Analysis of Drug Permeation Across Caco-2 Monolayer: Implication for Predicting *In Vivo* Drug Absorption

Shinji Yamashita,[1,2] Yoshihiro Tanaka,[1] Yoriko Endoh,[1] Yoko Taki,[1] Toshiyasu Sakane,[1] Tanekazu Nadai,[1] and Hitoshi Sezaki[1]

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Purpose. The aim of the present work is to characterize *in vitro* drug permeation processes across Caco-2 monolayer and to identify the advantages of this cultured cell system in predicting *in vivo* drug absorption after oral administration.

Methods. The passive permeability of various drugs through Caco-2 monolayer was measured using Ussing-type chambers and compared with that of the isolated rat jejunum and colon. The *in vivo* drug permeability to the intestinal membrane was estimated by means of an intestinal perfusion study using the rat jejunum.

Results. In Caco-2 monolayer, drug permeability increased with increasing drug lipophilicity and showed a good linear relationship with the *in vivo* permeability. In contrast, in the isolated jejunum and colon, the permeability of high lipophilic drugs was almost constant and, propranolol, a drug with the highest lipophilicity, hardly passed through the jejunal membrane *in vitro*. As a result, there was no significant relationship between *in vitro* and *in vivo* drug permeability in rat jejunum. However, the amount of drugs accumulated in the jejunal mucosa increased with increasing drug lipophilicity even under the *in vitro* condition.

Conclusions. The permeation and the accumulation studies suggested that the rate-limiting process of *in vitro* permeation of lipophilic drugs through the intestinal membrane differs from that of *in vivo* drug absorption. On the other hand, drug permeation through Caco-2 monolayer, which consists of an epithelial cell layer and a supporting filter, is essentially the same process as that of *in vivo* drug absorption. We concluded that the simple monolayer structure of a cultured cell system provides a distinct advantage in predicting *in vivo* drug absorption.

KEY WORDS: Caco-2 monolayer; intestinal membrane; drug lipophilicity; *in vitro* drug permeability; *in vivo* drug absorption.

INTRODUCTION

One of the most important factors in defining oral drug absorption should be drug permeability across the intestinal membrane. A number of *in vivo, in situ* and *in vitro* experimental procedures have been developed which examine intestinal drug permeability. Other than studies on the mechanisms of drug absorption, prediction of oral drug absorption in humans, the bioavailability of drugs, is the final goal of such studies. Amidon et al. have demonstrated that the permeability of several drugs in the rat small intestine obtained by single-pass perfusion correlates well with the fraction dose absorbed in humans. (1,2) This theoretically established relationship has indicated that drug absorption in humans can be predicted from studies in small experimental animals.

During the last few years, the epithelial cell lines such as Caco-2 and HT29 have received considerable attention from the pharmaceutical industry. They have been suggested for screening drug absorption at the early stage of drug development. Several advantages of applying them to pre-clinical studies have been suggested by many investigators including, the fact that they are of human origin (3–6). However, key issue is that drug permeation through the cultured cell monolayer must accurately reflect drug absorption in humans.

We have already described the usefulness of *in vitro* drug permeation studies using the isolated intestinal membrane of experimental animals, mounted in side-by-side chambers (Ussing type chambers) (7,8). Because the isolated intestinal membrane can be handled as a flat sheet, it might be possible to use it in the same manner as cultured monolayers. In fact, we showed that the higher electrical resistance of Caco-2 monolayer than that of the rat intestine is due to the difference in the number of cell-junctions per unit area (9). This derives from the monolayers being flat whereas the intestine is villous. By standardizing the areas of application, therefore, the paracellular permeability of Caco-2 monolayer and the isolated rat intestine were almost the same.

In this study, we compared *in vitro* drug permeation processes in three membranes, Caco-2 monolayer, rat jejunum and rat colon under the same *in vitro* conditions. From the correlation between the *in vitro* and *in vivo* permeability of various drugs, the advantages of Caco-2 monolayer use to predict *in vivo* drug absorption were discussed based upon the structural differences of those membranes.

MATERIALS AND METHODS

Materials

The Caco-2 cell line was obtained from American Type Culture Collection (Rockville, Md.) at passage 17. Dulbecco's modified Eagle medium (DMEM), non-essential amino acids (NEAA), fetal bovine serum (FBS), L-glutamate, trypsin (0.25%)-EDTA (! mM) and antibiotic-antimycotic mixture (10000 U/ml penicillin G, 10000 μg/ml streptomycin sulfate and 25 μg/ml amphotericin B in 0.85% saline) were purchased from Gibco Laboratories (Lenexa, KS). Cefazolin was donated by Fujisawa Pharmaceutical Co., (Osaka, Japan). All other reagents were of the highest purity.

Preparation of Caco-2 Monolayer

Caco-2 cells were grown in DMEM supplemented with 10% FBS, 1% L-glutamate, 1% NEAA and 5% antibiotic-antimycotic solution at 37°C in culture flasks (Nippon Becton Dickinson Co., Ltd., Tokyo Japan) in a humidified air-5% CO_2 atmosphere. Cells were harvested with trypsin-EDTA and seeded on polycarbonate filters (0.3 μm pores, 4.71 cm^2 growth area) inside Transwell cell culture chambers (Costar, Cambridge, MA) at a density of 3×10^5 cells/filter. The culture medium (1.5 ml in the insert and 2.6 ml in the well) was replaced every 48 h for the first 6 days and every 24 h thereafter (10). After 15–18 days in culture, the filter with Caco-2 mono-

[1] Faculty of Pharmaceutical Sciences, Setsunan University, Nagaotoge-cho, Hirakata, Osaka 573-01, Japan.

[2] To whom correspondence should be addressed. (e-mail: shinji-y@po.iijnet.or.jp)

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layer was removed from the well and mounted in Ussing type chambers for the following experiments (11).

Preparation of the Isolated Sheet of Rat Jejunum and Colon

Flat sheets of rat isolated jejunum or colon were prepared as described previously (9). Briefly, the lower jejunum or colon was removed from Wistar strain male rats and opened along the mesenterium to give a flat sheet. After washing the intestinal contents with ice-cold transport medium (see below), the muscle layer of the membrane was stripped. The obtained mucosal sheet was immediately mounted in Ussing type chambers.

Membrane Drug Permeability *In Vitro*

Both sides of the membrane in the chamber were filled with 11 ml of transport medium (Hank's balanced salts solution (HBSS) supplemented with 25 mM glucose) and stirred by bubbling with a 95% CO_2-5% O_2 mixture. The buffer pH was adjusted to 7.0 using HEPES. After 25 min, the transport medium containing the drug was introduced into the mucosal side. The initial concentration of drugs were 1 mM for sulfanilamide (SAM), sulfapyridine (SP), warfarin (WAR), and propranolol (PPR) and 10 mM for sulfanilic acid (SUA) and cefazolin (CEZ), respectively. Thereafter, aliquots of samples were taken from the serosal side every 10 min for 1 hr. The volume of the serosal solution was maintained by adding fresh transport medium. The mucosal-to-serosal permeability of each drug was calculated from its flux rate estimated as the rate of increase in the serosal concentration. This experiment proceeded at 37°C and the electrical resistance of the membrane (Rm) was monitored (12). The values were compatible with the reported Rm of each membrane (13).

Drug Accumulation in the Rat Intestinal Mucosa

The rat jejunal membrane was prepared as described above. After a defined period of incubation of the mucosal side with a drug (SUA, SAM or PPR), the membrane was removed from the chambers, washed twice in ice-cold mannitol solution (0.3 M), then blotted gently with filter papers to remove residual water. The mucosa of the membrane was scraped off with a cover glass, weighed and homogenized in 5 ml of ice-cold saline. The amount of drug accumulated was calculated from the concentration in the homogenate and expressed as μ moles/g wet tissue. The extracellular water space was calculated using ^{14}C-labeled inulin (data not shown). Before calculating drug accumulation, the amount remaining in the extracellular space was subtracted from the total amount in the homogenate.

Permeability of Rat Small Intestine *In Vivo*

The drug permeability of rat jejunum was evaluated by single-pass perfusion as described by Hu et al. (14). Rats were anesthetized with pentobarbital, then the abdominal cavity was opened and an intestinal loop (10–15 cm length) was made at the upper portion of the jejunum (beginning 2–4 cm below the ligament of Treitz) by canulation with a silicone tube (i.d. 3 mm). After removing the intestinal contents by a slow infusion of saline and air, transport medium containing each drug (1 mM) and FITC-dextran (M.W. = 4000, 0.1 mg/ml) was perfused using an infusion pump (Harvard Apparatus, Model 944, S. Natick, MA) at a flow rate of 0.191 to 0.764 ml/min. The effluent was corrected from 30 min after starting the perfusion to 90 min at 10 min intervals, because steady state absorption was usually achieved by 30 min under these conditions.

The *in vivo* drug permeability (Peff) was calculated from the ratio of outlet/inlet drug concentration according to the following equation where the effect of water transport during perfusion was corrected using the concentration ratio of a non-absorbable marker (FITC-dextran).

$$\text{Peff} = Q\,\frac{1 - \text{Cin/Cout} \times \text{Aout/Ain}}{2\,\pi\,\text{RL}}$$

where Cin, Cout and Ain, Aout represent the inlet and the outlet concentrations of the drug and FITC-dextran, respectively. R and L are the radius and the length of the intestine used for the perfusion. The length of the intestine was measured immediately after the perfusion by laying it flat without stretching. Since, under our conditions, a 10 cm loop of the small intestine was filled with 1 ml of water without expansion, its radius was assumed to be 0.178 cm and was used to calculate Peff in all studies.

Analytical Methods

The concentrations of SUA, SAM and SP were estimated spectro-photometrically as described by Kimura *et al.* (15). CEZ, WAR and PPR were detected spectrophotometrically (CEZ) or fluorometrically (WAR, PPR) by means of high performance liquid chromatography (HPLC, LC-6A Shimadzu Co., Kyoto, Japan) with a reversed phase column (Inertosil ODS-2, Gaskuro-kogyo, Tokyo, Japan) equipped with a variable wave-length ultraviolet detector (SPD-10A, Shimadzu Co., Kyoto Japan) or a fluorescence spectromonitor (RP-530, Shimadzu Co.). The analytical conditions for each drug were: CEZ: mobile phase, water containing 0.01 M ammonium acetate/methanol (77/23 by volume); flow rate, 1.2 ml/min; wave length, 272 nm; WAR: mobile phase, water containing 0.1% H_3PO_4/methanol (3/7 by volume); flow rate, 1.0 ml/min; wave length, 319 nm for excitation and 396 nm for emission; PPR: mobile phase, water containing 0.1% H_3PO_4/methanol (1/1 by volume); flow rate, 1.0 ml/min; wave length, 295 nm for excitation and 360 nm for emission. In all assay procedures using HPLC, the column was placed in a temperature controlled column oven at 40°C. The concentration of FITC-dextran was determined fluorometrically (495 nm for excitation and 514 nm for emission) using a spectrofluoro-photometer (RF-5300PC, Shimadzu Co., Kyoto Japan).

RESULTS

Table 1 shows the structures of the studied drugs with their molecular weights and partition coefficients between n-octanol and the transport medium (log PC). Log PC varied from −2.6 for CEZ to 1.0 for PPR, suggesting a difference in the permeability of these drugs through the lipid membrane.

Drug permeability for each membrane was measured in the chamber system and plotted in Fig. 1 against their log PC. The permeability of the hydrophilic drugs (CEZ, SUA) was highest in the jejunum and lowest in Caco-2 monolayer, as

Table 1. Structures of Drugs Tested

Drug	log PC	M.W.
Cefazolin (CEZ)	−2.620	454.50
Sulfanilic acid (SUA)	−1.987	173.84
Sulfanilamide (SAM)	−0.825	172.21
Sulfapyridine (SP)	−0.123	249.29
Warfarin (WAR)	0.772	308.32
Propranolol (PPR)	1.002	259.34

Note: The partition coefficient of the drug was measured in n-octanol/buffer at pH 7.0.

shown in the insert in Fig. 1. In Caco-2 monolayer, the drug permeability increased with increasing log PC, and PPR permeated the most. In contrast, the permeability of the lipophilic drugs for the jejunum and colon was not particularly increased. Except for PPR, the permeability of drugs having higher log PC than that of SAM (log PC = −0.825) was almost constant in both membranes. PPR, which is the most lipophilic, did not permeate the jejunal membrane very well.

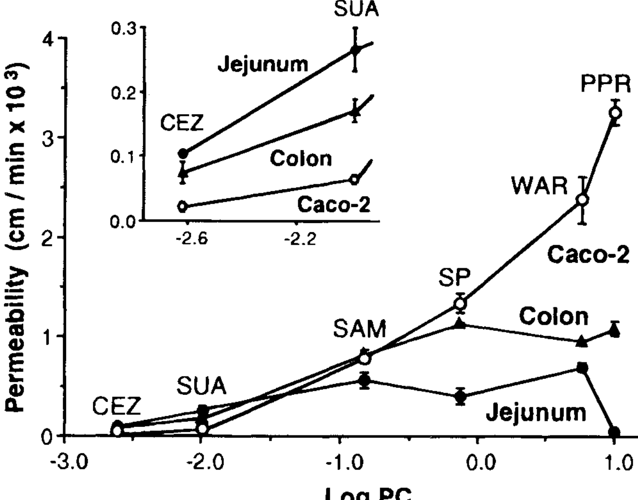

Fig. 1. *In vitro* drug permeability of the three membranes as a function of drug lipophilicity. The permeability of 6 drugs across Caco-2 monolayer (○), isolated rat jejunum (●) and colon (▲) were measured in Ussing chamber system under the same conditions *in vitro*. The log PC of each drug was measured in n-octanol/transport medium at pH 7.0. The small insert shows the permeability of CEZ and SUA at an enlarged scale. Each point represents the mean ± SE of at least three experiments.

The *in vivo* permeability of each drug was determined by means of an intestinal perfusion study using rat jejunum. Figure 2 shows the relationship between the *in vitro* and *in vivo* permeability of each drug. Since *in vivo* drug permeability through rat jejunum increased with increasing drug lipophilicity, the relationship to that of Caco-2 monolayer *in vitro* was quite linear (regression coefficient, R = 0.99). However, as deduced from the results in Fig. 1, there was no significant correlation (R = 0.20) between *in vivo* and *in vitro* permeability for rat jejunum. The correlation of the permeability for the isolated colon was better than of the jejunum (R = 0.88), however, the regression line had a large intercept on the y-axis. Figure 3 shows the ratio between the drug permeability of Caco-2 monolayer and that of rat jejunum *in vivo*. Although the drug permeability of Caco-2 monolayer showed a good linear relationship with the *in vivo* permeability of rat jejunum (Fig. 2), the permeability ratio varied from 0.16 to 0.37, which increased with increasing drug permeability.

To determine the reason for low permeability of lipophilic drugs through jejunum *in vitro*, we measured the amount of SUA, SP and PPR that accumulated in the jejunal mucosa under *in vitro* conditions. Figure 4 shows the time-course of accumulation of three drugs. Only a little amount of hydrophilic SUA was found and essentially, it did not accumulate in the jejunal mucosa. Accumulation of PPR reached a steady state within 40 min and its level was the highest among the three drugs tested.

DISCUSSION

In this study, we used drugs that are absorbed by the intestine through a passive diffusional mechanism, and com-

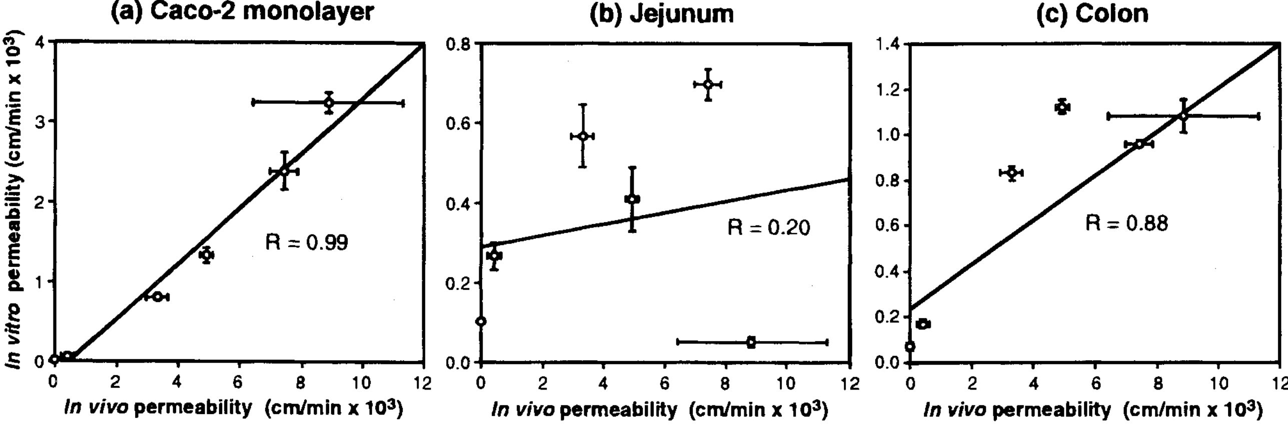

Fig. 2. Relationship between *in vitro* and *in vivo* drug permeability. The permeability of 6 drugs across Caco-2 monolayer (a), isolated rat jejunum (b) and colon (c) *in vitro* was correlated to that *in vivo* by determining the intestinal perfusion of the rat jejunum. Each point represents the mean with ± SE of at least three experiments.

pared the permeability of Caco-2 monolayer with that of isolated rat jejunum and colon *in vitro*. Membrane permeability was measured using side-by-side chambers (Ussing type chambers). The validity of using this type of chambers for permeation experiments with Caco-2 monolayer was confirmed in previous reports on hydrophilic drugs (9). Also, for lipophilic drugs, statistically the same level of permeability was obtained both in the Transwell and in the chamber system (data are not shown).

We have already characterized the paracellular permeation of drugs through Caco-2 monolayer using the same chamber system (9). We concluded that the higher electrical resistance of Caco-2 monolayer compared to that of the jejunum derives from its smaller surface area due to its flatter structure, rather than a difference in the tightness of the cell junctions. This structural difference also leads to low paracellular permeability of Caco-2 monolayer because there are fewer cell junctions per unit area. In this study, the permeability of CEZ and SUA was lowest in Caco-2 monolayer, moderate in the colon and highest in the jejunum (Fig. 1). Because both drugs are considered to permeate mainly through the paracellular route (8,9), this order of permeability reflects the differences in membrane resistance caused by differences in the effective surface area (9).

With increasing drug lipophilicity, permeation via the transcellular route becomes dominant and the total permeability of the membrane increased. As shown in Fig. 1, drug permeability to Caco-2 monolayer increased with increasing drug lipophilicity, which correlates well with the *in vivo* permeability of rat jejunum. Amidon *et al.* (1) have demonstrated a good correlation between drug permeability calculated from the rat intestinal perfusion study and the fraction dose absorbed in humans. Therefore, the results shown in Fig. 2 indicate the possibility of Caco-2 monolayer system predicting drug absorption in humans. However, the permeability of isolated rat jejunum *in vitro* did not significantly correlate with that *in vivo,* although the origin of the membrane was the same (Fig. 2 b). This conflict was obviously due to the low permeability of lipophilic drugs, especially PPR, through the jejunum *in vitro.*

One major difference between Caco-2 monolayer and rat jejunum is an absence of a mucus layer on the surface of Caco-

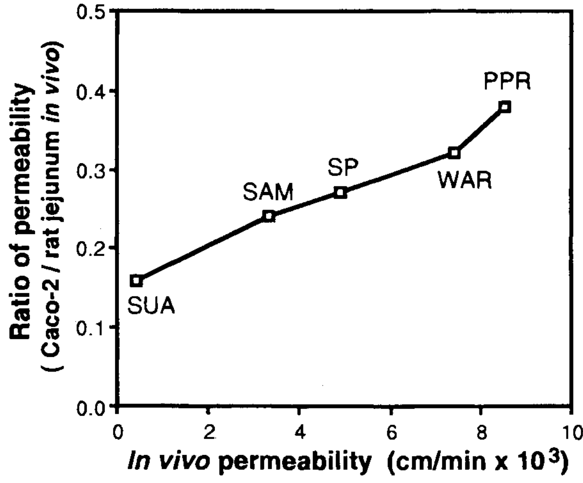

Fig. 3. Ratio of *in vitro* and *in vivo* permeability of drugs. The ratio of permeability was calculated by dividing the permeability of each drug for Caco-2 monolayer by that for rat jejunum *in vivo*. In this figure, CEZ data was omitted because its *in vivo* permeability was assumed to be zero in the intestinal perfusion study.

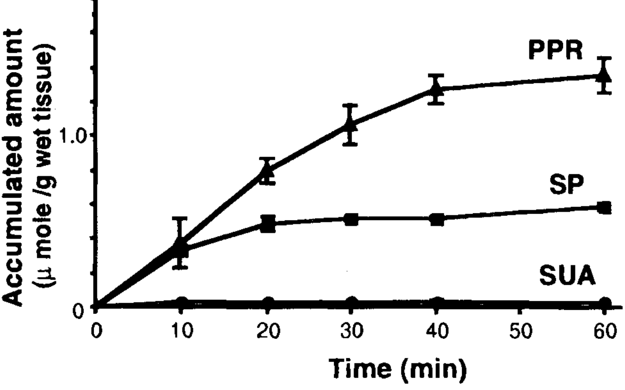

Fig. 4. Time course of *in vitro* drug accumulation in the isolated rat jejunum. The accumulated amounts of SUA (●), SP (■) and PPR (▲) in the rat jejunum were measured under the same *in vitro* conditions as those applied in the permeation study. The concentration of drugs introduced to the mucosal side of the membrane was 1 mM for SP and PPR and 10 mM for SUA. Each point represents the mean ± SE of at least three experiments.

2 cells due to the lack of mucin secreting cells (goblet cells) (16). Diffusion through the mucus layer, which forms a thick unstirred water layer, might be rate-limiting in the permeation of lipophilic drugs such as PPR. The significant effect of an unstirred water layer on intestinal drug absorption is well documented (17,18). However, if the mucus layer was the rate-limiting step of drug permeation across the jejunal membrane, this layer should have the same effect on *in vivo* absorption. In addition, more PPR than other drugs accumulated in the jejunal mucosa *in vitro.* This finding suggests that the unstirred water layer on the mucosal surface could not be the main barrier against PPR permeation. Furthermore, even *in vitro,* lipophilic drugs partitioned well to the cell membrane. The very low accumulation of SUA in the membrane corresponds to the fact that SUA permeates the membrane mainly via the paracellular route as reported (9).

From the high accumulation and the very low permeability of PPR, another rate-limiting process emerged in the *in vitro* permeation of lipophilic drugs through the rat jejunum. As illustrated in Fig. 5, the jejunal membrane has a villous structure that increases the effective surface area of absorption. Drugs can enter the blood circulation *in vivo,* just after crossing the epithelial absorptive cell layer from capillaries in the lamina propria of the villi (19). In contrast, *in vitro* permeation needs the diffusion of drugs from the mucosal to the serosal side of the membrane through the villi. Because the lipophilic drugs easily permeated the epithelial cell layer, this diffusive process, which is not involved in the *in vivo* absorption, should limit the rate of total permeation. The interaction of drugs with some villous components is also a factor in low permeability. Cationic drugs, such as PPR in this study, are known to have a high affinity for negatively charged components of the tissue, such as acidic phospholipids, and this might explain the extremely low permeability of PPR *in vitro.* In the colon, which does not have a villous structure, the presence of some folds and the remaining connective layer rate-limited the permeation of lipophilic drugs, although those were less limiting than the villous structure of the jejunum (see Fig. 1).

On the other hand, Caco-2 monolayer consists of a cell layer and a supporting membrane (polycarbonate filter with 0.3 μm pores). Because the polycarbonate filter does not limit the permeation of low molecular weight compounds, a drug that crosses the cell layer is quickly detected on the serosal side. The process of *in vitro* permeation across the monolayer system, therefore, is considered to be essentially the same as that of *in vivo* drug absorption for the intestinal membrane. In this respect, the Caco-2 monolayer system mimics and therefore predicts *in vivo* drug absorption better than isolated intestinal membranes.

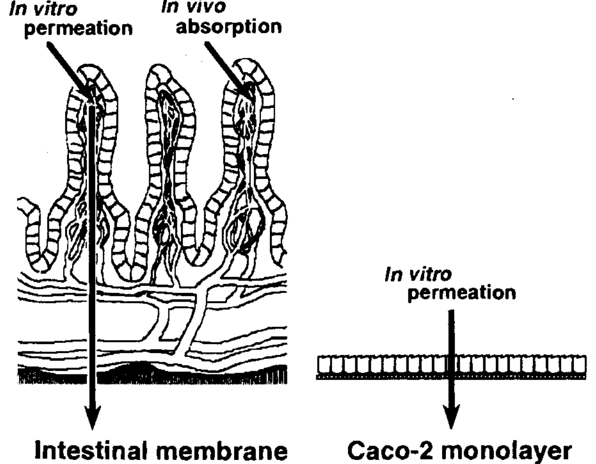

Fig. 5. Schematic representation of *in vitro* permeation and *in vivo* absorption of drugs.

The drug permeability of Caco-2 monolayer was always lower than that of rat jejunum *in vivo* as determined by perfusion experiments (Fig. 2). This might be a result of the smaller surface area of Caco-2 monolayer due to its flatter structure. However, the ratio of the permeability of both membranes was not constant (Fig. 3). We postulate that the effective surface area of drug absorption in the small intestine varies with drug permeability. Drugs with low permeability will diffuse down the length of the villi and be absorbed across the wide area of the membrane surface. In contrast, highly lipophilic drugs can be absorbed rapidly and completely through the tip of the villi, only a small part of the membrane. Because the effective area of Caco-2 monolayer is considered to be constant for all drugs, the differences in surface area for PPR and WAR between rat jejunum and Caco-2 monolayer become relatively small and cause a smaller difference in the permeability. In order to use Caco-2 monolayer to predict the oral absorption of drugs with various permeability, an appropriate correction is necessary in respect to the differences in the effective surface area affecting absorption.

Caco-2 monolayer has now received considerable attention from the pharmaceutical industry because the correlation between drug permeability of Caco-2 monolayer and *in vivo* drug absorption is very high (20). This study provides a theoretical rationale for using the cultured monolayer system to predict *in vivo* drug absorption. The simple concept shown in Fig. 5 is applicable not only to Caco-2, but also to other monolayer systems and is very important when considering differences between the *in vitro* permeation and the *in vivo* absorption of drugs.

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