

# Use of a Perfusion Co-culture System Consisting of Caco-2 and Hep G2 Cell Compartments for the Kinetic Analysis of Benzo[a]pyrene Toxicity

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**Summary** — Conventional cytotoxicity tests cannot usually include various metabolic processes in humans. We therefore developed a physiologically based, multi-compartment perfusion co-culture system, using a Caco-2 cell monolayer on a semi-permeable membrane and a microcarrier-based, three-dimensional culture of Hep G2 cells to mimic permeation across the small intestine and biotransformation of the small intestine and the liver. Stable operations allowed us to maintain various activities of both cells for at least 4 days. Co-cultivation improved the growth of Hep G2 cells and enhanced the cytochrome P450 1A1/2 capacities of both Hep G2 and Caco-2 cells. When benzo[a]pyrene (BaP) was loaded on the apical side of the Caco-2 cell layer, the enhanced P450 capacities produced a higher amount of BaP-7,8-hydrodiol, a precursor of the ultimate carcinogen of BaP, BaP-7,8-dihydrodiol-9,10-epoxide (BPDE). These phenomena led to the initially retarded, but later stronger, expression of BaP toxicity in the co-culture system than in pure cultures, which agreed with the actual load of BaP-7,8-hydrodiol to the Hep G2 cells. Because this kind of system can reproduce such complicated phenomena, including those influenced by organ–organ interactions, it is useful as a new *in vitro* experimental system, for understanding the unknown mechanisms involved in final toxicity in humans and thereby improving physiologically based pharmacokinetic (PBPK) simulation models.

**Key words:** *benzo[a]pyrene, Caco-2 cell, cell–cell interaction, co-cultivation, Hep G2 cell, perfusion culture.*

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## Introduction

One of the major reasons for the successful use of animals for the prediction of various effects of chemicals on humans is that final toxicities observed in such experiments are the result of *in vivo* metabolic processes involving absorption from the small intestine or lung epithelium, biotransformation by the liver, distribution through the systemic blood circulation, excretion by the kidney and accumulation in various organs. Conventional cytotoxicity tests cannot reproduce these metabolic processes occurring *in vivo*, because they use cultured cells of a single population.

To overcome this limitation, the integration of data concerning biological responses obtained from *in vitro* tests by a proper physiologically based pharmacokinetic (PBPK) model is presented (1). This approach is likely to become advantageous over whole-animal experiments in the near future, because it may compensate for the disadvantages of conventional single-cell-population-based cytotoxicity tests. However, such PBPK models can only incorporate toxicity expression mechanisms that have already been established, and they cannot discover unanticipated *in vivo* metabolism.

Recently, some researchers presented a new concept of *in vitro* experimental systems that incorpo-

rate these metabolic processes. Sweeney *et al.* first reported the development and utilisation of a multi-compartment cell culture system, referred to as a cell culture analogue (CCA) device (2, 3), focusing on liver and lung tissues. Such systems allow the observation of final toxicity, as activities in target cells result from various metabolic processes, without the numerical description of metabolic processes that is necessary in a PBPK model. More recently, as an extension of the above-mentioned research to small intestine and liver tissues, Brand *et al.* (4) developed a perfusion co-culture system for evaluation of the permeation across the small intestine and resulting toxicity in the liver tissue using two cell lines, Caco-2 and Hep G2 cells. However, the duration of their system is less than 1 day. Therefore, it is necessary to develop a more stable system that may be helpful in predicting mechanisms involved in acute toxicity at least in humans.

We here describe the development of a new stable perfusion co-cultivation system, incorporating a Caco-2 cell layer formed on semi-permeable membranes and Hep G2 cells cultured in macroporous microcarriers. We employed benzo[a]pyrene (BaP) as a model toxic chemical in this study. This is because our preliminary experiment with a simple double-layered static co-cultivation system and using Caco-2 cells and normal human diploid fibro-

blasts, TIG-1 cells, showed that final toxicity in the TIG-1 cells is sometimes influenced by the bio-transformation and/or polarised transport by the Caco-2 cells (5). BaP appears to be a suitable model chemical, because it requires metabolic activation through cytochrome P450 (CYP) 1A1/2 for the expression of its toxicity resulting from the formation of a highly reactive species, BaP-7,8-dihydrodiol-9,10-epoxide (BPDE; 6).

## Materials and Methods

### Cell culture and medium

Caco-2 and Hep G2 cells were obtained from the Riken Gene Bank (Tsukuba, Japan). They were routinely cultured in Dulbecco's modified minimum essential medium (DMEM; Nissui Pharmaceutical Co. Ltd, Tokyo, Japan) supplemented with 10% fetal bovine serum, 1% non-essential amino acid solution, 20mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES), and antibiotics. Both cell lines were subcultivated by using 0.25% trypsin/0.02% EDTA in phosphate-buffered saline.

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

Caco-2 cells were seeded onto polycarbonate membrane culture inserts (Transwell 3419, 75mm in diameter, culture surface area of 44cm<sup>2</sup>) precoated with type-I collagen (Nitta Gelatin Co. Ltd, Osaka, Japan) at an initial density of  $1.0 \times 10^5$  cells/cm<sup>2</sup> in the accompanying culture dish (Transwell 3419, Corning Inc., Corning, NY, USA). The monolayer of Caco-2 cells was developed until it reached an equilibrium state with the trans-epithelium electrical resistance (TEER) of approximately 200 $\Omega$ .cm<sup>2</sup> measured with a Millicell-ERS (Millipore Co. Ltd, Bedford, MA, USA), after subtracting the resistance of the membrane culture insert (80 $\Omega$ .cm<sup>2</sup>) from the measured values. The culture medium was replenished every two days, and the cell layer was used for experiments after 2 or 3 weeks of culture.

### Culture of Hep G2 cells in macroporous microcarriers

We used cellulose-made, macroporous microcarriers (MCs) coated with Type-I collagen (Cellsnow-CX, 0.7–1.0mm<sup>3</sup> per carrier, Kilin Brewery Co. Ltd, Tokyo, Japan) to attain high cell density. About 100mg of the carriers (1500 carriers) were washed, equilibrated to the culture medium, and put in a 60mm dish for suspension culture. Then,  $4 \times 10^6$  Hep G2 cells were inoculated and continuously shaken on a rotational shaker at a speed of 70rpm.

The culture medium was replenished every two days, and the cell-loaded MCs were used for experiments after 2 weeks of culture.

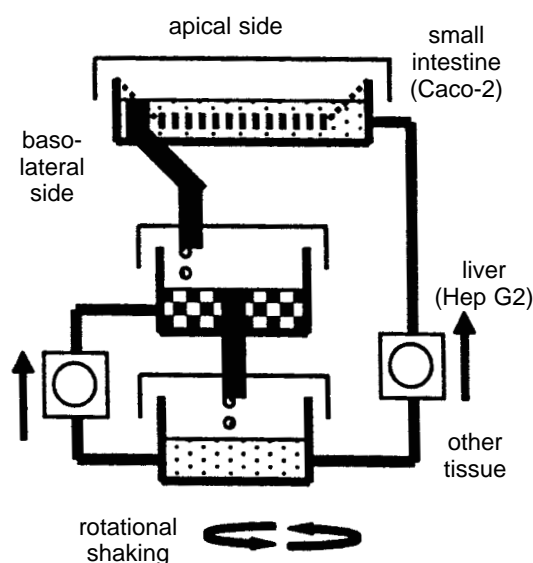
### Development of the perfusion co-cultivation system

The perfusion system consists of three compartments (Figure 1); the top compartment is for the Caco-2 cell membrane formed on a Coaster's culture insert; the second one is for MC-attached Hep G2 cells; the bottom one is a representation of other tissues. These three compartments can be combined and laid in a three-layered shelf made from aluminum, and can be shaken in a rotational manner. The three compartments are connected by two overflows and by Teflon tubing through two Teflon pumping systems (Metarol Pump, MRP-IX; Nikko Engineering Co. Ltd, Tokyo, Japan). All these compartments were made from glass and Teflon to avoid non-specific adsorption of BaP onto the inner surfaces of the system. We used a special CO<sub>2</sub> incubator equipped with a cooling system (BNR-110M, Tabai Espec Co. Ltd, Tokyo, Japan) to avoid an excess increase of temperature. The mechanical part of this system was manufactured by Y.M.A Scientific Co. Ltd, Tokyo, Japan. The glassware was manufactured by the Technical Support Center in our institute.

### System operation and BaP loading experiments

Before day one of cell addition, the set-up of the system was completed to confirm good operation. The

**Figure 1: Schematic representation of the perfusion co-culture system**



rotation speed was 70rpm and two flow rates of the culture medium were set at 0.1ml/minute. Then, on the next day, either the Caco-2 cell-loaded membrane culture insert or Hep G2-loaded MCs, or both, were put into the appropriate compartments. The standard volume of the culture medium is 9ml on the apical (Ap) side of the Caco-2 cell layer, 20ml in the basolateral (BL) side of the layer, and 8ml in the Hep G2 and other tissue compartments. The culture medium contained 2mM 3-methylcholanthrene (3MC; Wako Pure Chemicals Co. Ltd, Osaka, Japan) to enhance the CYP1A1/2 capacities of both cells. After 2 days of perfusion culture, fresh culture medium with 3MC (36ml) was supplied to all the compartments except the Ap side of the Caco-2 cell layer. Then, another portion of fresh culture medium (9ml, 20% of the whole volume) containing both 3MC and 50mM of BaP (Wako) was loaded to the Ap side of the Caco-2 cell layer. Thereafter, various metabolic functions of both cells and concentrations of BaP and of major metabolites in each compartment were measured for another 2 days of perfusion culture.

#### Measurement of cellular activities

TEER values for the Caco-2 cell layers were measured by the Millicell-ERS and used to monitor the integrity of the Caco-2 cell layers. The number of viable Hep G2 cells was measured in terms of intracellular acid phosphatase (AP) content (7). CYP1A1/2 capacities of both cells were measured in terms of ethoxyresorufin *O*-demethylase (EROD) activity in the presence of 10mM dicoumarol (8).

#### Measurement of the concentrations of BaP and its metabolites

20 $\mu$ l of culture medium was sampled from the various compartments of the perfusion culture system, and an equal volume of methanol was added. Then, the proteinaceous precipitate formed and cell debris were removed by centrifugation (15,000rpm, 10 minutes), and the supernatant was used to measure the concentrations of BaP, BaP-3-OH, BaP-9,10-hydrodiol and BaP-7,8-hydrodiol. These concentrations were detected with an HPLC system (HIC-6A, Shimadzu Co. Ltd, Kyoto, Japan) equipped with an octadecylsilil-silica (ODS) gel column (Shim-pack CLC-ODS; Shimadzu), a guard column (Shim-pack G-ODS) and a spectrofluorometric detector (RF-10A<sub>XL</sub>; Shimadzu). The conditions for the measurement were decided according to the manufacturer's recommendation and a previous report (9). The elution buffer was acetonitrile:water:acetic acid = 70:30:0.3 (v:v:v, pH 3–4), and the excitation and emission wavelengths were 295nm and 430nm, respectively. The standards for the three BaP metabolites were purchased from the National

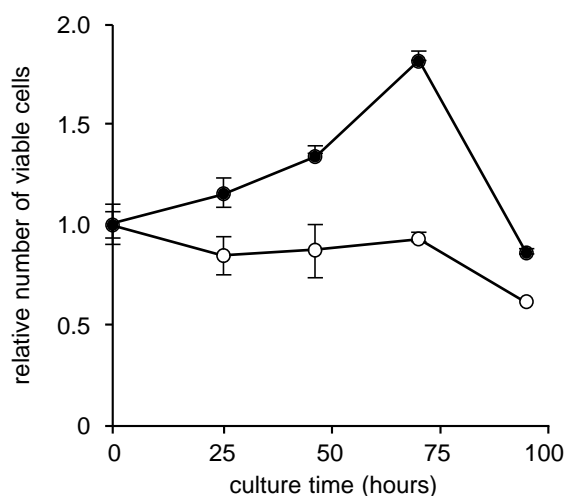
Cancer Institute (NCI, Chemical Carcinogen Reference Standard Repository, Midwest Research Institute, Kansas City, KS, USA).

## Results and Discussion

To investigate cell–cell interactions and their effects on BaP toxicity, three perfusion culture systems were simultaneously operated, i.e. a system having only a Caco-2 cell layer, one having both a Caco-2 and Hep G2 cells and another having only Hep G2 cells. When both cells were co-cultured in the system developed, the growth of the Hep G2 cells was strongly enhanced over that in pure culture (Figure 2). This growth continued up to day 3, even on day one after the commencement of BaP loading, but then, strong toxicity was observed on day 4. The TEERs of the Caco-2 cell layers in both cultures were almost maintained at the initial values to show no significant damage occurred in the Caco-2 cell layer (data not shown).

Interestingly, when co-cultured for 2 days in the 3MC-containing culture medium, the CYP1A1/2 (EROD) capacities activity of both cells were significantly enhanced when compared with those in pure cultures (Figure 3a, 3b1 and 3b2). In the case of Hep G2 cells, this enhancement in the co-cul-

**Figure 2:** Changes in the relative number of viable Hep G2 cells in the systems during induction by 3-methylcholanthrene (0–48 and 48–96 hours) and administration of benzo[a]pyrene (48–96 hours)



● = co-culture with Caco-2; ○ = pure culture.

Each point represents the mean  $\pm$  SD of three separate samples.

ture system was completely diminished by the BaP toxicity on days 3 and 4. This reduction of cellular activity in the co-culture system agreed with the rapid decrease in the viability of Hep G2 cells (Figure 2). The enhanced CYP capacity of the co-cultured Caco-2 cells was kept almost constant up to day 4. This suggested that Hep G2 is more sensitive to BaP toxicity compared with Caco-2 cells.

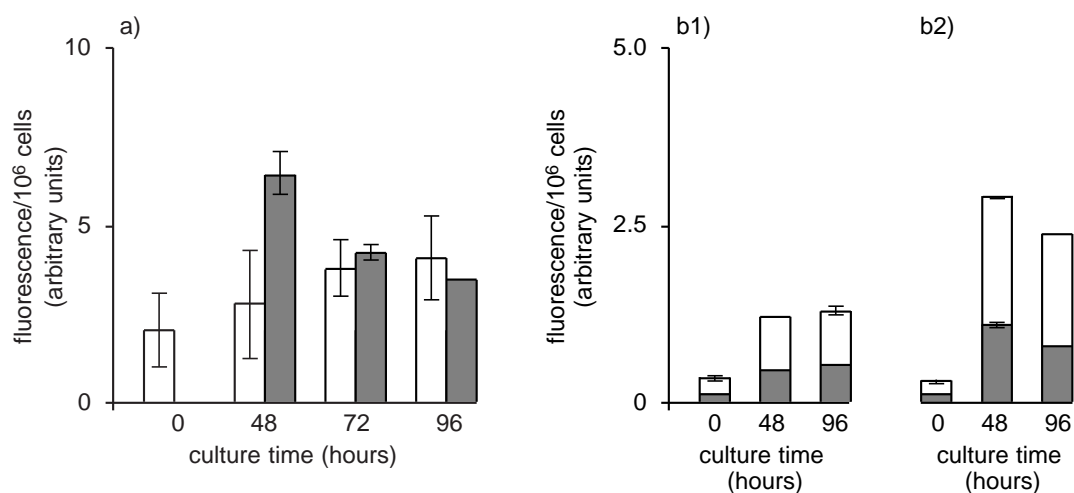
The phenomena described above, such as growth enhancement of Hep G2 cells or enhancement in CYP capacities of both cells in co-culture, clearly show that this kind of co-culture system that enables cells originated from different tissues to interact with each other in real-time in a physiological-situation-mimicking manner has an advantage over conventional pure culture systems. Although we have not yet elucidated the mechanisms of this enhancement in growth or CYP capacities, as far as we know, this is the first demonstration of such unexpected possible organ-organ interactions. Because the duration of previous co-culture systems by other investigators was very short (2–4), it was natural that the experiments in these systems did not allow them to observe the same types of phenomena as those observed in our system.

The concentration of BaP-7,8-hydrodiol in the liver compartment in the co-culture system was remarkably higher than those in the two types of pure cultures at 24 hours, and then it was rapidly lowered at 48 hours (Figure 4). This was partly explained by the fact that BaP-7,8-hydrodiol in

the Caco-2 cell layer was secreted rather to the BL side of the layer as opposed to the expectation by the polarised transport mechanism by P-glycoproteins (10), which really acted on other metabolites, such as BaP-3-OH or BaP-9,10-hydrodiol (data not shown). In addition, although we did not quantify it, there is a possibility that another important metabolic enzyme, epoxide hydrolase, which converts BaP-7,8-hydroxide to BaP-7,8-hydrodiol, was also up-regulated in the co-culture system. Consequently, the total load of this toxic chemical, area under the curve (AUC, time integral of the concentration), to the Hep G2 cells was accordingly a higher value, 3.2mM/hour, whereas it was 0.24mM/hour and 1.32mM/hour in the pure culture of Hep G2 and Caco-2, respectively. This measurement can explain the reason why the number of the viable Hep G2 cells in the co-culture system was rapidly reduced after 24 hours of BaP administration (Figure 2), although the cellular activities evidenced from the enhanced growth and high CYP content in the co-culture exceeded those in pure Hep G2 culture system (Figures 2 and 3a).

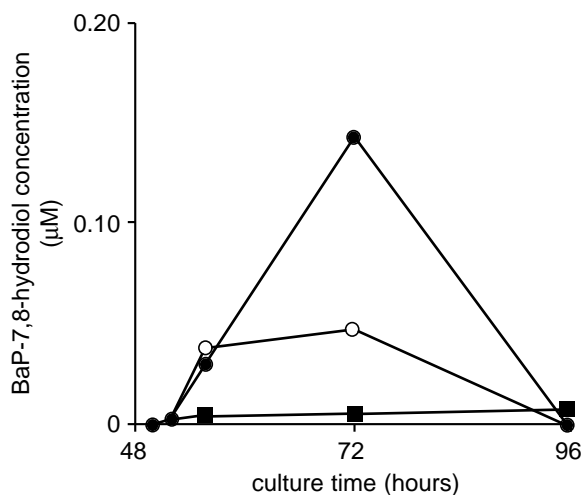
It is accepted that the ratio in the metabolic clearances of the small intestine and the liver are in the same order of magnitude (11). However, in our system, the contribution of Hep G2 cells to the formation of BaP-7,8-hydrodiol was remarkably lower than that of Caco-2 cells (Figure 4). This was mainly due to the lower CYP capacity of Hep G2 cells in MCs, even after 3MC induction (Figure 3a). In the next step, we have to enhance the con-

**Figure 3: Changes in CYP1A1/2 (EROD) capacities of Hep G2 cells in microcarriers (a) and Caco-2 cell membranes in pure- (b1) and co-culture (b2) systems during induction by 3-methylcholanthrene (0–48 and 48–96 hours) and administration of benzo[a]pyrene (48–96 hours)**



a)  $\square$  = pure Hep G2 culture;  $\blacksquare$  = co-culture with Caco-2 cells. b1) and b2)  $\square$  = apical and  $\blacksquare$  = basolateral sides of the Caco-2 cell layer. Each point represents the mean  $\pm$  SD of three separate samples.

**Figure 4: Changes in the concentration of BaP-7,8-hydrodiol in the liver compartment during the 2 days of benzo[a]pyrene loading (48–96 hours)**



● = co-culture; ○ = pure culture of Caco-2 cells;  
■ = pure culture of Hep G2 cells.

tribution of the liver compartment to the whole metabolic capacity of the system to mimic more physiologically relevant situations. Nevertheless, as discussed above, this kind of system has many advantages over conventional pure culture systems, because we could observe complicated or unknown phenomena and their contribution to final toxicity. Through combining such *in vitro* experimental systems with relevant PBPK models, more-quantitative predictions of chemical toxicities in humans will be attainable, partly without *in vivo* experiments.

## Conclusions

We developed a new *in vitro* perfusion co-culture system that mimics active and passive absorption across the small intestine and biotransformation of the small intestine and the liver, by using Caco-2 and Hep G2 cells arranged in a physiologically relevant manner. Stable operations enabled us to maintain the viability and other important metabolic capacities of the two cells for at least 4 days. In addition, many important but complicated phenomena, including possible organ–organ interactions, were successfully reproduced *in vitro*. Therefore, this type of co-culture system would be very helpful when we improve PBPK simulations through the understanding and modelling of the mechanisms of such phenomena and of their contribution to the final effects on humans.

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