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Human placental transport of vinblastine, vincristine, digoxin and progesterone: contribution of *P*-glycoprotein

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Abstract

To elucidate the role of *P*-glycoprotein in human placenta, we examined its expression in placenta, and the transcellular transport and uptake of *P*-glycoprotein substrates in cultured human placental choriocarcinoma epithelial cells (BeWo cells). The uptake of [^{3}H]vinblastine and [^{3}H]vincristine into BeWo cells was increased in the presence of a metabolic inhibitor, sodium azide. The basolateral-to-apical transcellular transport of [^{3}H]vinblastine, [^{3}H]vincristine and [^{3}H]digoxin was greater than the apical-to-basolateral transcellular transport. In the presence of cyclosporin A, the basolateral-to-apical transcellular transport of [^{3}H]vinblastine, [^{3}H]vincristine and [^{3}H]digoxin was significantly increased, and the apical-to-basolateral transcellular transport was decreased. The uptake of [^{3}H]vinblastine, [^{3}H]vincristine and [^{3}H]digoxin into BeWo cells was significantly enhanced in the presence of several inhibitors, such as verapamil or mouse monoclonal antibody anti-*P*-glycoprotein MX-MDR (MRK16) as well as cyclosporin A. Although progesterone significantly enhanced the uptake of [^{3}H]vinblastine, [^{3}H]vincristine and [^{3}H]digoxin into BeWo cells, the uptake of [^{3}H]progesterone was not affected by these inhibitors. Immunoblot analysis revealed that *P*-glycoprotein with a molecular weight of 172 kDa was expressed in BeWo cells and isolated trophoblast cells. Furthermore, *P*-glycoprotein was detected in human placental brush-border membrane vesicles, but not in human placental basolateral membrane vesicles. In conclusion, these data suggest that *P*-glycoprotein is expressed on the brush-border membrane (maternal side) of human placental trophoblast cells. *P*-Glycoprotein is considered to regulate the transfer of several substances including vinblastine, vincristine and digoxin from mother to fetus, and to protect the fetus from toxic substances.

Keywords: *P*-Glycoprotein; Placenta; BeWo cells; Blood–placental barrier; Drug disposition

1. Introduction

Vinblastine and vincristine, antineoplastic vinca alkaloids, are commonly used for the treatment of breast cancer and Hodgkin's disease, malignancies that are often found in gravida. Their teratogenic potencies have been examined in a variety of animal models (Joneja and Ungthavorn, 1969; Courtney and Valerio, 1968; Ferm, 1963; Tanaki et al., 1967). In hamsters, intravenous injection of vinblastine (0.25 mg/kg) or vincristine (0.1 mg/kg) was reported to induce malformations such as microphthalmia, anophthalmia, exencephaly and spina bifida (Ferm, 1963). Similar results were reported in mice (Joneja and Ungthavorn, 1969), rats (Tanaki et al., 1967) and rhesus monkeys (Courtney and Valerio, 1968). In humans, two cases of vinca alkaloid-induced teratogenicity were reported after maternal treatment with vinblastine or vincristine (Thomas, 1976; Mannuti et al., 1975). Digoxin was also reported to be teratogenic (Johnny and Menachem, 1987). However,

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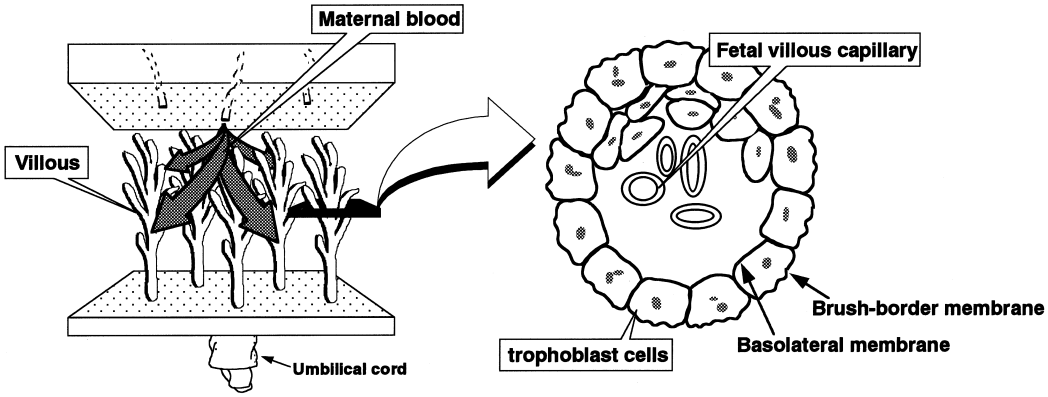

Fig. 1. Schematic diagram of the blood–placental barrier, which consists of trophoblast cells. The brush-border membrane of trophoblast cells face to the maternal blood.

the permeability and transport mechanisms of these drugs across the placenta remain to be investigated.

Throughout gestation, the placenta plays important roles in regulating the exchange of various materials between the maternal and the fetal circulations (Stulc, 1997). Many investigators have examined the transport mechanisms of nutrients, such as amino acids, vitamins and glucose, across the blood–placental barrier, which consists of trophoblast cells (placental microvillous membrane epithelial cells) shown in Fig. 1 (Hay, 1994). Several transport systems for amino acids, including the Na^+-dependent A or ASC system and the Na^+-independent L system, were reported to be present in trophoblasts (Furesz et al., 1993; Moe et al., 1994; Moe, 1995; Ramamoorthy et al., 1992). It was also reported that glucose transporter 1 (GLUT 1) was expressed both on the brush-border (maternal side) and the basal (fetal side) membranes, while glucose transporter 3 (GLUT 3) was expressed only on the brush-border membrane (Bissonnette, 1981; Reid and Boyd, 1994; Hahn and Desoye, 1996). Thiamine (vitamin B1) is transported in exchange for H^+ via a Na^+- and membrane potential-dependent transport system (Grassl, 1998). Biotin, lipoate and pantothenate are cotransported with Na^+ (Hu et al., 1994; Schenker et al., 1992; Prasad et al., 1998). However, little is known about the transport of drugs or xenobiotics across the blood–placental barrier, though, several drugs are known to cross the placenta and reach the developing fetus when administered during pregnancy. The fetal blood concentration of drugs is not always equal to the maternal blood concentration (Pacifici and Nottoli, 1995; van der Aa et al., 1998), suggesting that there are active transport systems for drugs in the placenta. In several tissues such as kidney, adrenal gland, vessels at blood–brain barrier sites, liver, intestine and testis, *P*-glycoprotein was found to be expressed and to extrude a range of hydrophobic natural products and drugs against a concentration gradient (Thiebaut et al., 1987; Tsuji et al., 1992; Cordon-Cardo et al., 1989; Sugawara et al., 1988, 1997). *P*-Glycoprotein is encoded by a multidrug resistance gene (MDR1), and can confer multidrug resistance by extruding a wide range of structurally unrelated, amphiphilic hydrophobic drugs from cells in an ATP-dependent manner (Juliano and Ling, 1976; Kartner et al., 1983). In recent studies, *P*-glycoprotein was shown to be expressed in the placenta (Sugawara et al., 1997; Nakamura et al., 1997). However, these reports were based on immunohistochemical techniques, and functional studies have not been carried out. Therefore, it is essential to investigate whether *P*-glycoprotein operates as a drug-efflux pump in the human placenta. Human placental choriocarcinoma epithelial cells (BeWo cells) are commonly used for studies of the blood–placental barrier, including transport mechanisms. BeWo cells have similar properties to normal trophoblasts in terms of morphology, biochemical markers and hormone secretion (Liu et al., 1997).

In the present study, we aimed to elucidate the role of *P*-glycoprotein in the placenta by means of immunoblotting studies and uptake and transcellular transport studies using BeWo cells. We employed vinblastine, vincristine, digoxin and progesterone as model drugs. Immunoblot analysis was carried out with cultured BeWo cells, isolated human placental trophoblast cells, human placental brush-border membrane vesicles and human placental basolateral membrane vesicles.

2. Methods

2.1. Materials and reagents

[^{3}H]Vinblastine sulphate (15.5 Ci/mmol) and [^{3}H]vincristine sulphate (5.70 Ci/mmol) were purchased from Amersham International (Buckinghamshire, UK). [^{3}H]Digoxin (19.0 Ci/mmol) and [1, 2-^{3}H]progesterone (52.0 Ci/mmol) were purchased from NEN Research Products (MA, USA). [1-^{14}C]D-mannitol (53 Ci/mmol) was purchased from Moravek Biochemicals (CA, USA). Trypsin was purchased from GIBCO BRL Life Technologies (Rockville, MD, USA). Collagenase Type I was purchased from Worthington Biochemical (Lakewood, NJ,

USA). DNase I was purchased from Sigma (St. Louis, MO, USA). Sodium azide and verapamil were purchased from Nacalai Tesque (Kyoto, Japan). Cyclosporin A was kindly supplied by Sandoz (Basel, Switzerland). Mouse monoclonal antibody anti-*P*-glycoprotein MX-MDR (MRK16) was purchased from Kyowa Medex (Tokyo, Japan). Rabbit anti-mouse immunogloblin G (IgG) was purchased from EY Laboratories (San Mateo, CA, USA). Mouse monoclonal antibody C219 was purchased from TFB (Tokyo, Japan). P170-Glycoprotein, multidrug-resistance-related clone JSB-1 was purchased from Monsan (Netherlands). Monoclonal anti-β-actin, mouse ascites fluid, was purchased from Sigma. Peroxidase-conjugated sheep affinity-purified antibody to mouse IgG was purchased from ICP Pharmaceuticals (Aurora, OH, USA). Horseradish peroxidase-conjugated rabbit anti-mouse IgG enhanced chemiluminescence (ECL) kit was purchased from Amersham (Oakville, ON, USA). All other chemicals used in the experiments were commercial products of reagent grade. BeWo cells were purchased from American Type Culture Collection (Rockville, MD, USA). KB/WT and KB/VJ-300 cells were kindly supplied by the Department of Biochemistry, Graduate School of Medical Sciences, Kyushu University.

2.2. Isolated trophoblast cells

Initial processing of human term placenta was performed essentially as described by Kliman et al. (1986). Normal term (about 42 weeks gestation) placentas were obtained immediately after uncomplicated caesarean section. Several cotyledons were removed from underlying fibrous elements and rinsed thoroughly in 0.9% NaCl at room temperature. The soft villous materials obtained from the maternal surface were cut away from connective tissue and vessels until approximately 100 g was collected. The tissue was coarsely minced, transferred to 100 ml of warmed Ca^{2+}- and Mg^{2+}-free Hank's solution (CMF Hank's) containing 25 mM HEPES, 0.125% trypsin, 0.3% collagenase and 0.2% DNase I, pH 7.4, and then incubated in a shaking water bath at 37°C for 30 min. The flask was set at an angle, and tissue fragments were allowed to settle for 1 min. The supernatant was layered over calf serum and centrifuged at $1000 \times g$ for 5 min at room temperature. The resultant pellets were resuspended in Dulbecco's modified Eagle's medium containing 25 mM HEPES and 25 mM glucose (DMEM-H-G) at room temperature. The suspension was layered over a preformed Percoll gradient made up in Hank's solution. The gradient was made from 70%, 60%, 40%, 20% and 5% Percoll (v/v) in steps of 3 ml each by dilution of 90% Percoll (9 parts Percoll:1 part 10× Hank's) with CMF Hank's solution, layered in a 50-ml conical polystyrene centrifuge tube. The gradient was centrifuged at $1200 \times g$ at room temperature for 30 min. After centrifugation, three regions were identified, and the middle layer was removed and resuspended in DMEM-H-G for tissue culture.

2.3. Preparation of human placental brush-border membrane vesicles

The procedure was a modification of that described by Smith et al. (1977). Human term placentas from uncomplicated pregnancies were obtained within 15 min after vaginal or caesarean delivery and placed in 0.9% NaCl at 4°C. After removal of the cord, amniochorion, and decidua, placental tissue was cut from the maternal side and washed in 300 mM mannitol, 10 mM HEPES–Tris at pH 7.4 (MHT buffer). The mince was stirred for 1 h to loosen the microvilli and filtered through two layers of woven cotton gauze. A sample of this starting mince was taken for enzyme analysis. The filtrate was centrifuged at $800 \times g$ for 10 min. The pellet was discarded and $MgCl_2$ was added to the supernatant to a final concentration of 10 mM. After 10 min, with occasional stirring, the supernatant was centrifuged at $10{,}500 \times g$ for 10 min. The pellet was discarded, and the supernatant was centrifuged at $20{,}000 \times g$ for 20 min. The pellet from this run was suspended in MHT buffer with 25-gauge syringe needle. All the subsequent procedures were performed at 4°C.

2.4. Preparation of human placental basolateral membrane vesicles

Human placental basolateral membrane vesicles were prepared by the method previously described by Kelley et al. (1983) with minor modifications. Human term placentas from uncomplicated pregnancies were obtained within 15 min after vaginal or caesarean delivery and placed in 0.9% NaCl at 4°C. After removal of the cord, amniochorion, and decidua, placental tissue was cut from the maternal side and washed in phosphate buffered saline (PBS) (−). Tissue was stirred in PBS (−) for 30 min and collected on a nylon mesh. The filtrate was washed three times with cold 50 mM Tris–HCl (pH 7.4), collected on a 250-μm pore size nylon mesh and divided into several equal portions. Each portion was sonicated in 100 ml of the same Tris buffer using a 3/4-in. high-gain probe for 10 s at 240 W (Vibra-cell, Sonics and Materials, CT, USA). The suspensions were kept on ice. The sonication procedure selectively removes any remaining brush-border membrane. Sonicated tissue was collected on the mesh, washed three times with 5 mM Tris–HCl (pH 7.4) and then stirred gently for 60 min in the same buffer. Tissue was then collected on the nylon mesh and washed again in the same buffer. This procedure disrupts and removes the intracellular components, thus exposing the basolateral membrane. Tissue portions of 25–30 g were resuspended in about 100 ml of 50 mM Tris–HCl (pH 7.4) containing 10 mM EDTA and 250 mM sucrose and incubated for 30

min with occasional stirring. Portions were then sonicated twice for 20 s at 250 W to release the basolateral membrane. Suspensions were strained through nylon mesh and the supernatant was centrifuged at $3300 \times g$ for 10 min to remove debris. The supernatant from this spin was recentrifuged at $80{,}000 \times g$ for 40 min to yield the basolateral membrane pellet, which was resuspended, using a Dounce homogenizer, in 25 mM HEPES–Tris (pH 7.4) containing 1 mM EDTA and 275 mM sucrose. This fraction was further purified by centrifugation on a discontinuous gradient of 10% (w/v) Ficoll (Pharmacia) in the resuspension buffer overlaid with 4% Ficoll (as described by Kelley et al., 1983) prepared in 25 mM HEPES–Tris (pH 7.4) containing 1 mM EDTA and 275 mM sucrose. Ficoll gradient tubes were spun at $90{,}000 \times g$ for 1 h. The material at the density gradient interfaces was collected, washed and resuspended in 25 mM HEPES–Tris (pH 7.4) containing 275 mM sucrose. The suspension from this run was resuspended in the same buffer with a 25-gauge syringe needle. All the operations were carried out at 4°C.

2.5. Enzymatic analysis

Purity was evaluated by assaying the specific activity in human placental brush-border membrane vesicles or human placental basolateral membrane vesicles relative to that in the homogenate. Alkaline phosphatase and γ-glutamyl transpeptidase activities or dihydroalprenolol binding activity were selected as markers enzyme of microvillous membrane (Hulstaert et al., 1973; Sawabu et al., 1980) or basal membrane (Williams et al., 1976), respectively. Protein was quantitated by the method of Lowry et al. (1951).

2.6. Cell culture

BeWo cells were cultured in F12K modification of Ham's medium (Sigma) supplemented with 10% fetal calf serum, 50 mg/ml streptomycin and 50 U/ml penicillin G under conditions of 37°C and 5% CO_2/95% air (Pattilo et al., 1986). For the uptake or transport study, the cells were seeded at 4×10^4 cells/well on 96-well multidishes (Nunc, Denmark) or 1×10^7 cells/well on polycarbonate membrane (3.0 μm pore size) Transwell™ cluster (Corning Coster Japan, Japan). The cells were grown for 4 or 12 days, and used for the uptake or transcellular transport study. In the transport study, we used BeWo cells after monitoring membrane resistance across the BeWo monolayer, and examining the transport of mannitol as a paracellular transport marker. KB/WT and KB/VJ-300 cells were kindly supplied by the Department of Biochemistry, Graduate School of Medical Sciences, Kyushu University. KB/WT and KB/VJ-300 cells were cultured in MEM medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% newborn calf serum, 0.04 mg/ml kanamycin and 100 U/ml penicillin G under the conditions of 37°C and 5% CO_2/95% air (Kohno et al., 1988).

2.7. Detection of P-glycoprotein

P-Glycoprotein was detected by Western blot analysis. Sample cells, KB/VJ-300 (positive control) and KB/WT (negative control) were centrifuged, and suspended in the lysis buffer containing 100 mM Tris HCl (pH 7.6), 150 mM NaCl, 1 mM $CaCl_2$, 1% Triton, 0.1% sodium dodecyl sulfate (SDS), 0.1% Np-40, 1 mM phenylmethyl sulfonyl fluoride, 0.01 mg/ml leupeptin, 0.01 mg/ml aprotinin and 1 mM sodium vanadate, and incubated for 30–45 min at 4°C. After incubation, the suspension was centrifuged at $15{,}000 \times g$ for 15 min at 4°C. SDS-polyacrylamide gel electrophoresis (PAGE) was performed according to the method of Laemmli (1970). The proteins were transferred electrophoretically onto a 0.2 μm pore size Clear Blot Membrane-P (Atto, Tokyo, Japan). Blots were blocked overnight at 4°C with 5% nonfat powdered milk in PBS (−). The membranes were washed three times with PBS (−)-Triton, and incubated with C219 or JSB-1 for 1 h at 37°C. The antibody was diluted in PBS (−) containing 1% nonfat powdered milk. Horseradish peroxidase-conjugated rabbit anti-mouse IgG was used as the secondary antibody. Detection was done with ECL reagents according to the manufacturer's instructions.

2.8. Transcellular transport experiments by BeWo cell monolayer

After reaching confluency, BeWo cells were washed three times with transport buffer (140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM D-glucose and 25 mM HEPES for pH 7.4; the pH was adjusted with NaOH at 37°C) before the transcellular transport study. Transport buffer was put into both the receiver (1.5 ml) and donor (0.5 ml) sides. Then 30 nM [^{3}H]vinblastine, 50 nM [^{3}H]vincristine, 50 nM [^{3}H]digoxin, 10 nM [^{3}H]progesterone or 800 nM [^{14}C]mannitol, a paracellular transport marker, was loaded into the donor or receiver side of the cell insert. At the designated time, 0.5 ml samples of the basolateral and 0.2 ml samples of the apical side solutions were sampled and subsequently equal volumes of transport buffer were used as replacements. To assay the radiolabeled compounds, all samples were transferred into counting vials, mixed with 4 ml of scintillation fluid (Clear-sol I, Nacalai Tesque) and put in a liquid scintillation counter (LS6500, Beckman Instruments, Fullerton, CA, USA). The amount of protein in the cells was measured by Lowry's method (Lowry et al., 1951). The real permeability coefficient (P_{trans}) of all compounds was calculated by use of the following equation:

$$1/P_{app} = 1/P_{filter} + 1/P_{trans}$$

where P_{app} and P_{filter} are the apparent permeability coefficients estimated by transport study in the presence and absence of BeWo cells, respectively.

2.9. Cellular uptake experiments by BeWo cells

The culture medium was removed and washed three times with 100 μl of incubation buffer (140 mM NaCl, 5.4 mM KCl, 1.8 mM CaC_{l2}, 0.8 mM $MgSO_4$, 5 mM D-glucose and 25 mM HEPES, pH 7.4) at 37°C or 4°C. Uptake experiments were performed in the incubation buffer containing [^{3}H]vinblastine, [^{3}H]vincristine, [^{3}H]digoxin or [^{3}H]progesterone in the presence of an inhibitor at 37°C or 4°C. In the metabolic inhibition experiments, cells were preincubated with the incubation buffer containing 10 mM sodium azide for 10 min at 37°C. The cells were washed four times with 100 μl of ice-cold buffer to stop the uptake. Cells were dissolved in 3 N NaOH (200 μl), then neutralized with 6 N HCl (100 μl) and scintillation cocktail (Clear-sol I, Nacalai Tesque) was added. The radioactivity of intracellular [^{3}H]vinblastine, [^{3}H]vincristine, [^{3}H]digoxin or [^{3}H]progesterone was then determined with a liquid scintillation counter (LS6500, Beckman Instruments). The amount of protein in the cells was measured by Lowry's method (Lowry et al., 1951). The uptake of [^{3}H]vinblastine, [^{3}H]vincristine, [^{3}H]digoxin or [^{3}H]progesterone was expressed as the ratio of uptake amount per mg protein of the cells relative to the drug concentration (μl/mg protein).

2.10. Data analysis

All of the data are expressed as mean ± S.E.M. Statistical analysis was performed by using Student's *t*–test or analysis of variance (ANOVA) followed by Duncan's test. The differences between means were considered to be significant when the P values were less than 0.05.

3. Results

3.1. Purity of human placental brush-border membrane vesicles and human placental basolateral membrane vesicles

The purity of human placental brush-border membrane vesicles or human placental basolateral membrane vesicles was confirmed by examining the enzyme activities of alkaline phosphatase and γ-glutamyl transpeptidase or dihydroalprenolol binding activity, respectively. In the case of human placental brush-border membrane vesicles, the activities of alkaline phosphatase and γ-glutamyl transpeptidase for the vesicles and homogenate were 7.14 ± 0.03 and 0.41 ± 0.01 (pmol/mg protein/min) and 26.79 ± 0.43 and 1.30 ± 0.21 (IU/mg protein), respectively. In the case of human placental basolateral membrane vesicles, the dihydroalprenolol binding activities of the vesicles and homogenate were 1.49 ± 0.03 and 0.047 ± 0.01 (pmol/mg protein), respectively. These data indicate that the two types of membrane vesicles were purified approximately 20-fold from the whole villous tissue. The dihydroalprenolol binding activity of human placental brush-border membrane vesicles (homogenate) and the activity of alkaline phosphatase for human placental basolateral membrane vesicles (homogenate) were 0.041 ± 0.003 (0.086 ± 0.0004 pmol/mg protein/min) and 1.68 ± 0.04 (0.565 ± 0.07 pmol/mg protein/min), respectively.

3.2. Effect of metabolic inhibitor on [^{3}H]vinblastine or [^{3}H]vincristine uptake by BeWo cells

In the presence of 10 mM sodium azide, a metabolic inhibitor, initial uptake rate of [^{3}H]vinblastine or [^{3}H]vincristine by BeWo cells was significantly enhanced to 6.54 or 2.57 μl/mg protein/min in comparison with the control values of 4.84 or 1.66 μl/mg protein/min, respectively (Fig. 2A,B).

3.3. Immunodetection of P-glycoprotein

Western blot analysis was employed to detect *P*-glycoprotein in BeWo cells, isolated trophoblast cells, human placental brush-border membrane vesicles and human placental basolateral membrane vesicles. Using the monoclonal antibody C219 or JSB-1, *P*-glycoprotein of 172 kDa

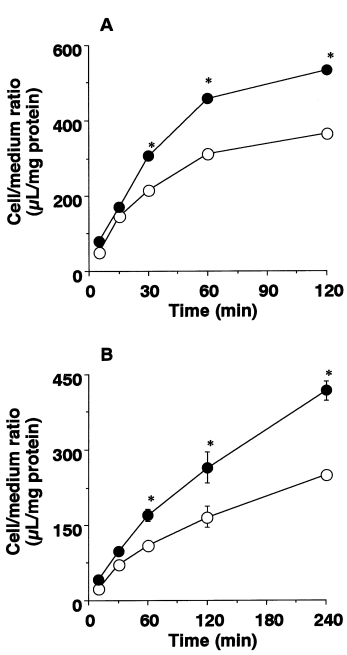

Fig. 2. The uptake of 10 nM [^{3}H]vinblastine (A) or 50 nM [^{3}H]vincristine (B) by BeWo cells in the presence (closed circles) or absence (open circles) of 10 mM sodium azide as a metabolic inhibitor. Each point represents the mean ± S.E.M. of four experiments. Significant differences from the control were determined by Student's *t*-test ($^*P < 0.05$).

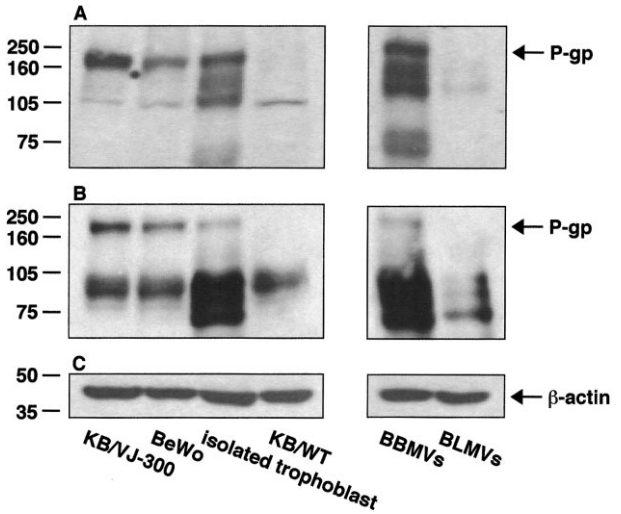

Fig. 3. Immunodetection of *P*-glycoprotein. Protein samples, KB/VJ-300 10 μg, BeWo 50 μg, isolated trophoblasts 50 μg, KB/WT 10 μg, human placental brush-border membrane vesicles 50 μg and human placental basolateral membrane vesicles 50 μg, were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) with a 7.5% polyacrylamide gel and transferred onto Clear Blot Membrane-P (Atto). Immunoblots were performed with MAb C219 (A), JSB-1 (B) or a β-actin Mab (C) and developed with the ECL detection reagent, as described in Section 2.

was detected in BeWo cells, isolated trophoblast cells and human placental brush-border membrane vesicles as well as KB/VJ-300 cells (positive control), but not in human placental basolateral membrane vesicles or KB/WT cells (negative control) (Fig. 3). β-Actin of 41 kDa was also detected in all samples.

3.4. Effect of cyclosporin A on the transcellular transport of [^{3}H]vinblastine, [^{3}H]vincristine, [^{3}H]digoxin or [^{3}H]progesterone across the BeWo monolayer

The basolateral-to-apical transport of [^{3}H]vinblastine, [^{3}H]vincristine or [^{3}H]digoxin was significantly greater than transport in the opposite direction (Fig. 4A). The values of apical-to-basolateral permeability coefficient of [^{3}H]vinblastine, [^{3}H]vincristine and [^{3}H]digoxin were 0.218 ± 0.046, 0.542 ± 0.096 and 0.107 ± 0.035, while those of the basolateral-to-apical transport were 1.34 ± 0.229, 2.01 ± 0.295 and 0.537 ± 0.087 (μl min/mg protein/min), respectively (Fig. 4B). In the presence of 20 μM cyclosporin A, the apical-to-basolateral transport of [^{3}H]vinblastine was increased, and the basolateral-to-apical transport of [^{3}H]vinblastine, [^{3}H]vincristine or [^{3}H]digoxin was significantly reduced (Fig. 4A). The values of apical-to-basolateral permeability coefficient of [^{3}H]vinblastine, [^{3}H]vincristine and [^{3}H]digoxin were 0.625 ± 0.032, 0.779 ± 0.062 and 0.122 ± 0.019, while those of the basolateral-to-apical transport were 0.678 ± 0.105, 0.660 ± 0.078 and 0.274 ± 0.034 (μl/min mg protein/min) in the presence of cyclosporin A, respectively. [^{3}H]Progesterone was not transported directionally in the presence or absence of cyclosporin A. The transport of [^{14}C]mannitol was negligible compared with the transport of all test drugs (permeability coefficient of 0.0625 ± 0.002, 0.0834 ± 0.004 and 0.0452 ± 0.004 μl/min mg protein/min), and was not affected by cyclosporin A (permeability coefficient of 0.0678 ± 0.002, 0.085 ± 0.007 and 0.0544 ± 0.002

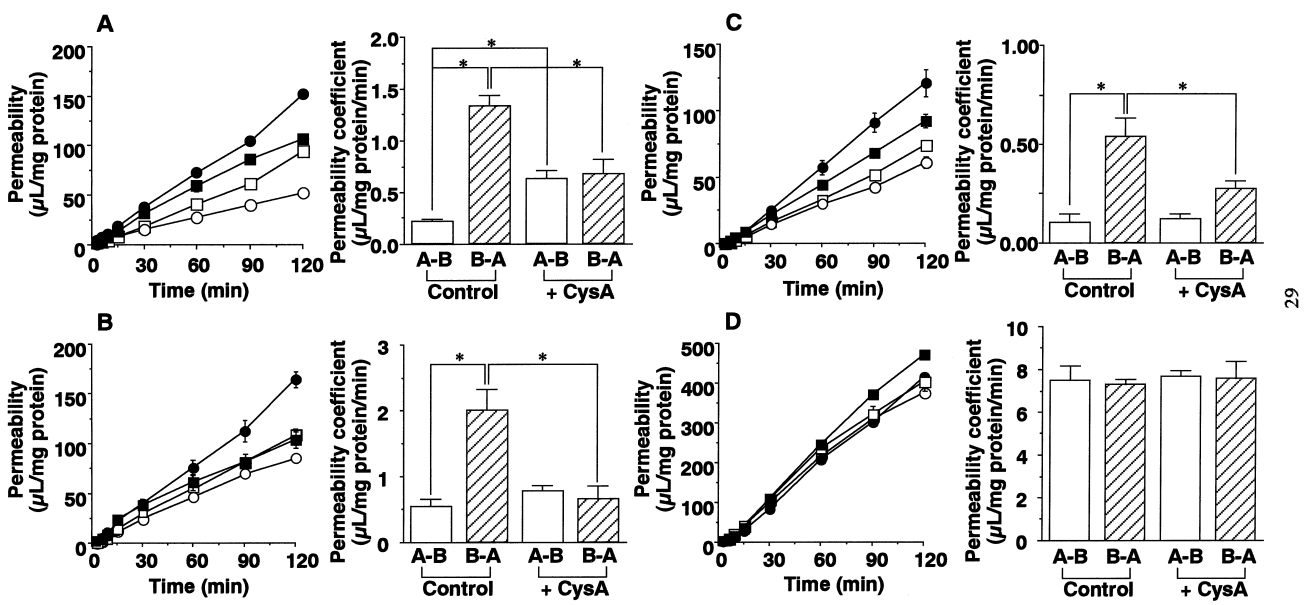

Fig. 4. Effects of 20 μM cyclosporin A on the transcellular transport of 50 nM [^{3}H]vinblastine (A), [^{3}H]vincristine (B), [^{3}H]digoxin (C) or [^{3}H]progesterone (D) across the BeWo monolayer. The apical-to-basolateral (open symbols) and basolateral-to-apical (closed symbols) transport of each drug was measured in the presence (squares) or absence (circles) of cyclosporin A. Permeability coefficients for the apical-to-basolateral (open columns) and basolateral-to-apical (hatched columns) transport of each drug in the presence or absence of cyclosporin A are also shown. A–B or B–A represents the maternal-to-fetal or fetal-to-maternal flux, respectively. Each point represents the mean ± S.E.M. of three experiments. Significant differences from the control were determined by ANOVA followed by Duncan's test ($^*P < 0.05$).

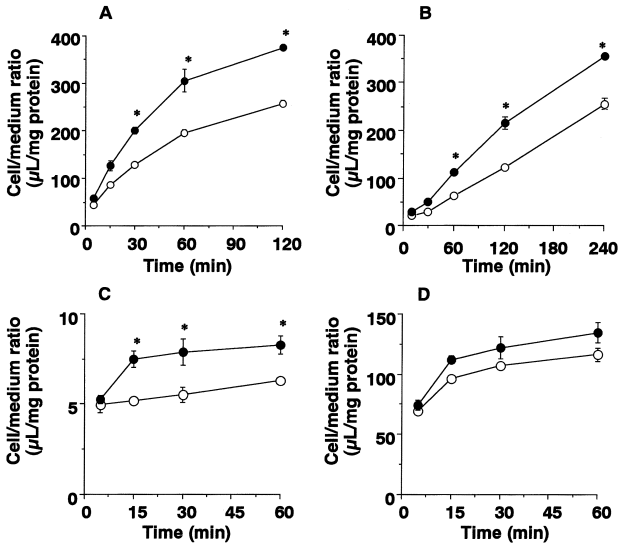

Fig. 5. Effect of cyclosporin A on the uptake of *P*-glycoprotein substrates. The uptake of 10 nM [^{3}H]vinblastine (A), 50 nM [^{3}H]vincristine (B), 50 nM [^{3}H]digoxin (C) or 10 nM [^{3}H]progesterone (D) by BeWo cells in the presence (closed circles) and absence (open circles) of 20 μM cyclosporin A is shown. Each point represents the mean ± S.E.M. of four experiments. Significant differences from the control were determined by Student's *t*-test ($^*P < 0.05$).

μl/min mg protein/min), suggesting that leakage through the cell monolayer is negligible during the cyclosporin A treatment.

3.5. Effect of cyclosporin A on the uptake of [^{3}H]vinblastine, [^{3}H]vincristine, [^{3}H]digoxin or [^{3}H]progesterone by BeWo cells

We examined the effect of 20 μM cyclosporin A on the uptake of [^{3}H]vinblastine, [^{3}H]vincristine, [^{3}H]digoxin or [^{3}H]progesterone by BeWo cells. The uptake levels of [^{3}H]vinblastine, [^{3}H]vincristine or [^{3}H]digoxin was significantly enhanced in the presence of cyclosporin A, while that of [^{3}H]progesterone was not changed (Fig. 5).

3.6. Effects of verapamil, progesterone or MRK16 on the uptake of [^{3}H]vinblastine, [^{3}H]vincristine, [^{3}H]digoxin or [^{3}H]progesterone by BeWo cells

The effects of several *P*-glycoprotein inhibitors were examined. The uptake levels of [^{3}H]vinblastine, [^{3}H]vincristine or [^{3}H]digoxin were enhanced in the presence of 20 μM verapamil or 200 μM progesterone, and also

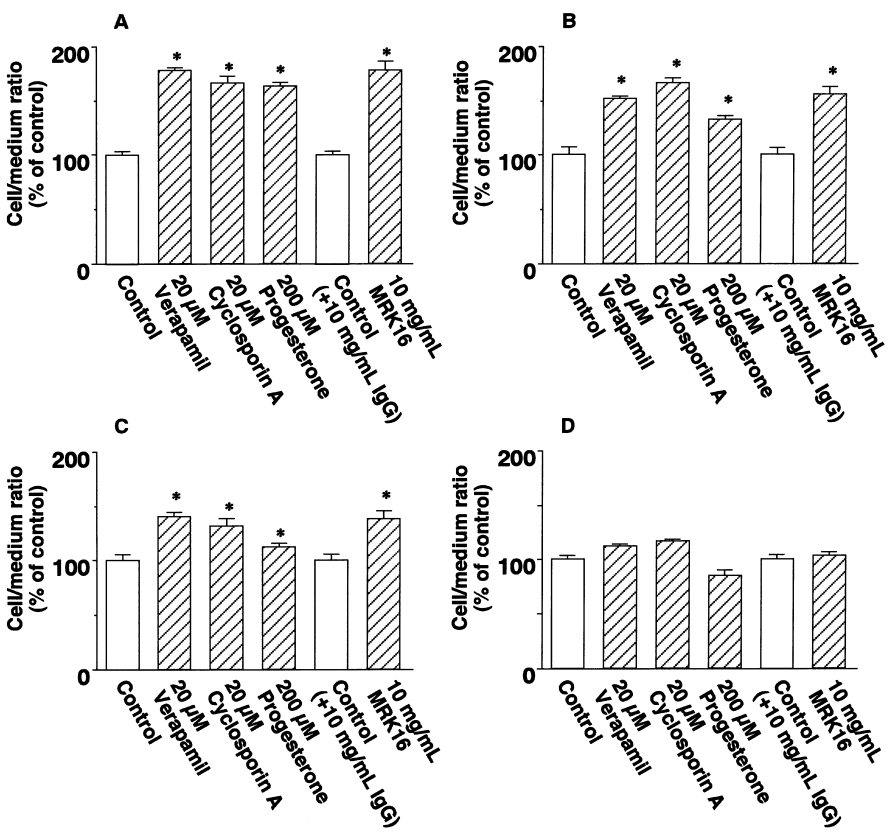

Fig. 6. Effects of several inhibitors on the uptake of 10 nM [^{3}H]vinblastine (A), 50 nM [^{3}H]vincristine (B), 50 nM [^{3}H]digoxin (C) or 10 nM [^{3}H]progesterone (D) by BeWo cells in the presence (hatched columns) and absence (open columns) of several inhibitors (20 μM verapamil, 20 μM cyclosporin A, 200 μM progesterone and 10 μg/ml MRK16). Each point represents the mean ± S.E.M. of four experiments. Significant differences from the control were determined by ANOVA followed by Duncan's test ($^*P < 0.05$).

increased in the presence of 10 μg/ml MRK16 (monoclonal antibody to *P*-glycoprotein), as well as 20 μM cyclosporin A. The uptake of [^{3}H]progesterone was not affected by these inhibitors (Fig. 6).

4. Discussion

P-Glycoprotein can confer multidrug resistance by extruding a wide range of structurally unrelated, amphiphilic hydrophobic drugs from cells in an ATP-dependent manner (Juliano and Ling, 1976; Kartner et al., 1983). In recent studies, *P*-glycoprotein was reported to be expressed in trophoblast cells but not endothelial cells of the placenta (Sugawara et al., 1997; Cordon-Cardo et al., 1989; Nakamura et al., 1997). Therefore, *P*-glycoprotein in the trophoblast cells is likely to be involved in the blood–placental barrier function. In this study, immunoblot analysis showed 172 kDa signals in BeWo cells and isolated human placental trophoblast cells (Fig. 3). The same signal was detected in KB/VJ-300 cells, but not in KB/WT cells. KB/VJ-300 cells are a vincristine-resistant human cancer KB cell line with increased expression of multidrug-resistance gene (Kohno et al., 1988). Anti *P*-glycoprotein monoclonal antibodies, C219 and JSB-1, have been produced for characterization and analysis of *P*-glycoprotein (Kartner et al., 1985; Schepter et al., 1988), and it was reported that *P*-glycoprotein has a molecular size of about 170 kDa (Gottesman et al., 1996). Overall, the results suggest that *P*-glycoprotein is expressed in the trophoblast cells of placenta.

Moreover, it was detected in human placental brush-border membrane vesicles, but not in human placental basolateral membrane vesicles (Fig. 3). Since the alkaline phosphatase and γ-glutamyl transpeptidase enzymatic activities of our placental brush-border membrane vesicles and the dihydroalprenolol binding activity of the placental basolateral membrane vesicles were enriched approximately 20-fold, in agreement with previous reports (Smith et al., 1977; Sawabu et al., 1980; Kelley et al., 1983), the two kinds of vesicles appear to have been purified to comparable extents. β-Actin (40 kDa) was detected in all samples. Therefore, we conclude that *P*-glycoprotein is localized on the brush-border (maternal side) membrane of the trophoblast cells, but not on the basolateral membrane.

BeWo cells were established by Pattilo et al. (1986) in order to evaluate the function of placental trophoblast cells. Morphologically, they are cytotrophoblastic, not syncytial. In this study, placental brush-border membrane vesicles and basolateral membrane vesicles were prepared from full-term human placenta, so that both membranes were almost syncytiotrophoblastic. However, considering that fetal toxicity may depend on the period of exposure to toxic compounds, transfer of materials from mother to fetus during the first trimester may be most important. Therefore, our use of BeWo cells should be relevant to the in vivo situation. It is noteworthy that *P*-glycoprotein was detected in BeWo cells, isolated trophoblast cells and human placental brush-border membrane vesicles, suggesting that it is expressed at all stages of gravida.

No study has previously been conducted to investigate the functional contribution of *P*-glycoprotein to transport across trophoblast cells. Vinblastine, vincristine (Hunter et al., 1993; Wils et al., 1994) and digoxin (Tanigawara et al., 1992) are well-known substrates of *P*-glycoprotein. In this study, the basolateral-to-apical (fetal-to-maternal) transport of [^{3}H]vinblastine, [^{3}H]vincristine and [^{3}H]digoxin across the BeWo monolayer was shown to be greater than transport in the opposite direction (Fig. 4). It was reported that an MDR1 transfectant cell line (LLC-GA5-COL150) exhibited markedly greater basolateral-to-apical transport and less apical-to-basolateral transport of *P*-glycoprotein substrates than the host cell line (LLC-PK1) (Tanigawara et al., 1992). This increase was suppressed in the presence of 20 μM cyclosporin A, concomitantly with an increase in transport in the opposite direction. Cyclosporin A was reported to inhibit multidrug-resistance (Twentyman, 1988). Therefore, it appears that *P*-glycoprotein acts to extrude these drugs from the fetal to the maternal circulation. Since the uptake levels of [^{3}H]vinblastine and [^{3}H]vincristine by BeWo cells were increased in the presence of a metabolic inhibitor (Fig. 2), the transporter that excludes these drugs in BeWo cells, is ATP-dependent.

The uptake levels of [^{3}H]vinblastine, [^{3}H]vincristine or [^{3}H]digoxin were significantly enhanced in the presence of 20 μM cyclosporin A (Fig. 5). Furthermore, the uptake of [^{3}H]vinblastine, [^{3}H]vincristine and [^{3}H]digoxin was also significantly increased in the presence of various *P*-glycoprotein inhibitors, i.e., 20 μM verapamil, 200 μM progesterone or 10 μg/ml anti-*P*-glycoprotein mouse monoclonal antibody MRK16, as well as cyclosporin A (Fig. 6). Verapamil competitively inhibits the transport of antitumor drugs by *P*-glycoprotein (Tsuruo et al., 1981). MRK16 was reported to recognize the extracellular epitope of human *P*-glycoprotein and to increase intracellular accumulation of vincristine and actinomycin D (Hamada and Tsuruo, 1986). Therefore, the increase of the uptake of [^{3}H]vinblastine, [^{3}H]vincristine and [^{3}H]digoxin caused by these *P*-glycoprotein inhibitors suggests that they inhibited efflux of these drugs from BeWo cells. It has been reported that digoxin has a low fetal transfer ratio (fetal/maternal ratio $= 0.36 \pm 0.04$) (Derewlany et al., 1991), suggesting that transfer to the fetus from the mother is regulated by *P*-glycoprotein. In contrast, the uptake of [^{3}H]progesterone in the steady-state was not affected by these inhibitors. In the presence of progesterone itself, the steady-state uptake of [^{3}H]progesterone was slightly reduced. These results are consistent with previous findings that progesterone is a potent inhibitor of *P*-glycoprotein, but is not transported by *P*-glycoprotein (Ueda et al., 1992; Barnes et al., 1996). We found, however, that both uptake

into and efflux from BeWo cells of progesterone were saturated in a concentration-dependent manner (data not shown). Further experiments are needed to identify the transport mechanism of progesterone in the placenta.

In conclusion, the immunoblot analysis revealed that *P*-glycoprotein, with a molecular weight of 172 kDa, is expressed in BeWo cells and localized at the brush-border membrane of placental trophoblast cells. The flux of [^{3}H]vinblastine, [^{3}H]vincristine or [^{3}H]digoxin across a BeWo monolayer in the fetal-to-maternal direction was decreased in the presence of 20 μM cyclosporin A. Furthermore, several inhibitors of *P*-glycoprotein significantly suppressed the efflux of *P*-glycoprotein substrates from BeWo cells. These data suggest that *P*-glycoprotein is expressed on the brush-border membrane (maternal side) of placental trophoblast cells and functionally regulates the transfer of xenobiotics into the fetus from the mother, thereby protecting the developing fetus from toxic factors in the maternal circulation.

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