Effect of PCB-126 on Intracellular Accumulation and Transepithelial Transport of Vinblastine in LLC-PK1 and Its Transformant Cells Expressing Human P-Glycoprotein

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ABSTRACT. The effects of 3, 3', 4, 4', 5-pentachlorobiphenyl (PCB-126), which is the most toxic congener of coplanar polychlorinated biphenyls (Co-PCBs), on intracellular accumulation and transepithelial transport of vinblastine were examined in porcine kidney cells, LLC-PK1, and its transformant cells expressing human P-glycoprotein (LLC-MDR1). The accumulation decreased less than one-tenth in LLC-MDR1 compared to LLC-PK1. In both cells, the accumulation increased with the addition of PCB-126 and cyclosporine A (CYA), which are P-glycoprotein modulators, though the magnitudes were different in these two cell groups as well as for these two chemicals. Thus, PCB-126 might inhibit extrusion of vinblastine through the drug extrusion system as does CYA. In both the cells, there might be an endogenous drug extrusion system other than P-glycoprotein that was inhibited by CYA or PCB-126. The net basal-to-apical transepithelial transport of vinblastine increased 1.7-fold more in LLC-MDR1 than in LLC-PK1. By adding PCB-126 on the apical side, the transport was greatly decreased by -76% in the monolayer of both cells. By adding PCB-126 and CYA on the basal side in LLC-MDR1 monolayer, the transports increased -1.7-fold, so that PCB-126 might inhibit the extrusion of vinblastine on both the apical and basal sides. One of the causes to be considered for the adverse effects of Co-PCBs, in addition to the binding with an aryl hydrocarbon receptor, might be the modification of drug transport by its interaction with the drug transport system.

KEY WORDS: LLC-PK1, 3,3',4,4',5-pentachlorobiphenyl, P-glycoprotein, transepithelial transport, vinblastine.

Coplanar polychlorinated biphenyls (Co-PCBs) are environmental pollutants, and soil, rivers and oceans have been widely degraded by them. Due to the food chain and bioaccumulation, these chemicals might have become highly accumulated in humans and animals [1, 3, 6, 12, 22]. It was considered that almost the toxic effects of Co-PCBs were initiated by binding with the aryl hydrocarbon receptor (AhR) [18, 24], and these chemicals induced estrogenic and antiestrogenic responses via the crosstalk between AhR and estrogen receptor [8, 31]. Thus, the toxicity of Co-PCBs has appeared in the form of endocrine disruptors and has been found to cause many adverse effects such as reproductive disorders, malformation and cancer [7, 16, 17, 20, 22]. In addition to such toxicities, Co-PCBs might affect some other parts of the animal body. During our survey to determine whether Co-PCBs were transported by a drug extrusion pump such as P-glycoprotein, we found that Co-PCBs seemed to inhibit the extrusion of other chemicals in human epidermoid carcinoma cells, such as KB-3, expressed with P-glycoprotein [2, 11].

P-glycoprotein transports lipophilic chemicals, and thus it plays an important role in transporting metabolites and extruding toxic chemicals from epithelial cells [4, 5, 14, 28]. There are also substrates of P-glycoprotein which are not transported, but are bound to P-glycoprotein and inhibit the transport of other chemicals [4, 25]. Therefore, there is a possibility that Co-PCBs are the substrates for the binding of P-glycoprotein, and inhibit drug extrusion in KB-3 cells. In our previous experiments, we measured drug accumulation in KB-3 cells [2, 11]. Thus, doubts may arise as to whether the values reflected inhibition of transport or competition of drug adsorption by lipophilic Co-PCBs, because lipophilic compounds were readily adsorbed on the plasma membrane in a non-specific fashion. Therefore, we employed both measurements for the intracellular accumulation and transepithelial transport in the present experiment. One of the present authors showed that P-glycoprotein existed at the apical membrane in epithelial cells, and mediated transepithelial transport in the epithelial monolayer cells [30], so that it is important to examine the effects of Co-PCBs on the epithelial monolayer.

3,3',4,4',5-pentachlorobiphenyl (PCB-126) is the most toxic among the congeners of Co-PCBs, and has been detected in human blood [7, 23]. Here we report the effect of PCB-126 on the accumulation and transepithelial transport of vinblastine in a porcine kidney epithelial cell line, LLC-PK1, and its transformant cells expressed with human P-glycoprotein. Vinblastine is among the anticancer drugs, which has been well examined as a substrate of P-glycoprotein for transport [15, 19, 29]. Cyclosporine A (CYA) was employed as a positive control for an inhibitor of P-glyco-
protein, which has been shown to compete with vinblastine for a binding site of P-glycoprotein [25]. The measurement of the effects of Co-PCBs on the intracellular accumulation of the drug might reflect the cell toxicity, and the examination of the effect on the transepithelial transport could reveal a modification of drug transport across the epithelium.

MATERIALS AND METHODS

**Chemicals:** PCB-126 was purchased from Kanto Kagaku (Tokyo, Japan). CYA was obtained from Wako (Osaka, Japan), and vinblastine was obtained from Sigma (St. Louis, MO, USA). [G-3H]-vinblastine sulphate (470 GBq/mmol) was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA), and [carboxy-14 C]-inulin (92.5 GBq/g) was purchased from American Radio Labeled Chemical (St. Louis, MO, USA).

**Cells:** Porcine kidney epithelial cells, LLC-PK1, and their transformant cells expressing human P-glycoprotein, LLC-GA5-COL150 (LLC-MDR1), were employed as reported previously [27]. LLC-PK1 was maintained in medium 199 supplemented with 10% fetal calf serum, and LLC-MDR1 was cultured in that medium containing 150 µM colchicine in an atmosphere of 5% CO2 at 37°C. In LLC-PK1, the inhibition dose for 50% cell growth in four days (ID50) in the medium with colchicine and vinblastine was 28 nM and 6.2 nM, respectively. These ID50s with colchicine and vinblastine were increased by 19- and 52-fold, respectively, in LLC-MDR1.

**Accumulation of vinblastine:** For a determination of the intracellular accumulation of vinblastine, a cover slip and 24-well multi-dish (Nalge Nunc International, Rochester, NY, USA) were used as reported previously [9, 10]. The cells were seeded at 5 × 10^4 cells/well in the medium, and incubated in 5% CO2 at 37°C. After 6 days of incubation, the medium was replaced with a fresh medium without colchicine, and the cells were incubated for 6 hr. Then the medium was replaced with 750 µl fresh medium containing 11 nM [3H]-vinblastine (5.16 kBq/ml) with or without CYA or PCB-126. After incubation for 1, 2 and 3 hr, the cover slip was removed and the cells were washed 3 times with PBS and lysed with lysate buffer; their radioactivity was then measured with a liquid scintillation counter. The accumulation of vinblastine in the cells was expressed as pmol/mg protein.

**Transepithelial transports:** Transepithelial transports, both basol-to-apical and apical-to-basal, were measured in the cell monolayer on a bottom-filtered well (12 mm diameter, Transwell, 3402, Costar, Cambridge, MA, USA) as reported previously [27, 30]. The cells were seeded on the bottom-filtered well in the same manner as in the accumulation experiment. After a 6-day incubation, the medium on either the basal or apical side of the monolayers was replaced with 750 µl fresh medium containing 11 nM [3H]-vinblastine (5.16 kBq/ml) with 43.2 µg/ml [14 C]-inulin (4.0 kBq/ml) with or without 1 µM CYA or PCB-126. An aliquot (25 µl) of the receiver side medium was collected within 3 hr, and its radioactivity was measured with a liquid scintillation counter. The transepithelial transport was indicated as pmol per well. The paracellular fluxes were monitored by measuring the appearance of inulin on the other side, which turned out to be less than 5% at 3 hr as reported earlier [26].

**Statistical analysis:** Following the F-test, Student’s t-test was employed to examine the statistical significance of the accumulations and transepithelial transports of those with and without inhibitors.

**RESULTS**

Figure 1 shows the intracellular accumulation of vinblastine as a function of time in the medium with or without CYA and PCB-126 in LLC-PK1 (A), and ratios of the accumulations without and with these chemicals after a 3-hr incubation (B). The accumulations hyperbolically increased in all the mediums with or without CYA and PCB-126, though the accumulation was higher in the medium with these chemicals than without them. The accumulation was 4.14 pmol/mg protein in the medium without these chemicals after 3-hr incubation, and it was increased by 2- and 1.8-fold in the medium with CYA and PCB-126, respectively, but there was no difference between the accumulations with CYA and PCB-126. Therefore, the extrusion of vinblastine was decreased by adding these chemicals in LLC-PK1.

Figure 2 shows the intracellular accumulation of vinblastine as a function of time in the medium with or without CYA and PCB-126 in LLC-MDR1 (A), and ratios of the accumulations without and with those chemicals at a 3-hr incubation (B). The accumulation was decreased to 0.30 pmol/mg protein after a 3-hr incubation in the medium with-
out chemicals in LLC-MDR1: it was decreased by 93% compared to that in LLC-PK1 (Fig. 1A). In LLC-MDR1, the accumulations of vinblastine in the medium with CYA and PCB-126 were 6.1- and 2.5-fold higher, respectively, compared to that in the medium without these chemicals. Thus, PCB-126 inhibited the extrusion of vinblastine the same as CYA did, although the absolute magnitude of the inhibition with PCB-126 was lower than that with CYA.

Figure 3 shows the effect of CYA (A, B) and PCB-126 (C, D) at the apical (A, C) and basal sides (B, D) on the transepithelial transports of vinblastine in LLC-PK1, together with a comparison of ratios for the net basal-to-apical transport of without and with these chemicals (E). The basal-to-apical transport decreased and the apical-to-basal transport was increased by adding CYA at either the basal or apical side (A, B) in LLC-PK1. Thus, the net basal-to-apical transports with CYA on the apical and the basal sides decreased by 65 to 100% (E). By adding PCB-126 on the apical side, the basal-to-apical transport was greatly decreased by 85% at a 1-hr incubation, whereas the apical-to-basal transport was slightly increased at all time points (C). Thus, the net basal-to-apical transport with CYA on the apical side was unchanged from that without the chemical, whereas there was a slight increase in the apical-to-basal transport with CYA on the apical side (A), but one that was too small to affect the net basal-to-apical transport. With CYA on the basal side, the basal-to-apical transport increased significantly, but the increase in the apical-to-basal transport was very small (B). Thus, the net basal-to-apical transport increased by around 1.4-fold when CYA was added on the basal side in LLC-MDR1 (E). With PCB-126 on the apical side, the basal-to-apical transport was greatly reduced and the apical-to-basal transport was unchanged at a 1-hr incubation, so the net basal-to-apical transport decreased by 55% compared to that without PCB-126 (C, E), but the basal-to-apical transports were unchanged in 2- to 3-hr incubation. Thus, no difference was found for the net transepithelial transports when using the medium with and without PCB-126 at the later time points. With PCB-126 on the basal side, the basal-to-apical transport was increased, but the apical-to-basal transport always remained unchanged (D). Thus, the net basal-to-apical transport increased to 1.7-fold when PCB-126 was added to the basal side in LLC-MDR1, yielding the same result as in the experiment with CYA (E).

DISCUSSION

In LLC-MDR1, the accumulation of vinblastine was greatly decreased compared to LLC-PK1 as reported by Horio et al. [15], indicating the P-glycoprotein was responsible for extruding the chemical. Although it was possible that the values for accumulation were the result of adsorption in the cell membrane but not that of uptake, the decrease in accumulation coincided with the result of the ID50. The ID50 of vinblastine in LLC-MDR1 was greater by 52-fold than that in LLC-PK1 in the present experiment, and its ID50 was within the range mentioned in the previous report [26], so that the decreased accumulation reflected the increased ID50.

The increase in the accumulation of vinblastine by adding CYA in LLC-MDR1 might be considered due to the inhibition of the extrusion through P-glycoprotein binding with CYA as reported earlier [19, 25]. Nevertheless, the effect of CYA was detected not only in LLC-MDR1 but also in LLC-PK1, and the absolute magnitude of the inhibition with CYA was greater in LLC-PK1 than in LLC-MDR1. P-glycoprotein was detected only on the apical side in the monolayer of LLC-MDR1, but it was not detected in LLC-PK1 with anti-human P-glycoprotein antibody which is able to detect porcine P-glycoprotein [30]. Therefore, CYA might inhibit
some drug extrusion system other than P-glycoprotein in LLC-PK1. There are many drug extrusion systems in cell membranes [4, 14], so that endogenous drug extrusion systems might be inhibited by binding with CYA in LLC-PK1. Since the effect of CYA on the accumulation in LLC-MDR1 was smaller than that in LLC-PK1, the inhibition of CYA on the drug extrusion though P-glycoprotein was not defined in this experiment.

By adding PCB-126, an inhibition of the decrease in drug accumulation was also detected, suggesting that PCB-126 might act as an inhibitor of that drug extrusion system. Since Co-PCBs seem to readily accumulate in a lipophilic cell membrane, there is the possibility that Co-PCBs act as the binding substrate of P-glycoprotein and hamper drug extrusion in human and animal cell membranes [21]. As a matter of fact, the chemicals of which very little was transported were potent inhibitors of the transport of other transport substrates. One of the congeners of Co-PCBs, 3, 3', 4, 4'-tetrachlorobiphenyl (PCB-77), was hardly transported at all in the monolayer of LLC-MDR1 and was highly accumulated in the cells [9, 10]. Therefore, there is the possibility that Co-PCBs may hamper transport of drugs or metabolites through some drug extrusion systems including P-glycoprotein in cell membranes, thus exerting adverse effects on the cells. However the effect of PCB-126 on the transport in LLC-MDR1 was less than that in LLC-PK1, so that the effect might be due not to the inhibition of P-glycoprotein, but to another drug extrusion system.

The net basal-to-apical transport of vinblastine in the monolayer cells increased in LLC-MDR1 compared to LLC-PK1 as reported earlier [10, 19], thus indicating that the P-glycoprotein was responsible for the net basal-to-api-
EFFECT OF PCB-126 ON DRUG TRANSPORT

By adding CYA and PCB-126 to the basal side of the monolayer of LLC-MDR1, the basal-to-apical transports were increased, thereby increasing the net basal-to-apical transepithelial transports. This might be due to the enhanced uptake of the chemical on the basal side by the inhibition of some drug extrusion systems. There might be an endogenous drug transport system on the basal side which was inhibited by CYA or PCB-126 in LLC-MDR1. In LLC-PK1, the basal-to-apical transport was also enhanced with PCB-126 on the basal side, but not with CYA, suggesting that the effects of PCB-126 and CYA on a drug transport system may differ from each other.

A decrease in the basal-to-apical transport and an increase in the apical-to-basal transports by adding CYA and PCB126 on the apical side in LLC-PK1 might be interpreted as an inhibition of the extrusion of vinblastine by these chemicals on the apical side. Therefore, the net basal-to-apical transport was reduced by these inhibitors on the apical side in LLC-PK1, though its magnitude was decreased after a 3-hr incubation with PCB126 (Fig. 3-C). Therefore, PCB-126 might affect the apical side at an early stage, then the chemical moves to the basal side and ham-

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**Fig. 4.** Effect of CYA (A, B, filled symbols) and PCB-126 (C, D, filled symbols) at the apical (A, C) or basal side (B, D) on the basal-to-apical (circles) and apical-to-basal (squares) transepithelial transport of vinblastine in LLC-MDR1, and ratios of the net basal-to-apical transports without (Cont) and with CYA or PCB-126 on the apical (Api, slanted columns) or basal (Bas, filled columns) sides (E). Values are the means and SD of four experiments. *, Statistical significance of the transport without and with CYA or PCB-126 on the apical and basal sides in LLC-MDR1 (p<0.05).
pers the extrusion of the chemical on that side at a later stage in LLC-MDR1, so that this influx on the basal side might increase later, increasing the net basal-to-apical transport. In LLC-MDR1, a similar effect was also detected when PCB-126 was added on the apical side (Fig. 4-C), though no effect was found subsequently. The apical-to-basal transport was basically very low in LLC-MDR1 (Fig. 4), and the absolute magnitude of the inhibition of the drug extrusion was not so high (Fig. 2A), so that the minor effect on the apical side might fail to affect the net basal-to-apical transport due to the considerable magnitude of the basal-to-apical transport.

In this experiment, the effects of CYA and PCB-126 on the accumulation or transepithelial transport in LLC-MDR1 and LLC-PK1 were different; in most cases, the effects were less in the former cells than in the latter. One interpretation would be that these inhibitions affected mainly transport systems other than P-glycoprotein, and all drug extrusion systems were modified in LLC-MDR1 due to the transfection of the vector with the inserted human P-glycoprotein gene. Sometimes these unexpected effects were observed after gene transfection. For example, in the cells transfected with the K-Cl cotransporter (KCC) gene, ion transport activity other than KCC was enhanced [13]. This was considered to be due to the modification of total gene expression by the transfection of one gene, or the change in the relationship or crosstalk among the protein induced and the other endogenous proteins.

In conclusion, PCB-126 inhibited the accumulation and transepithelial transport of vinblastine as effectively as CYA did, possibly due to an inhibition of drug extrusion systems by PCB-126. Both PCB-126 and the other Co-PCBs are highly lipophilic and accumulated in animal and human organs [1, 3, 12, 22]. Therefore, Co-PCBs may inhibit the function of the drug extrusion systems in many organs, as shown in this experiment. Though the principal toxicities of Co-PCBs are induced by binding with AhR [18, 24], the effects of Co-PCBs on drug transport systems have to be considered one of the possible causes of the adverse effects of these chemicals.

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