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Development of a Simple Double-layered Cell Culture System Using Caco-2 and TIG-1 Cells as a New Cytotoxicity Test

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Abstract

We investigated the effect of a Caco-2 cell barrier on toxicity expression in cultured human diploid fibroblasts, TIG-1 cells, in a simple double-layered culture system. The experimental setup used a 12-well plate for the TIG-1 cells and a companion membrane culture insert for the Caco-2 cells. Four model chemicals, *i.e.*, caffeine, diclofenac sodium, indomethacin, and paraquat, which have different levels of *in vivo* human absorbability, were employed to test the feasibility of the culture system. In transport studies using a Caco-2 cell layer, not only initial apparent permeation coefficients of the four chemicals but also *in vitro* absorption ratios in equilibrium phases were well correlated with previously reported *in vivo* human absorbability. This phenomenon was explained by the differences in the permeation coefficients between the two directions across the Caco-2 cell layer. Changes in the dose-response relationships in terms of TIG-1 cell growth on the ninth day in the double-layered culture system were largely predicted from the cell growth in the single-layered system (without a Caco-2 cell layer) and the *in vitro* absorbability. However, small but significant discrepancies between the observed and the predicted dose-response relationships were found in the loading of diclofenac sodium and paraquat. The first discrepancy suggests the involvement of possible enhanced detoxification of diclofenac sodium by the Caco-2 cells. The latter discrepancy reflects the specific permeation kinetic of paraquat across the Caco-2 cell layer. Final toxicity in terms of TIG-1 cell growth in the double-layered system reflected *in vivo* toxicity better than that of the single-layered system without the Caco-2 cell layer. These results demonstrate that a Caco-2-cells-based permeation test combined with a single-cell-population-

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based cytotoxicity test is more useful in estimating *in vivo* human oral toxicity.

Keywords: *Cocultivation, Caco-2 cells, Permeation, Human diploid fibroblast, Growth inhibition.*

Introduction

In vitro cytotoxicity tests are very promising in meeting current demands from society to reduce the number of experiments on animals. Prediction of acute toxicity by simple short-term *in vitro* cytotoxicity tests has been established by multi-center validation programs such as MEIC (Clemedson and Ekwall, 1999; Ekwall, 1999) or JSAAE projects (Ohno *et al.*, 1998). However, there are still a great number of experiments on animals that should be replaced or reduced in the near future. One of the important reasons for the continued use of animals is that the toxicities observed in such animal experiments are the results of *in vivo* metabolic processes involving absorption from the small intestine or lung epithelium, biotransformation by the liver, distribution over organs through the systemic blood circulation, and accumulation in target organs. Conventional cytotoxicity tests use cultured cells of a single population. Therefore, the metabolic processes occurring *in vivo* cannot be reproduced

To overcome this limitation of conventional cytotoxicity tests, integration of dose-response data obtained from *in vitro* tests by a suitable physiologically-based pharmacokinetic (PBPK) model was presented (DeJongh, 1999). Although this approach is still in a preliminary stage, it is presumed that it will soon be advantageous over whole animal experiments, because it may compensate for the above-mentioned disadvantages of conventional single-cell-population-based cytotoxicity tests. However, such PBPK models can incorporate toxicity expression mechanisms that have already been clarified, and they cannot discover unanticipated *in vivo* metabolism of a focused chemical. In addition, description by PBPK models usually requires many adjustable parameters whose values must inevitably be decided by curve fittings (Shuler *et al.*, 1996).

Recently, some researchers presented a new concept of an experimental system that incorporates metabolic processes occurring in humans. Sweeney *et al.* (1995) first reported development of a multi-compartment cell culture system referred to as a "Cell Culture Analogue" (CCA) device. This system consisted of liver cell-, lung cell-, and other tissue-(reservoir) compartments that were connected with a physiologically relevant perfusion circuit. They tested their system's efficacy in evaluating the toxicity expression processes of some model chemicals such as naphthalene (Sweeney *et al.*, 1995; Shuler *et al.*, 1996) and 2,3,7,8-TCDD (Mufti *et al.*, 1998). Such kinds of experimental systems can observe final toxicity expression as the activities in the target cells resulting from different metabolic processes or organ-to-organ interactions without numerical description of the metabolic processes required in a PBPK model. Although such a system will certainly be required in the future, the operation of such a complicated perfusion bioreactor system seems to be very difficult (Sweeney *et al.*, 1995). Thus, at this moment they may cost a lot in comparison with experiments on small animals such as rats or mice (Shuler *et al.*, 1996). Therefore, a much simpler culture system incorporating actual *in vivo* processes is needed.

In this article, we investigated the feasibility of a simple multi-layered culture system incorporating an absorption process across the

small intestine. Caco-2 cells (Fogh *et al.*, 1977) cultured on membrane culture inserts were used to mimic an absorption barrier of the human small intestine. Although this cell line was established from human colon carcinoma tissue, it can differentiate into a polarized cuboidal epithelium monolayer mimicking that of the small intestine after being cultured on a collagen-coated semipermeable membrane for a certain period. This *in vitro* culture model has been widely accepted as a simple tool for the prediction of *in vivo* human absorbability of a wide range of chemicals (Artursson and Karlsson, 1991). Normal human diploid fibroblasts, TIG-1 cells (Ohashi *et al.*, 1980) established from a Japanese female embryo, were employed as the target cells.

Materials and Methods

Cell culture and Medium

Caco-2 (Fogh *et al.*, 1977) and TIG-1 cells (Ohashi *et al.*, 1980) were obtained from the Riken Gene Bank (Tsukuba, Japan) and the Health Science Research Resources Bank (HSRRB; Osaka, Japan), respectively. They were routinely cultured in Dulbecco's modified Minimum Essential Medium (DMEM) with high glucose content (Nissui Pharm. Co., Ltd.; Tokyo, Japan) supplemented with 10% fetal bovine serum (Filtron; Altona, Australia), 1% non-essential amino acid solution (GIBCO, Life Technol., Grand Island, NY), 25 mM hydroxyethylpiperazine-N'2-ethanesulfonic acid (HEPES; Dojindo, Kumamoto, Japan), 100 units -penicillin/mL (Wako), 100 μg-streptomycin/mL (Wako), and 1.0 μg -amphotericin B/mL (Sigma). This culture medium was used in all experiments involving chemical transport studies. Both cell lines were subcultivated using 0.25% trypsin in phosphate-buffered saline. Caco-2 cells were used between the 50th and 60th passages. TIG-1 cells were used below the 40th passage.

Formation of a Caco-2 cell layer on a membrane support

Culture of Caco-2 cells was essentially performed following the procedure of Chong *et al.* (1996). Briefly, the cells were first seeded onto polyester membrane culture inserts (Transwell 3460, culture surface area of 1.0 cm^2, 0.4- μm pore; Coaster, Cambridge, MA) precoated with Type-I collagen (Nitta Gelatin, Osaka, Japan) at an initial density of 1.0×10^5 cells/cm^2. The monolayer of Caco-2 cells was then allowed to develop until it reached equilibrium with the trans-epithelium electrical resistance (TEER) of over 400 ohms $\cdot$ cm^2 measured with a Millicell-ERS (Millipore Corp., Bedford, MA). This resistance included those of the membrane culture insert and the culture medium (approximately 100 ohms $\cdot$ cm^2).Under our culture conditions, the cells usually took about two weeks to archive such tight monolayers.

Transport studies

A culture medium containing four model chemicals, caffeine (1.00 mM), indomethacin (0.25 mM), diclofenac sodium (0.25 mM), and paraquat (0.25 mM), was added to the apical (Ap) side of the Caco-2 monolayer (0.8 mL), and a chemical-free culture medium was added to the basolateral (BL) side (1.0 mL). The chemicals were all purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). The first three chemicals were dissolved in DMSO, then diluted 200 times with culture medium. The paraquat was dissolved directly in the culture medium. Chemical transport from the apical (Ap) side to the basolateral (BL) side was measured during 72 hours of incubation. Transport in the opposite direction (BL to Ap) was also measured. The concentrations in the BL sides in the transport studies were measured in both directions.

Measurement of chemical concentrations

For chemical analysis, 20 μl of culture medium in the BL compartment was sampled at each incubation period. An equal volume of methanol was then added to the sample, and it

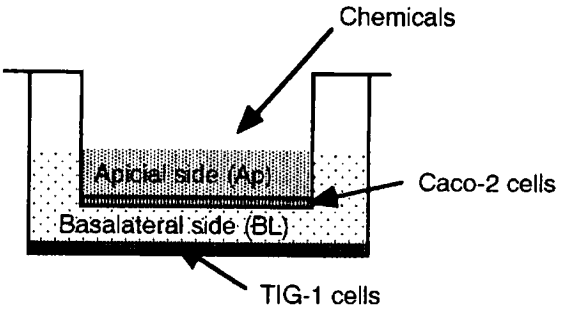

Fig. 1 Schematic representation of a double-layered culture system consisting of a Caco-2 cell-loaded membrane and TIG-1 cells.

was centrifuged at 15,000rpm for 10 min to remove cell debris and plastid protein. This procedure did not significantly affect chemical quantification. Chemical concentration in the medium samples from the BL side was detected with an HPLC system (HIC-6A, Shimadzu, Osaka, Japan) equipped with an octadecylsilil-silica (ODS) gel column (Shim-pack CLC-ODS; Shimadzu) and a spectrophotometric detector (SPD-6AV; Shimadzu).

Measurement conditions were set up according to the manufacturer's recommendations. For caffeine, the elution buffer was acetonitrile : 10 mM sodium phosphate buffer (pH 7.2) : chloroform = 10 : 90 : 0.2 (v : v : v), the flow rate was 1.5 mL/min, and the detection wavelength was 270 nm. For diclofenac sodium, the elution buffer was methanol : 10 mM sodium phosphate buffer (pH 7.2) = 80 : 20 (v : v), the flow rate was 1.5 mL/min, and the detection wavelength was 270 nm. For indomethacin, the elution buffer was methanol : 10 mM sodium phosphate buffer (pH 7.2) = 80 : 20 (v : v), the flow rate was 1.5 mL/min, and the detection wavelength was 320 nm. For paraquat, the elution buffer was methanol : water : triethylamine : phosphoric acid : sodium octasulfate = 30 : 70 : 1.4 : 0.8 : 0.3 in a volumetric ratio, and the detection wavelength was 250 nm. The column temperature was maintained at 30℃ for the measurement of all four chemicals. The peak area was used for chemical quantification.

Culture of TIG-1 cells with a Caco-2 cell layer

Caco-2 cell layers were prepared in the same manner as in the transport study. TIG-1 cells were seeded in 12-well plates (3513, culture surface area of 3.7 cm^2; Costar) at an initial cell density of 1.0×10^4 cells/cm^2 with 1 mL of the culture medium. After one day of culture, the prepared membrane culture insert with a Caco-2 cell layer was put into the well having the TIG-1 cells, as shown in Fig. 1. The culture medium (0.8 mL) containing the varied concentrations of chemicals was then added to the apical (Ap) side of the Caco-2 cell layer. The medium was replenished every three days. Specifically, the chemical was loaded into the Ap side of the Caco-2 cells at three-day intervals. Simultaneously, TIG-1 cells were cultured in a single-layered system without Caco-2 cells in the same manner as that employed in the double-layered culture system. The volume of the culture medium with or without the chemicals was set at 1.0 mL, which was the same as that employed in the transport study. Chemical loading was repeated until the ninth day.

Measurement of TIG-1 cell growth

The number of viable TIG-1 cells on the ninth day was measured in terms of intracellular acid phosphatase (AP) content (Connolly *et al.*, 1986). This assay was shown to be one of the most sensitive and easy methods of measuring the number of viable cells in a culture

(Martin and Clynes, 1993). The TIG-1 cells attached to the bottom surface of the 12 well plates were rinsed once with PBS, and soaked in 1 mL of sodium acetate buffer (pH 5.5) containing 0.037 g-p-nitrophenol phosphate/L and 0.1% Triton X-100. After two hours of incubation at 37℃, an equal volume of 10-mM NaOH was added to the well, and absorbance at 405 nm was measured with a spectrophotometer (UV-160, Shimadzu; Tokyo, Japan). The absorbance and the number of living cells were confirmed to be in a linear relationship in the ranges of cell density between 1.0×10^4 (inoculum cell density) and 2×10^5 cells/cm^2 (maximal at a confluence). All the data on the number of living cells was standardized with that in the control culture and indicated as relative cell growth.

Results and Discussion

Long term transport of four chemicals through a Caco-2 layer

When *in vivo* absorbability is estimated from an *in vitro* permeation study using a Caco-2 layer formed on a semipermeable membrane support, the apparent permeability coefficients of the initial phase of permeation are usually used, and *in vitro* absorbability in equilibrium phases is not given much attention. However, in a double-layered culture system (Fig. 1) where target cells are loaded with a chemical after it permeates to the basolateral (BL) side, once it reaches equilibrium, the chemical concentration in the BL side seems to be significant in determining the toxicity observed in TIG-1 cells.

Therefore, we first examined the permeation kinetics of four chemicals across a Caco-2 cell layer during a somewhat longer time, that is, 72 h after commencing the loading of the chemicals (Fig. 2). In the conventional kinetic description of the *in vivo* absorption process from the small intestine, the concentration in the blood of a relevant chemical in the BL side of the small intestinal epithelia is assumed to be zero (zero sink assumption) compared with that in the Ap side (Yuasa *et al.*, 1996). In a double-layered culture system, however, the BL concentration may not be negligible in determining the total amount that permeates the Caco-2 cell layer. Therefore, in Fig. 2, the horizontal lines at a concentration of 0.444 mM for caffeine and 0.111 mM for the remaining three chemicals indicate an assumed equilibrium concentration, $C_{assumed,\infty}$, defined as follows,

$$C_{assumed,\infty} = (C_{Ap,0} \cdot V_{Ap}) / (V_{Ap} + V_{BL}) = 0.444 \cdot C_{Ap,0}$$

where, V_{AP} and V_{BL} is the volume of the culture medium in the Ap (0.8 mL) and BL (1.0 mL), respectively, and $C_{Ap,0}$ is the chemical concentration in the Ap side at t = 0 min. This $C_{assumed,\infty}$ is the concentration at $t = \infty$ when only a passive mechanism operates in the chemical transport across the Caco-2 layer. If that is the case, the chemical concentration in the BL side, $C_{BL,\infty}$, should become equal to $C_{Ap,\infty}$ ($C_{assumed,\infty} = C_{BL,\infty} = C_{Ap,\infty}$).

Changes in the BL concentrations in transport studies in both directions (Ap ➡ BL and BL ➡ Ap) were examined. The chemical concentrations employed were less than their toxic ranges to the Caco-2 cells in terms of TEER values (data not shown). The *in vivo* absorbabilities in humans of the four chemicals were reported as 99% for caffeine, 75% for diclofenac sodium, 50% for indomethacin, and 15% for paraquat (Nishi *et al.*, 1994).

After a rapid increase or decrease for eight hours, the BL concentration seemed to reach equilibrium in the studies of both directions except for paraquat (Fig. 2 (D)). For the first three chemicals, the BL concentrations became almost the same after 24 hours of culture irrespective of the direction of permeation (Fig. 2 (A-C)). However, for paraquat loading to the Ap side, the BL concentration still continued to increase after 24 h, and there was a

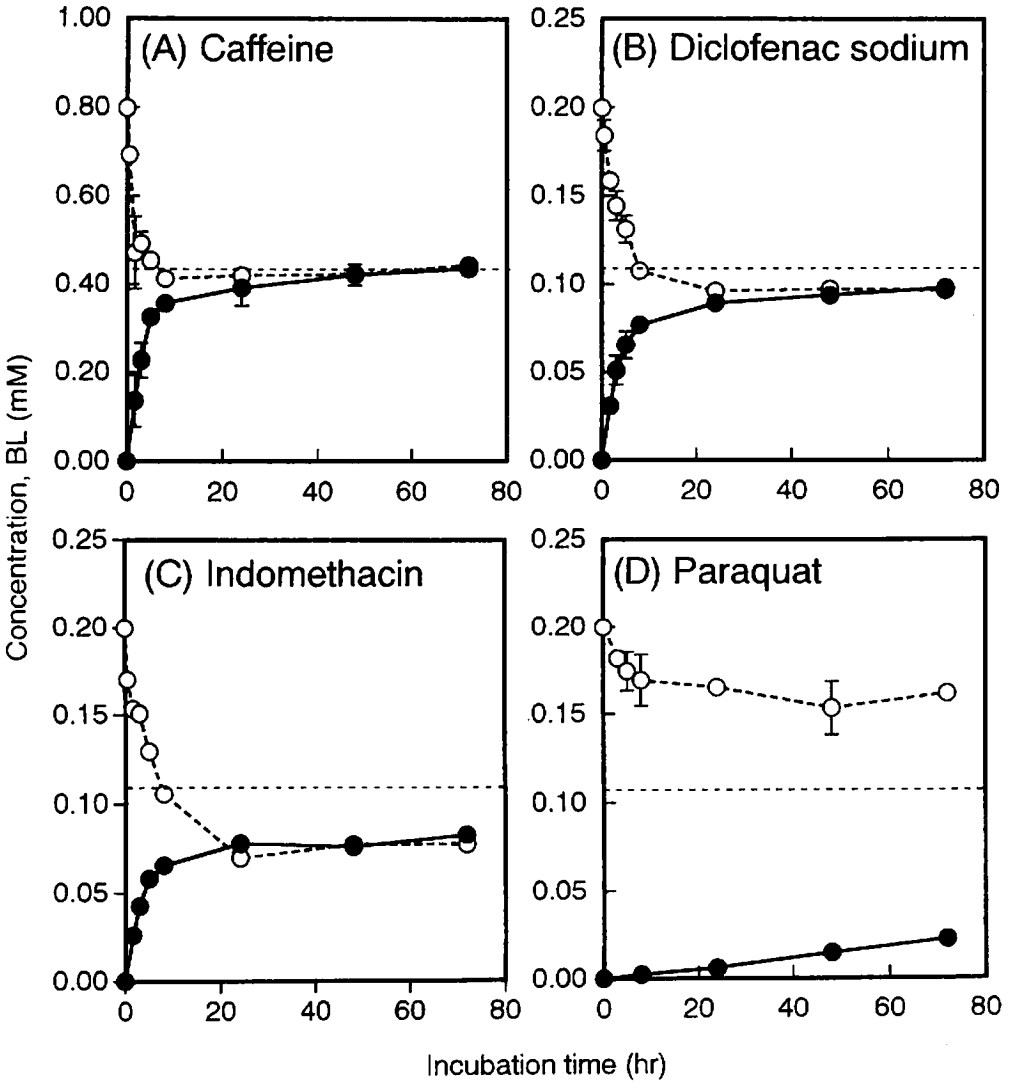

Fig. 2 Permeation kinetics of four model chemicals across Caco-2 cell layers. (A), caffeine; (B), diclofenac sodium; (C), indomethacin; and (D), paraquat. The data indicates the changes in the basolateral (BL) concentration (C_{BL}) when the chemicals are loaded in the apical (Ap) (●) and BL (○) sides. The initial caffeine concentrations were 1.00 mM for the Ap side (C_{Ap}) and 0.80 mM for the BL side. The initial concentrations of the other three chemicals were 0.25 mM for the Ap side and 0.20 mM for the BL side. The horizontal lines at the concentration of 0.444 mM for caffeine and 0.111 mM for the other three indicate an assumed equilibrium concentration when no active transport mechanism operates ($C_{BL} = C_{Ap}$), as defined in the text. Each point represents the mean ± SD of the four cultures.

large difference in the BL concentrations between the Ap and BL side additions even at 72 h (Fig. 2 (D)).

The correlation between the *in vivo* and *in vitro* absorbabilities was examined when we used two different indices for *in vitro* absorbability, that is, the initial apparent permeation coefficient, P_{app} in the Ap → BL direction (Fig. 3 (A)) and the *in vitro* absorbability in at equilibrium, after 72 h (Fig. 3 (B)). P_{app} was calculated by a simple equation as follows (Artursson and Karlsson, 1991).

$$V_{BL} (dC_{BL,t} / dt) = P_{app} \cdot A \cdot C_{Ap,0}$$

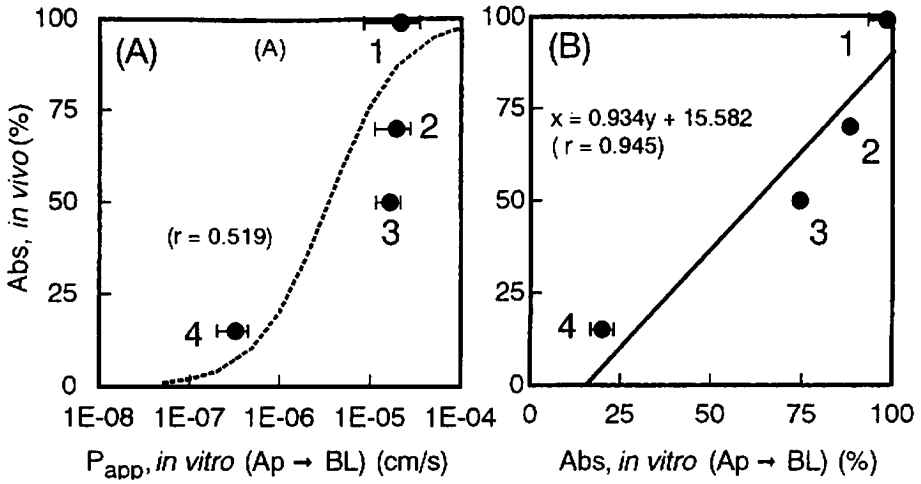

Fig. 3 Predictivity of *in vivo* absorption ratios in humans (Nishi *et al.*, 1994) by apparent permeability coefficients (P_{app}) in the Ap → BL direction (A) and by relative *in vitro* absorption ratios determined by Caco-2 cell layers at 72 h (B). (1), caffeine; (2) diclofenac sodium; (3), indomethacin; and (4), paraquat. An established experimental calibration curve (Chong *et al.*, 1991) between P_{app} and *in vivo* absorbability in humans is also shown in Fig. 2 (A). Each point represents the mean ± SD of the four cultures.

where A is the area of the Caco-2 layer (1.0 cm^2). The initial BL concentrations during three hours of incubation were used in calculating P_{app} except for paraquat, where the concentrations during the initial eight hours of incubation were used. *In vitro* absorbability was calculated from $C_{BL,1}$ at t = 72 h using the following equation:

$$\text{Absorption, } in\ vitro\ (\%) = (C_{BL,\,72\,h} / C_{\,assumed,\,\infty}) \cdot 100$$

where $C_{\,assumed,\,t=\infty}$ has been previously defined in the text.

If we use an empirical description based on a sigmoidal A_{max} model, P_{app} was shown to predict well the *in vivo* absorbabilities of passively-transported chemicals (Artursson and Karlsson, 1991), as follows:

$$\text{Absorption, } in\ vivo = (A_{max} \cdot P_{App}{}^{\gamma}) / (P_{App,\,50}{}^{\gamma} + P_{App}{}^{\gamma})$$

where A_{max} is set at 100%, and $P_{App,\,50}$ represents the permeability coefficient that achieves 50% absorption *in vivo*. According to Chong *et al.* (1996), who examined permeation of ten passively-transported chemicals using Caco-2 cell layers, $P_{App,\,50}$ and γ were determined to be 3.5 x 10^{-6} cm/s and 1.1, respectively.

Although the correlation coefficient between the P_{app} values measured in this study and the established curves was not so high (r = 0.519), the tendency shown in both plots is the same (Fig. 3 (A)). The absolute permeability coefficients were reported to have large variations among laboratories (Chong *et al.*, 1996; Rubas *et al.*, 1993). Therefore, the observed difference in P_{app} values is considered acceptable. The *in vitro* absorption ratios determined from the BL concentrations at 72 h correlated very well (r = 0.945) with the *in vivo* absorbabilities for the four chemicals (Nishi *et al.*, 1994) (Fig. 3 (B)). This result regarding *in vitro* absorbabilities demonstrates the involvement of active transport mechanisms in the permeation phenomena in the cultured Caco-2 cell layer.

Contributions of passive and active mechanisms to chemical transport

To further examine the contributions of both passive and active transport mechanisms to the overall transport phenomena of the four chemicals, we calculated passive and active perme-

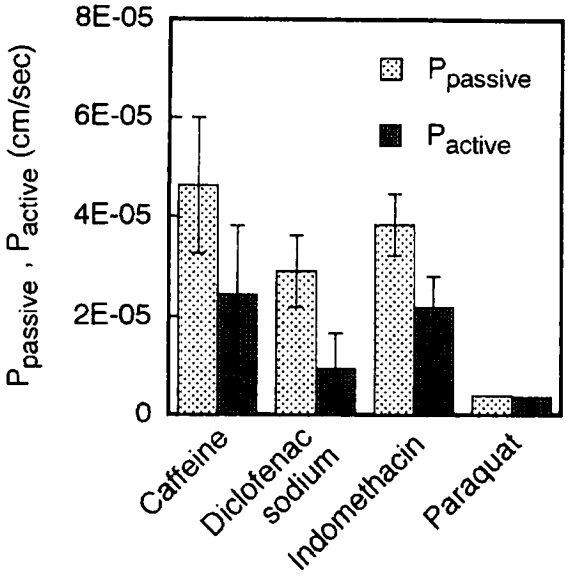

Fig. 4 Contributions of passive and active transports to the apparent permeation phenomena through Caco-2 cell layers. Passive ($P_{passive}$) and active (P_{active}) permeation coefficients were calculated from P_{app} (Ap ➡ BL) and P_{app} (BL ➡ Ap) values using the equations described in the text. Each bar represents the mean ± SD of the four cultures.

ation coefficients ($P_{passive}$ and P_{active}, respectively) using the initial changes in concentration both in the Ap ➡ BL and BL ➡ Ap transport studies. According to the study of Gan *et al.* (1996), the relationships among $P_{passive}$, P_{active}, P_{app} (Ap ➡ BL), and P_{app} (BL ➡ Ap) are as follows:

$$P_{passive} = (P_{app}(Ap \rightarrow BL) + P_{app}(BL \rightarrow Ap)) / 2$$

$$P_{active} = (P_{app}(BL \rightarrow Ap) - P_{app}(Ap \rightarrow BL)) / 2$$

In our study, P_{app} (BL ➡ Ap) was calculated from the following equation:

$$V_{Ap}(dC_{Ap,t}/dt) = V_{BL}(dC_{BL,t}/dt) = P_{app}(BL \rightarrow Ap) \cdot A \cdot C_{BL,0}$$

According to this analysis, active transport mechanisms causing efflux from the BL to the Ap side clearly acted in all four chemicals examined (Fig. 4). This agrees well with the asymmetric partition of the chemical in the Ap and BL sides in the equilibrium phase of the transport study (24 - 72 h in Figs. 2 and 3 (B)). For caffeine, diclofenac sodium, and indomethacin, the passive permeation coefficients were larger than the active transport coefficients whereas, in paraquat, the passive and active permeation coefficients were almost the same. An active transport mechanism causing an efflux from the Ap to BL sides appears to act relatively well compared with the passive permeation of paraquat across the Caco-2 cell layer. This result is in good agreement with the very low absorbability and specific permeation kinetics of paraquat (Fig. 2 (D)).

Toxicities in single- and double-layered culture systems

In terms of TIG-1 cell growth, dose-response (DR) relationships of the four chemicals were measured both in the single- and double-layered culture systems (Fig. 5). The relationships observed in the single-layered

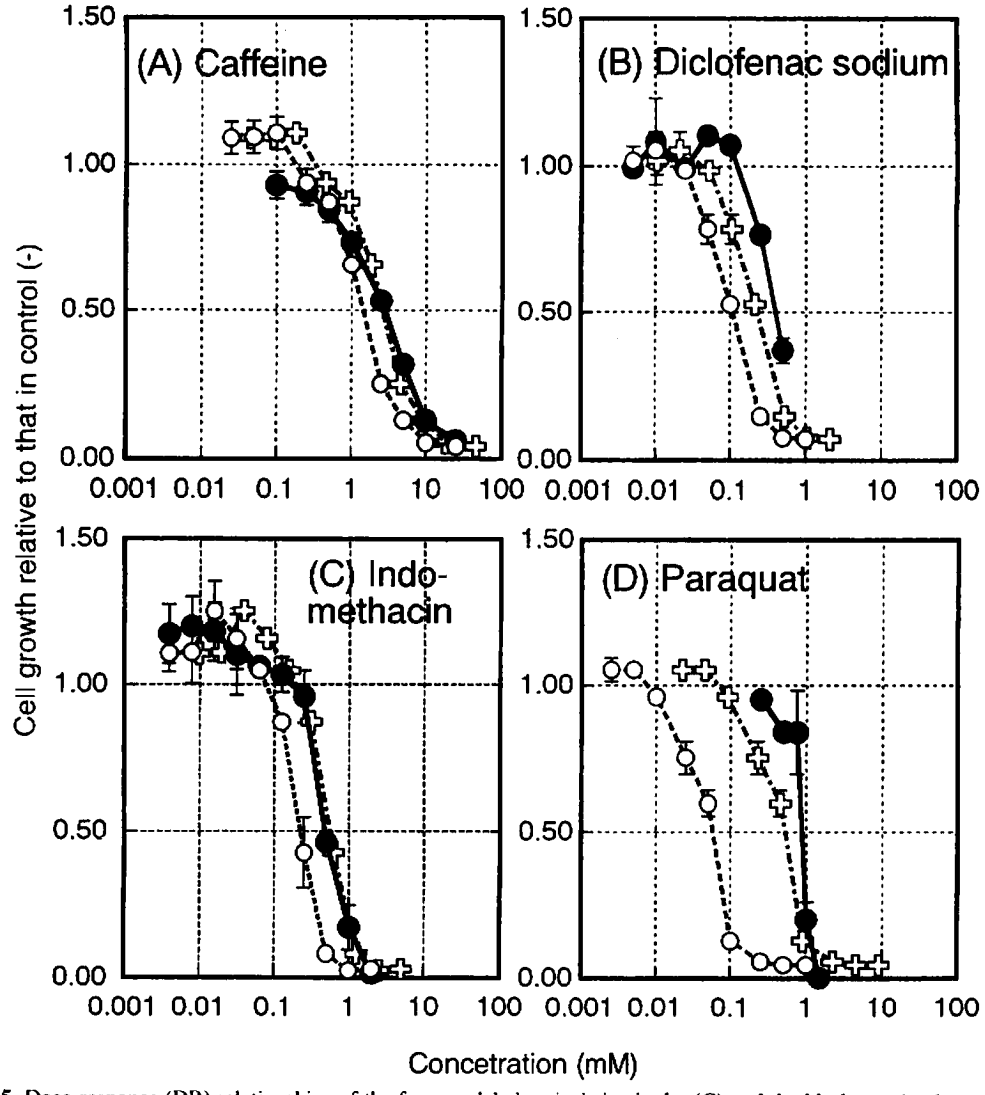

Fig. 5 Dose-response (DR) relationships of the four model chemicals in single- (○) and double-layered cultures (●), together with the DR curves predicted (✙) from those in the single-layered culture and the *in vitro* absorption ratios measured in the transport study (Fig. 2). (A), caffeine; (B), diclofenac sodium; (C), indomethacin; and (D), paraquat. Each point represents the mean ± SD of the four cultures.

systems clearly shifted toward the ranges of higher concentration in the double-layered system for all four chemicals. However, the differences between the two DR curves were probably a reflection of the absorbability of the relevant chemical, as depicted in Fig. 3 (B).

To further clarify this, another DR curve based on the absorbability of each chemical was calculated, as shown in Fig. 5. The predicted curve was simply obtained by replotting the data on relative cell growth in the single-layered culture system against the BL side concentration predicted from the *in vitro* absorbability (Fig. 3 (B)) and the chemical concentration added to the Ap side. The trans-epithelium electrical resistance (TEER) of the Caco-2 cells was confirmed to remain almost constant (between 400-600 ohms · cm^2) during the nine days of coculture with TIG-1 cells (data not shown). Thus, the permeation characteristics were not likely to change during the

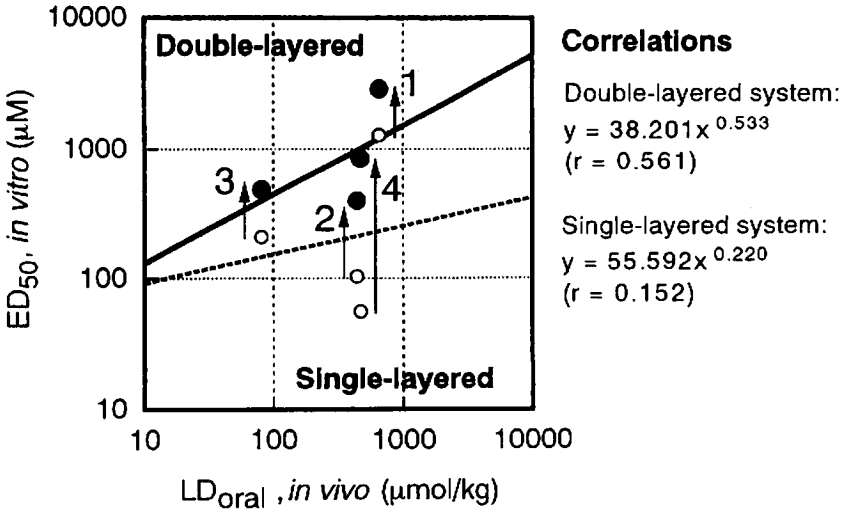

Fig. 6 Correlation between acute oral lethal doses (LDs) in rats or mice and ED_{50}s determined in single- (○) and double-layered (●) cultures. (1), caffeine; (2) diclofenac sodium; (3), indomethacin; and (4), paraquat. The animal data was referenced to the Chemical Health & Safety Database by National Toxicology Program (2000).

nine days. Generally, these predicted DR curves showed tendencies similar to those observed in the double-layered system. This demonstrates that the addition of a Caco-2 cell layer in *in vitro* cytotoxicity testing systems is advantageous over conventional single-cell-population-based systems, because such a Caco-2 barrier can restrict the actual chemical load on the target cells. However, for diclofenac sodium and paraquat, the discrepancies between the predicted and the observed DR curves in the double-layered system did not seem to be neglected (Fig. 5 (B) and (D)).

The reason for a level of toxicity of diclofenac sodium lower than predicted in the double-layered system has not yet been elucidated (Fig. 5 (B)). The most plausible cause for the discrepancy is the contribution of enhanced detoxification that occurs in the Caco-2 cell layer in the double-layered culture system. Of the four chemicals examined in this study, the literature reports that diclofenac sodium is most actively detoxified by cytochrome P450s such as 2C9 or 3A4 by further conjugation with glutathione except in a very rare case where hepatotoxicity is observed (Tang *et al.*, 1999). Cultured Caco-2 cells were shown to contain some cytochrome P450s such as the 1A (Boulenc *et al.*, 1992) or 3A families (Carriere *et al.*, 1994). In the transport study, we observed several HPLC peaks that were different from that of the original diclofenac sodium. However, over 90% of the amount added at the initiation of the transport study was recovered after 72 h is the total amount in the Ap and BL sides (data not shown). We could not identify the possible metabolites because these chemicals are not commercially available. Therefore, involvement of the biotransformation in Caco-2 cells, particularly in the double-layered system, requires further detailed research.

The toxicity of paraquat observed in the double-layered system was also slightly lower than predicted (Fig. 5 (D)). As presented in the transport study (Fig. 2 (D)), the permeation kinetics of paraquat across the Caco-2 cell layer are different from those of the other three chemicals, *i.e.*, equilibrium was not reached even after 72 h of incubation. Therefore, the most plausible reason for the discrepancy is that the actual paraquat load (concentration multiplied by time) during the nine days was lower than that predicted from its *in vitro*

absorbability (14%), which was the basis of the predicted DR curve in Fig. 5 (D).

To test the efficacy of the double-layered culture system in predicting *in vivo* toxicity, ED_{50} values determined from the DR curves presented in Fig. 5 were compared with reported acute oral lethal doses (LD) in rats or mice (Chemical Health & Safety Database by National Toxicology Program (2000)) (Fig. 6). We used the data on these animals, because data on human oral LD is only available for caffeine and paraquat. As confirmed in Fig. 5, ED_{50}s from the double-layered culture system were higher than those in the single-layered system, and the ED_{50} for least absorbable paraquat increased most significantly in the double-layered culture system. In addition, there was remarkable improvement in the predictivity of *in vivo* acute toxicity as can be seen in the comparison of the correlation coefficients, r, between the two culture systems (0.561 in the double-layered system versus 0.152 in the single-layered system) (Fig. 6). In the future, we must further check the feasibility of the double-layered culture system in terms of its *in vivo* predictivity using a large number of chemicals whose data on human oral lethal dose are available. This is because many factors are concerned in the final toxicity expression in humans, besides intake through the small intestine. In addition, the scale of the commercially available culture system employed in this study determined the number of cells and the volume of the culture medium, and they are quite different from those in an actual human body.

One significant advantage of this culture system as an alternative for experiments on animals is that it can modify the toxicity observed in conventional single-cell-population-based methods by considering absorbability through the small intestine. It thereby provides a simple screening method that is useful for the qualitative estimation or ranking of chemicals. As partly demonstrated in this study (Fig. 5), by comparing the DR curves of a focused chemical in the single- and double-layered culture systems, we can obtain valuable information about its absorbability and possible metabolism when it permeates through the small intestine. Such a comparison is particularly advantageous for estimating the toxicity of foods or environmental samples. This is because that, to determine the toxicity of these samples, it is reasonable to observe their biological effects, such as the inhibition of cell growth, instead of identifying or determining what chemicals and metabolites they contain.

Conclusions

We developed a new cytotoxicity test incorporating absorption by the small intestine using a double-layered culture of Caco-2 and TIG-1 cells. In terms of TIG-1 cell growth, final toxicity in the double-layered system with a Caco-2 cell layer reflected *in vivo* toxicity better than that obtained in single-layered system without a Caco-2 cell layer. Involvement of possible biotransformation by the Caco-2 cells was considered by comparing the dose-response relationships observed in the double-layered system with those predicted from dose-responses in the single-layered system and from *in vitro* absorbability.

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