Lack of Contribution of P-Glycoprotein (P-gp) to Transport *via* the Mouse Blood-Cerebrospinal Fluid Barrier

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The blood-brain barrier (BBB) and the blood-cerebrospinal fluid (CSF) work effectively to restrict the free exchange of compounds between blood and brain. It is well known that P-glycoprotein (P-gp) that is expressed in brain microvessel endothelial cells contributes to the BBB as an efflux transporter. Western blot analysis has shown that P-gp is also expressed in the choroid plexus forming the blood-CSF barrier. The purpose of this study was to examine the role of P-gp in the efflux transport of its substrates across the blood-CSF barrier. The CSF concentration of etoposide and digoxin after intracerebroventricular administration, and the CSF concentration of ^{99m}Tc-sestamibi after intravenous administration were compared between wild-type and P-gp knockout mice. After intracerebroventricular administration, there was no significant difference in the remaining concentration of etoposide and digoxin in CSF between wild-type and P-gp knockout mice, respectively. After intravenous administration of ^{99m}Tc-sestamibi, its CSF-to-serum concentration ratio in P-gp knockout mice was not significantly different from that in wild-type, whereas its brain-to-serum concentration ratio was significantly increased in P-gp knockout mice compared with wild-type mice (1.62-fold, $p < 0.05$). In addition, immunohistochemical analysis showed a low membrane expression of P-gp in mouse choroid plexus, compared with brain microvessels. These results suggest that P-gp does not play an important role in the transport of its substrates across the blood-CSF barrier.

Key words —— blood-cerebrospinal fluid barrier, P-glycoprotein, drug transport

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INTRODUCTION

The choroid plexus (CP), which forms the blood-cerebrospinal fluid (CSF) barrier, secretes CSF and regulates its composition through active and selective transport processes.[1] The anatomical entity of the blood-CSF barrier consists of choroid epithelial cells that are tightly connected to each other and polarized to form brush-border and basolateral membranes. In addition to this physical barrier, metabolic enzymes and transporters on this barrier also play important roles in a barrier function on the CP.[2,3]

It is well known that P-glycoprotein (P-gp), a 170-kD membrane protein that is responsible for the multidrug resistance of tumor cells, plays important role at the blood-brain barrier (BBB).[4,5] In the case of a wide range of compounds, including anti-cancer agents, such as etoposide and cardiovascular agents, such as digoxin, P-gp is known to restrict their brain uptake directly, thereby protecting the brain against xenobiotic invasion.[3] Also, P-gp may be involved in the pathogenesis of Alzheimer's disease, by affecting the transport of substances such as beta-amyloid, suspected of playing a role in neurodegenerative disease.[6,7]

Interestingly, the expression of P-gp has also been confirmed in the CP of rodents and monkeys and it has been hypothesized that P-gp may contribute to the blood-CSF barrier of the CP.[8,9] Piwnica-Worms and his co-workers have shown that P-gp is expressed in the CP of rodents by Western blot analysis and it is suspected that P-gp functionally acts as a drug-permeation barrier for the CP, based on results involving the *in vitro* transport of ^{99m}Tc-sestamibi and [^{3}H]Taxol in cultured rat CP epithelial cells.[8] However, the P-gp inhibitor, Cyclosporine A, does not appear to significantly increase the CSF penetration of doxorubicin in monkeys at a concentration sufficient to alter the systemic clearance of doxorubicin.[9]

These reports prompted us to examine the involvement of P-gp in the transport of its substrates across the blood-CSF barrier. For this purpose, *in vivo* kinetic analyses of model substrates of P-gp in the CSF after intracerebroventricular (etoposide and digoxin) and intravenous administration (^{99m}Tc-sestamibi) were carried out using P-gp knockout mice and wild-type control mice. In addition, the plasma membrane expression of P-gp in the mouse CP was examined and compared with expression of Breast cancer resistance protein (Bcrp) by immunohistochemical analysis.

MATERIALS AND METHODS

Chemicals and Experimental Animals —— [^{3}H]etoposide and [^{14}C]mannitol were purchased from Moravek Biochemicals (Brea, CA, U.S.A.). [^{3}H]digoxin was purchased from PerkinElmer Life Sciences (Boston, MA, U.S.A.). ^{99m}Tc-sestamibi was purchased from Daiichi Radioisotope Laboratories Ltd. (Tokyo, Japan). All other chemicals were commercial products of analytical grade. Male P-gp knockout mice (Mdr1a/1b double knockout) and their matched wild-type controls were purchased from Taconic (Germantown, NY, U.S.A.) and maintained under standard conditions with a reverse dark-light cycle. Food and water were available *ad libitum*.

Elimination of [^{3}H]Etoposide and [^{3}H]Digoxin from CSF —— Before surgery, mice were anaesthetized intraperitoneally with a mixture of ketamine and xylazine (100 and 10 mg/kg, respectively, 0.1 ml/25 g body weight) and secured in a stereotaxic device. An incision was made in the scalp to expose the bregma. Intracerebroventricular injections of [^{3}H]etoposide and [^{3}H]digoxin with passive elimination marker, [^{14}C]mannitol, were performed according to the previously described method.[10,11] At 15 min after intracerebroventricular administration, aliquots of CSF (5–10 μl) were withdrawn by cisternal puncture, and their radioactivity was determined in a liquid scintillation counter (LS 6000SE; Beckman Coulter, Fullerton, CA, U.S.A.). The remaining concentration ratio of [^{3}H]etoposide and [^{3}H]digoxin to [^{14}C]mannitol was used to characterize the elimination of substrates, and determined by the following equation:

$$\text{The remaining concentration ratio} = \frac{\text{Concentration of model compound in CSF}}{\text{Concentration of mannitol in CSF}}$$

Statistical significance was determined by the unpaired two-tailed Student's *t*-test at $\alpha = 0.05$.

CSF Distribution of ^{99m}Tc-Sestamibi after Intravenous Administration —— After mice were anaesthetized with a mixture of ketamine and xylazine (100 and 10 mg/kg, respectively, intraperitoneally), ^{99m}Tc-sestamibi in 0.9% saline (1 mCi/28 g bw) was injected into a tail vein. For tissue distribution studies, mice were sacrificed 60 min after intravenous administration and the radioactivity associated with serum, brain and CSF was measured in a gamma scintillation counter (Beckman Coulter) to calculate the tissue-to-serum concentration ratios.

Immunohistochemical Analysis of P-gp at the Mouse CP —— Cryosections of mouse brain samples were used for immunohistochemical analysis. Because it has been reported that Bcrp is expressed in the brain capillary endothelial cells as a new type of efflux transporter,[12] double immunohistochemical staining of P-gp and Bcrp was performed using C219 [P-gp antibody; 1 : 40 dilution in 1% bovine serum albumin/phosphate buffered saline (BSA/PBS), from Signet, Dedham, MA, U.S.A.] and Bxp-53 (Bcrp antibody; 1 : 40 dilution in 1% BSA/PBS, from Signet). After incubation with primary antibody (4°C, overnight), sections were incubated with appropriate Alexa Fluor secondary antibodies (Molecular Probes, Eugene, OR, U.S.A.) and Topro3 (DNA dye, Molecular Probes, Hilversum, Netherlands) for 1 hr at room temperature and mounted in Vectashield mounting medium® (Vector Laboratories, Burlingame, CA, U.S.A.) and visualized under a Zeiss confocal fluorescence microscope. Negative control staining without primary antibody was also conducted to check for false positive staining in the immunohistochemical analysis.

RESULTS AND DISCUSSION

CSF concentrations of [^{3}H]etoposide and [^{3}H]digoxin 15 min after intracerebroventricular administration in wild-type and P-gp knockout mice are shown in Fig. 1. The concentration ratios of remaining [^{3}H]etoposide and [^{3}H]digoxin, normalized by the simultaneously administered [^{14}C]mannitol,

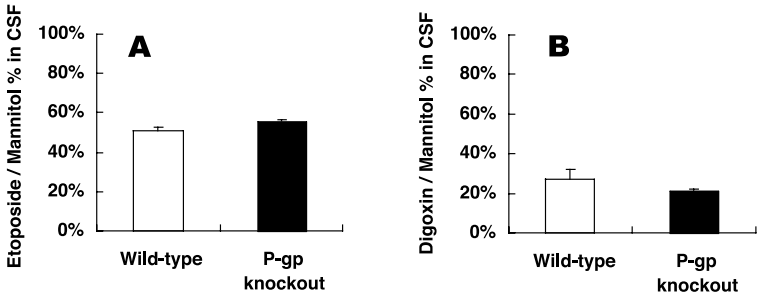

Fig. 1. The Concentration Ratios of Remaining [^{3}H]Etoposide (A) and [^{3}H]Digoxin (B) in the CSF 15 min after Intracerebroventricular Administration in Wild-Type and P-gp Knockout Mice (mean ± S.E., n = 3–4)

were compared between wild-type and P-gp knockout mice (Fig. 1). The elimination of [^{3}H]etoposide and [^{3}H]digoxin from CSF after intracerebroventricular administration was faster than that of mannitol which is eliminated by CSF turnover and simple diffusion into ependyma[13] both in wild-type and P-gp knockout mice (Fig. 1A and 1B). At 15 min after administration, the remaining concentration ratio of [^{3}H]etoposide in wild-type and P-gp knockout mice was 51.1 ± 1.9 and 55.8 ± 0.6% of the [^{14}C]mannitol concentration, respectively, and the corresponding values for [^{3}H]digoxin in wild-type and P-gp knockout mice were 27.3 ± 4.8 and 21.4 ± 0.9%, respectively. These results suggest that knockout of P-gp did not make any difference to the elimination of etoposide and digoxin across the blood-CSF barrier after intracerebroventricular administration.

The brain-to-serum and CSF-to-serum concentration ratios (K_p) of ^{99m}Tc-sestamibi 60 min after intravenous administration is shown in Fig. 2. ^{99m}Tc-sestamibi [Hexakis(2-methoxyisobutylisonitrile) technetium-99m], originally developed as a radiopharmaceutical for myocardial perfusion, is a well known lipophilic cationic compound for the functional evaluation of P-gp-mediated transport activity *in vivo*.[8]

The brain-to-serum concentration ratios of ^{99m}Tc-sestamibi were increased in P-gp knockout mice, compared with those in wild-type mice, suggesting that the P-gp restricts the brain uptake of ^{99m}Tc-sestamibi at the BBB (Fig. 2, $p < 0.05$; $n = 3$). However, the CSF-to-serum concentration ratios of ^{99m}Tc-sestamibi were not significantly different between wild-type and P-gp knockout mice, suggesting that P-gp has little effect on the CSF uptake of ^{99m}Tc-sestamibi (0.082 ± 0.026 *vs*. 0.109 ± 0.029 ml/g tissue) (Fig. 2).

The expression of P-gp and Bcrp in mouse brain

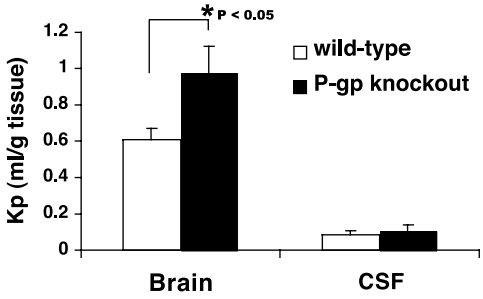

Fig. 2. The Brain-to-Serum and CSF-to-Serum Concentration Ratios of ^{99m}Tc-Sestamibi in Wild-Type and P-gp Knockout Mice 60 min after Intravenous Administration (mean ± S.E., n = 3)

microvessels and CP was evaluated by immunohistochemical analysis. A consistent staining of brain microvessel capillaries for P-gp (red) and Bcrp (green) throughout the mouse brain was observed (Fig. 3A). However, no signal for P-gp was not detected in the CP, although P-gp was detected in the CP using Western blot analysis in a previous report.[8] This result suggests a relatively low level of membrane expression of P-gp in mouse CP, compared with that in brain microvessels (Fig. 3B). However, Bcrp was found in the CP as well as in brain microvessels, although its cellular localization in epithelial cells of the CP was unclear (Fig. 3B). Recently, Bcrp has been investigated actively as a new type of efflux transporter which is involved in the intestinal absorption and hepatobiliary elimination of its substrates.[14]

The previous report by Rao *et al*. has shown that P-gp is expressed in the CP and treatment of GF120918, P-gp and Bcrp co-inhibitor, modifies the transport of ^{99m}Tc-sestamibi across the blood-CSF barrier in human and cultured rat choroid plexus.[8] However, the present study using P-gp knockout mice shows that the knockout of P-gp does not af-

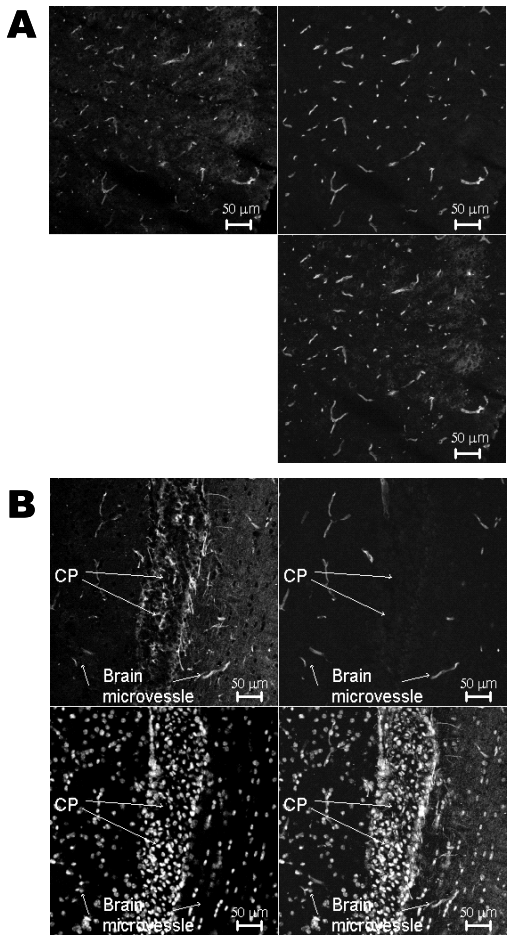

Fig. 3. Expression of P-gp (red) and Bcrp (green) in Brain Microvessels (A) and CP (B)

The nucleus was stained blue to show the location of CP in the brain (B). A merged image is shown below right (A and B). Scale bar = 50 μm.

fect the apparent transport of [^{3}H]etoposide, [^{3}H]digoxin and ^{99m}Tc-sestamibi across the blood-CSF barrier. In addition, expression of P-gp in mouse CP is quite low or undetectable, compared with that in brain microvessels. Interestingly, Bcrp is clearly present in the CP as well as brain microvessels. These results suggest that the contribution of P-gp in the CP to the blood-CSF barrier may be minor. Other efflux transporter(s) that are expressed in the CP, such as Bcrp and multidrug resistance associated protein 4,[15] may play a more important role in the blood-CSF barrier.

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