Evaluation of the Establishment of a Tight Junction in Caco-2 Cell Monolayers Using a Pore Permeation Model Involving Two Different Sizes

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The tight junction formation in Caco-2 cell monolayers was compared after 9 and 13 d of culture. Four different sized paracellular markers were simultaneously applied to the apical side of the monolayers. The transepithelial resistance and permeability coefficient of mannitol for the monolayers cultured for 13 d were 17.4 and 0.095 times those after 9 d, respectively. The tight junction structure developed during that period. The pore permeation model involving two different sizes was used to quantitatively evaluate the paracellular pathways. The results suggest that there were two-different sized (large and small) pathways in the monolayers cultured for 13 d, while there was only a single large pathway in those cultured for 9 d. The small pathway in the monolayers cultured for 13 d might be a major permeation pathway for the paracellular permeation of urea and the equivalent cylindrical pore radius of the small pathway was less than 0.4 nm, suggesting development of the tight junctions after 13 d.

Key words paracellular transport; Renkin function; Caco-2 cell; permeability; tight junction; diffusion

Cultured epithelial cell monolayers are widely used for drug permeation studies as alternatives to experimental animal models. Generally, *in vitro* permeation studies are better than the *in vivo* absorption studies for examining the kinetic properties and mechanism of drug permeation. The cells are seeded on transwells or similar devices, and then cultured until confluent before the permeation study. For small lipophilic drugs, since transcellular (intracellular) permeation is a major route of drug absorption, establishment of tight junctions between each cell is checked by measurement of transepithelial resistance (TER) and/or the permeability of paracellular markers, such as mannitol, before use. Since well-established tight junctions have a low permeability for drugs and the area occupancy compared with that of the epithelial surface is very low, the paracellular (intercellular) permeation is negligible for small lipophilic drugs under optimum conditions. On the other hand, for large hydrophilic drugs, such as peptide and protein drugs, the paracellular permeation is a major route of absorption even under optimum conditions because of the difficulty such drugs have in passing through cell membranes.[1)] Therefore, establishment of the tight junctions of cultured cell monolayers should be carefully evaluated as far as the use of large hydrophilic drugs is concerned.[2,3)]

In recent years, our knowledge of the structure of tight junctions has advanced dramatically and their molecular structure involving occludin, claudins and ZO proteins has been described.[4—7)] During the culturing process of epithelial cells, these components assemble between each cell, and then arrange themselves so that the cell monolayer forms a tight barrier for hydrophilic drugs. Studies of the structure of claudins suggest the existence of aqueous pores in the tight junctions[8)] and at least two different sized pores are assumed to be present.[9,10)] Since the permeability of large hydrophilic drugs in the tight junctions is dependent on the size and area occupancy of each pore, the establishment of the tight junction structure during the culturing process needs to be evaluated quantitatively.

In a previous study, we used the Renkin molecular sieving function to characterize the paracellular pathway in the tight junctions of Caco-2 cell monolayers.[11)] The equivalent cylindrical pore radius (R) and pore occupancy/length ratio (ε/L) of the monolayers were determined based on this function. Assuming the existence of two different sized pore pathways, the function can be arranged in a parallel circuit. In this study, a permeation model involving two different sized pores was used to evaluate the formation of the tight junctions in Caco-2 cell monolayers. Four different sized paracellular markers, urea, mannitol, 5(6)-carboxyfluorescein (CF) and rhodamine isothiocyanate dextran-10 (RD10), were simultaneously applied to the apical side of Caco-2 cells cultured for 9 or 13 d on transwells. Since the TER of the Caco-2 cell monolayer was dramatically changed about 10 d after seeding in a preliminary study, 9 and 13 d were chosen for the culturing periods. Since 21 d of culture of Caco-2 cells is usually needed for permeation studies, the monolayers cultured for 13 d might represent an advance in the development of the tight junction structure. In this study, we tried to evaluate the values of R and ε/L, as parameters of the large and small pathways for each Caco-2 cell monolayer based on this model.

MATERIALS AND METHODS

Materials RD10 (MW 11000), diacetyl monoxime and mannitol-1-^{13}C were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Thiosemicarbazide was obtained from Wako Pure Chemical Industries (Osaka, Japan). CF was purchased from Acros Organics (NJ, U.S.A.). All other chemicals were of reagent grade and used as received.

Cell Culture Caco-2 cells were obtained from the Riken Gene Bank (Ibaraki, Japan). The cells were maintained in DMEM containing 10% heat-inactivated fetal calf serum, 40 μg/ml gentamicin and 1% nonessential amino acids, in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Only cells from passage number 37—38 were used to avoid

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the effect of the cell passage number on the paracellular permeability.[12] The cells were seeded (4.5×10^5cell/cm^2) on polycarbonate filter inserts (pore size 0.4 μm, area 4.7 cm^2, Transwell, Costar) and cultivated in the medium for 9 or 13 d. Before starting the drug transport experiments, the culture medium was changed to Hanks balanced salt solution (HBSS), and then the TER was measured using a Millicell®-ERS (Millipore, MASS, U.S.A.).

Drug Transport Experiments Simultaneous transport of urea, mannitol, CF and RD10 through the Caco-2 cell monolayers was observed at 37 °C. HBSS in the apical side was removed and then a mixed solution (1.5 ml) containing urea (8 mM), mannitol (18 mM), CF (10 μg/ml) and RD10 (1.0%) in PBS (pH 7.4) was applied to each well. HBSS (2.5 ml) was used as the solution for the basolateral side. The HBSS was changed every 30 min for 2 h.

Analytical Methods For quantitative determination of urea, the sample solution (0.3 ml) was mixed with an acidic solution (0.45 ml) which was prepared by mixing 20% sulfuric acid, 999 ml, and 56.7% phosphoric acid containing 0.12 M $FeCl_3$, 1 ml, and then the resulting solution was mixed with a solution (0.3 ml) containing 61.7 mM diacetyl monoxime and 3.4 mM thiosemicarbazide. After heating in a boiling water bath for 10 min, the absorbance at 520 nm was determined.

An HPLC-MS/MS system was used for determination of mannitol. A packed column (Shodex Asahipak NH2P-50, 4.6$\phi\times$150 mm, Showa Denko, Tokyo, Japan) was used in the HPLC system (LC-10Ai, Shimadzu Corporation, Kyoto, Japan) and acetonitrile : 10 mM ammonium acetate = 5 : 2 was used as the mobile phase. The separation was carried out at a flow rate of 0.3 ml/min and a column temperature of 40 °C. The eluting solution was introduced into the MS/MS system (PE SCIEX API300, CA, U.S.A.) by valve switching 12 min after the sample injection. Atmospheric Pressure Chemical Ionization (APCI) was used for the ion source and the generated ions were monitored in negative ion mode. Mannitol-1-^{13}C was used for the internal standard.

The concentrations of CF and RD10 in the samples were determined using a fluorescence spectrophotometer (RF-5000, Shimadzu Corporation). The wavelengths of excitation and emission were 488 and 514 for CF and 550 and 580 for RD10, respectively.

Characterization of Caco-2 Cell Monolayers The apparent permeability coefficient of penetrant i ($Papp_i$) was calculated using the following Eq. 1:

$$Papp_i = \frac{\Delta M_i/\Delta_t}{A\cdot C_{0,i}} \tag{1}$$

where ($\Delta M_i/\Delta t$) is the rate of the penetrant i appearing on the receiver side, A is area of the monolayers and $C_{0,i}$ is the initial concentration of i. The Renkin function and $Papp_i$ through a cylindrical porous pathway are expressed by the following Eqs. 2 and 3[13,14]:

$$F\left(\frac{r_i}{R}\right)=\left(1-\left(\frac{r_i}{R}\right)\right)^2\left[1-2.14\left(\frac{r_i}{R}\right)+2.09\left(\frac{r_i}{R}\right)^3-0.95\left(\frac{r_i}{R}\right)^5\right] \tag{2}$$

$$Papp_i=\left(\varepsilon/L\right)\cdot D_i\cdot F\left(\frac{r_i}{R}\right) \tag{3}$$

where r_i is the molecular radius of penetrant i which can be calculated from the diffusion coefficient (D_i) as the Stokes–Einstein radius. When the pore size is much larger than r_i, the Renkin function will be unity and the $Papp_i$ will be expressed by the following Eq. 4:

$$Papp_i=(\varepsilon/L)\cdot D_i \tag{4}$$

In order to decide whether to choose Eqs. 3 or 4, the ratio of $Papp_i$ of two paracellular markers (small one/large one) is calculated and compared with the ratio of the D_i values. If both of them are similar, Eq. 4 is applicable and we do not know the size of the pathway. If the ratio of $Papp_i$ is higher than that of D_i, this means that Eq. 3 should be used, and we will be able to calculate r_i of the pathway using Eq. 2.

In the pore permeation model involving two different sizes, the large pore pathway is characterized initially using the $Papp_i$ of two large paracellular markers, CF and RD10 in this study, and then the $Papp_i$ values of two small paracellular markers, urea and mannitol in this study, through the large pathway are calculated using Eqs. 3 or 4 characterized by the two large markers. If the calculated values are similar to the observed values, this means there are no small sized pathways. When a small sized pathway is present to the extent that it can be observed, the observed values are higher than the calculated values and the difference can be expressed by Eq. 3 for the small pathway.

RESULTS AND DISCUSSION

The porous diaphragm method or the chromatographic broadening method was used for the determination of the diffusion coefficient (D_i) as previously reported.[15] The D_i and the Stokes–Einstein radius (r_i) of four different sized paracellular markers are listed in Table 1. When RD10 is compared with urea, for which there is a 180-fold difference in MW, the value is 0.09 times for D_i and 11 times for r_i.

Table 2 shows the $Papp_i$ of the paracellular markers through three different Caco-2 cell monolayers cultured for 9 d. The monolayers looked confluent under an optical microscope, but the TER values were very low ($83.7\pm25.4\ \Omega\cdot$cm, mean$\pm$S.D.). The ratio of the $Papp_i$ (CF/RD10) for each monolayer (2.2—3.0) was lower, rather than higher, than that of D_i (3.76), suggesting that the size of the pathway was far bigger (2.1 nm$<<R$) than that of RD10. The low ratio of the $Papp_i$ (CF/RD10) might be due to the repulsive interaction of anionized CF to the paracellular pathway or the contribution of smaller RD10 molecules to the permeation because RD10 is polydisperse in nature. The interaction of CF could reduce the $Papp_i$ of CF ($Papp_{CF}$) and the permeation of the smaller RD10 could increase the $Papp_i$ of RD ($Papp_{RD10}$). The ε/L for each monolayer was calcu-

Table 1. Diffusion Coefficient (D_i) and Stokes–Einstein Radius (r_i) of Drugs Used at 37 °C

	MW	D_i (cm^2/s)$\times10^6$	r_i (nm)
Urea	60.1	17.5[a]	0.19
Mannitol	182.2	9.56[a]	0.34
CF	376.3	5.87[b]	0.56
RD10	11000	1.56[b]	2.09

a) Porous diaphragm method. *b*) Chromatographic broadening method.

Table 2. *Papp* and TER of Caco-2 Cell Monolayers Cultured for 9 d

#	*Papp* (cm/s)×10^6				TER (Ω·cm)
	Urea	Mannitol	CF	RD10	
9-1	13.7	8.20	5.06	1.82	79.9
9-2	14.9	11.7	5.84	1.98	110.7
9-3	8.46	6.13	4.43	2.04	60.3

Table 3. Characteristic Parameters of the Permeation Pathway in Caco-2 Cell Monolayers Cultured for 9 d and the Calculated *Papp* of Urea and Mannitol Using these Parameters

#	ε/L (cm^{-1})	R (nm)	Calculated *Papp* (cm/s)×10^6	
			Urea	Mannitol
9-1	0.862	2.1≪	15.1	8.24
9-2	0.995	2.1≪	17.4	9.51
9-3	0.755	2.1≪	13.2	7.22

Table 4. *Papp* and TER of Caco-2 Cell Monolayers Cultured for 13 d

#	*Papp* (cm/s)×10^6				TER (Ω·cm)
	Urea	Mannitol	CF	RD10	
13-1	7.62	0.571	0.298	0.0991	1493
13-2	8.59	0.287	0.139	0.0711	1751
13-3	14.5	1.02	0.794	0.343	1119
13-4	25.6	1.75	1.52	0.592	1548
13-5	15.2	0.477	0.294	0.135	1362

Table 5. Characteristic Parameters of the Permeation Pathway in Caco-2 Cell Monolayers Cultured for 13 d and the Calculated *Papp* of Urea and Mannitol Using these Parameters

#	Large pathway		Small pathway	
	ε/L (cm^{-1})	R (nm)	ε/L (cm^{-1})	R (nm)
13-1	0.0508	2.1≪	7.63	0.39
13-2	0.0237	2.1≪	11.2	0.37
13-3	0.135	2.1≪	—	<0.34
13-4	0.259	2.1≪	—	<0.34
13-5	0.0501	2.1≪	—	<0.34

#	Calculated *Papp* (cm/s)×10^6				Contrib. of small pathway to urea permeation[a)]
	Large pathway		Small pathway		
	Urea	Mannitol	Urea	Mannitol	
13-1	0.889	0.485	6.73	0.0859	0.88
13-2	0.416	0.227	8.18	0.0597	0.95
13-3	2.37	1.29	12.2	—	0.84
13-4	4.53	2.47	21.1	—	0.82
13-5	0.876	0.479	14.3	—	0.94

a) Estimated contribution of the small pathway to urea permeation through the Caco-2 cell monolayer assuming that the contribution of UT-B urea transporter is negligible; calculated *Papp* through the small pathway/observed *Papp* of urea.

lated using Eq. 4 and also the $Papp_{CF}$, although CF is not an ideal paracellular marker because of its anionic nature. Table 3 shows the calculated ε/L values and the calculated $Papp_i$ of urea and mannitol ($Papp_u$ and $Papp_m$) from the ε/L and corresponding D_i. The calculated $Papp_u$ and $Papp_m$ were similar to the observed values for each monolayer (there were no significant differences between calculated and observed values for urea and mannitol in the paired *t*-tests, $p>0.05$), suggesting that CF is available for the paracellular marker in this case. The repulsive interaction might be negligible for such a large pathway. The result also suggests that there is no smaller pathway in Caco-2 cell monolayers cultured for 9 d on the transwells. These results suggest that the culture period of 9 d in our method is not enough for confluency and no tight junctions are established during this period.

Table 4 shows the $Papp_i$ of the paracellular markers through five different Caco-2 cell monolayers cultured for 13 d. The monolayers looked confluent and the TER values (1454±234Ω·cm) were higher than those after 9 d of culture, but the $Papp_i$ of each paracellular marker differed between the individual monolayers. The ratio of $Papp_i$ (CF/RD10) for each monolayer (2.0—3.0) was also lower than that of D_i, suggesting that the size of the pathway was far bigger than that of RD10. The $Papp_u$ and $Papp_m$ values through the pathway were calculated from the ε/L obtained from the $Papp_{CF}$ for each monolayer, under the assumption that the repulsive interaction of CF is negligible, and then compared with the observed values (Table 5). For samples 13-1 and -2, the observed values were higher than the calculated values for both urea and mannitol, suggesting the presence of a small pathway for both.[9] On the other hand, for samples 13-3, -4 and -5, the observed values were higher only for urea, suggesting the presence of a small pathway, the size of which was smaller than for mannitol ($R<0.34$ nm). The $Papp_u$ and $Papp_m$ values through the small pathway were calculated as the difference between the observed and calculated values for each monolayer, although a contribution of UT-B urea transporter to the transport of urea through Caco-2 cell monolayers has been reported.[16] The ε/L and R of the small pathway for samples 13-1 and -2 were calculated using the Renkin function. When the calculated $Papp_u$ through the small pathway was compared with the observed total $Papp_u$ for each monolayer, the contribution of the small pore pathway to urea permeation was over 80% if the contribution of the UT-B urea transporter is negligible, suggesting that the small pathway is the major permeation pathway for such small hydrophilic molecules. In the comparison of the area occupancy between the large and small pathways for the samples 13-1 and -2, the ε/L was 0.0508 : 7.63 (1 : 150) and 0.0237 : 11.2 (1 : 473), respectively. The tight junction, as a permeation barrier for large hydrophilic molecules, can be formed but it is different for each monolayer at 13 d.

In this study, the tight junction formation in the Caco-2 cell monolayers was compared between 9 and 13 d of culture. The TER and $Papp_m$ values for monolayers cultured for 13 d were 17.4 and 0.095 times the values for 9 d, respectively. The tight junction structure developed at various levels during that period. The pore permeation model involving two different sizes is useful for evaluating the paracellular pathways in a more quantitative way. There were two-different sized pathways in the monolayers cultured for 13 d, while there was only a single large pathway in those cultured for 9 d. The R of the small pathway in the Caco-2 cell monolay-

ers cultured for 13 d was less than 0.4 nm, suggesting the development of tight junctions. As mentioned above, the paracellular markers used in this study are not ideal, in terms of the nature or characteristics for Caco-2 cell monolayer permeation, *e.g.* CF has a negative charge and RD10 is polydisperse. This might be related to the discrepancy in the pore size of the large pathway compared with that reported by Knipp *et al.*[14] Therefore, the parameters obtained in this study should be evaluated in relative terms. However, the methodology provided in this study is valuable for characterizing the individual monolayers. Since the paracellular permeability of the Caco-2 cell monolayers can be affected by not only the culturing conditions but also the cell passage number,[12] validation will be needed for any quantitative evaluation of the permeation of large hydrophilic drugs. Our results provide important information for establishing such a validation method. The choice of adequate paracellular markers for the accurate determination of the parameters and examination of the relationship between these parameters and the tight junction molecular assembly will be the subject of the next stage of our research.

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