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Restricted transport of cyclosporin A across the blood–brain barrier by a multidrug transporter, P-glycoprotein

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Abstract—The blood–brain barrier permeability of cyclosporin A (CsA), an immunosuppressive cyclic peptide, is restricted despite its highly lipophilic nature. In this study, the uptake of CsA by primary cultured bovine brain capillary endothelial cells (BCEC) was investigated in order to clarify whether P-glycoprotein (P-gp), an ATP-dependent drug efflux pump expressed in the luminal surface of BCEC, causes the decreased transport of CsA into the brain. P-gp, having a molecular mass of 130–140 kDa, was detected with anti-P-gp monoclonal antibody, C219, using western blot analysis of the membrane fraction isolated from the bovine brain capillary. The uptake of CsA by primary cultured bovine BCEC was time-dependent, and the steady-state uptake of CsA was increased in the presence of several multidrug resistance reversing agents including verapamil and steroid hormones and the substrate of P-gp in BCEC, vincristine. The steady-stage uptake was increased significantly to approximately 4-fold when cellular ATP was depleted by treating with 2,4-dinitrophenol, suggesting that the efflux process is ATP dependent. Furthermore, in the presence of an anti-P-gp monoclonal antibody, MRK16, at a 10 μg/mL concentration, the uptake of CsA was increased approximately 3-fold. These results suggest that the low permeability of CsA into the brain is caused by the active efflux from BCEC by P-gp present in the luminal surface of cells.

Cyclosporin A (CsA*), a cyclic undecapeptide of fungal origin, has a powerful immunosuppressive effect in the mammal due to its selective and reversible inhibition of T-lymphocytic action [1, 2]. Its cyclic structure with an internal hydrogen-bond results in a highly lipid-soluble character with the logarithm of the partition coefficient being 3.0 in octanol/buffer (pH 7.4) [3]. Therefore, it is expected that this peptide traverses the blood–brain barrier (BBB) freely in a manner similar to that of other highly lipid-soluble substances [4, 5]. However, while CsA accumulates in other tissues [6, 7], limited distribution of CsA into the brain has been reported. Several mechanisms have been proposed as the reasons for such an unusual BBB permeability of CsA: (i) that the lipid solubility of CsA transiently drops in the transmembrane transport process [3], (ii) that CsA is largely in the bound form with plasma proteins or with red blood cells [8], and (iii) that CsA is effectively trapped in the capillary endothelial cells of the brain [9].

Recently, P-glycoprotein (P-gp), which functions as an ATP-dependent pump that transports drugs out of multidrug-resistant (MDR) tumor cells, has been detected in brain capillary endothelial cells (BCEC) [10, 11]. Moreover, it has been reported that P-gp localizes in the luminal membrane of BCEC and transports vincristine (VCR), a *Vinca* alkaloid anticancer drug, and rhodamine 123 out of cells [12–14]. As observed in MDR tumor cells, the transport of VCR mediated by P-gp is metabolic-energy dependent and is inhibited by various MDR-reversing agents including verapamil and steroid hormones. The transfer of VCR from blood into the brain is limited despite its high lipid-solubility (log P = 2.8) [5]. The results described above led to the idea that P-gp in BCEC functions as a transport barrier against toxic substances that are highly lipid soluble, with the consequence of low permeation of VCR across the BBB. Since it has already been shown that CsA competitively inhibits *Vinca* alkaloid binding to P-gp and thereby reverses the MDR in tumor cells [15], it is likely that P-gp in BCEC transports CsA, which enters cells by passive diffusion, out of cells into the bloodstream, resulting in the restricted permeation of CsA across the BBB.

The purpose of the present study was to examine whether the reduced transport of CsA from the circulating blood into the brain is ascribed to P-gp function in BCEC. Primary cultured bovine BCEC were used in this study because they are highly viable, form a polarized monolayer of endothelial cells [16, 17], and express P-gp specifically in the luminal membrane [12].

Materials and Methods

Chemicals. [G-^{3}H]Vincristine sulfate ([^{3}H]VCR, 4.3 Ci/mmol) and [mebmt-β-^{3}H]cyclosporin A ([^{3}H]CsA, 8.8 Ci/mmol) were purchased from Amersham International plc (Buckinghamshire, U.K.), and [^{14}C(U)]sucrose (3.7 Ci/mol) was from New England Nuclear (Boston, MA). Unlabeled cyclosporin A was a gift from Sandoz AG (Basel, Switzerland). Horse serum was purchased from GIBCO (Grand Island, NY), rat tail collagen (type I) from Collaborative Research Inc. (Bedford, MA), human fibronectin from Boehringer Mannheim GmbH (Mannheim, F.R.G.), bovine serum albumin (Fraction V, BSA) from the Sigma Chemical Co. (St. Louis, MO), and Clear-sol I (xylene based liquid scintillation fluid) from Nacalai Tesque Inc. (Kyoto, Japan). All other chemicals were of reagent grade and commercially available.

Electrophoresis and immunoblot. Bovine brain capillaries were prepared by the method of Pardridge *et al.* [18]. Plasma membranes of brain capillary endothelial cells, human leukemic cells, K562, and its drug-resistant cells, K562/ADM, were prepared by the method of Naito *et al.* [19]. Cells were harvested, suspended in 10 mM Tris–HCl (pH 7.4), 10 mM NaCl, 1.5 mM $MgCl_2$ and 0.02 mM phenylmethylsulfonyl fluoride, and homogenized with a Potter–Elvehjem homogenizer. The cell homogenate was centrifuged, and the membrane fraction was resuspended in 10 mM Tris–HCl (pH 7.4) and 250 mM sucrose. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed by the method of Laemmli [20] in a 7.5% polyacrylamide gel. The separated proteins were transferred electrophoretically onto polyvinylidene difluoride membrane and then reacted with anti-P-gp monoclonal antibody C219 (100 ng/mL) as the primary antibody according to the avidin-biotin-peroxidase complex

* Abbreviations: BBB, blood–brain barrier; BCEC, brain capillary endothelial cells; BSA, bovine serum albumin; CsA cyclosporin A; DNP, 2,4-dinitrophenol; MDR, multidrug resistance; P-gp, P-glycoprotein; and VCR, vincristine.

(ABC) method [21]. Human leukemic cells, K562 and K562/ADM, were used as the negative and the positive control of P-glycoprotein, respectively.

Isolation and culture of BCEC. BCEC were isolated from cerebral gray matter of bovine brains as described previously [22]. The isolated BCEC were stored at −100° in the culture medium containing 20% horse serum and 10% dimethyl sulfoxide until used for cell culture. Isolated BCEC were seeded on dishes that were coated with rat tail collagen and human fibronectin and cultured at 37° with 95% air and 5% CO_2. Transport experiments were performed when cells reached confluence in 10–12 days. These primary cultured cells were identified to be capillary endothelial cells by the immunostaining method using Factor-VIII related antigen ([23]; data not shown).

Transport experiment. Uptake of ^3H- or ^{14}C-labeled compounds into primary cultured monolayers of BCEC was studied by a method reported previously [22]. Briefly, the cultured cells were washed three times with 1 mL of the incubation solution [141 mM NaCl, 4 mM KCl, 2.8 mM $CaCl_2$, 0.5 mM $MgSO_4$, 10 mM D-glucose, 10 mM HEPES, 0.1% BSA, pH 7.4, 300 mOsM] at 37°. Cultured BCEC were preincubated at 37° for 30 min in the incubation solution. Immediately after the preincubation, the solution was removed by suction, and the incubation solution (250 μL) containing [^3H]VCR (190 nM) or [^3H]CsA (90 nM) was added into each well to initiate transport. [^{14}C]Sucrose was used as the extracellular space marker. To terminate the transport reaction, cells were washed five times with 1 mL of ice-cold incubation solution at the designated time.

Analytical method. Cells in each well were solubilized by incubating them in 300 μL of 1 N NaOH at room temperature for 60 min. After neutralization with 60 μL of 5 N HCl, each solution was put into a counting vial. The radioactivity was measured by a liquid scintillation counter. Protein content in cultured cells was determined by the method of Lowry *et al.* [24] using BSA as a standard. Uptake, expressed as the cell-to-medium concentration ratio (μL/mg protein), was obtained by dividing an apparent uptake amount per milligram of protein by the substrate concentration in the incubation medium and by correcting an extracellularly adsorbed amount of substrates estimated from the apparent uptake of [^{14}C]sucrose.

Results and Discussion

The expression of P-gp in the membrane fraction of brain capillary was determined with anti-P-gp monoclonal antibody C219 using the ABC method. As shown in Fig. 1, the protein that reacted with C219 had a molecular mass of about 130–140 kDa in the case of plasma membrane fraction from bovine brain capillary. The band of about 170 kDa was stained in the membrane of K562/ADM representing MDR tumor cells, but no specific staining was observed in the membrane of K562, the drug-sensitive parental cells. The size of P-gp in bovine brain capillary is relatively smaller than that of MDR cells; however, the size is comparable with that of bovine adrenal gland [25]. Therefore, the present result, together with our previous observations that fresh bovine brain tissue and primary cultured BCEC are immunohistochemically stained by using two different anti-P-gp monoclonal antibodies, C219 and MRK16 [12], demonstrated that P-gp is present in the brain capillary endothelial cell membrane.

To investigate whether CsA interacts with P-gp present in BCEC, we examined the effect of increasing CsA concentrations from 0.1 to 50 μM on the steady-state uptake of [^3H]VCR, which has already been confirmed to be excluded by P-gp out of the primary cultured bovine BCEC [12]. As clearly shown in Fig. 2, CsA increased the uptake of [^3H]VCR in a concentration-dependent manner. This result suggests that CsA competitively inhibits the binding of VCR to P-gp, that is, specifically displaces VCR binding to P-gp in BCEC as well as in MDR tumor cells [15]. Accordingly, it is likely that CsA is also transported

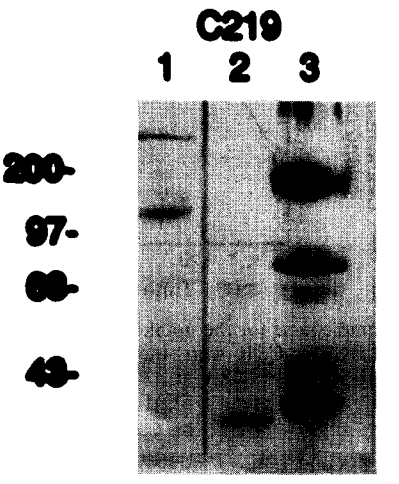

Fig. 1. Immunoblots of plasma membrane proteins using anti-P-gp monoclonal antibody C219. The proteins separated on 7.5% SDS–PAGE were transferred and stained by the ABC method with the use of C219. Lanes 1, 2 and 3 are the plasma membrane from bovine brain capillary, K562 (negative control) and K562/ADM (positive control) cells, respectively. The molecular weight is indicated on the left.

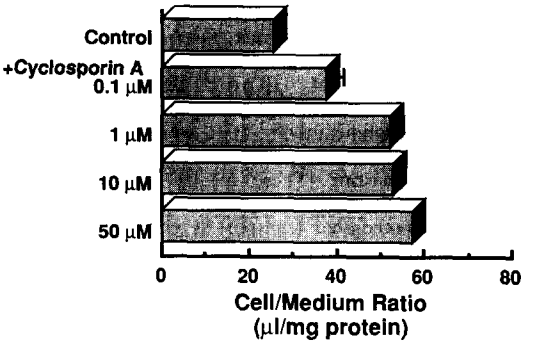

Fig. 2. Concentration-dependent effect of CsA on the uptake of VCR by primary cultured BCEC. Cells were pretreated with CsA at 37° for 30 min. The uptake of [^3H]-VCR (190 nM) was measured for 60 min. Each value represents the mean ± SEM (N = 4).

by P-gp in BCEC. In the following experiments, the uptake of CsA by BCEC was measured in order to characterize the mechanism for CsA transport.

Figure 3 shows the time course of the uptake of [^3H]-CsA by BCEC at a concentration of 90 nM at 37° and pH 7.4. The accumulation of [^3H]CsA was time dependent, and a steady-state level was attained by 60 min. Accordingly, the uptake at 60 min was studied in the following experiments to evaluate the efflux of [^3H]CsA mediated by P-gp.

Recently, we reported that MDR-reversing agents, such as verapamil, bind to P-gp in BCEC and inhibit the efflux of VCR mediated by P-gp in a manner analogous to that reported in MDR tumor cells, resulting in an increased steady-state uptake of [^3H]VCR by BCEC [12]. Therefore, the effects of several MDR-reversing agents on the steady-state uptake of [^3H]CsA by BCEC were studied in order to determine whether P-gp in BCEC transports CsA. As shown in Table 1, in the presence of 500 μM verapamil, the steady-state uptake of [^3H]CsA increased significantly to approximately 3-fold. Moreover, the initial uptake of

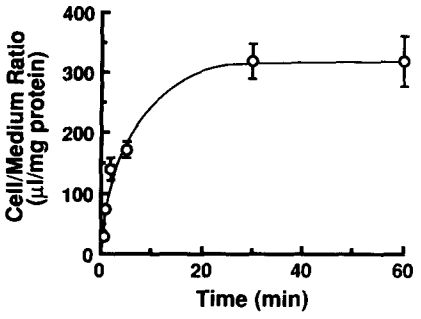

Fig. 3. Time course for the uptake of [^{3}H]CsA by primary cultured BCEC. Cells were incubated with a medium containing 90 nM [^{3}H]CsA at 37°. Each point represents the mean ± SEM (N = 6).

[^{3}H]CsA (15 sec) showed no significant difference in the presence and absence of verapamil [18.2 ± 2.2 and 18.7 ± 2.2 μL/mg protein, respectively (mean ± SEM, N = 6)]. However, after BCEC were loaded with [^{3}H]CsA for 60 min at 37° and the efflux was studied with the same procedure as described previously [12], the remaining amount of BCEC at 60 min was increased significantly in the presence of verapamil to 150 ± 15.7% (N = 6, P < 0.05) compared with that in the absence of verapamil (100%, 0.74 ± 0.05 pmol/mg protein, N = 6). Consequently, by measuring the steady-state uptake of [^{3}H]CsA in the presence of MDR-reversing agents, it is possible to detect the P-gp-mediated efflux. Furthermore, quinidine, chlorpromazine, testosterone and progesterone significantly increased the uptake of [^{3}H]CsA by BCEC at the steady-state (Table 1). Additionally, the uptake of [^{3}H]CsA was also increased significantly by VCR, which has been shown to be a substrate of P-gp in BCEC [12, 13], and by its analogue, vinblastine. These results suggest that P-gp participates in the BBB transport of CsA.

Since P-gp-mediated transport is characterized by its ATP dependence [12, 19, 26, 27], the effect of ATP depletion on CsA uptake was tested. When BCEC were pretreated with the metabolic inhibitor 2,4-dinitrophenol (DNP) in the presence of 2-deoxy-D-glucose, an unmetabolizable sugar analogue, the uptake of [^{3}H]CsA increased significantly to approximately 4-fold (Table 1). The observed metabolic-energy dependence of the uptake of CsA was consistent with the characteristics of P-gp. Moreover, to confirm the participation of P-gp in CsA transport, we examined the effect of a specific binder to P-gp, a monoclonal antibody (MRK16) which inhibits P-gp-mediated transport by binding to an external domain of P-gp [12, 28], on the uptake of CsA. As shown in Table 1, by pretreating BCEC with MRK16 at a concentration of 10 μg/mL, the steady-state uptake of CsA increased about 3-fold, whereas no increase was observed when BCEC were treated with mouse IgG at the same concentration. These results strongly indicate that CsA is ejected from BCEC by P-gp.

In the present study, we demonstrated that CsA is transported out of the primary cultured BCEC by P-gp, whose molecular mass is approximately 130–140 kDa. Since we have already reported that P-gp localizes at the luminal membrane of freshly isolated bovine brain capillary endothelial cells [12], P-gp must exclude CsA out of BCEC into the bloodstream *in vivo*. Until now, several mechanisms have been proposed to explain the exceptionally low distribution of CsA into the brain, including the binding to blood components and trapping in BCEC [3, 8, 9]. However, P-gp-mediated efflux from BCEC is a more likely mechanism than the others reported previously, because our proposed mechanism gives us a more general idea of the restricted transport into the brain of several compounds that have lower permeability across the BBB than expected from their lipid solubility [5] and because some of these compounds with restricted BBB permeability are known to inhibit the function of P-gp in MDR tumor cells. We therefore believe that the function of P-gp in the luminal surface of BCEC is to restrict the transport into brain of toxic substances that are highly lipophilic, thereby serving as the BBB against such compounds. The extent of contributions of the P-gp function remains to be confirmed *in vivo*.

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Table 1. Effects of various agents on the uptake of CsA by primary cultured BCEC

Agents	Relative uptake (% of control)
Control	100*
Verapamil (500 μM)	326 ± 12.8†
Quinidine (500 μM)	241 ± 2.1†
Chlorpromazine (100 μM)	138 ± 4.9†
Testosterone (100 μM)	227 ± 9.2†
Progesterone (100 μM)	152 ± 14.7†
Vincristine (100 μM)	226 ± 12.8†
Vinblastine (100 μM)	166 ± 7.2†
2,4-DNP (1 mM) + 2-deoxyglucose (10 mM)	399 ± 22.8†
MRK16‡ (10 μg/mL)	344 ± 13.5†
Mouse IgG‡ (10 μg/mL)	125 ± 21.6

After cells were pretreated at 37° for 30 min with each compound, the uptake of [^{3}H]CsA (90 nM) was measured at 37° for 60 min. Each value represents the mean ± SEM (N = 3–6).

* Control uptake was 386 ± 34.0 μL/mg protein (mean ± SEM, N = 6).

† Significantly different from control (P < 0.05).

‡ Pretreated at 4° for 30 min; then the uptake was measured at 37° for 60 min.

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