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Research Paper

Comparison of Albumin Uptake in Rat Alveolar Type II and Type I-like Epithelial Cells in Primary Culture

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Purpose. To elucidate and compare the activity and mechanism of albumin uptake in primary cultured alveolar type II and type I-like epithelial cells.
Materials and methods. Type II epithelial cells isolated from rat lungs were cultured for 2 days at 5×10^6 cells/35-mm dish or for 6 days at 2×10^6 cells/35-mm dish. The mRNA expression of marker genes and FITC-albumin uptake were examined.
Results. The cells cultured for 2 days exhibited cuboidal type II epithelial morphology with lamellar bodies inside the cells, while the cells cultured for 6 days exhibited squamous type I epithelial morphology. These morphological characteristics were consistent with the changes in mRNA expression pattern of marker genes. FITC-albumin uptake in both cells was temperature-dependent and was inhibited by metabolic inhibitors and bafilomycin A_1. The rate of uptake was much higher in type II cells than type I-like cells. In both cells, FITC-albumin uptake was inhibited by clathrin mediated-endocytosis inhibitors, but not by caveolae mediated-endocytosis inhibitors.
Conclusions. These findings indicate that albumin in alveolar lining fluid is internalized into type II and type I epithelial cells via clathrin-mediated endocytosis, and the rate of albumin uptake is higher in type II cells than type I cells.

KEY WORDS: albumin; alveolar epithelial cell; endocytosis; primary culture; transdifferentiation.

INTRODUCTION

The alveolar epithelium is comprised of two morphologically and functionally different cell types, type I and type II. The squamous type I epithelial cells cover more than 90% of the alveolar surface area and are essential for normal gas exchange (1). On the other hand, the cuboidal type II cells outnumber type I cells, although the type II cells occupy only 5–10% of the surface area (1). Type II cells have multifunctions including surfactant production and secretion (2). In addition, type II cells serve as progenitor cells of type I cells, and transdifferentiate into type I cells to repair the epithelium when it is injured (2). The alveolar epithelial surface is covered by the thin fluid layer which contains various proteins such as albumin, immunoglobulin G, and transferrin. The albumin concentration in the alveolar lining fluid is extremely low compared with that in the plasma under normal condition (<10% of plasma), but reaches around the plasma level in lung injury pulmonary edema (1,3). Albumin clearance from the alveolar space is essential for the recovery from pulmonary edema. Understanding the detailed mechanisms of alveolar albumin clearance may therefore provide novel therapeutic approaches for pulmonary edema. In addition, it may provide important information for the development of new pulmonary delivery systems of protein drugs into the systemic circulation.

Protein transport across alveolar epithelia has been widely investigated (3,4). The most possible mechanism of protein transport, including that of albumin, is endocytosis (transcytosis) in alveolar epithelial cells. It has been reported that gp60 albumin-binding protein and caveolae are involved in albumin transport in primary cultured type II alveolar epithelial cells isolated from rats (5). On the other hand, we have recently reported that the uptake of albumin is mediated by clathrin-mediated endocytosis, but not by caveolae-mediated endocytosis, using alveolar type II epithelial cell line RLE-6TN (6). Thus, the uptake mechanism of

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ABBREVIATIONS: ANOVA, analysis of variance; BAF, bafilomycin A_1; CINC-1, chemokine-induced neutrophilic chemoattractant-1; CPZ, chlorpromazine; DMEM/F-12, Dulbecco's modified Eagle medium-nutrient mixture F-12 (1:1); DMSO, dimethyl sulfoxide; DNP, 2,4-dinitrophenol; 2DOG, 2-deoxy-D-glucose; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; FITC-albumin, fluorescein isothiocyanate-labeled bovine serum albumin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IGFBP6, insulin-like growth factor binding protein 6; IND, indomethacin; MCD, methyl-β-cyclodextrin; mdr1a, multidrug resistance protein 1a; NaN_3, sodium azide; NYS, nystatin; PAO, phenylarsine oxide; PBS, phosphate-buffered saline; RTI40, rat type I cell 40-kDa protein; RT-PCR, reverse transcription-polymerase chain reaction; SDS, sodium dodecyl sulfate; SP-B, surfactant protein B.

albumin in alveolar type II cells is still controversial. In addition, it is possible that type I epithelial cells, which occupy more than 90% of the alveolar surface area, may play an important role in overall albumin clearance from the alveolar surface. Bur *et al.* (7) examined albumin transport across human alveolar type I-like cells, and showed that the flux of albumin was strongly direction-dependent with apical-to-basolateral flux being higher than the reverse direction. However, there is few study in which the activity and mechanism of albumin transport is directly compared between alveolar type II and type I cells.

In past 30 years, various isolation methods of alveolar epithelial type II cells have been reported, and cell yields and viabilities have been improved (8). Isolated alveolar epithelial type II cells reportedly undergo transdifferentiation into type I-like cells that exhibit morphologic characteristics of type I cells (9,10). In this study, we attempted to examine and compare the activity and mechanism of albumin transport in type II and type I cells, using rat primary cultured alveolar epithelial type II cells and transdifferentiated type I-like cells. For this purpose, we firstly optimized culture conditions either to maintain type II cells or to facilitate the transdifferentiation into type I-like cells. Then, the transport of FITC-albumin was investigated and compared between these two types of cells.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle medium-nutrient mixture F-12 (1:1; DMEM/F-12), penicillin-streptomycin, fungizone, and trypsin (1:250, powder) were purchased from Invitrogen Corp. (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Daiichi Pure Chemicals (Tokyo, Japan), and Percoll was from GE Healthcare Bio-Science Corp. (Piscataway, NJ). Deoxyribonuclease I from bovine pancreas, FITC-labeled bovine serum albumin (FITC-albumin), calf thymus DNA (Type I, fibrous), bafilomycin A_1, phenylarsine oxide, indomethacin, and nystatin were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Bisbenzimide H 33258 fluorochrome trihydrochloride (Hoechst 33258), sodium azide (NaN_3), 2,4-dinitrophenol, and chlorpromazine were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). 2-Deoxy-D-glucose was purchased from Kanto Chemical Co.,Inc. (Tokyo, Japan). Tylosin and methyl-β-cyclodextrin were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Nucleic acid purification kit (Mag Extractor-RNA), RT-PCR kit (Rever Tra Dash), and SYBR® Green Realtime PCR Master Mix were purchased from TOYOBO CO., LTD. (Osaka, Japan). All other chemicals were of the highest grade commercially available.

Isolation, Purification and Culture of Rat Alveolar Type II Epithelial Cells

Experiments with animals were performed in accordance with the "Guide for Animal Experimentation" from Hiroshima University and the Committee of Research Facilities for Laboratory Animal Sciences, Hiroshima University. Alveolar type II epithelial cells were isolated from specific pathogen-free Sprague–Dawley male rats weighing 120–200 g as previously reported with a slight modification (11). Rats were anesthetized by intraperitoneal injection of pentobarbital (30 mg/kg) and given heparin (500 U/kg) via jugular vein. A tracheotomy was performed and a cannula (o.d. 1.52 mm) was fixed in the trachea, and then inferior aorta and vena cava were cut. The lungs were perfused with 0.15 M NaCl using a 50-ml syringe fitted with a 23-gauge needle through the right ventricle until the lungs were perfectly turned to white. The lungs were excised with trachea, and were lavaged six times with 0.15 M NaCl through tracheal cannula to remove macrophages, and then completely filled with 0.25% trypsin in solution B (133 mM NaCl, 5.2 mM KCl, 1.89 mM $CaCl_2$, 1.29 mM $MgSO_4$, 2.59 mM phosphate buffer, 10.3 mM HEPES buffer, 1 mg/ml glucose, pH 7.4). After discarding the solution, the lungs were filled again with trypsin solution and suspended in the bath containing 0.15 M NaCl and continuously topped up with trypsin solution via 50-ml syringe attached to tracheal cannula for 30 min at 37°C. Usually, a total of 100 ml trypsin solution was used. The trachea, main bronchi and large airways were discarded and the lungs were blotted onto the paper. Each lung was minced with sharp scissors and choppers into 1–2 mm cubes in a petri dish. FBS (5 ml) was added to stop trypsin reaction and the tissue suspension was prepared to a final volume of 20 ml by solution A (solution B without calcium and magnesium salts) containing deoxyribonuclease I (250 μg/ml). The minced lung tissue was transferred to a plastic 250-ml Erlenmeyer flask and shaken in a water bath at 130 strokes/min for 4 min at 37°C. The suspension was filtered through two and then four layers of cotton gauze followed by 150- and 15-μm nylon mesh into a 50-ml conical plastic centrifuge tube on ice.

The cell suspension was layered on a sterile discontinuous Percoll gradient, the composition of which was as follows: heavy density (1.089 g/ml; 1 ml 10×solution A containing 5% FBS, 6.49 ml Percoll, and 2.51 ml distilled water), light density (1.040 g/ml; 1 ml 10×solution A containing 5% FBS, 2.72 ml Percoll, 6.23 ml distilled water, and 50 μl phenol red). Final concentration of phenol red in the light density gradient was 0.001% (w/v). The preparation was centrifuged at 250 *g* for 25 min at 4°C. The layer containing type II cells at the interface of the two gradients was collected and resuspended in solution A containing deoxyribonuclease I (50 μg/ml) and tylosin (120 μg/ml) to a final volume of 40 ml, followed by centrifugation at 200 *g* for 5 min at 4°C. The cells obtained were suspended in DMEM/F-12 containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml fungizone at 37°C. The yield of type II cells per rat was approximately 30×10^6 cells and the cell viability estimated by trypan blue exclusion was more than 90%. The cells were grown at 37°C in 5% CO_2/95% air and culture medium was replaced every 2 days. For the experiments, the cells were seeded 5×10^6 cells/35-mm culture dish and used at day 2, or seeded 2×10^6 cells/35-mm culture dish and used at day 6.

Evaluation of mRNA Expression Pattern in Alveolar Epithelial Cells

Total RNA was extracted from the cells at day 2 or day 6 with Mag Extractor-RNA (TOYOBO, Osaka, Japan) as described previously (6). The total RNA (0.55 μg, *caveolin-*

1; 0.1 μg, others) in a final volume of 10 μl was reverse-transcribed into cDNA by using Rever Tra Ace (TOYOBO, Osaka, Japan). Real-time PCR was performed on a BioFlux LineGene system (TOYOBO, Osaka, Japan) using SYBR Green. Reaction mixtures consisted of 2 μl cDNA, 10 μl SYBR Green and primers in a final volume of 20 μl. The PCR conditions were; initial denaturation in one cycle of 1 min at 95°C followed by 40 cycles of 5 s at 95°C (denaturation), 5 s at 60°C (annealing) and 15 s at 72°C (extension). After the cycles, a melting curve was checked to confirm the single product. The primers used in the present study were shown in Table I. The expression level of each mRNA was normalized by that of *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), a housekeeping gene.

Uptake of FITC-albumin

Uptake experiments were carried out as described previously (6) at day 2 or day 6 after seeding. Cells were washed twice with phosphate-buffered saline (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.1 mM $CaCl_2$, and 0.5 mM $MgCl_2$, pH 7.4; PBS buffer) supplemented with 5 mM D-glucose (PBS-G buffer) and preincubated in the absence or presence of the inhibitor for 10 min at 37°C. Preincubation conditions in the presence of the inhibitor were as follows: 10 mM NaN_3 plus 5 mM 2-deoxy-D-glucose or 1 mM 2,4-dinitrophenol in PBS buffer for 10 min, 100 nM bafilomycin A_1 in PBS-G buffer containing 0.1% dimethyl sulfoxide (DMSO) for 30 min, 3 μM phenylarsine oxide or 54 μM nystatin in PBS-G buffer containing 0.5% DMSO for 10 min, 84.4 μM chlorpromazine, 100 μM indomethacin or 5 mM methyl-β-cyclodextrin in PBS-G buffer for 10 min. The same vehicles were used for each control experiment. After preincubation, FITC-albumin (50 μg/ml) with or without the inhibitor was added, and the cells were incubated for 60 min at 37°C or 4°C. Bafilomycin A_1 and phenylarsine oxide were added only during preincubation. The treatment for hypertonicity and potassium depletion was conducted as reported previously (6,12). The control buffer used for these experiments consisted of 140 mM NaCl, 10 mM KCl, 20 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mg/ml D-glucose (pH 7.4). For the hypertonicity experiment, 450 mM sucrose was added to the control buffer and the osmolarity was measured to be 920 mOsm. For the potassium depletion experiment, KCl was omitted from the control buffer. After incubation, cells were rinsed three times with ice-cold PBS buffer followed by scraping with a rubber policeman. Following the wash by centrifugation at 9,838 *g* for 3 min at 4°C twice, the pellet was solubilized in 0.1% Triton X-100 in PBS buffer without $CaCl_2$ and $MgCl_2$ for 30 min at room temperature and centrifuged at 5,600 *g* for 3 min. The supernatant was used for fluorescence and protein assays. The fluorescence of FITC-albumin was measured using a Hitachi fluorescence spectrophotometer F-3000 (Tokyo, Japan) at excitation and emission wavelengths of 500 and 520 nm, respectively. Protein content was determined by the Lowry method (13) with bovine serum albumin as the standard.

Table I. Primer Sequences for Real-Time PCR

Gene Name	Accession Number	Sequence (F: forward R: reverse)	Position of Amplified DNA (bp)	Product Size (bp)
GAPDH	NM_017008	F: AGCCCAGAACAT CATCCCTG R: CACCACCTTCTT GATGTCATC	676–856	181
RTI40	U07797	F: GCCATCGGTGCGC TAGAAGATGATCTT R: GTGATCGTGGTCG GAGGTTCCTGAGGT	306–500	195
IGFBP6	BC099742	F: CCGCAGACACTTGG ATTCAGT R: TTGCTCCGCCTCTG AAGAC	458–519	62
mdr1a	AF257746	F: GATGGAATTGATAA TGTGGACA R: GTACGTCGTCATC CAGAGT	1997–2138	142
caveolin-1	Z46614	F: CAGCATGTCTGGGG GTAAAT R: TGCTTCTCATTCAC CTCGTCT	25–147	123
SP-B	BC072466	F: GCTGAGCGTTACAC AGTACTTCTAC R: ACCAGGCCACAGA CTAGCT	723–796	74
CINC-1	D11444	F: GGGTGTCCCCAAGT AATGGA R: CAGAAGCCAGCGT TCACCA	312–383	72

DNA Content in Alveolar Epithelial Cells

DNA contents in isolated alveolar type II cells and in the cells cultured for 2 or 6 days were measured as previously reported with a slight modification (14). Briefly, the cells cultured for 2 or 6 days after seeding were washed twice with ice-cold 0.9% NaCl, and were scraped in ethylenediaminetetraacetic acid (EDTA) solution (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1.1 mM D-glucose and 0.613 mM EDTA, pH 7.4) at 37°C with a rubber policeman. The cell suspension obtained and the freshly isolated alveolar type II cells of known number were centrifuged at 1,000 *g* for 10 min at room temperature. Following the wash by centrifugation at 1,000 *g* for 10 min at room temperature twice, the pellet was solubilized in 1% sodium dodecyl sulfate (SDS). The solubilized cell solution was diluted to a final SDS concentration of 0.01% with standard saline citrate buffer (154 mM NaCl, 15 mM Na_3-citrate, pH 7.0). Hoechst 33258 in standard saline citrate buffer (0.8 μg/ml, 1 ml) was added to the solution (2 ml), and after incubation for 10 min at room temperature in the dark, the fluorescence of Hoechst 33258 coupled with DNA was measured using a Hitachi fluorescence spectrophotometer F-3000 (Tokyo, Japan) at excitation and emission wavelengths of 360 and 450 nm, respectively. The calf thymus DNA was used as the standard.

Confocal Laser Scanning Microscopy

Alveolar type II cells isolated from rat lung were grown on 35-mm glass bottom culture dishes for 2 days. The cells were incubated with FITC-albumin (50 μg/ml) for 60 min at 37 or 4°C as described above. After the cells were washed with ice-cold PBS buffer three times for 5 min each, fluorescence in the cells was observed by confocal laser scanning microscopy (LSM5 Pascal, Carl ZEISS).

Statistical Analysis

Data are expressed as the mean±SE. Statistical analysis was performed by unpaired Student's *t* test or by one-way ANOVA followed by the Dunnett's test for multiple comparisons.

RESULTS

Characteristics of Rat Primary Cultured Alveolar Epithelial Cells

As a first step to determine the culture conditions with which the characteristics of either type II or type I-like epithelial cells can be studied, isolated type II cells were seeded at various densities (1–8×10^6 cells/35-mm dish) and cultured for various days (1–8 days). The essential criteria we employed were morphological features of the culture cells (cuboidal cells containing lamellar bodies for type II cells, and squamous cells without lamellar bodies for type I-like cells), which were observed by phase-contrast microscopy. We also measured alkaline phosphatase activity, which is one of characteristic markers of alveolar type II cells, as described previously (6). When isolated type II cells were seeded at a density of four ×10^6 cells/35-mm dish, the activities were 27.8±1.8, 21.7±2.1, 12.0±0.1, 15.0±3.6, 10.2±0.8, 8.3±2.5 nmol/min/mg protein (*n*=3–5) at day 0 (isolated cells before seeding), 2, 3, 4, 5, and 6, respectively, indicating that type II phenotype would be lost fairly quickly, especially after 3 days in culture. Based on these preliminary experiments, two culture conditions were selected. Fig. 1a shows a phase-contrast micrograph of rat alveolar epithelial cells cultured for 2 days after seeding the isolated type II cells at a density of 5×10^6 cells/35-mm dish. The cells grew to be almost confluent, and were cuboidal in shape. In most of the cells, lamellar bodies, a characteristic feature of alveolar type II cells, were clearly observed inside the cells. Fig. 1b shows the morphology of the cells cultured for 6 days after seeding 2×10^6 cells/35-mm dish. The cells became flattened and were squamous in shape, and possessed practically no lamellar bodies, as observed in the lung type I cells. The expression pattern of marker gene mRNAs for type I and type II cells was examined using real-time PCR analysis in order to further characterize these cells. As shown in Table II, the expression of mRNAs reported as markers for type I cells

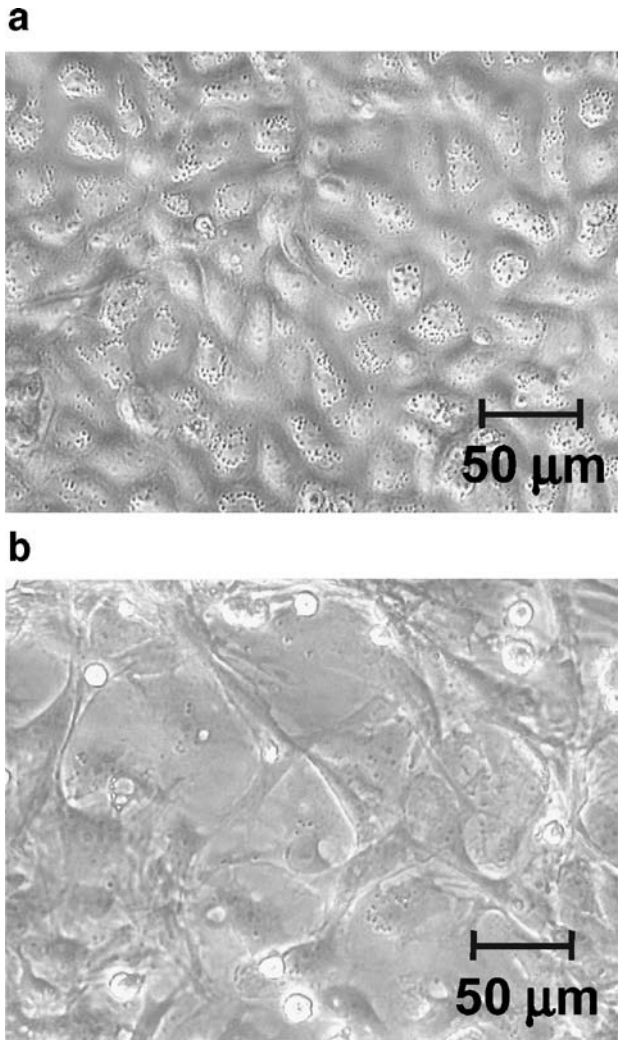

Fig. 1. Phase-contrast micrographs at day 2 (**a**) and day 6 (**b**) after seeding isolated rat alveolar type II epithelial cells. The cells were seeded 5×10^6 cells/35-mm dish (**a**), or 2×10^6 cells/35-mm dish (**b**). magnification×200.

Table II. Change in the Expression Level of Marker Gene mRNA in Rat Alveolar Epithelial Cells During Culture

Gene Name	Day 2	Day 6	Ratio (day 6/day 2)
Type I cell marker			
RTI40	0.12±0.07	1.55±0.19**	12.6
IGFBP6	0.37±0.07	2.00±0.46*	5.5
mdr1a	0.15±0.10	2.17±0.65*	14.2
caveolin-1	0.21±0.15	11.26±1.67**	52.5
Type II cell marker			
SP-B	1.26±0.12	0.19±0.02**	0.15
CINC-1	1.17±0.12	0.03±0.01**	0.03

Total RNA was extracted from the cells cultured for 2 or 6 days after seeding 5 or 2×10^6 cells/35-mm dish, respectively. Each value represents the mean±SE of relative expression level normalized by *GAPDH* (n=3).
*p<0.05
**p<0.01 vs. day2.

including *rat type I cell 40-kDa protein* (*RTI40*; 15), *insulin-like growth factor binding protein 6* (*IGFBP6*; 15), *multidrug resistance protein 1a* (*mdr1a*; 16) and *caveolin-1* (15,16) was markedly higher in the cells 6 days after seeding than in those 2 days after seeding. On the other hand, the mRNA expression of *surfactant protein B* (SP-B; 15) and *chemokine-induced neutrophilic chemoattractant-1* (*CINC-1*; 15), type II cell markers, decreased markedly 6 days after seeding. These results indicate that when isolated alveolar type II cells were seeded at a density of 5×10^6 cells/35-mm dish and cultured for 2 days, the characteristics of type II cells would be retained. On the other hand, when the cells were seeded at a density of 2×10^6 cells/35-mm dish and cultured for 6 days, type II cells would be transdifferentiated into type I-like cells. These cells were used as type II cells and type I-like cells, respectively, in the following experiments.

General Characteristics of Albumin Uptake by Type II and Type I-like Cells

Fig. 2a shows the time course of FITC-albumin uptake by cultured type II and type I-like epithelial cells. The uptake of FITC-albumin at 37°C was increased with time up to 60 min in both cells and the rate of uptake per mg cell protein was much higher in type II cells than type I-like cells (about five to sixfold at 60 min). The uptake for 60 min was markedly suppressed at 4°C in each type of cells (Fig. 2b). Fig. 2c shows temperature-dependent uptake of FITC-albumin per cell, which was estimated from the data in Fig. 2b using DNA content/cell and DNA content/mg cell protein in each type of cells. FITC-albumin uptake per cell was more than twofold higher in type II cells than that in type I-like cells.

The uptake of FITC-albumin and the effect of temperature were also examined in type II cells by confocal laser scanning microscopy. When type II cells were incubated with FITC-albumin at 37°C for 60 min, punctate localization of fluorescence was observed in the cells (Fig. 3a). This phenomenon was not observed when incubation was at 4°C (Fig. 3b).

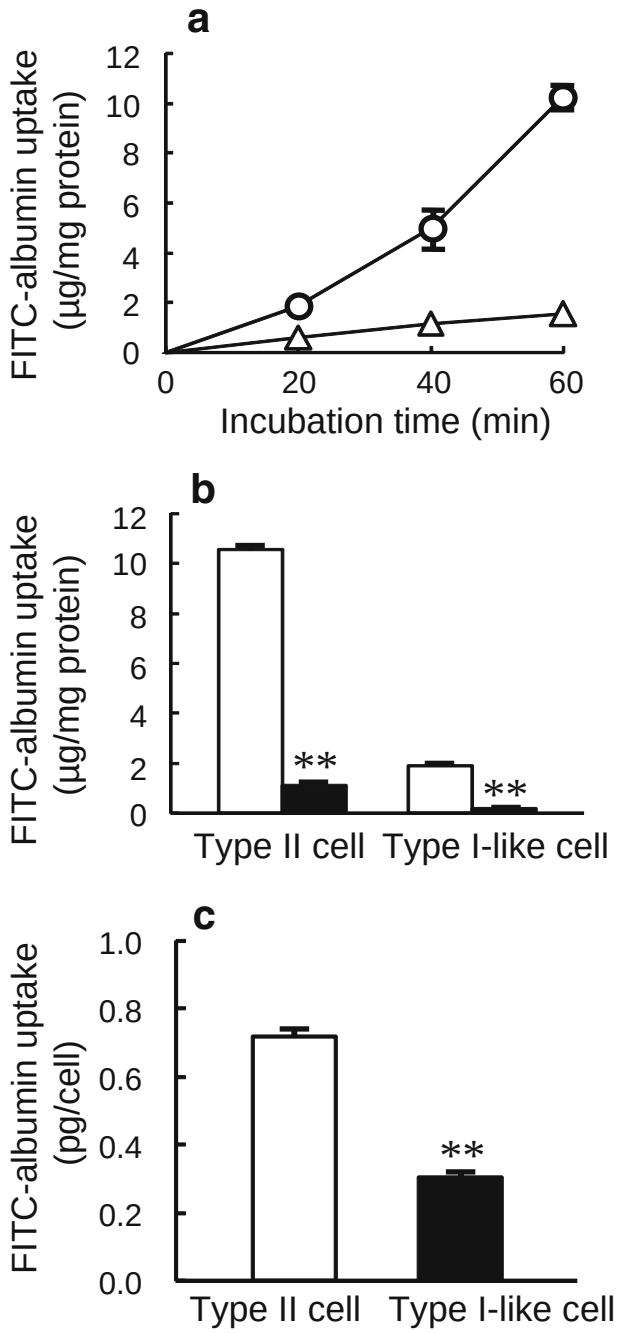

Fig. 2. Time course (**a**) and temperature-dependence (**b**) of FITC-albumin uptake and uptake activity per cell (**c**) in rat alveolar epithelial type II and type I-like epithelial cells. **a** The uptake of FITC-albumin (50 μg/ml) was measured at 37°C in type II (*open circles*) and type I-like (*open triangles*) cells. **b**: The uptake of FITC-albumin (50 μg/ml) was measured at 37°C (*open column*) or 4°C (*closed column*) for 60 min in type II and type I-like cells. **c**: Temperature-dependent uptake (uptake at 4°C was subtracted from that at 37°C) of FITC-albumin per cell for 60 min was estimated from the data in Fig. 2b, using DNA content/cell and DNA content/mg cell protein in each type of cells. Each point or column represents the mean±SE (n=3–5). *Double asterisks* in **b** indicate p<0.01 vs. 37°C and *Double asterisks* in **c** indicate p<0.01 vs. type II cell.

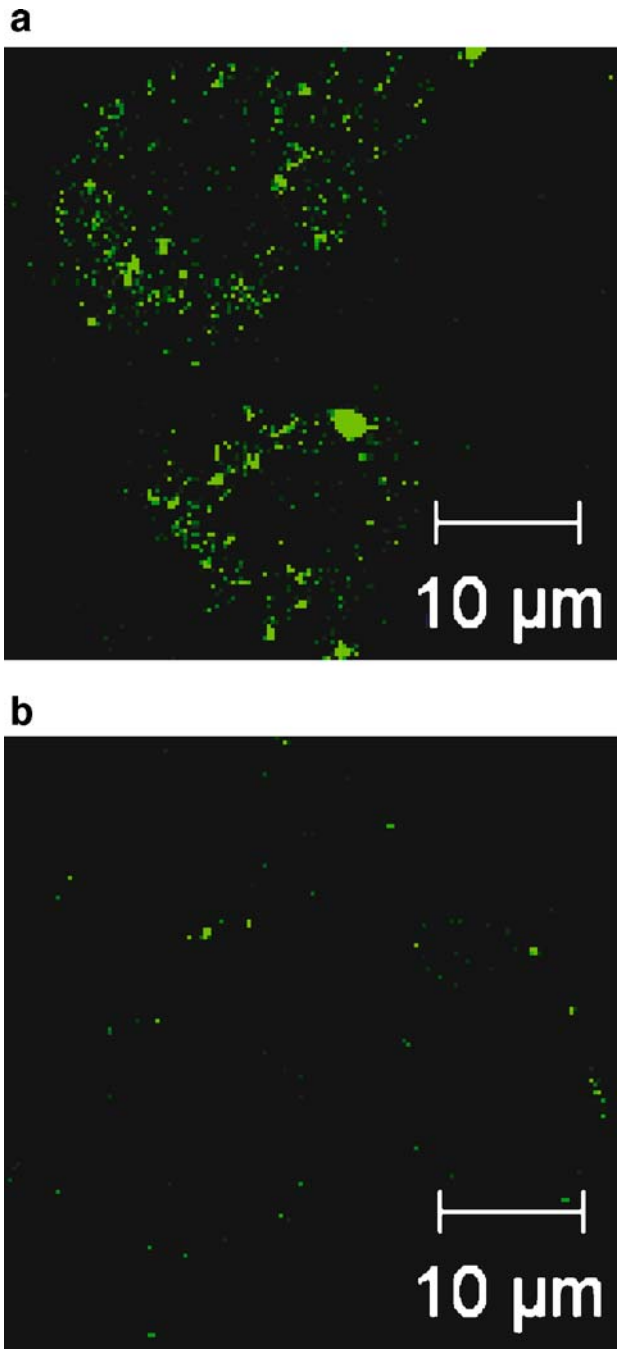

Fig. 3. Confocal laser scanning micrographs of type II epithelial cells. After FITC-albumin (50 μg/ml) uptake by type II cells grown on glass bottom culture dishes for 60 min at 37°C (**a**) or at 4°C (**b**), cells were observed by confocal laser scanning microscopy.

Effects of metabolic inhibitors on FITC-albumin uptake were examined (Fig. 4). Treatment of the cells with NaN_3 plus 2-deoxy-D-glucose or 2,4-dinitrophenol potently inhibited FITC-albumin uptake in both type II and type I-like cells. Similarly, FITC-albumin uptake was inhibited by the pretreatment with bafilomycin A_1, an inhibitor of vacuolar H^+-ATPase, in both cells.

Endocytic Pathways of Albumin Uptake by Type II and Type I-like Cells

Next, the endocytic pathway(s) involved in FITC-albumin uptake by type II and type I-like cells were examined. Fig. 5 shows the effects of clathrin-mediated endocytosis inhibitors, chlorpromazine and phenylarsine oxide, on FITC-albumin uptake. The uptake of FITC-albumin was inhibited by the treatment with chlorpromazine or phenylarsine oxide, and the inhibitory potencies of these inhibitors were somewhat stronger in type II cells. We further examined the effects of hypertonicity and potassium depletion (Fig. 6). These treatments were known to prevent clathrin-coated pits formation and inhibit the clathrin-mediated endocytic pathway. In both cells, hypertonicity resulted in the potent inhibition of FITC-albumin uptake. Potassium depletion also inhibited the albumin uptake significantly, though the inhibitory effect was much weaker than that by hypertonicity.

The effects of caveolae-mediated endocytosis inhibitors on FITC-albumin uptake were examined (Fig. 7). FITC-albumin uptake in type II cells was not affected by indomethacin and methyl-β-cyclodextrin, while that in type

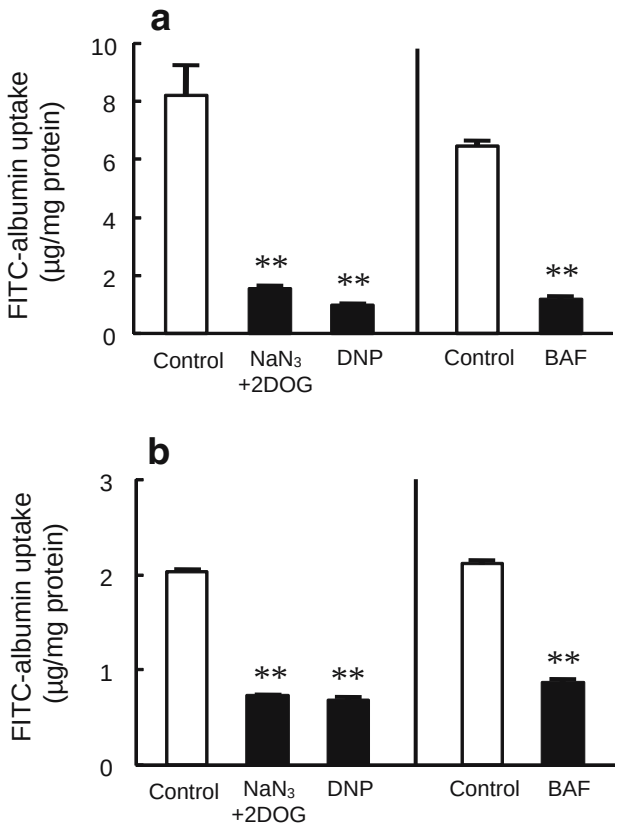

Fig. 4. Effect of metabolic inhibitors and a vacuolar H^+-ATPase inhibitor on FITC-albumin uptake by rat alveolar type II (**a**) and type I-like (**b**) epithelial cells. The uptake of FITC-albumin (50 μg/ml) was measured at 37°C for 60 min. Cells were treated with 10 mM sodium azide (*NaN_3*) plus 5 mM 2-deoxy-D-glucose (*2DOG*), 1 mM 2,4-dinitrophenol (*DNP*), or 100 nM bafilomycin A_1 (*BAF*) as described in Materials and Methods. Each column represents the mean±SE (n=3–6). *Double asterisks* indicate $p<0.01$ vs. control.

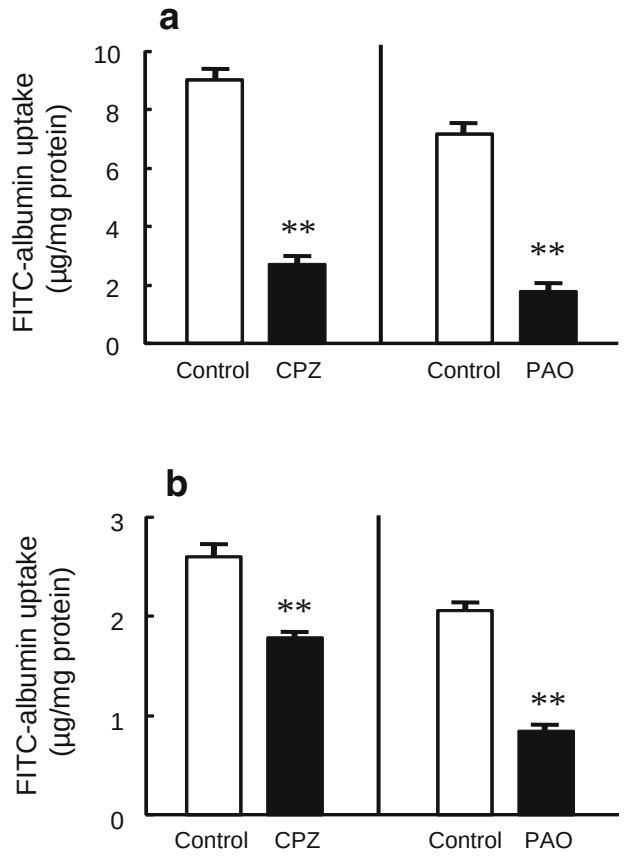

Fig. 5. Effect of inhibitors of clathrin-mediated endocytosis on FITC-albumin uptake by rat alveolar type II (**a**) and type I-like (**b**) epithelial cells. The uptake of FITC-albumin (50 μg/ml) was measured at 37°C for 60 min. Cells were treated with 84.4 μM chlorpromazine (*CPZ*) or 3 μM phenylarsine oxide (*PAO*) as described in Materials and Methods. Each column represents the mean±SE (n=3). *Double asterisks* indicate p<0.01 vs. control.

I-like cells was slightly stimulated. No inhibitory effect of nystatin on FITC-albumin uptake was observed in either type of cells.

DISCUSSION

In the present study, we elucidated and compared the activity and mechanism of albumin transport in two types of alveolar epithelial cells, type I and type II cells. Alveolar type II epithelial cells were isolated from rat lung. The yield of cells obtained was approximately 30×10^6 cells/rats, and the viability was more than 90%, being comparable to those reported by Richards *et al.* (11). Primary cultured alveolar type II epithelial cells plated on a culture dish are known to be transdifferentiated into type I-like cells (9,10). Reynolds *et al.* (17) reported that factors such as seeding density and extracellular matrix could affect the transdifferentiation of type II cells to type I-like cells, and indicated that high density cultures would be better for retaining type II cell characteristics longer than low density cultures. In addition, Goodman *et al.* (18) reported that at low plating densities, the cells never grew to confluence. Based on the information above, we firstly examined the effect of cell density on the morphology of cells plated on the culture dish, as a preliminary experiment. Isolated alveolar type II cells were plated at the cell densities of $1–8\times10^6$ cells/35-mm dish, and were observed microscopically for 1–8 days. Among these culture conditions, we chose the cells cultured for 2 days after seeding 5×10^6 cells/35-mm dish and the cells cultured for 6 days after seeding 2×10^6 cells/35-mm dish as the cells for studying type II and type I-like cells, respectively. As shown in Fig. 1, these cells showed morphological similarities to alveolar type II (Fig. 1a; cuboidal epithelial cells containing lamellar bodies inside the cells) and type I cells (Fig. 1b; squamous epithelial cells without lamellar bodies), respectively.

In order to characterize the cells cultured under the conditions described above, mRNA expression of marker genes for type I and type II cells was examined by real-time PCR analysis. The mRNA expressions of four type I cell marker genes, *RTI40* (15,16), *IGFBP6* (15), *mdr1a* (16) and *caveolin-1*(15,16), were markedly higher in the cells cultured for 6 days (about 5- to 50-fold) than those in the cells cultured for 2 days. On the other hand, the mRNA expressions of two type II cell marker genes, *SP-B* and *CINC-1* (15), decreased markedly in the cells cultured for 6 days (3 to 15%) compared with the cells cultured for 2 days

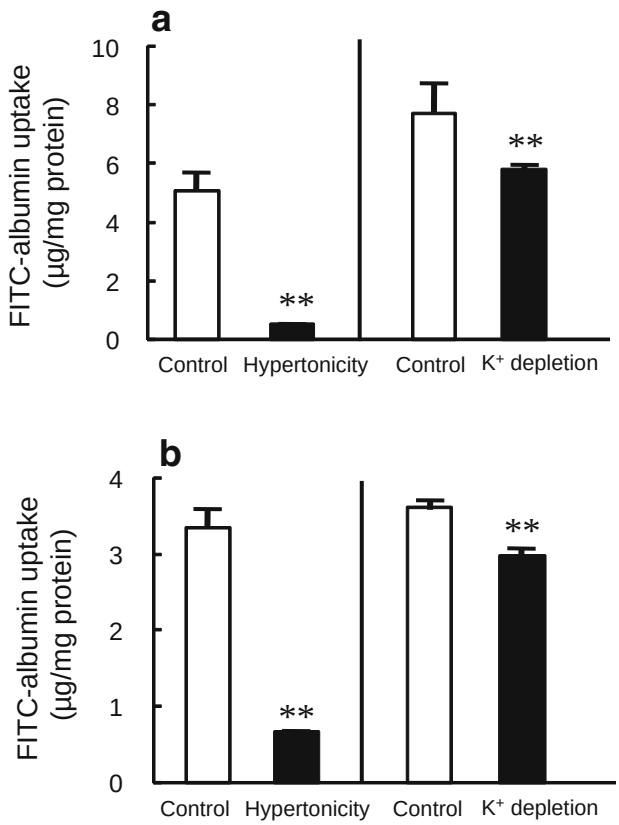

Fig. 6. Effect of hypertonicity and potassium depletion on FITC-albumin uptake by rat alveolar type II (**a**) and type I-like (**b**) epithelial cells. The uptake of FITC-albumin (50 μg/ml) was measured at 37°C for 60 min. Each treatment was carried out as described in Materials and Methods. Each column represents the mean±SE (n=3). *Double asterisks* indicate p<0.01 vs. control.

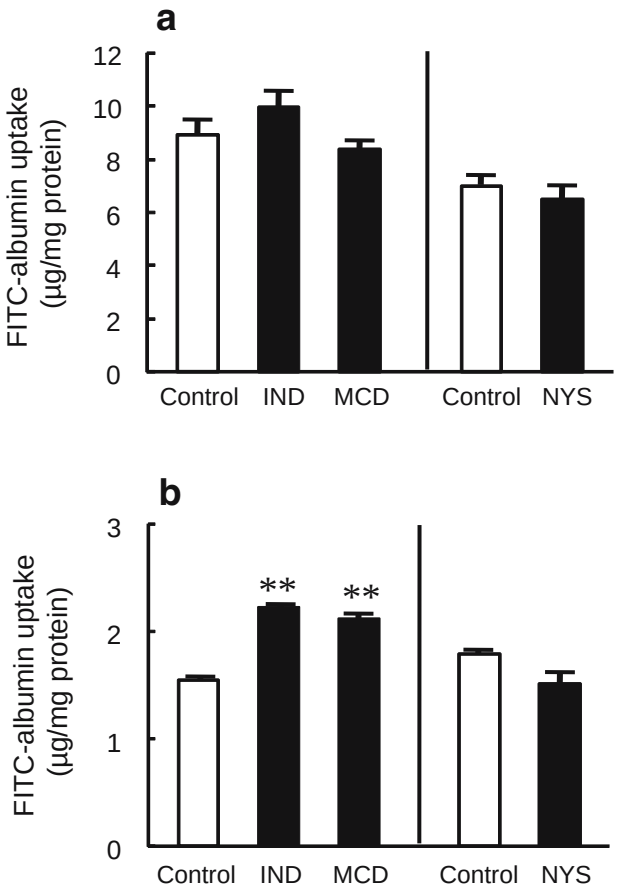

Fig. 7. Effect of inhibitors of caveolae-mediated endocytosis on FITC-albumin uptake by rat alveolar type II (**a**) and type I-like (**b**) epithelial cells. The uptake of FITC-albumin (50 μg/ml) was measured at 37°C for 60 min. Cells were treated with 100 μM indomethacin (*IND*), 5 mM methyl-β-cyclodextrin (*MCD*), or 54 μM nystatin (*NYS*) as described in Materials and Methods. Each column represents the mean±SE (n=3–6). *Double asterisks* indicate $p<0.01$ vs. control.

(Table II). Taken together, the cells cultured for 2 days after seeding isolated alveolar type II cells at 5×10^6 cells/35-mm dish would retain the characteristics of type II cells, while the cells cultured for 6 days after seeding 2×10^6 cells/35-mm dish would be transdifferentiated into type I-like cells. Therefore, the former cells were used as type II cells, and the latter cells as type I-like cells in the present study.

Using type II and type I-like epithelial cells cultured as described above, the transport of albumin was investigated. FITC-albumin uptake was increased with time up to 60 min at 37°C in type II and type I-like cells and the temperature-dependence (37°C vs. 4°C) was observed in both cells (Fig. 2a and b). In addition, the rate of FITC-albumin uptake per mg cell protein at 37°C was much higher (five to sixfold at 60 min) in type II cells than type I-like cells (Fig. 2a and b). In order to extrapolate these *in vitro* findings to *in vivo*, it should be necessary to evaluate albumin uptake activity of type II and type I-like cells per cell or per cell surface area, because information concerning the relative number or the surface area of each type of cells in alveolar epithelia is reportedly available. Therefore, we attempted to estimate the number of cells cultured for 2 days (type II cells) or 6 days (type I-like cells) by measuring DNA content per cell and per mg protein. The DNA content per cell in freshly isolated type II alveolar epithelial cells was 7.0±0.2 pg/cell (n=6). This value was almost the same as that reported previously, and would be fairly constant irrespective of cell types (19). The DNA contents per mg protein were 92.5±1.2 and 39.8±1.2 μg/mg protein (n=6) in type II and type I-like cells, respectively. Using these values, temperature-dependent uptake of FITC-albumin for 60 min in each type of cells was expressed as uptake amount (pg)/cell (Fig. 2c). As a result, FITC-albumin uptake per cell was found to be more than twofold higher in type II cells than that in type I-like cells. Assuming that the number of type II cells in alveolar epithelia is about 1.5-fold of that of type I cells (1), the contribution of type II cells to total albumin uptake by alveolar epithelial cells was calculated to be more than 75%. Thus, though the surface area occupied by type II cells is much smaller than that by type I cells, type II cells may significantly contribute to albumin uptake in the lung.

The uptake of FITC-albumin into the type II cells and its temperature-dependence were also observed by confocal laser scanning microscopy (Fig. 3). When type II cells were incubated with LysoTracker red, an acidic organelle-selective cell-permeant fluorescence probe, lamellar bodies were strongly stained (data not shown), probably due to the acidic internal pH of the lamellar bodies (20). The intracellular localization of the lamellar bodies observed by confocal laser scanning microscopy, however, was quite different from that of FITC-albumin, suggesting that FITC-albumin taken up by type II cells would not be localized in the lamellar bodies. FITC-albumin may be partly localized in lysosomes, as we previously observed in cultured alveolar type II epithelial cell line RLE-6TN (6).

The effects of metabolic inhibitors (NaN_3 plus 2-deoxy-D-glucose, and 2,4-dinitrophenol) and bafilomycin A_1 on FITC-albumin uptake were studied. FITC-albumin uptake in both types of the cells was inhibited by these inhibitors (Fig. 4). Bafilomycin A_1 is a specific inhibitor of vacuolar H^+-ATPase. Vacuolar H^+-ATPase localized in the endosomal membrane is responsible for lowering pH inside the endosome, which is an essential process for the dissociation of ligands and receptors after receptor-mediated endocytosis (21). Inhibition of vacuolar H^+-ATPase results in a decreased activity of the receptor-mediated endocytosis (22,23). Thus, these results suggest that FITC-albumin is taken up by type II and type I-like alveolar epithelial cells by receptor-mediated endocytosis. Our findings in type II cells are consistent with other reports indicating that albumin is taken up by receptor-mediated endocytosis in alveolar type II epithelial cells isolated from rats (24,25).

We then examined the possible endocytic pathway of FITC-albumin in both cells. Two well-known endocytic pathways are clathrin-mediated and caveolae-mediated endoytosis (26–28). Firstly, the role of clathrin-mediated endocytosis in FITC-albumin uptake was examined in both types of the cells using two inhibitors. One is chlorpromazine, which inhibits the process by inducing the loss of coated pits from the cell surface probably by interacting with AP-2 binding to membranes (29), and the other is phenylarsine oxide, which reacts with vicinal sulfhydryls to form stable ring structures (30). Both chlorpromazine and phenylarsine

oxide significantly inhibited FITC-albumin uptake in type II as well as in type I-like cells, though the degree of inhibition was somewhat smaller in type I-like cells (Fig. 5). Furthermore, effects of hypertonicity and potassium depletion on FITC-albumin uptake were examined. These treatments are known to inhibit clathrin-mediated endocytosis by preventing clathrin-coated pit formation (6,12). Both treatments lead to the significant inhibition of FITC-albumin uptake in type II and type I-like cells, though the inhibitory effect of potassium depletion was weaker than that of hypertonicity (Fig. 6). In addition, the expression of clathrin heavy chain mRNA was observed in both type II and type I-like cells (data not shown). Taken together, clathrin-mediated endocytosis would be involved in the uptake of FITC-albumin in type II and type I-like cells. We recently reported that clathrin-mediated endocytosis was involved in FITC-albumin uptake in alveolar type II epithelial cell line RLE-6TN (6), corresponding with the present findings in type II cells.

Next, the role of caveolae-mediated endocytosis in FITC-albumin uptake in type II and type I-like cells was examined. Indomethacin, methyl-β-cyclodextrin, and nystatin were employed as inhibitors of caveolae-meidated endocytosis. Indomethacin is known to suppress the pathway by inhibiting the internalization of caveolae and the return of plasmalemmal vesicles to the cell surface (31), while methyl-β-cyclodextrin and nystatin inhibit the process by interacting with cholesterol in the plasma membrane (32,33). However, these compounds did not inhibit FITC-albumin uptake in type II and type I-like cells (Fig. 7), indicating that caveolae-mediated endocytosis is not involved in the uptake of FITC-albumin in these alveolar epithelial cells. In contrast, John et al. (5) reported that albumin uptake in the cells cultured for 2 days after seeding isolated alveolar type II cells was mediated by gp60 and caveolae. The reason for this apparent discrepancy is not known. However, the expression of *caveolin-1* mRNA was very low in type II cells (Table II). Also, the expression of caveolin-1 protein was reportedly low in alveolar type II cells (34). In addition, as described above, we reported that clathrin-mediated endocytosis, but not caveolae-mediated endocytosis, was involved in FITC-albumin uptake in alveolar type II epithelial cell line RLE-6TN (6). Therefore, at least in type II cells, the involvement of caveolae-mediated endocytosis in albumin uptake seems to be unlikely.

The results in the present study suggest that albumin is taken up by clathrin-mediated, but not by caveolae-mediated, endocytosis in both type II and type I-like cells. However, it is not clear whether albumin uptake observed in type I-like cells may represent the intrinsic characteristics of type I cells themselves, or may reflect residual characteristics of type II cells, because both cells showed similar characteristics in terms of albumin endocytosis. In addition, the receptor(s) involved in albumin uptake observed in the present study is not known at this stage. One of the possible receptors is gp60. However, as described above, it was suggested that gp60 was involved in caveolae-mediated endocytosis of albumin in alveolar epithelial cells (5,26), which was not the case in the present study. Therefore, it is unlikely that gp60 is involved in albumin uptake by alveolar type II and type I-like cells under the present experimental conditions. Other possible receptors are megalin and cubilin. Megalin and cubilin are known to play an important role in the clathrin-mediated endocytosis of proteins including albumin, especially in the kidney (35,36). Megalin, a member of the low-density lipoprotein receptor gene family, is an endocytic receptor that recognizes a large number of protein ligands and is abundantly expressed and located in clathrin-coated pits in the apical membrane of renal proximal tubular epithelial cells (37–39). Cubilin is also a multiligand endocytic receptor coexpressed with megalin in renal proximal tubules, although its structure is quite different from megalin and has no transmembrane domain (40). Therefore, megalin is assumed to mediate internalization of cubilin and its ligands bind to cubilin (35,41). Kolleck *et al.* (42) showed that megalin and cubilin were expressed in alveolar type II cells. In addition, we also confirmed the mRNA expressions of megalin and cubilin in type II and type I-like cells by RT-PCR (data not shown). Therefore, these receptors may be involved in the clathrin-mediated endocytosis of albumin in alveolar type II and type I cells. However, there is no direct evidence supporting this hypothesis at this moment, and further studies are needed to clarify the receptors for albumin endocytosis in alveolar epithelium.

In conclusion, albumin in alveolar lining fluid was suggested to be internalized into type II and type I cells via clathrin-mediated endocytic pathway with much higher uptake rate in type II cells than in type I cells.

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