Evidence for Diminished Functional Expression of Intestinal Transporters in Caco-2 Cell Monolayers at High Passages

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Purpose. To investigate the effects of passaging on the intrinsic membrane transport parameters of compounds absorbed by means of passive and carrier-mediated processes in the Caco-2 cell line.

Methods. Caco-2 cells at low (28–36) and high (93–108) passage numbers were used to evaluate the transport characteristics of model compounds for paracellular diffusion (mannitol), transcellular diffusion (progesterone) and carrier-mediated transport (cephalexin, cephradine, phenylalanine, proline, and taurocholic acid) using side-by-side diffusion chambers. Intrinsic intestinal transport parameters were determined by correcting the effective permeability for potential biases introduced by the microporous filter and aqueous boundary layer. Intrinsic maximal flux (J_{max}), Michaelis constant (K_m) and carrier permeability (P_c) were determined as a function of passage number.

Results. Compared to the low passaged cells, the high passaged Caco-2 cells were characterized by less morphological heterogeneity, higher transepithelial electrical resistance, higher transcellular diffusion, lower paracellular diffusion, lower carrier-mediated transport and lower alkaline phosphatase activity. The use of effective transport parameters overestimated the K_m and underestimated P_c but had no effect on J_{max}.

Conclusions. The current results provide experimental evidence that the passaging process significantly affects the biological characteristics and transport properties of Caco-2 cell monolayers. The effects are consistent with a reduction in the functional expression of a brush border enzyme and several transport proteins as passage number is increased. The underlying basis for this appears to be a selection of fast-growing subpopulations from the original heterogeneous Caco-2 cell line during passaging.

KEY WORDS: aqueous boundary layer; brush border enzymes; carrier-mediated transport; diffusion chamber; heterogeneity; permeability.

INTRODUCTION

The Caco-2 cell line has been widely used as an *in vitro* model for studying intestinal absorption (1,2). The transport properties of the monolayer are influenced by many culture-related factors including the type of clone, the passage number, the seeding density, the composition of the medium, and the type of microporous filter (1,3). The laboratory-to-laboratory variability in the transport properties of cell monolayers limits the useful application of Caco-2 permeability data such as published correlations between permeability and the extent of drug absorption (4). While the systematic evaluation of the transport properties of the Caco-2 cell monolayer as a function of culturing conditions is generally lacking, Lu *et al.* (5) recently demonstrated that the transport characteristics of several model compounds with different absorption mechanisms did not change with passage number. However, since it is widely known that brush border enzyme expression varies with culturing factors (6) it is also likely that the functional expression of intestinal transport proteins is similarly affected. Therefore, in the current study, the effect of passaging on the transport properties of Caco-2 cell monolayers, particularly the functional expression of intestinal transport proteins, is evaluated by characterizing the intrinsic transport parameters of several carrier-mediated compounds.

MATERIALS AND METHODS

Materials

^{3}H-Phenylalanine, ^{3}H-progesterone and ^{3}H-proline were purchased from Amersham USA (Arlington Heights, IL), and ^{3}H-taurocholic acid and ^{14}C-mannitol from Du Pont-NEN (Boston, MA). All medium components and reagents for cell culture were obtained from Gibco Life Technologies, Inc. (Grand Island, NY). All other chemicals were purchased from Fisher Scientific, Inc. (Fair Lawn, NJ) or Sigma Chemical Company (St. Louis, MO) and were used as received.

Cell Culture

The Caco-2 cell line was obtained from American Type Culture Collection (Rockville, MD) at passage 18. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% heat-inactivated fetal bovine serum, 1% non-essential amino acids, penicillin (100 U/ml) and streptomycin (100 mg/ml), in an atmosphere of 95% air and 5% CO_2 at 37°C. Stock cells were passaged every three to five days through passage 108 by trypsinizing cells at 90% confluency using 0.05% trypsin and 0.53 mM EDTA at 37°C for 10 min. To seed cells onto the Snapwell™ polycarbonate filters with 0.4 μm pore diameter (Corning-Costar Corporation, Cambridge, MA), cells were harvested at 90% confluency and seeded at a density of 70,000/cm^2. Cells at passage number 28–36 were designated as the "low" passage range (C2LO) and 93–108 as the "high" passage range (C2HI). All cells used for transport were cultured in parallel under identical conditions for four weeks since previous reports showed that the differentiation of Caco-2 cells was complete 25 to 30 days after confluence was achieved (7).

Measurement of TEER and Cell Density

The transepithelial electrical resistance (TEER) of cell monolayers was measured every week up to four weeks post seeding using the EVOM™ epithelial voltohmmeter and EndOhm-12™ tissue resistance measurement chamber (World Precision Instruments, Sarasota, FL). Cells grown on Snapwell™ filter clusters were rinsed three times with Ringer's buffer (pH 7.4) containing 25 mM glucose at room temperature 20 min before the TEER was measured in this buffer. The resistance of a blank filter was used to correct the TEER values

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determined for cell monolayers. Cell density was determined by trypsinizing and counting the cells using a hemacytometer.

Enzyme Assays

Brush border membrane vesicles were isolated by modifying Pinto's method (8): a 1 M solution of $CaCl_2$ replaced solid $CaCl_2$ and ultrasonication was omitted. Protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA). The activity of alkaline phosphatase was determined as previously described (9). The enzyme activity is expressed as milliunits per mg of brush border protein and one unit is defined as the activity that hydrolyzes 1 μmole of substrate per min under experimental conditions.

Transport Studies

A side-by-side diffusion chamber system (Precision Instrument Design, Tahoe City, CA) was used in transport experiments. The apical buffer was pH 6.5 MES Ringer's buffer and the basolateral buffer was pH 7.4 Ringer's buffer. Both buffers contained 25 mM glucose and were adjusted to 290 mOsm/Kg. For transport experiments using filters with cell monolayers, the Snapwell™ inserts were rinsed three times with Ringer's buffer containing 25 mM glucose over a 20 min period at room temperature. The TEER was measured for each monolayer before they were mounted between two half-chambers and placed into the warming blocks preheated to 37°C. The apical chamber was filled with 7 ml of preheated apical buffer containing the compound to be tested and the basolateral side was filled with 7 ml of basolateral buffer. The fluid in the chamber was circulated using a gas lift mechanism with 5% CO_2-95% O_2 at a constant flow rate of 20 ml/min monitored with a J&W ADM2000 flow meter (Fisher Scientific, Fair Lawn, NJ). Starting 30 min after the initiation of the experiment, samples were taken from the basolateral chamber every 15 min for 105 min. For transport experiments using blank filters, samples were taken every 10 min for 50 min starting at 10 min.

Calculation of Intrinsic Transport Parameters

The effective permeabilities, P_{eff}, of filters with cell monolayers were calculated from the experimental data using the equation:

$$P_{eff} = \frac{V_r}{A\,C_o}\frac{dC}{dt} \tag{1}$$

where V_r is the volume of the receiver chamber, A is the surface area of the filter, C_o is the initial drug concentration in the donor (apical) phase and dC/dt is the change in drug concentration in the receptor (basolateral) phase per unit time. The effective permeabilities of blank filters, P_{eff}^{blank}, were determined in an analogous manner.

For a solute which is transported by a mixed mechanism, the total flux through the cell monolayer can be considered as the sum of two parallel mechanisms, a saturable carrier-mediated and a non-saturable simple diffusion mechanism:

$$J_{eff} = \frac{J'_{\max}C_o}{K'_m + C_o} + P'_m C_o \tag{2}$$

or, rewritten in terms of permeability:

$$P_{eff} = \frac{J'_{\max}}{K'_m + C_o} + P'_m = \frac{P'_c}{1 + \dfrac{C_o}{K'_m}} + P'_m \tag{3}$$

The conventional approach is to determine J_{eff} or P_{eff} as a function of C_o, and to regress the data using Eqn. (3) to determine the biased values (denoted by the prime) of the maximal flux, J'_{max}, the Michaelis constant, K'_m, the membrane permeability, P'_m, and the carrier permeability P'_c. These parameters are biased by the resistances of the filter and the aqueous boundary layer (ABL). In order to obtain the intrinsic transport parameters, the permeability through the cell monolayer P_{mono} and the concentration at the surface of the monolayer, C_w, are used:

$$P_{mono} = \frac{J_{\max}}{C_w + K_m} + P_m \tag{4}$$

and

$$P_{mono} = \frac{P_c}{1 + \dfrac{C_w}{K_m}} + P_m \tag{5}$$

C_w can be calculated using the following method originally introduced by Johnson and Amidon (10):

$$C_w = \left(1 - \frac{P_{eff}}{P_{aq}}\right) C_o \tag{6}$$

The P_{aq} can be measured by conducting multi-flow rate experiments as previously described (11).

The P_{mono} must be separated from the permeabilities of the filter and the ABL. A detailed analysis on this subject will be published separately (11). Briefly, the effective permeability can be defined as:

$$\frac{1}{P_{eff}} = \frac{1}{P_{eff}^{blank}} + \frac{1}{P_{mono}} \tag{7}$$

By rearranging Eqn. (7), P_{mono} is easily calculated and used in Eqns. (4 and 5) to obtain the intrinsic transport parameters.

Calculation of Carrier Permeability

For phenylalanine, P_{eff}'s were measured at the following concentrations: 0.0001, 0.001, 0.01, 0.1, 0.3, 1.0, 10 and 100 mM. Previously obtained $P_{eff}^{blank} = 2.35 \times 10^{-4}$ cm/s and $P_{aq} = 5.36 \times 10^{-4}$ cm/s (11) were used in the calculations of P_{mono} and C_w using Eqns. (7) and (6), respectively, and P_c was obtained by performing a non-linear regression analysis on Eqn. (5). For cephalexin, cephradine, proline and taurocholic acid, a simplified approach was used to estimate P_c. A low and a high concentration, both significantly distant from reported K_m's, were chosen for each compound. P_{eff} and P_{eff}^{blank} were then measured at these concentrations and P_{mono} was calculated using Eqn. (7). Finally, the P_c's were approximated by the difference of P_{mono}'s at low and high concentrations. The reported K_m's through Caco-2 monolayers are 8.3 mM, 4.7 mM, 9.2 mM and

~50 μM for cephalexin (12), cephradine (13), proline (14) and taurocholic acid (2,15,16), respectively. The concentrations used in the experiments were: 0.3 mM and 40 mM, 0.05 mM and 25 mM, 0.01 mM and 30 mM, and 3.9 μM and 500 μM, respectively. For mannitol and progesterone, the apical concentrations were 20 mM and 1×10^{-7} mM, respectively.

Analytical Methods

All radiolabeled chemicals were analyzed using a Wallac 1409 liquid scintillation counter (Rockville, MD). Cephradine and cephalexin were analyzed by reverse phase HPLC using previously established methods (17).

Statistical Analysis

Results are reported as the mean ± standard error of the mean (SEM). All data analyses and statistical calculations were performed using Lotus 1-2-3 for Windows (Lotus Development Corp., Cambridge, MA). Non-linear regressions were performed using Micromath Scientist for Windows 2.0 (Salt Lake City, UT) with a weighing scheme of 1/(relative standard deviation)^2. A minimum p value of 0.05 was used as the significance level for t-tests.

RESULTS AND DISCUSSION

Comparisons of TEER and Cell Density

The TEER values for the C2HI cells consistently displayed significantly higher TEER values ($p \leq 0.05$) than C2LO (C2HI: 139 ± 17, 197 ± 4, 313 ± 8, and 353 ± 7 Ω·cm^2 for weeks 1, 2, 3, and 4 respectively; C2LO: 86 ± 9, 173 ± 3, 269 ± 8, and 314 ± 10 Ω·cm^2 for weeks 1, 2, 3, and 4 respectively). Correspondingly, the C2HI cells demonstrated significantly higher cell density ($5.83 \pm 0.61 \times 10^4$ cells/cm^2) than the C2LO cells ($3.25 \pm 0.20 \times 10^4$ cells/cm^2) on day 7, suggesting better cell attachment and a faster rate of growth. Since the tight junction at the end of week 1 was not fully established, the relatively higher cell density of C2HI cell layers appears to be consistent with its higher TEER value. After confluence is reached, however, the TEER is a comprehensive reflection of the function of ion transporters on cell surfaces, the integrity of the tight junction and the cell density. It remains to be explained what caused the passage difference in TEER at a later stage of culture.

Comparisons of Passive Diffusion

Two model compounds, mannitol for paracellular diffusion and progesterone for transcellular diffusion, were selected as passive markers. As shown in Fig. 1, the monolayer permeability (P_{mono}) of mannitol through the C2HI was significantly (36%) lower than through C2LO, consistent with the change in TEER. In contrast to the paracellular marker, the P_{mono} of progesterone was 35% higher through C2HI in the apical (AP) to basolateral (BL) direction, a statistically insignificant difference. For C2LO and C2HI, the BL to AP P_{mono}'s of progesterone were consistently lower than the corresponding AP to BL permeabilities (Fig. 1), suggesting the absence of the p-glycoprotein-driven efflux at the apical surface.

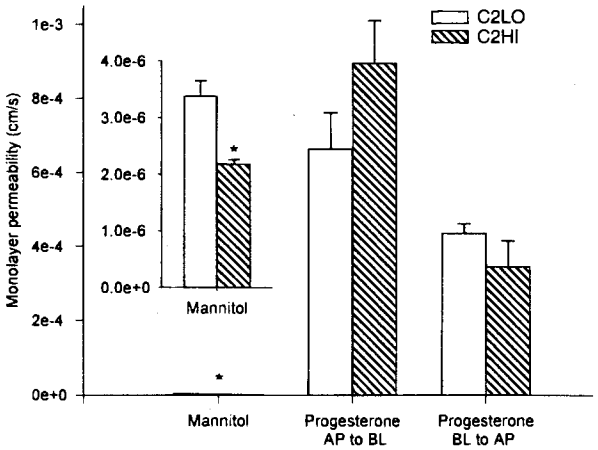

Fig. 1. Effect of passaging on the monolayer permeabilities of marker compounds for passive diffusion. Apical (AP) to basolateral (BL) transport of mannitol and bidirectional transport of progesterone were measured using Snapwell-grown Caco-2 cell monolayers on day 28 post seeding. Monolayer permeabilities were obtained by correcting the P_{eff} for the biases of the ABL and filter resistances. Data represents mean ± SEM (n = 3). Inset shows enlargement of mannitol data. Asterisks indicate significant difference between populations ($p < 0.05$, t-test).

Effect of Passaging on the Activity of Alkaline Phosphatase

The effect of passaging on cell differentiation was evaluated by examining the activity of alkaline phosphatase, a commonly used intestinal brush-border enzyme marker (8). As shown in Fig. 2, the 2-week-old C2HI cells showed a significantly lower (28.6%) activity of alkaline phosphatase than the C2LO cells. By four weeks post seeding, the difference increased to 66.9%.

Effect of Passaging on the Intrinsic Transport Parameters

A complete evaluation of the potential effects of passaging on carrier-mediated transport requires comparisons of the P_c,

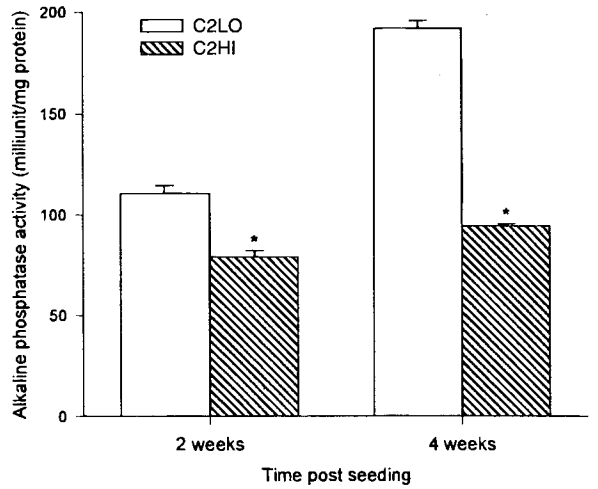

Fig. 2. Effect of passaging on the activity of alkaline phosphatase. Data represents mean ± SEM from three measurements from the same batch of culture. Asterisks indicate significant difference between populations ($p < 0.05$, t-test).

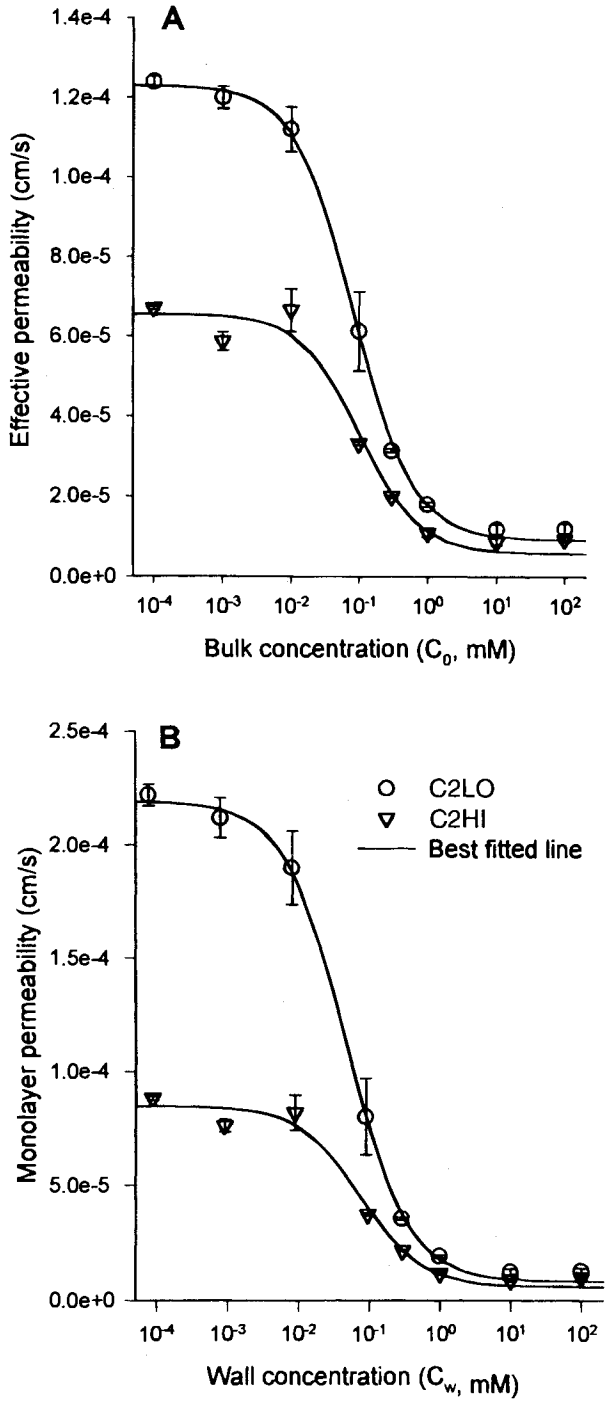

Fig. 3. Concentration dependence of phenylalanine permeability across C2LO and C2HI monolayers. **A,** P_{eff} vs. C_o; **B,** P_{mono} vs. C_w. Apical to basolateral transport was measured using Snapwell-grown Caco-2 cell monolayers on day 28 post seeding. Data represents mean ± SEM (n = 3).

J_{max} and K_m. Phenylalanine was chosen for such an evaluation because its P_{aq}, which is necessary to obtain the intrinsic parameters, was previously obtained (11). The biasing effect due to the resistances of the filter and ABL on the transport parameters was examined first. The P_{eff} and P_{mono} were plotted against C_o and C_w, respectively (Fig. 3A and B), and the effective and intrinsic J_{max}, K_m, P_c and P_m were obtained by regressing each set of data using Eqns. (3–5). As shown in Table 1, the use of P_{eff} and C_o resulted in a significant overestimate of K_m and an underestimate of P_c, but had no significant effect on the J_{max} and P_m, consistent with previously published results using animal tissue (18,19). In a cell culture transport experiment, both the microporous filter and the ABL serve as sources of bias on the K_m and P_c. The current results clearly demonstrate the importance of using the intrinsic permeabilities to obtain unbiased transport parameters for studying carrier-mediated transport. Comparisons between the C2LO and C2HI intrinsic transport parameters indicate that, as the passage number increases, the J_{max} and the P_c are reduced by 40.4% and 62.6%, respectively (Table 1). The insignificant difference in K_m suggests a change in the number rather than the type of the amino acid transporters on the apical and/or basolateral membranes during the passaging of Caco-2 cells. The effective K_m of phenylalanine reported in this paper is approximately five to seven fold lower than that reported by Hidalgo and Borchardt (0.56 mM) (15). A meaningful comparison on J_{max} is prohibited without knowing the protein content of the cell monolayer used in their study. Although the stagnant Transwell™ system used in their study is presumed to generate a larger ABL effect on the K_m than the side-by-side diffusion chamber (20), potential differences in the culturing conditions, experimental conditions and passage number represent additional factors that can affect the K_m value. Since the transport parameters of phenylalanine are a measure of the combined functioning of multiple transporters (21), any changes in these factors may significantly affect the expression of the transporters and the ratio between each of them. Additional carrier-transported compounds, cephalexin, cephradine, proline and taurocholic acid, were also studied using intrinsic transport parameters (Table 2). Comparisons of the approximated P_c values between C2LO and C2HI showed that, as the passage number increases, the P_c's are reduced by 88.7%, 82.7%, 78.1% and 64.5% for cephalexin, cephradine, proline and taurocholic acid, respectively (Fig. 4), suggesting a reduction in the functional expression of the corresponding transporters.

As the best-described cell culture model system for studying intestinal drug transport, Caco-2 cells have been used at passage numbers ranging from the low 20's to over 100. A few studies, however, have revealed passage-related differences in enzymatic differentiation (22), glucose transporter expression (23) and glycoprotein expression (24) in Caco-2 cells. A phenotypic drift of the transport of taurocholic acid with increasing passage numbers was observed by Chandler et al. (16), suggesting that the transport properties of this cell line may be affected by passaging. Walter and Kissel (25) also compared the transport properties of Caco-2 cells from different clones at different passage numbers, showing the active transport for thyrotropin-releasing hormone at high passage numbers. The only systematic study on passaging effects was reported by Lu et al. (5) in which they compared Caco-2 cells at the early passages (passages 35–47) and later passages (passages 87–112). In their study, the TEER values of high passage cells were more-than-doubled (1423 $\Omega \cdot cm^2$ comparing to 664 $\Omega \cdot cm^2$ at low passages) and a dedifferentiation of the cell was suggested. No significant differences, however, were found with respect to transport properties using a limited number of marker compounds for passive and carrier-mediated transport. In con-

Table 1. Comparisons of the Maximal Flux (J_{max}), Michaelis Constant (K_m), Carrier Permeability (P_c) and Passive Permeability (P_m) of L-Phenylalanine Obtained from C2LO and C2HI Monolayers

Parameter	Effective[a]		Intrinsic[b]		Difference (%)[c]	
	C2LO	C2HI	C2LO	C2HI	C2LO	C2HI
J_{max} ($\times 10^{-6}$ μmole·cm^{-2}·s^{-1})	9.51 ± 0.89	6.05 ± 0.58	9.76 ± 0.18	5.82 ± 0.49	2.6	−3.8
K_m (μM)	83.4 ± 6.7	101 ± 47.5	46.2 ± 4.54	73.7 ± 30.5	−44.6	−27.0
P_c ($\times 10^{-6}$ cm/s)	114 ± 2.39	59.8 ± 5.71	211 ± 5.12	78.9 ± 6.62	85.1	31.2
P_m ($\times 10^{-6}$ cm/s)	9.08 ± 2.37	5.74 ± 4.77	8.20 ± 3.26	5.79 ± 5.60	−9.7	0.9

[a] Non-linear regression (NLR) output from Fig. 3A, using P_{eff} and C_o, before the correction for the effects of the resistances of the filter and ABL.
[b] NLR output from Fig. 3B, using P_{mono} and C_w, after the correction for the effects of the resistances of the filter and ABL.
[c] Calculated by [(intrinsic value − effective value)/effective value] × 100.

Table 2. Measured Effective Permeabilities of Marker Compounds for Carrier-Mediated Transport Across Blank Filters (P_{eff}^{blank}) and Filters with Caco-2 Monolayers (P_{eff}) in the Diffusion Chamber at a Flow Rate of 20 ml/min (mean ± SEM, n = 3)

Compounds	P_{eff}^{blank} ($\times 10^{-6}$ cm/s)[a]	Conc. (mM)	P_{eff} ($\times 10^{-6}$ cm/s)		P_{mono} ($\times 10^{-6}$ cm/s)	
			C2LO	C2HI	C2LO	C2HI
Cephalexin	164 ± 2.00	0.30	16.6 ± 3.16	2.51 ± 0.11	18.6 ± 3.96	2.55 ± 0.11
		40	1.40 ± 0.12	0.60 ± 0.10	1.41 ± 0.12	0.60 ± 0.10
Cephradine	162 ± 1.00	0.05	8.96 ± 0.42	2.03 ± 0.01	9.49 ± 0.47	2.05 ± 0.01
		25	0.76 ± 0.05	0.54 ± 0.02	0.76 ± 0.05	0.54 ± 0.02
L-proline	310 ± 6.97	0.01	41.2 ± 0.86	23.0 ± 1.20	47.6 ± 1.14	24.8 ± 1.40
		30	20.2 ± 0.11	18.0 ± 1.05	21.6 ± 0.13	19.2 ± 1.19
Taurocholic acid	126 ± 0.00	0.00039	57.8 ± 5.11	28.9 ± 1.37	109.0 ± 17.0	37.6 ± 2.27
		0.5	4.15 ± 0.34	0.44 ± 0.12	4.29 ± 0.36	0.44 ± 0.12

[a] Concentrations used: 0.1 mM, 0.1 mM, 0.1 mM and 0.001 mM for cephalexin, cephradine, proline and taurocholic acid, respectively.

trast, all comparisons conducted in the current study between C2LO and C2HI cells, including morphological homogeneity, TEER, passive diffusion, carrier-mediated transport, and brush-border enzyme activity, demonstrate that passaging significantly affects the biological characteristics and transport properties of the cultured Caco-2 cell monolayer. After extensive passaging, the Caco-2 cells are characterized by reduced paracellular diffusion and functional expression of enzymes and carriers in the apical membrane.

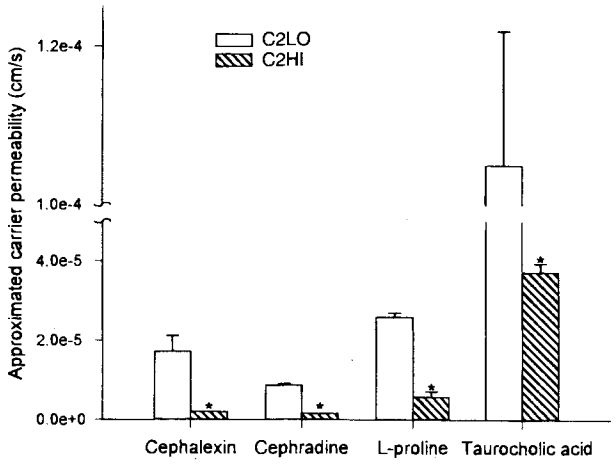

Fig. 4. Effect of passaging on the approximated carrier permeability of marker compounds for carrier-mediated transport. See "Methods" section for details on the concentrations used and the approximation of P_c. Data represents mean ± SEM (n = 3). Asterisks indicate significant difference between passages ($p < 0.05$, t-test).

The heterogeneity of the Caco-2 cell line seems to be the biological basis underlying the passaging effect. The phenotypic heterogeneity of Caco-2 cell line has been increasingly recognized by investigators (2,3,24,26). Since Caco-2 cells are comprised of subpopulations with differing rates of proliferation, the passaging process will favor the proliferation of faster-growing subpopulations. Morphologically, the cell populations in this study exhibited a decrease in heterogeneity with higher passage number (data not shown). Therefore, as with other cell lines, the population and, subsequently, the transport properties of the Caco-2 cells will change after repeated passaging (3,27,28). The higher initial growth rate and less heterogeneous cell composition characterized by the C2HI cells support this hypothesis. In spite of the lower heterogeneity in the C2HI, they appeared to be less differentiated as evidenced by the decreased carrier permeabilities for the five marker compounds (Figs. 3 and 4, Table 1) and by the reduction in the activity of alkaline phosphatase (Fig. 2). However, the current studies cannot resolve if the reduction is due to "dedifferentiation" as suggested by Lu et al. (5).

In summary, the current results provide experimental evidence showing that the passaging process significantly affects the biological characteristics and transport properties of Caco-2 cell monolayers. Since the passaging process would favor fast growing subpopulations, it is likely that these effects are related to the heterogeneous nature of the cell line.

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