of PAP, respectively, suggesting that the progression of PAP *per se* and/or PAP-associated infection contributed to poor prognosis. The use of corticosteroid therapy and a diffusing capacity of the lung for carbon monoxide of less than 44% were predictive of poor prognosis.

Conclusion: Development of sPAP during the course of MDS may be an important adverse risk factor in prognosis of patients with mild MDS.

Keywords: Proteinosis, Myelodysplastic syndrome, GM-CSF, WPSS, Secondary pulmonary alveolar proteinosis, MDS, PAP, Refractory anemia

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