Identification of P-Glycoprotein and Transport Mechanism of Paclitaxel in Syncytiotrophoblast Cells

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Abstract

When chemotherapy is administered during pregnancy, it is important to consider the fetus chemotherapy exposure, because it may lead to fetal consequences. Paclitaxel has become widely used in the metastatic and adjuvant settings for women with cancer including breast and ovarian cancer. Therefore, we attempted to clarify the transport mechanisms of paclitaxel through blood-placenta barrier using rat conditionally immortalized syncytiotrophoblast cell lines (TR-TBTs). The uptake of paclitaxel was time- and temperature-dependent. Paclitaxel was eliminated about 50% from the cells within 30 min. The uptake of paclitaxel was saturable with $K_m$ of 168 $\mu$M and 371 $\mu$M in TR-TBT 18d-1 and TR-TBT 18d-2, respectively. $[^3H]$Paclitaxel uptake was markedly inhibited by cyclosporine and verapamil, well-known substrates of P-glycoprotein (P-gp) transporter. However, several MRP substrates and organic anions had no effect on $[^3H]$paclitaxel uptake in TR-TBT cells. These results suggest that P-gp may be involved in paclitaxel transport at the placenta. TR-TBT cells expressed mRNA of P-gp. These findings are important for therapy of breast and ovarian cancer of pregnant women, and should be useful data in elucidating teratogenicity of paclitaxel during pregnancy.

Key Words: Paclitaxel, Pregnancy, Syncytiotrophoblast, TR-TBT cells, Blood-placental barrier, P-glycoprotein

INTRODUCTION

Cancer during pregnancy is uncommon, occurring in approximately 1/1,000 to 1/2,000 pregnancies (Van Calsteren et al., 2010). This incidence is expected to increase, given the trend for women to delay becoming first-time moms. Therefore, chemotherapy is regularly administered in pregnant women with cancer. When chemotherapy is administered during pregnancy, it is important to consider the fetus chemotherapy exposure, which may lead to fetal consequences including malformations, fetal growth retardation, and death. The placenta which regulates nutrient and waste exchange between the mother and the fetus may serve as a protective barrier for the fetus against maternal blood-borne toxins (Sai et al., 2008). Therefore, it is important to clarify the transfer of drugs from maternal blood to fetus across the blood-placenta barrier (BPB), which is composed of syncytiotrophoblast cells when chemotherapy is administered during pregnancy.

Among active drugs in breast, gynecological, and lung neoplasms that may occur during pregnancy, paclitaxel display a favorable toxicity profile during the second and third trimesters (Mir et al., 2010). To date, available human data suggest that the risk of congenital anomalies or premature birth as a result of paclitaxel use is low (De Santis et al., 2000; Sood et al., 2001; Méndez et al., 2003; Gonzalez-Angulo et al., 2004). Maternal side effects (mainly alopecia, nausea and vomiting, hematological toxicity) seem to be mild and manageable, as seen in non-pregnant women (Mir et al., 2010). These reports suggested the feasibility of use of paclitaxel to pregnant cancer patients. Therefore, the aim of this study is to characterize the uptake mechanism of paclitaxel at the blood-placenta barrier (BPB). Recently, TR-TBT cells are established from pregnant transgenic rats as an in vitro blood-placenta barrier (BPB) model (Kitano et al., 2004). It has been reported that the transport functions of the syncytiotrophoblast layer are similar in human and rat placenta (Takata and Hirano, 1997). Therefore, TR-TBTs are a good model for analysis of the placental transport of nutrients and chemicals. TR-TBTs exhibit typical properties of syncytiotrophoblast cells and TR-TBT 18d-1 and 18d-2 are derived from syncytiotrophoblast I and syncytiotrophoblast II, respectively (Kitano et al., 2004), so we adopted TR-TBT cells as a model cell lines for the present study.
MATERIALS AND METHODS

Materials
Radiolabeled [o-benzamido-3H]taxol ([3H]paclitaxel, 1.0 mCi/ml) was obtained from Moravek Biochemicals (Mercury Lane, Brea, California, USA). Cyclosporine, verapamil, probenecid, cefmetazole, methotrexate and mitoxantrone were purchased from Sigma Chemical (St. Louis, MO, USA). All other chemicals and reagents were commercial products of reagent grade.

Cell culture
TR-TBT cells were established from pregnant transgenic rat harboring the temperature-sensitive simian virus 40 large T-antigen genes at 18 days of gestation by Kitano et al. (2004). TR-TBT 18d-1 and 18d-2 have the histological characteristics of syncytiotrophoblast I and II, respectively (Kitano et al., 2004). The TR-TBT cells were cultured with Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS; Invitrogen), 100 U/ml penicillin (PC, Invitrogen), and 100 µg/ml streptomycin (SM; Invitrogen) at 33°C in a humidified atmosphere of 5% CO2/air. On rat tail collagen type I-coated 24 well culture plates (IWAKI, Tokyo, Japan) initial seeding was done at 1×10⁵ cells/well and the cultures became confluent after seeding. Then the cells were incubated at 37°C for 2 days to deactivate SV40 temperature-sensitive T-antigen.

Functional studies
A confluent monolayer of TR-TBT cells was washed three times with 1 ml extracellular fluid (ECF) buffer consisting of 122 mM NaCl, 25 mM NaHCO3, 3 mM KCl, 1.4 mM CaCl2, 1.2 mM MgSO4, 0.4 mM K2HPO4, 10 mM D-glucose and 10 mM Hepes adjusted to pH 7.4 at 37°C. Uptake was initiated by applying 200 µl ECF buffer containing 1 µCi [3H]paclitaxel at 37°C or 4°C in the presence or absence of inhibitors. After appropriate time periods, the applied solution was removed to terminate uptake and the cells were immersed in ice-cold ECF buffer. To measure the efflux of [3H]paclitaxel in TR-TBTs, after incubating the cells with 1 ml extracellular fluid (ECF) buffer consisting of 122 mM NaCl, 25 mM NaHCO3, 3 mM KCl, 1.4 mM CaCl2, 1.2 mM MgSO4, 0.4 mM K2HPO4, 10 mM D-glucose and 10 mM Hepes adjusted to pH 7.4 at 37°C. Uptake was initiated by applying 200 µl ECF buffer containing 1 µCi [3H]paclitaxel at 37°C for 60 min, the reaction was stopped by adding ice-cold ECF buffer. Then the cells were incubated again with ECF buffer alone or containing unlabeled inhibitors at 37°C for designated time. After that, the cells were then solubilized in 750 µl of 1 N NaOH and radioactivity was measured in a liquid scintillation counter (LS6500; Beckman, Fullerton, CA, USA).

Data analysis
For kinetic studies, the Michaelis-Menten constant (Km) and the maximum uptake rate (Vmax) of [3H]paclitaxel were estimated from Equation (1):

\[ V = V_{\text{max}} \cdot C / (K_m + C) + K_d \cdot C \]  

(1)

where V and C are the initial uptake rate of [3H]paclitaxel at 5 min and the concentration of paclitaxel, \( V_{\text{max}} \) is the maximum uptake rate for the saturable component, and \( K_d \) is the first-order constant for the non-saturable component respectively. Statistical analyses were carried out by one-way ANOVA with Dunnett’s post-hoc test.

RESULTS
Time-course of [3H]paclitaxel uptake and efflux by TR-TBT cells
We examined the uptake of [3H]paclitaxel for 60 min in TR-TBTs in order to investigate characterization of paclitaxel transport at the BPB. [3H]Paclitaxel uptake increased in a time-dependent manner it was linear for 5 min (Fig. 1). So, [3H]Paclitaxel uptake was measured at 5 min in the following kinetic and inhibition studies. [3H]Paclitaxel uptake was markedly decreased at 4°C condition.
In addition, [3H]paclitaxel was eliminated by TR-TBTs. As shown in Fig. 2, the amount of intracellular [3H]paclitaxel was decreased in a time-dependent manner up to 30 min in the cells. [3H]paclitaxel efflux was also significantly inhibited by the addition of 500 μM unlabeled paclitaxel (Fig. 2). These results suggest that paclitaxel transport in TR-TBTs is involved in specific efflux transport systems.

Kinetic analysis of [3H]paclitaxel uptake by TR-TBT cells
To characterize the kinetics of [3H]paclitaxel uptake by TR-TBT cells, we analyzed the concentration dependence of [3H]paclitaxel uptake. The transport process was saturable with a Michaelis-Menten constant (K_m) of 168 μM and a maximum rate of uptake (V_max) of 216 pmol/mg protein/min in TR-TBT 18d-1 cells and K_m of 371 μM and V_max of 191 pmol/mg protein/min in TR-TBT 18d-2 cells (Fig. 3).

Inhibitory effects of various compounds on [3H]paclitaxel uptake by TR-TBT cells
To determine the substrate selectivity of paclitaxel transport system in TR-TBT cells, we performed inhibition studies with derivatives (Table 1). These experiments revealed that [3H]paclitaxel uptake was markedly inhibited by excess unlabeled paclitaxel and also by cyclosporine and verapamil, which are well-known substrate of P-glycoprotein (P-gp) transporter. [3H]Paclitaxel uptake was not significantly inhibited by probenecid and cefmetazole, substrates of MRP4. Novobiocin, a substrate of organic anion transporter (OAT) did not significantly inhibit the uptake of [3H]paclitaxel. And several amino acids, adenosine and glycine did not inhibit [3H]paclitaxel uptake.

The gene expression of p-glycoprotein in TR-TBT cells, rat and human placenta
Total RNA from cultured TR-TBTs and rat placenta was isolated by the RNeasy kit from Quiagen, according to the manufacturer’s instruction. The expression of mdr1a, mdr1b and hMDR1 was analyzed by RT-PCR using total RNA isolated from rat placenta and TR-TBTs (Fig 4). The mdr1a at 97 bp, mdr1b at 846 bp and hMDR1 at 502 bp were amplified in rat placenta as a positive control.

DISCUSSION
The goal of this study is to investigate transport mechanisms of paclitaxel from maternal uptake across to the fetus through the blood placenta barrier. We used TR-TBT 18d-1 and 18d-2 as in vitro model of the BPB.

Conditionally immortalized rat syncytiotrophoblast cell line, TR-TBT was established from pregnant transgenic rats harboring the temperature-sensitive simian virus 40 large T-antigen genes at 18 days of gestation by Faculty of Pharmacy, Keio University (Kitano et al., 2002). The cells exhibited the typical characteristics of in vivo syncytiotrophoblast (Kitano et al., 2004). Especially, they were testified to form a polarized layer of cells with the apical and basolateral membranes having functional similarities to those in rat placenta (Kitano et al., 2002; Kitano et al., 2004). In addition, TR-TBT cells express many transporter genes found in human placenta and also exhibit the uptake activity of taurine and GABA through the several transporters. Recently, our group characterized the BPB uptake mechanism of choline and 6-mercaptopurine (6-MP)
via choline transporter-like protein 1 (CTL1) and equilibrative nucleoside transporters (ENTs) using these cells, respectively (Lee et al., 2009; Lee et al., 2011). Also, it is reported that uridine and adenosine are taken up via ENTs in TR-TBT cells (Chishu et al., 2008; Sato et al., 2009). Therefore, TR-TBT cells were considered to be a suitable model for the analysis of paclitaxel transport activities in the BPB. In rat, the labyrinthine part of the placenta is the principal site of maternal-fetal exchange. The labyrinthine wall is composed of two syncytiotrophoblast layers, the maternal-side (SynI) and fetal-side syncytiotrophoblast layers (SynII) (Kitano et al., 2004). TR-TBT 18d-1 and 18d-2 have the histological characteristics of syncytiotrophoblast I and II respectively (Kitano et al., 2004). According to these reason, we investigated paclitaxel transport using both cell lines in this study.

It has been reported that paclitaxel is a substrate of P-gp (Murray et al., 2012). Indeed, the P-gp plays a critical role in protecting the organism against xenobiotics, decreasing their tissue accumulation in an ATP-dependent manner (Fromm, 2004). In our results, mRNA of P-gp, mdr1a and mdr1b was expressed in TR-TBT cells and rat placenta (positive control) (Fig. 4). [3H]Paclitaxel uptake was time dependency and inhibited unlabeled paclitaxel (Fig. 1). Therefore, it is suggested that paclitaxel can be delivered by specific transporter from TR-TBT cell lines. Also, about 50% of the amount of intracellular [3H]paclitaxel was decreased in a time dependent manner up to 30 min in the TR-TBT cells (Fig. 2). [3H]Paclitaxel efflux was also significantly inhibited by the addition 500 μM unlabeled paclitaxel (Fig. 2). Verapamil and cyclosporine are well-known inhibitors of P-gp transport system(s) (Staud et al., 2012), respectively, they had significant inhibitory effect, indicating that P-gp is involved in paclitaxel uptake by TR-TBT cells. Breast cancer resistance protein (BCRP) transports a variety of drugs including mitoxantrone and cefmetazole (Mao, 2008), however, these drugs had no effect on paclitaxel transport in TR-TBT cells. [3H]Paclitaxel uptake was not significantly inhibited by probenecid and cefmetazole, substrates of MRP4. Novobiocin, a substrate of organic anion transporter (OAT) did not significantly inhibited the uptake of [3H]paclitaxel. And several amino acids, adenosine and glycine did not inhibited [3H]paclitaxel uptake (Table 1). Kinetic analysis revealed that paclitaxel uptake by TR-TBT cells was saturable (Fig. 3). The K_{m} value for paclitaxel uptake in TR-TBT 18d-1 and 18d-2 were approximately 168 μM and 371 μM, respectively (Fig. 3). Taken together, P-gp might play an important role in paclitaxel transport across the BPB from maternal plasma. Indeed, it has been reported that intravenous administration of paclitaxel to pregnant dams revealed a 16-fold higher trans-placental transfer rate in mdr1a/1b (-/-) knockout mice than in wild-type mice (Smit et al., 1999). In a recent report, paclitaxel could not be detected in fetal plasma at ninety minutes after IV injection in pregnant mice (Van Calsteren et al., 2011). Therefore, we hypothesize that the placental P-gp reduces the trans-placental transfer of paclitaxel, making their clinical use possible during the 2nd and 3rd trimesters of pregnancy. So far, 25 cases have been described using paclitaxel during pregnancy (Mir et al., 2010) and no malformation was reported. Also, data concerning maternal hematologic toxicity were not available in most cases (Mir et al., 2010). TR-TBT cells were originated from rat. It has been reported that transport functions in the BPB are similar in human and rat placenta (Takata and Hirano, 1997). In our results, mRNA of P-gp, was expressed in TR-TBT cells, rat and human placenta (Fig. 4). Therefore, we expected to exist similar mechanisms of paclitaxel uptake by TR-TBT cells was measured in the absence (control) or presence of compounds for 5 min at 37°C. Each value represents the mean ± S.E.M. (n=4). *p<0.05, **p<0.01, ***p<0.001; significantly different from control.

Table 1. Effect of several transporter inhibitors on [3H]paclitaxel uptake in TR-TBT cells

| Substrate          | Concentration (mM) | TR-TBT 18d-1 | TR-TBT 18d-2 |
|--------------------|--------------------|--------------|--------------|
| Control            |                    | 100.0 ± 6.0  | 100.0 ± 4.0  |
| Paclitaxel         | 0.5                | 23.8 ± 1.1***| 13.8 ± 1.8***|
| Cyclosporin        | 0.5                | 20.3 ± 1.0***| 54.4 ± 2.7***|
| Verapamil          | 1                  | 42.1 ± 1.8***| 22.7 ± 3.9***|
| Probenecid         | 1                  | 106.0 ± 8.0  | 108.0 ± 6.0  |
| Cefmetazole        | 1                  | 110.0 ± 6.0  | 101.0 ± 10.0 |
| Methotrexate       | 1                  | 75.2 ± 10.4* | 88.0 ± 10.3  |
| Mitoxantrone       | 1                  | 91.0 ± 6.9   | 89.6 ± 9.5   |
| Novobiocin         | 1                  | 93.6 ± 8.5   | 102.0 ± 13.0 |
| Adenosine          | 1                  | 92.9 ± 4.0   | 105.0 ± 6.0  |
| Glycine            | 1                  | 90.7 ± 5.2   | 85.3 ± 8.5   |

**Fig. 4.** Expression of mdr1a, mdr1b, MDR1 and GAPDH by RT-PCR in TR-TBT cells and placenta in rat and human. Total RNA (1 μg) was reverse-transcribed and cDNA (0.1 μg) was amplified by PCR. Products were electrophoresed on 5% acrylamide gel and visualized by ethidium bromide staining. Products of isolated cells were observed at the expected sizes. (+) and (-) represent the presence or absence of reverse transcriptase (RT), respectively.
taxel transport in human placenta.

In conclusion, P-gp expressed at the BPB is involved in the transport of paclitaxel. Our findings are important for therapy of breast and ovarian cancer of pregnant women, and should be useful data in elucidating teratogenicity of paclitaxel during pregnancy.

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