Permeability of Peptides and Proteins in Human Cultured Alveolar A549 Cell Monolayer

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Purpose. The transport of peptides or proteins across the alveolar cell monolayer was studied *in vitro* in order to elucidate their transport pathway. **Methods.** The permeability of 14 peptides or proteins and 6 dextrans with MW 1,000~150,000 was measured in cultured human lung adenocarcinoma A549 cell monolayers at 37°C or 4°C. The stability of the tested peptides and proteins was also evaluated. **Results.** The permeability coefficients of these macromolecules across the A549 cell monolayer at 37°C ranged from 10^{-5} to 10^{-7} (cm/sec), and exhibited a good inverse correlation with molecular weight. All macromolecules were stable throughout the transport experiment, and degradation by proteases was minimal. Permeability at 4°C did not differ from that at 37°C. Clear selectivity for direction of transport was not observed. **Conclusions.** These results suggested that the tested peptides and proteins appeared to penetrate the A549 cell monolayer *via* a paracellular route by passive diffusion.

KEY WORDS: peptide transport; epithelial permeability; A549 cells; alveolar absorption.

INTRODUCTION

The pulmonary route has potential for noninvasive systemic administration of peptides and proteins (1, 2). Fujita *et al.* (3) and Yamahara and Lee (4) established *in vitro* experimental systems using cultured alveolar epithelial cells for determination of drug absorption. The transport of dextrans was studied in human lung adenocarcinoma A549 cells (3), and that of insulin in rat alveolar primary cultured cells (4). Such *in vitro* systems are useful for the analysis of the mechanisms of pulmonary absorption of macromolecular drugs and for dosage form evaluation in drug development. In this paper, we report the *in vitro* permeability of various water soluble macromolecules, *i.e.* peptides and proteins with MW 1,000~150,000 across A549 cell monolayers, which are thought to resemble alveolar type II cells (5,6), using dextrans with MW 4,400~150,000 as a reference marker.

MATERIALS AND METHODS

Materials

The following commercially available macromolecules were used: human bradykinin (hBK) from Peptide Institute Inc., Japan; synthetic salmon calcitonin (sCT) from Bachem, USA; human recombinant epidermal growth factor (hEGF) from Genzyme, USA; human urokinase (hUK) from Protogen AG, Switzerland; human factor XIII (hFXIII) from Behringwerke AG, FRG; FITC-labeled human immunoglobulin G (FITC-IgG) from Cappel Organon Teknika Corp., USA; recombinant human interleukin-2 (hIL-2), -6 (hIL-6) and -8 (hIL-8) from Amersham, UK; FITC-labeled human transferrin (FITC-TF) and human albumin (FITC-HSA) from Inter-cell Technologies, Inc., USA; recombinant human transforming growth factor β-2 (hTGF-β-2) from Austral Biologicals, USA; recombinant human interleukin-6 receptor (hIL-6R) and recombinant human platelet-derived growth factor (hPDGF) from R&D Systems, USA; and FITC-labeled dextrans of various molecular weights (FITC-dextrans) and bovine serum albumin (BSA, fatty acid free) from Sigma Chemical Co., USA.

Assay

FITC-labeled macromolecules were assayed with a fluorescence spectrometer. The others were assayed using commercially available kits: hBK was assayed with Markit-A Bradykinin (Dainippon Pharmaceutical, Japan), hUK with Tint Elize uPA (Biopool AB, Sweden), hEGF, hIL-2, hIL-6, hIL-8 and hTGF-β-2 with Biotrak (Amersham), hIL-6R and hPDGF with Quantikine (R&D Systems), and hFXIII with Iatron-FL FXIII (Iatron, Japan). sCT was assayed by ELISA using mouse anti-sCT monoclonal antibody (Teikoku Seiyaku, Japan), rabbit-anti-sCT antibody (UCb Bioproducts, USA) and purified peroxidase-labeled goat anti-rabbit IgG (Kirkegaard & Perry Laboratories, USA). All assays were performed in duplicate.

Cell Cultivation

The human lung adenocarcinoma A549 cell line kindly provided by Riken Cell Bank was cultured in Dulbecco's modified Eagle:Ham F-12 (1:1) medium (Sigma Chemical Co.) supplemented with 10% fetal calf serum (Gibco), which was subcultured once a week. The passage number of the cell line for experiments was between 88 and 94.

Transport Experiment

The transepithelial permeation experiment using the A549 cell line was performed using the method of Fujita *et al.* (3), except that a Transwell-COL chamber (Costar, MA; collagen membrane 12 mm in diameter and 0.4 μm in pore size) which allows microscopic observation of cultured cells was used instead of the Transwell chamber manufactured by the same company (polycarbonate membrane 24 mm in diameter and 3 μm in pore size). An A549 cell suspension prepared by treatment with trypsin and EDTA was plated onto the collagen membrane at 2~4 × 10^6 cells/cm^2 and cultured at 37°C in a humidified atmosphere of 5% CO_2-95% air. The culture medium was exchanged every 2 days. The cells became completely confluent on day 4 of cultivation and were used for experiments on day 6. The transepithelial resistance (R; 100 μA of pulse current at 50 Hz, 10 msec

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duration) of the cell monolayer reached equilibrium on and after day 4 and was 764 ± 29 Ω (n = 10) on day 6.

The cell monolayer on the collagen membrane was washed twice with Earle's balanced salt solution containing 0.1% BSA and equilibrated by incubation with the same solution in a 5% CO_2-96% air atmosphere at 37°C for 1 hr. For FITC-HSA, BSA-free equilibrating salt solution was used. The volume of equilibrating solution was 0.5 ml for the upper reservoir (apical side, A) and 1.5 ml for the lower reservoir (basolateral side, B). A macromolecular solution was placed in the upper or lower reservoir as the donor fluid at a desired final concentration, and allowed to stand in a humidified atmosphere of 5% CO_2-95% air at 37°C or 4°C. At 15, 30, 60, 120 and 180 min later, the concentration of macromolecules in 10 μl of the apical receiver fluid or in 50 μl of the basolateral receiver fluid was determined in order to estimate transport from the apical side to the basolateral side (A→B) and that in the reverse direction, B→A, at 37°C and A→B at 4°C. A control experiment was performed using the collagen membrane alone without the monolayer. The apparent permeability coefficient (P_{app}) for each macromolecule was calculated using the following equation:

$$dJ/dt = -P_{app}A\,(C_d - C_r) \quad (1)$$

The following Equation (2) is obtained by solving Equation (1):

$$C_r = \frac{C_0 V_d}{(V_d+V_r)} \cdot \left[1-\exp\left\{-P_{app}A\left(\frac{1}{V_d}+\frac{1}{V_r}\right)t\right\}\right] \quad (2)$$

where dJ is the change in the solute diffusion flux, t is the time, P_{app} the apparent permeability coefficient, A the area of collagen membrane, C_d the concentration of macromolecules in the donor fluid, C_r the concentration of macromolecules in the receiver fluid, C_0 the initial macromolecule concentration in the donor fluid, V_d the volume in the donor reservoir, and V_r the volume in the receiver reservoir.

Metabolic Degradation

The stability of the macromolecular drugs was evaluated using the same chamber. A peptide/protein solution of the same concentration as used for the transport experiment was placed in both the upper and lower reservoirs of the Transwell TW-COL chamber with A549 cell monolayers and incubated in a humidified atmosphere of 5% CO_2-95% air at 37°C for 3 hr in order to determine the remaining drug concentrations in both reservoirs. A control experiment was performed using the collagen membrane alone without the monoloyer.

RESULTS AND DISCUSSION

Permeability Coefficient for Each Macromolecule

The permeability coefficient (P_{app}) was calculated from experimental values using Equation (2), and curve fitting was performed with the Levenberg-Marquardt algorithm using a computer. Tables 1 and 2 summarize the P_{app} values for peptides and proteins and those for dextrans, respectively. The coefficient of regression obtained on curve fitting was 0.980 or higher for each macromolecule. The time courses of changes in permeation values for hBK, sCT, hIL-2, hPDGF, hUK and hFXIII are depicted in Fig. 1 as representative of peptides and proteins. The $P_{app(A\rightarrow B)}$ values for peptides/proteins at 37°C ranged between 10^{-5} and 10^{-7} (cm/sec), and those for FITC-dextrans approximated to those reported by Fujita *et al.* (3) However, the P_{app} values we obtained were far higher than the *in vivo* or *in situ* pulmonary P_{app} values (including those estimated from clearance rates) obtained by other researchers, and in some cases differences of about an order of magnitude were noted (7-10). Difficulty in obtaining true P_{app} values in *in vivo/in situ* experimental systems is thought to be a reason for these differences, as Byron and Phillips have reported (11). Furthermore, in *in vivo* experiments, the viscosity of alveolar lining fluid is thought to affect drug absorption as an unstirred water layer, but can be excluded in the *in vitro* cultured cell system. This might be another reason for the differences in values obtained. A third possible reason for these differences is the tight junctions of A549 cells, which might be more leaky than those of normal alveolar type II cells, since A 549 cells are malignant cells. The latter possibility was suggested by Mulin and McGinn in their study of renal cell lines (12).

The P_{app} for insulin reported by Yamahara and Lee is at least ten times smaller than ours for sCT, which has a molecular weight similar to that of insulin (4). The transepithelial resistance of their primary alveolar type II monolayer cell (2000 Ω or more) was 2-3 times higher than that of our A549 cell system. The difference in the strength of tight junctions in their system might have resulted in the differences in P_{app} values from our own. Thwaites and coworkers reported that the permeability for thyrotropin-releasing hormone (TRH) declined to 1/5~1/6 the initial value when the resistance was nearly doubled in a transepithelial transport experiment using cultured cell monolayers of Caco-2, *etc.* (13). Yamahara and Lee observed differentiation of type II cells to type I cells during cultivation in their experiment (4). Since various types of intercellular tight junctions exist in alveolar epithelium (14), the difference in properties of type I-type I cell tight junctions and type II-type II cell tight junctions might have resulted in differences in the strength of tight junctions as barriers. Examination of the morphological differences between the two systems may explain their differences in permeability.

Correlation Between Permeability Coefficient and Molecular Weight

As Fig. 2 shows,the molecular weight of each macromolecule exhibited a good inverse correlation with $P_{app(A\rightarrow B)}$ at 37°C (R = 0.968). This tendency has already been reported in *in vivo* and *in situ* studies (11,15,16), while *in vitro* experiments using peptides/proteins have not yet been reported. Effros and Mason (15) studied *in vivo* pulmonary epithelial permeability and reported the existence of an inverse correlation between log molecular weight and log pulmonary clearance in which clearance changed dramatically over the molecular weight range of 1,000~100,000. In our study, a similar tendency was also found, and no clear permeability threshold was observed for change in molecular weight.

Table 1. Permeability Coefficient for Peptides and Proteins in A549 Cell Monolayers

Compound (m.w.)	Direction of transport	Concentration	Permeability coefficient (10^{-6} cm/sec)[a] 37°C (R^b)	4°C (R^b)
hBK (1,000)	A → B	100 nM	8.4 ± 0.4[c] (0.99)	8.0 ± 0.5 (0.99)
		1 μM	8.0 ± 0.3 (0.99)	—[d]
	B → A	100 nM	7.5 ± 0.6 (0.98)	—
sCT (3,400)	A → B	100 nM	2.5 ± 0.2[c] (0.99)	2.4 ± 0.2 (0.99)
		1 μM	2.6 ± 0.1 (0.99)	—
		10 μM	2.5 ± 0.2 (0.99)	—
	B → A	100 nM	1.9 ± 0.04 (0.99)	—
hEGF (6,000)	A → B	10 nM	1.3 ± 0.09[c] (0.99)	1.3 ± 0.1 (0.99)
		100 nM	1.4 ± 0.09 (0.99)	—
	B → A	10 nM	0.5 ± 0.06 (0.98)	—
hIL-8 (8,500)	A → B	1 nM	2.2 ± 0.2[c] (0.96)	2.2 ± 0.3 (0.97)
		10 nM	2.3 ± 0.2 (0.97)	—
	B → A	1 nM	0.8 ± 0.06 (0.98)	—
hIL-2 (14,700)	A → B	10 nM	1.7 ± 0.09[c] (0.99)	1.8 ± 0.2 (0.99)
		100 nM	1.8 ± 0.09 (0.98)	—
	B → A	10 nM	1.4 ± 0.05 (0.99)	—
hIL-6 (20,000)	A → B	1 nM	1.1 ± 0.07[c] (0.99)	0.9 ± 0.1 (0.99)
		10 nM	1.0 ± 0.09 (0.99)	—
	B → A	1 nM	0.65 ± 0.02 (0.99)	—
hTGF-β-2 (25,000)	A → B	1 nM	0.64 ± 0.05[c] (0.99)	0.60 ± 0.03 (0.99)
		10 nM	0.65 ± 0.06 (0.98)	—
	B → A	1 nM	0.67 ± 0.08 (0.99)	—
hPDGF (27,000)	A → B	1 nM	0.73 ± 0.07[c] (0.99)	0.84 ± 0.07 (0.99)
		10 nM	0.73 ± 0.07 (0.99)	—
	B → A	1 nM	0.65 ± 0.05 (0.99)	—
hIL-6R (34,000)	A → B	1 nM	0.59 ± 0.04[c] (0.99)	0.57 ± 0.05 (0.99)
		10 nM	0.61 ± 0.04 (0.99)	—
	B → A	1 nM	0.54 ± 0.05 (0.99)	—
hUK (54,000)	A → B	10 nM	0.57 ± 0.05[c] (0.99)	0.54 ± 0.03 (0.99)
		100 nM	0.55 ± 0.05 (0.99)	—
	B → A	100 nM	0.38 ± 0.03 (0.99)	—
FITC-HSA (64,000)	A → B	0.5 μM	1.0 ± 0.09[c] (0.99)	0.99 ± 0.1 (0.98)
		5 μM	1.1 ± 0.5 (0.99)	—
		0.5 μM[e]	1.1 ± 0.1 (0.99)	—
	B → A	5 μM	0.74 ± 0.03 (0.98)	—
FITC-TF (80,000)	A → B	5 μM	0.97 ± 0.08[c] (0.99)	0.91 ± 0.04 (0.99)
	B → A	5 μM	0.39 ± 0.03 (0.99)	—
hFXIII (83,000)	A → B	1 μM	0.42 ± 0.04[c] (0.98)	0.39 ± 0.03 (0.99)
FITC-IgG (150,000)	A → B	1 μM	0.30 ± 0.02[c] (0.97)	0.29 ± 0.03 (0.99)
		1 μM[e]	0.30 ± 0.03 (0.99)	—
	B → A	1 μM	0.28 ± 0.04 (0.98)	—

[a] Mean ± S.E. (N = 4).
[b] Regression coefficient.
[c] These values were used in Fig. 2.
[d] Not tested.
[e] Non-labeled HSA and IgG were simultaneously added at 500 μM and 1 mM, respectively.

Permeation Mechanism

As shown in Tables 1 and 2, the P_{app} value for each macromolecule changed only slightly over a ten-fold change in donor concentration. There was no clear difference in P_{app} between cultures at 37°C and 4°C. P_{app} values for FITC-HSA and FITC-IgG remained unchanged even in the presence of high concentrations of unlabeled compounds. No lag time was observed in the permeation of peptides and proteins. These findings indicate that the peptides and proteins used in this study penetrated the epithelial monolayer *via* the paracellular pathway, *i.e. via* aqueous channels, by a passive diffusion mechanism, as is the case for dextrans. This conclusion is supported by the finding of an inverse correlation between molecular weight and P_{app} (Fig. 1) (16). It has also been reported that the absorption of TRH is passive and occurs *via* a paracellular route in Caco-2 cell monolayers (13).

Patton and coworkers reported that the *in vivo* rate of pulmonary absorption of peptides 22 Kd or less in molecular

Table 2. Permeability Coefficient for FITC-Dextrans in A549 Cell Monolayers

Compound (m.w.)	Direction of transport	Concentration	Permeability coefficient (10^{-6} cm/sec)[a] 37°C (R^b)	4°C (R^b)
F-4 (4,400)	A → B	1 μM	3.4 ± 0.2[c] (0.98)	3.3 ± 0.3 (0.99)
		10 μM	3.4 ± 0.3 (0.99)	—[d]
	B → A	1 μM	2.8 ± 0.2 (0.99)	—
F-10 (9,400)	A → B	1 μM	2.2 ± 0.02[c] (0.99)	1.4 ± 0.1 (0.99)
		10 μM	2.0 ± 0.04 (0.99)	—
F-20 (19,600)	A → B	1 μM	1.2 ± 0.1[c] (0.99)	1.3 ± 0.1 (0.99)
		10 μM	1.4 ± 0.06 (0.99)	—
	B → A	1 μM	0.84 ± 0.07 (0.99)	—
F-40 (38,900)	A → B	1 μM	0.75 ± 0.07[c] (0.98)	0.73 ± 0.03 (0.99)
		10 μM	0.71 ± 0.03 (0.98)	—
F-70 (71,200)	A → B	1 μM	0.61 ± 0.04[c] (0.99)	0.59 ± 0.02 (0.99)
		10 μM	0.60 ± 0.05 (0.99)	—
	B → A	1 μM	0.42 ± 0.02 (0.98)	—
F-150 (147,800)	A → B	1 μM	0.25 ± 0.03[c] (0.99)	0.22 ± 0.02 (0.99)
		10 μM	0.24 ± 0.05 (0.99)	—
	B → A	10 μM	0.19 ± 0.05 (0.99)	—

[a] Mean ± S.E. (N = 4).
[b] Regression coefficient.
[c] These values were used in Fig. 2.
[d] Not tested.

weight was not affected by molecular weight (18), and suggested that the absorption of peptides and proteins in the alveolar epithelium occurs mainly by a process of transcytosis (1,18). However, the independence of permeability from molecular weight in their experiments might have been due to metabolic barriers such as peptidases and proteases in the alveolar epithelium in the living body. Indeed, the absorption of several peptidase-resistant synthetic peptides in the rat lung has been found to depend on molecular weight (19). It remains to be determined whether A549 cells have a capacity for transcytosis equal to that of alveolar epithelial cells *in vivo*.

Metabolic Degradation

In studies of epithelial absorption of peptides and proteins, the metabolic barrier due to proteolytic enzymes has to be taken into consideration. When the stability of peptides/proteins in the present transport system was determined, it was found that the residual rate of all tested macromolecules was 95% or higher after 3 hr contact with the cell monolayer. This finding indicated that degradation by proteases did not occur to a degree that would affect the amount of permeation.

Peptides and proteins usually undergo *in vivo* degradation by extra- or intracellular proteases upon transport through the alveolar epithelium, though the degree of degradation may differ considerably. The system we used with cultured A549 monolayers is considered to involve very weak enzymatic barriers.

Insulin has been reported to undergo degradation during incubation in a system using primary alveolar type II cells (4), but such degradation of peptides and proteins was not observed in our system. The large difference in P_{app} between the two systems may depend on the existence of metabolic barrier.

Direction of Permeation

$P_{app(A \rightarrow B)}$ was about two or three times as high as $P_{app(B \rightarrow A)}$ for hEGF, hIL-8, hIL-6, hUK and FITC-TF. For the other peptides and proteins, however, large differences were not observed between the two directions. Molecular weight also appeared to be unrelated to directional differences. In the study by Yamahara and Lee, the $P_{A \rightarrow B}/P_{B \rightarrow A}$ ratio for insulin was 2 at 0.15 nM, and there was no difference between the two directions within the range 14~75 μM (4). Since detailed studies of the difference in direction of drug permeation have yet to be performed, a clear conclusion could not be obtained from the results of the present study alone as to whether the A549 cell monolayer has selectively on the direction of permeation for peptides and proteins.

The findings of the present study clearly show that peptides and proteins are transported through A549 cell monolayers in a molecular weight-dependent manner *via* a paracellular route by passive diffusion. The mechanism of *in vivo* absorption of peptides and proteins may in the future be elucidated by comparative studies of their absorption in the lung and *in vitro* permeability. Absorption enhancers targetting tight junctions and protease inhibitors appear to be useful tools for determining the contribution of the paracellular route and molecular weight-dependency to *in vivo* pulmonary absorption of peptides and proteins. In our previous study (20), several absorption enhancers were found to be effective in enhancing pulmonary absorption of salmon calcitonin in rats. The system we used with cultured cell monolayers also appears useful for evaluation of the potency of absorption enhancers. We are now studying *in vitro* absorp-

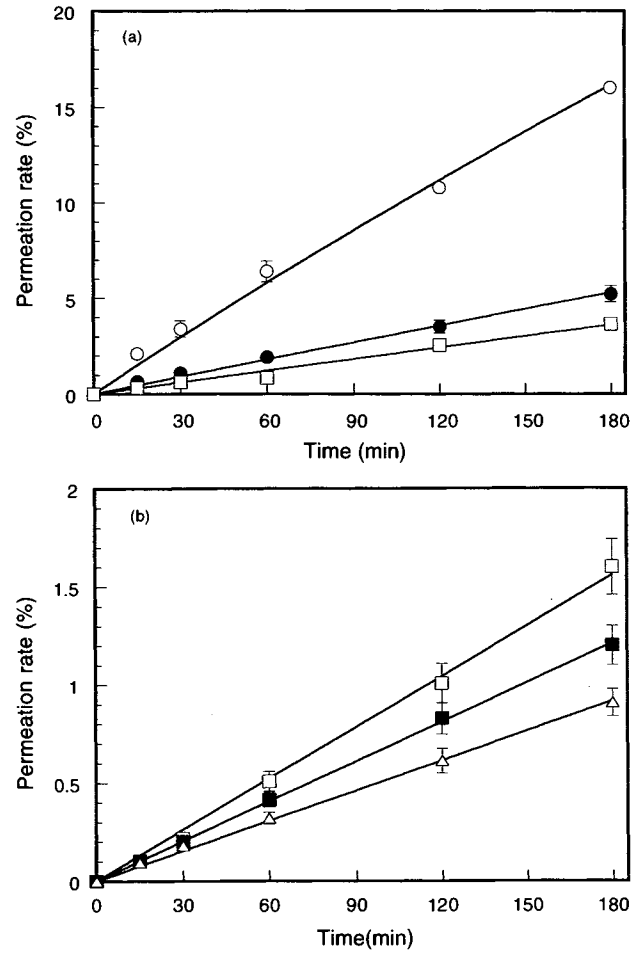

Fig. 1. Permeation of peptides and proteins in A549 cell monolayers from apical side to basolateral side at 37°C. (a) ○, hBK at 100 nM; ●, sCT at 100 nM; □, hIL-2 at 10 nM, (b) □, hPDGF at 1 nM; ■, hUK at 10 nM; △, hFXIII at 1 μM. Each point represents the mean ± SE (n = 4).

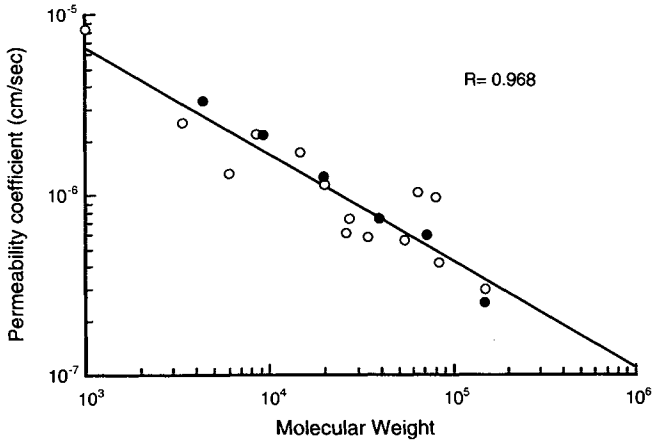

Fig. 2. Correlation between permeability coefficient and molecular weight of peptides/proteins and dextrans. ○, peptides/proteins; ●, dextrans. Each point represents the mean of four experiments shown in Tables 1 and 2 (values with a superscript of *c*).

tion enhancement in A549 cell monolayers and the mechanisms of pulmonary absorption and absorption enhancement.

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