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In vitro system for assessing dioxin absorption by intestinal epithelial cells and for preventing this absorption by food substances

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Abstract

A system for assessing intestinal dioxin absorption was established by applying a Caco-2 cell monolayer and stable dioxin-responsive cell line. The stable dioxin-responsive cell line was established by introducing a plasmid incorporating the human CYP1A1 promoter into human hepatic HepG2 genomic DNA upstream of the luciferase gene. 2,3,7,8-Tetrachlorodibenzodioxin (TCDD) was added to the apical side of differentiated human intestinal epithelial Caco-2 cell monolayers that had been cultured on a semipermeable membrane. The basal medium was taken after an appropriate incubation time and added to the dioxin-responsive cells, the TCDD content then being analyzed by a luciferase assay. The amount of TCDD in the basal medium increased in a dose- and time-dependent manner, the results being sufficiently sensitive and reproducible. The inhibition of TCDD permeability to the Caco-2 cell monolayer by such food substances as chlorophyll, insoluble corn fiber and tea dregs were observed by this *in vitro* assessment system. The system will therefore be useful to identify food substances having a preventive effect on the intestinal absorption of dioxins.

Abbreviations: AhR – aryl hydrocarbon receptor; 3-MC – 3-methylcholanthrene; MDR1 – multi-drug resistance 1; MRP2 – multi-drug resistance- associated protein 2; MXR – mitoxantrone resistance protein; RLU – relative light unit; TBT – tributyltin; TCDD – 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; XRE – xenobiotic responsive element

Introduction

Polychlorodibenzo-*p*-dioxins (PCDDs) and polychlorodibenzofurans (PCDFs), collectively referred to as dioxins, are notorious xenobiotics with such toxic consequences as endocrine disruption, and teratogenic and carcinogenic effects (Tanabe et al. 1987; Landers 1991; Whitlock Jr. 1991; Safe 2001). They are released as industrial compounds or as by-products of industry through the process of combustion, and are easily absorbed at the intestinal epithelium and accumulated in fatty tissues due to

their lipophilicity (Poland and Knutson 1982). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is known as the most toxic compound among the dioxins, and has been extensively investigated as a potent ligand of the aryl hydrocarbon receptor (AhR) (Hankinson 1995; Hahn 1997; Sogawa and Fujii-Kuriyama 1997).

Prior to ligand binding, AhR is present in the cytoplasm as a complex with hsp90, co-chaperone p23 and immunophilin-like protein XAP2 (AIP/ARA9) (Mayer et al. 1998; Kazlauskas et al. 1999; Mayer and Perdew 1999). In binding to TCDD, AhR releases hsp90, p23 and XAP2, this being followed by nuclear transduction and dimerization with the AhR nuclear translocator (Arnt) (Ko et al. 1996). Transactivation of a battery of AhR-responsive genes is inspired by binding this heterodimer to xenobiotic-responsive elements (XREs) that are located in the regulatory regions of these genes and by interacting with Sp1, CBP/p300 for the Arnt coactivator, RIP140 and SRC-1 for the AhR coactivator, and general transcription factors (GTFs) (Kobayashi et al. 1996; Kobayashi et al. 1997; Kumar et al. 1999; Kumar and Perdew 1999). Several xenobiotic-metabolizing enzymes, as typified by cytochrome P450 1A1 (CYP1A1), are expressed by these means, and they then activate latent endogenous or exogenous carcinogens (Nebert et al. 2000). In addition, the AhR/Arnt heterodimer has been reported to modulate ER-mediated oestrogen signaling which causes endometriosis and oestrogen-dependent tumors (Ohtake et al. 2003).

High resolution gas chromatography–high resolution mass spectrometry (HRGC–HRMS) or an immunochemical assay has been used to determine the amount of dioxins commencing with TCDD. The former method is extremely precise, but requires a long analysis time and high denomination. In contrast, the latter method only requires a short analysis time, but at the expense of accuracy to some extent. However, both of these methods target TCDD, and are unable to assess about 200 kinds of related compounds.

Although more than 90% of dioxins absorbed by the body enter orally *via* food (Djien Liem et al. 2000), it is difficult and costly to eliminate dioxins from food. Several food substances, however, have been reported to enhance the fecal excretion of dioxins (Morita et al. 1997, 2001; Aozawa et al. 2001). Chlorophyll derived from chlorella has been reported to inhibit dioxin absorption from the gastrointestinal tract and to accelerate dioxin excretion in rats (Morita et al. 2001), and several types of dietary fiber have been reported to enhance the fecal excretion of dioxin isomers in mice (Aozawa et al. 2001). However, a concise method for assessing dioxin absorption in the small intestine and the effect of food factors on this absorption has not been established. Although we have previously reported an assessment system for this purpose (Natsume et al. 2003) by using the AhR-mediated TCDD toxicity expression mechanism just described, a quantitative evaluation of TCDD permeability by this system was not always successful, probably because the transfection efficiency was different among individual wells.

We now report improved accuracy, sensitivity and repeatability with the AhR-mediated assessment system by applying a stable dioxin-responsive cell line. The reactivity of this new system to several xenobiotics other than TCDD was also investigated, and the system was used to investigate the suppressive effect of food substances on the intestinal absorption of TCDD.

Materials and methods

Materials

The Caco-2 and HepG2 cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA), and Dulbecco's modified Eagle's medium (DMEM) was purchased from Sigma (St. Louis, MO, USA). Penicillin–streptomycin (10,000 U ml^{-1} and 10 mg ml^{-1} in 0.9% sodium chloride, respectively), non-essential amino acids, Lipofectamine and the PLUS reagent were purchased from Gibco (Gaithersburg, MD, USA). Fetal bovine serum was purchased from Asahi Technoglass (Chiba, Japan), and Nucleo-Bond AX was purchased from Marcherey-Nagel (Easton, PA, USA). Glasswares – 60-mm dishes, 12-well Transwell inserts and 24-well plates were purchased from Corning-Coster Japan (Tokyo, Japan), and G418 disulfate was purchased from Nacalai Tesque (Kyoto, Japan). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was purchased from Wako Pure Chemical Industries (Osaka, Japan), and the Dual-Luciferase^{TM} Reporter

assay system was purchased from Promega (Madison, WI, USA).

Food samples

Chlorophyll was presented by Chlorophyll Japan (Tokyo, Japan), insoluble dietary fiber (corn dietary fiber) was purchased from Nihon Shokuhin Kako Co. (Tokyo, Japan), and tea dregs were presented by Coca-Cola (Japan) Co. (Tokyo, Japan).

Cell culture

Caco-2 cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in air with a culture medium consisting of DMEM, 10% fetal bovine serum, 1% non-essential amino acids, 200 U ml^{-1} of penicillin, and 200 $\mu g\ ml^{-1}$ of streptomycin. For establishing the stable cell line derived from HepG2, the non-essential amino acids were excluded and conc.G418 was added to the same medium as that used for culturing the Caco-2 cells. The stable cells were seeded at 0.5×10^5 cells $well^{-1}$ in a 24-well plate that had been precoated with collagen, and were used after 1 day of culture.

To form the Caco-2 monolayers, the cells were seeded at 2×10^5 cells $well^{-1}$ in a 12-well Transwell insert that had been precoated with collagen and were cultured for 2 weeks to obtain an integrated cell monolayer with a transepithelial electrical resistance (TER) of more than 150 ohm cm^2. The Caco-2 cells used in this study were between passages 40 and 60.

Reporter plasmid and expression vector

The dioxin-responsive plasmid (pLUC1A1) that includes the human CYP1A1 promoter upstream of the luciferase gene was kindly presented by Dr. R. H. Tukey (University of California, San Diego, USA) (Postlind et al. 1993). The plasmid was prepared according to the instruction manual from NucleoBond AX, and a stable dioxin-responsive cell line was established by introducing this plasmid into the HepG2 cell line derived from human hepatic carcinoma.

Stable integration and selection of G418-resistant colonies

HepG2 cells were transfected with pLUC1A1 by the lipofection technique with Lipofectamine and the PLUS reagent (Gibco, Gaithersburg, MD, USA). The HepG2 cells were grown in 10% FBS-DMEM until 80% confluent and transferred to 60-mm dishes 24 h before transfection. The dishes were seeded with an appropriate number of cells to be 50–60% confluent at the time of transfection. The medium in the dishes was replaced with a serum-free medium 1 h before transfection. Plasmid DNA (2 μg) and the PLUS reagent (8 μl) were diluted in 250 μl of the serum-free medium in each dish and then incubated at room temperature for 15 min. Lipofectamine (12 μl) was diluted in 250 μl of the serum-free medium. The plasmid and lipid dilutions were combined, gently mixed and then incubated at room temperature for 15 min. Meanwhile, the medium in the dishes was replaced with 2 ml of the serum-free medium. A 500 μl portion of the DNA/lipid suspension was added to each dishes and gently mixed.

The transfection process was followed by neomycin selection to ensure stable integration of plasmid DNA. After incubating for 3 h, the cells were cultured for 2–3 weeks in a medium containing 0.4 mg ml^{-1} of G418 disulfate (the neomycin analog) until colonies appeared. The G418-resistant colonies were selected and expanded in a 12-well plate. Randomly selected colonies were tested for the presence of recombinants by treating with 1 nM TCDD for 24 h, before being analyzed for their luciferase activity.

Evaluation of the intestinal permeability for TCDD

An appropriate amount of a TCDD solution (1 μM, diluted with ethanol) was added to the apical chamber (500 μl) of a Caco-2 cell monolayer to give final TCDD concentrations of 0.5–2.5 nM. After incubating for an appropriate period of time, the basal solution (1500 μl) was recovered. A 500 μl amount of this solution was added to each wells of a 24-well plate in which stably integrated HepG2 cells had been seeded. The luciferase assay was conducted after 24 h of incubation according to the instruction manual for the Dual-Luciferase^{TM} Reporter assay system

(Promega). The 24-well plate was washed twice with PBS, and the contents dissolved in a 1 × passive lysis buffer. The lysate was centrifuged at 15,000 rpm for 5 min, the resulting supernatant being transferred to a fresh tube and served for the luciferase assay.

To investigate the effects of dietary components on the TCDD permeability of the Caco-2 cell monolayers, TCDD was applied to the apical side of Caco-2 cell monolayers together with a food sample. The TCDD permeability was calculated by the formula obtained from Figure 1a (0–0.5 nM TCDD).

$$x = (y - 0.1974)/299.74$$

where x is TCDD in the basal medium (nM) and y is the firefly luciferase activity (RLU, $\times 10^4$)

$$z = (x \times 321.97 \times 1.5/1000)/0.415 \times 100$$

where z is the permeability (% of applied TCDD), 321.97 is the molecular weight of TCDD; the total volume of the basal medium; 1.5 (ml) the amount of TCDD added to the apical side of Caco-2 cell monolayers; 0.415 (ng)

Preparation of food samples by the freeze–thaw procedure

Quick freezing was performed by using a Sanyo MDF-792AT freezer (−80 °C preset temperature; 1.5 h processing time; 15 min freezing time). Slow freezing was performed by using a Hitachi EC-43MHP freezer (−3 °C preset temperature; 6 h processing time; 5 h freezing time). After the freeze–thaw procedure, each food sample was melted and dried at 90 °C for 10 h, before being pulverized with a National MK-K47 unit. These samples were fractionated according to their size with a sizing machine, and each fraction that was less than 500 μm in size was used as a food sample.

Results

Construction and characterization of the stable dioxin-responsive cell line

Having reported that the pLUC1A1 plasmid could direct a high level of luciferase expression by *trans*-activation of the CYP1A1 promoter and 5′-flanking sequence, we developed a stable cell line and investigated its characteristics for TCDD response. TCDD (0–2.5 nM) was added to the 24-well plate that the stable cells had been seeded on, and the luciferase assay was conducted 24 h later. The luciferase assay was also conducted by using cells exposed to TCDD (1 nM) after an appropriate period of incubation (0–24 h). The results demonstrate that the stable cell line had high sensitivity to TCDD, expressing luciferase in a dose- and time-dependent manner (Figure 1a and b). The induction of luciferase activity appeared to reach a plateau at about 0.5 μM TCDD and after 12 h of incubation. The protein contents of the control and TCDD-treated cells were no different (data not shown), suggesting that the TCDD-treatment had not affected the growth of the cells.

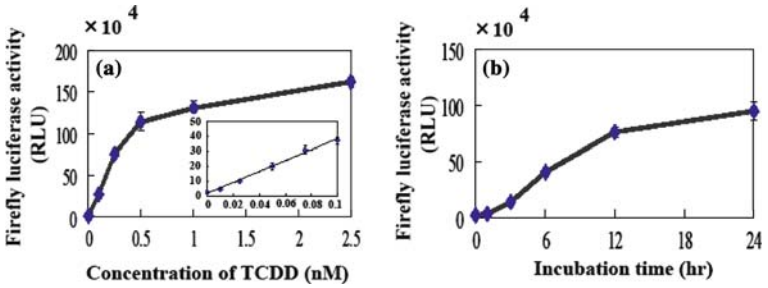


Figure 1. Established stable cell line responding to TCDD. (a) The stable dioxin-responsive cell line expressed luciferase in a TCDD dose-dependent manner. A known concentration of TCDD was added to dioxin-responsive cells cultured in a 24-well plate, before being analyzed 24 h later by a luciferase assay. The inserted graph shows the linearity between the luciferase activity and TCDD concentration ranging from 0 to 0.1 nM. The formula obtained from this graph is not same as that described in the manuscript, due to the difference of the cell line. (b) The stable dioxin-responsive cell line expressed luciferase in a time-dependent manner. About 1 nM TCDD was added to the dioxin-responsive cells that had been cultured in a 24-well plate for a known period of time before the luciferase assay. Each value is the mean ± SE (n = 4).

To investigate the effect of other xenobiotics on the TCDD-responsive cells, the cells were treated with 3-methylcholanthrene (3-MC: 0–100 μM), benzo[α]pyrene (0–1.5 nM), cadmium (0–5 μM) or tributyltin (TBT: 0–100 nM) (Figuer 2). 3-MC and benzo[α]pyrene are well-known AhR ligands and have been reported to induce CYP1A1 (Postlind et al. 1993). 3-MC induced luciferase activity that reached a plateau at a similar RLU level to that of TCDD did (Figure 2a), but the luciferase activity induced by benzo[α]pyrene was lower than that observed with the TCDD treatment (Figure 2b). In contrast, cadmium and TBT did not significantly induce luciferase activity (Figure 2c and d), indicating that they were not AhR ligands. These results demonstrate the usefulness of this cell line for evaluating the toxicity of AhR ligands.

Evaluation by Caco-2 cell monolayers of the intestinal permeability for TCDD

Human intestinal Caco-2 cell monolayers and the stable dioxin-responsive cell line were combined to construct an *in vitro* system for evaluating the intestinal absorption of TCDD. To investigate the dose–response characteristics, different concentrations of TCDD (0–5 nM) were added to the apical side of the Caco-2 cell monolayers. The basal medium (500 μl) was recovered after 24 h of incubation and added to a 24-well plate in which the dioxin-responsive cells had been seeded. A luciferase assay was conducted after incubating this plate for 24 h. The assay results indicated that the TCDD permeability increased linearly in a dose-dependent manner (Figure 3a). Its time-course characteristics were also investigated by adding 2.5 nM TCDD to the apical side of the Caco-2 cell monolayers, incubating for an appropriate period of time (0–36 h) and measuring TCDD in the basal solution by the same procedure as that for the experiment resulting in Figure 3a. The TCDD permeability also increased linearly in a time-dependent manner up to at least 36 h of incubation (Figure 3b). The integrity of the Caco-2 cell monolayers was evaluated by measuring the transepithelial electrical resistance (TER), and no significant differences were apparent between the TCDD-treated and control cells (data not shown). The experimental system and conditions used for evaluating the TCDD permeability are summarized in Figure 4.

Effects of food substances on the transepithelical transport of TCDD in Caco-2 cell monolayers

After considering the results just described, the medium that contained 2.5 nM TCDD and a food

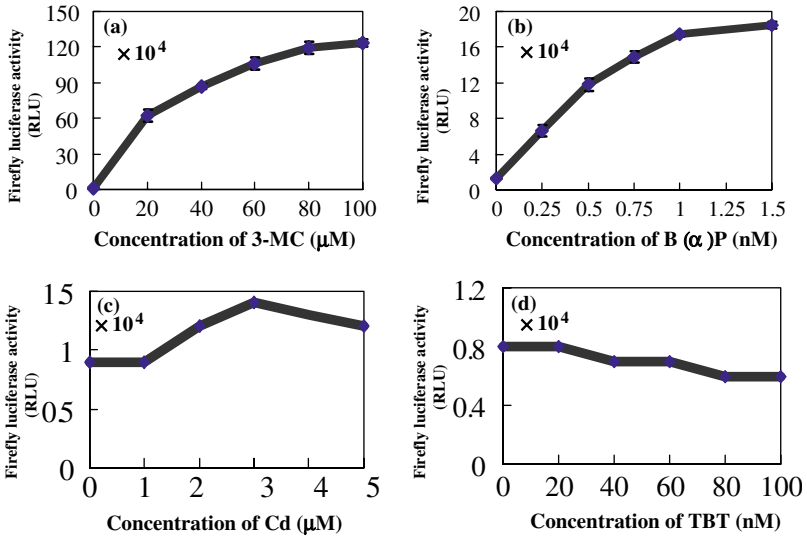


Figure 2. Effects of toxic substances on the luciferase activity. AhR ligands ((a) 3-MC and (b) B[α]P) or non-AhR ligands [(c) Cd and (d) TBT] were added to dioxin-responsive cells that had been cultured in a 24-well plate, before being analyzed by a luciferase assay 24 h later. Each value is the mean ± SE (n = 4).

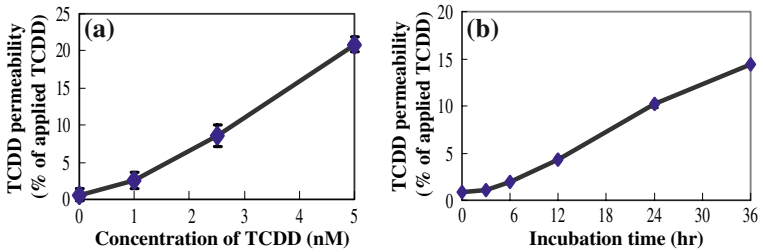
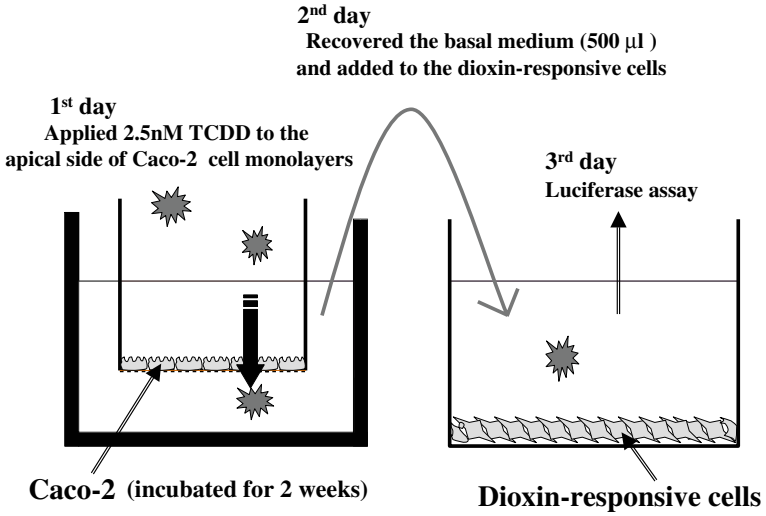


Figure 3. Transepithelial transport of TCDD across Caco-2 cell monolayers. (a) Induction of luciferase activity by TCDD in a dose-dependent manner. A known concentration of TCDD was added to the apical side of a Caco-2 cell monolayer, and the basal TCDD concentration after 24 h of incubation was evaluated by the luciferase assay. Each value is the mean ± SE ($n = 6$). (b) Induction of luciferase activity by TCDD in a time-dependent manner. 2.5 nM TCDD was added to the apical side of a Caco-2 cell monolayer, and the basal TCDD concentration after incubating for a defined period of time was estimated by the luciferase assay. Each value is the mean ± SE ($n = 4$).



Figrue 4. Experimental scheme and conditions, 1st day: The medium containing TCDD (dissolved in ethanol) and a food substance was added to the apical side of a differentiated Caco-2 cell monolayer that had been incubated for 2 weeks on a Transwell insert. Dioxin-responsive cells were seeded at 1×10^5 cells/well in a 24-well plate that had been precoated with collagen. 2nd day: The basal medium of the Caco-2 cell monolayer was recovered and 500 μL was transferred to each well of a 24-well plate in which the TCDD-responsive cells had been seeded. 3rd day: The luciferase assay was carried out.

sample to be tested was added to the apical side of the Caco-2 cell monolayers and incubated for 24 h to investigate the effect of food components on the intestinal absorption of TCDD. Chlorophyll and insoluble corn fiber, which had already been reported to increase the fecal excretion of orally administered TCDD in *in vivo* experiments on rats, were first tested (Aozawa et al. 2001; Morita et al. 2001). When chlorophyll or insoluble corn fiber (0–1% w/v) was added together with TCDD, the amount of TCDD in the basal medium decreased in a dose-dependent manner (Figures 5 and 6). About 7% of applied TCDD permeated the Caco-2 monolayers in 24 h, whereas the TCDD permeability respectively decreased to 5% and 2% in the presence of 0.5% and 1% (w/v) chlorophyll. Like the effect of chlorophyll, the insoluble corn fiber, respectively reduced the TCDD permeability to 3% and 1% in the presence of 0.5% and 1% insoluble corn fiber. However, soluble corn fiber (0–1% w/v) failed to inhibit the transepithelial transport of TCDD (data not

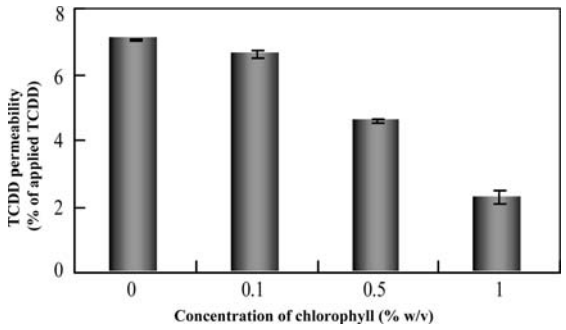


Figure 5. Suppressive effect of chlorophyll on the TCDD permeability of Caco-2 cell monolayers. A known concentration of chlorophyll and 2.5 nM TCDD were added to the apical side of a Caco-2 cell monolayer, and the basal TCDD concentration after 24 h of incubation was evaluated by the luciferase assay. Each value is the mean ± SE (n = 6).

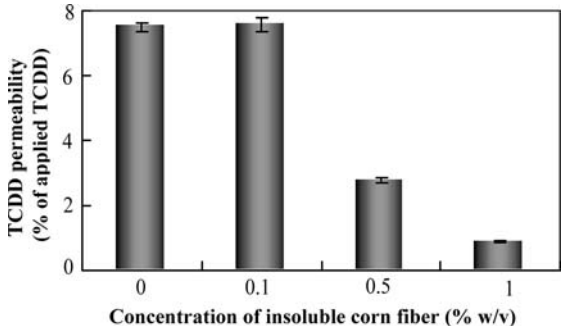


Figure 6. Suppressive effect of insoluble corn fiber on the TCDD permeability of Caco-2 cell monolayers. A known concentration of insoluble corn fiber and 2.5 nM TCDD were added to the apical side of a Caco-2 cell monolayer, and the basal TCDD concentration after 24 h of incubation was evaluated by the luciferase assay. Each value is the mean ± SE (n = 6).

shown). No significant differences were apparent between the food substance-treated and control cells in TER value (data not shown). The consistency between the results obtained with our *in vitro* assessment system and those from the *in vivo* experiments previously reported (Aozawa et al. 2001; Morita et al. 2001) indicates that the present system would provide a useful means for evaluating the inhibition of TCDD absorption.

Effect of tea dregs on the transepithelial transport of TCDD

Tea dregs are by-products of the tea industry and are produced in a large quantity by the manufacture of bottled and canned tea beverages. Since tea dregs contain fiber, chlorophyll and polyphenol they may provide effective materials for inhibiting TCDD absorption at a low price. We applied tea dregs to our evaluation system as one of the possible substances for inhibiting TCDD absorption. A significant reduction in TCDD permeability by tea dregs was apparent, although the effect was not pronounced (Figuer 7, dotted line). We then examined the effect of tea dregs to increase the inhibitory activity. Compared with the untreated samples, the freeze–thaw-treated tea dregs showed higher inhibitory activity, the quick-frozen sample decreasing the TCDD permeability more effectively than the slow-frozen sample (Figure 7). Dose-dependent inhibition (0–1% w/v) of the TCDD permeation by these samples was also observed. No significant differences were apparent between the tea dregs-treated and control cells in TER value (data not shown). The effect of these tea dregs was also examined by using Caco-2 free Transwell and the same tendency was obtained (data not shown), which implies the capture of TCDD by these samples.

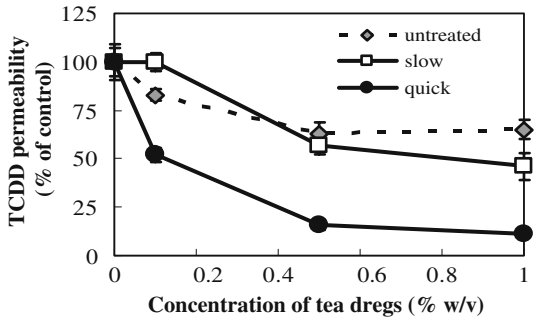


Figure 7. Effect of the freeze–thaw treatment of tea dregs on the TCDD permeability of Caco-2 cell monolayers. Normal tea dregs, slow-frozen tea dregs and quick-frozen tea dregs were used as food samples. A known concentration of a sample and 2.5 nM TCDD were added to the apical side of the Caco-2 cell monolayer, and the basal TCDD concentration after 24 h of incubation was evaluated by the luciferase assay. The permeability of 100% of control is; untreated: 4.1%, slow-frozen: 6.6%, quick-frozen: 6.2%. In this experiment using the untreated sample, the permeability was slightly low probably due to the difference of Caco-2 cell line. Each value is the mean ± SE (n = 6).

Discussion

A luciferase assay, using dioxin-responsive cells, has been widely used to screen food substances that directly affect the toxicity of dioxins. Several agonists/antagonists of AhR and food factors that affect AhR transformation have been discovered by this method (Amakura et al. 2002; Anoek et al. 2003; Chan et al. 2003). However, the method cannot be used to screen food factors that interact with the small intestine and alleviate dioxin toxicity. The intestines are important for the absorption of nutrients and as a physical and biological barrier for protecting the body. Modulating the behavior of dioxins in the intestines must therefore be effective for reducing the risks of orally administered dioxins. Three types of modulation by food substances need to be considered. First, intestinal epithelial cells possess such membrane transporters as MDR1 (P-glycoprotein), MRP2 and MXR which are involved in the cellular transport (influx and efflux) of lipophilic compounds or their conjugates (Litman et al. 2001). Since dioxins could be substrates for these transporters (Rosenberg and Leff 1993; Fardel et al. 1996), food substances that modulate these transporters might affect the flux of dioxins across the Caco-2 cell monolayer. Secondly, food substances that induce phase II enzymes might enhance the efflux of dioxins, because compounds that are conjugated are more easily excreted. Third, food substances that could capture dioxins in the small intestinal tract may reduce dioxin absorption by the intestinal epithelium. Some food substances have been found useful for decreasing the toxicity of dioxins by preventing their absorption. To investigate food factors having these modulatory functions, we combined the Caco-2 cell monolayer and a stable dioxin-responsive cell line derived from HepG2 to construct a system for assaying the dioxin absorption by intestinal epithelial cells. A differentiated Caco-2 cell monolayer expresses several features of intestinal epithelial cells (Hidalgo et al. 1989) and it is widely used as an intestinal epithelial cell model. We have previously attempted to evaluate the amount of TCDD on the basal side of Caco-2 cell monolayers by using HepG2 cells transiently transfected by a plasmid with XRE sequences (Natsume et al. 2003). However, this method was not suitable for quantitatively determining the intestinal permeability for TCDD, because the transfection efficiency varied each time. To obtain an experimental system with higher sensitivity and reproducibility, we constructed in the present study a stable dioxin-responsive cell line for a quantitative analysis. Good linearity of the dose-dependent increase in TCDD concentration of the basal solution was obtained in this work (Figure 3), whereas the basal TCDD concentration did not linearly increase when increasing the apical TCDD concentration from zero to 5 nM in the previous study (Natsume et al. 2003). The better reproducibility and sensitivity of the present assay system by using stable transfected HepG2 cells enabled us to more accurately evaluate food substances for their TCDD-absorption inhibitory activity. A dose-dependent effect of chlorophyll and insoluble corn fiber on the TCDD permeability of the Caco-2 cell monolayer, for example, was clearly observed (Figures 5 and 6).

This new system was used in this study to observe the suppressive effect of tea dregs on the TCDD permeability of the Caco-2 cell monolayer. Green tea leaves have been reported to contain AhR antagonists and flavonoids that modulate CYP1A1 expression and activity (Williams et al. 2000, 2003; Feng et al. 2002; Palermo et al. 2003). Our results indicate that not only a green tea extract, but also tea dregs would have a suppressive effect on the TCDD permeability. The presence of chlorophyll (Morita et al. 2001) and insoluble dietary fiber (Aozawa et al. 2001) in tea dregs may be involved in this suppressive effect. We also attempted to apply the physicochemical treatment of freeze–thawing to enhance the inhibitory activity of tea dregs, and evaluated the effect of freeze–thawing by using our assay system. It is known that a freeze–thaw treatment can produce cavernous food, and that the number and size of the pores can be regulated by the freezing rate. To investigate the effect of the freeze–thaw treatment, three different types of tea dregs (untreated, slow-frozen and quick-frozen) were tested for their TCDD-absorption inhibitory activity. The inhibitory effect varied among the three samples, the quick-frozen tea dregs showing significantly higher activity than the others. The difference among the three samples is thought to have depended on the structural changes caused by the freeze–thaw treatment. The results suggest that these tea dreg samples reduced the amount of

TCDD in the apical solution by capturing TCDD by direct interaction, and not by affecting the multi-drug resistance or expression of the conjugation enzymes in the intestinal epithelial cells. The effects of these tea dreg samples *in vivo* have not yet been investigated and should be evaluated on animals in the future.

In conclusion, the results of our present study indicate the validity of this improved assessment system for the first screening of food factors that inhibit TCDD absorption by the small intestine. They also indicate that a freeze–thaw treatment can enhance the suppressive effect of tea dregs on the TCDD permeability of the Caco-2 cell monolayer, probably by trapping TCDD. The tea dregs found in this study as a material for inhibiting TCDD absorption may provide a useful food ingredient having a preventive effect on dioxin absorption. It has been reported that cow's milk and its products, bovine adipose tissue, hen's eggs and fish mainly contribute to human dioxin exposure in adults (Hallikainen and Vartiainen 1997), and our results suggest that secondary agricultural products such as tea dregs may be useful to produce meat or poultry with a lower dioxin content by feeding the animals with these freeze–thaw-treated materials.

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