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Effect of cyclosporin A or tacrolimus on the function of blood–brain barrier cells

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Abstract

Recently, it has been reported that continuous treatment with cyclosporin A or tacrolimus induces encephalopathy in transplant patients. The mechanism of immunosuppressant-induced encephalopathy is unclear. We investigated the cytotoxicity to brain capillary endothelial cells and the effect of these two drugs on *P*-glycoprotein function using mouse brain capillary endothelial (MBEC4) cells. The transcellular transport of [^{3}H]sucrose was significantly increased and the cellular viability, based on 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay and trypan blue exclusion test, was decreased by cyclosporin A (approximately 50% at 5 μM; $P < 0.005$), while tacrolimus showed a much smaller effect. These findings indicate that the toxicity of cyclosporin A was greater than that of tacrolimus. The uptake of [^{3}H]vincristine, a substrate of *P*-glycoprotein, was increased by these two drugs. The expression of *P*-glycoprotein in MBEC4 cells was reduced, but there was no effect on *mdr1b* mRNA levels. The decrease in the expression of *P*-glycoprotein may be due to the inhibition of the turnover of *P*-glycoprotein, which involves translation. In conclusion, the direct cytotoxic effect on the brain capillary endothelial cells and the inhibition of *P*-glycoprotein may be partly involved in the occurrence of immunosuppressant-induced encephalopathy.

Keywords: Encephalopathy; Cyclosporin A; Tacrolimus; Blood–brain barrier; Cytotoxicity; *P*-glycoprotein

1. Introduction

Cyclosporin A and tacrolimus are immunosuppressants widely used in organ transplantation to prevent fatal graft-vs.-host disease or to suppress rejection symptoms (Powels et al., 1980; Kahan, 1992; Borel et al., 1995). However, the immunosuppressive potency of these two drugs is high, and it is well-known that they induce multiple organ toxicity (Hamilton et al., 1981; Adu et al., 1983; Gupta et al., 1991). Recently, the occurrence of encephalopathies such as seizure, confusion, motor paralysis and cortical blindness in transplant patients using these two drugs was reported (De Groen et al., 1987; Humphreys and Leyden, 1993; Shimizu et al., 1994; Appignani et al., 1996; Hinchey et al., 1996). The mechanism of immunosuppressant-induced encephalopathy is unclear. However, several investigators suggested that increased blood drug levels, due to hypocholesterolemia or concurrently used drugs, enhanced the distribution of drugs to the brain, that nephrotoxicity or hypertension induced by the two drugs caused edema of the white matter, and that virus infection due to the immunosuppressive effect caused virus encephalopathy (Brat et al., 1992; Shimizu et al., 1994; Worthmann et al., 1994; Hinchey et al., 1996). It has also been reported that cyclosporin A inhibits cell growth and induces apoptosis characterized by chromatin condensation, DNA ladder and shrinkage of the cell body in rat C6 glioma cells in a dose-dependent manner (Mosieniak et al., 1997), suggesting that the direct toxicity of the immunosuppressants entering the brain may trigger the encephalopathy.

Cyclosporin A and tacrolimus are known as substrates and inhibitors of *P*-glycoprotein, a multi-drug efflux pump, (Takeguchi et al., 1993; Tanaka et al., 1996). The transport of cyclosporin A across the blood–brain barrier is restricted by *P*-glycoprotein, so that the distribution of cyclosporin A in the brain is low despite its high lipophilicity

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(Tsuji et al., 1993; Shirai et al., 1994). *P*-glycoprotein is widely expressed in various tissues such as the brush-border membrane of renal proximal tubules, the bile canalicular membrane of hepatocytes and the capillary endothelial cells of the brain (Rothenberg and Ling, 1989; Postan et al., 1991), and *P*-glycoprotein could be related to the regulation of the pharmacokinetic behavior of drugs including self-defense. *P*-glycoprotein in brain capillary endothelial cells is localized on the apical surface and serves as an efflux pump for many lipophilic agents transported by passive diffusion across the cell membrane such as antitumor agents, cyclosporin A and tacrolimus in an ATP-dependent manner (Sugawara et al., 1990). In this study, we hypothesized that the permeability of blood–brain barrier to cyclosporin A and tacrolimus is enhanced and that encephalopathy is induced as a result of the enhanced brain distribution of these two drugs as a result of direct injury of the brain capillary endothelial cells and/or the depressed function of *P*-glycoprotein.

To clarify the mechanism of immunosuppressant-induced encephalopathy, we examined the effect of cyclosporin A or tacrolimus, using the mouse brain endothelial (MBEC4) cells as a model of brain capillary endothelial cells (Tatsuta et al., 1992; Tatsuta et al., 1994), on the function of the blood–brain barrier (cell viability and permeability of test substances by simple diffusion or *P*-glycoprotein-mediated active efflux transport, and expression of *P*-glycoprotein and its mRNA level).

2. Materials and methods

2.1. Reagents and antibodies

Cyclosporin A and tacrolimus were kindly supplied by Sandoz (Basel, Switzerland) and Fujisawa Pharmaceutical (Osaka, Japan), respectively. [^{3}H]Sucrose (specific activity; 12.3 Ci/mmol) and [^{3}H]vincristine sulphate (specific activity; 10.5 Ci/mmol) were purchased from Amersham International (Buckinghamshire, UK), and [α-^{32}P]dCTP (specific activity; 3000 Ci/mmol) was purchased from NEN Research Products (MA, USA). Polyclonal anti-*mdr* (Ab-1) was purchased from Oncogene Research Products (MA, USA). Alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG) was purchased from Organon Teknika (PA, USA). MTT (3-(4, 5-dimethylthiazol-2—yl)-2, 5-diphenyl tetrazolium bromide) was purchased from Chemicon International (CA, USA). All other chemicals were commercial products of reagent grade. Cyclosporin A and tacrolimus were dissolved in ethanol and diluted in serum-free culture medium (0.1% as the final ethanol concentration).

2.2. Cell cultures

Mouse brain capillary endothelial (MBEC4) cells, which were isolated from BALB/c mice brain cortices and immortalized by SV40-transformation (Tatsuta et al., 1992), were cultured in Dulbecco's modified Eagle's medium (Nikken Bio Medical Lab, Japan) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were grown in 12-well Transwell™ (Costar, MA, USA), 6-well, 24-well Collagen type 1 plates (Iwaki Glass, Chiba, Japan) or 96-well plates (Costar) in a humidified atmosphere of CO_2/air (5%/95%). After the cells were cultured for 3 days, they were washed three times with serum-free medium and treated with various concentrations of cyclosporin A or tacrolimus for 24 h. In parallel, cells were treated with serum-free medium containing the corresponding amount of ethanol as the control. MBEC4 cells retained the morphological and biochemical characteristics of brain capillary endothelial cells and expressed *P*-glycoprotein mainly encoded by the *mdr1b* gene (Tatsuta et al., 1992, 1994), so they were a useful and convenient model to evaluate the specific effect of the blood–brain barrier.

2.3. Transcellular transport of [^{3}H]sucrose

The effect of cyclosporin A or tacrolimus on MBEC4 cell monolayer permeability was tested as the transcellular transport of [^{3}H]sucrose. MBEC4 cells (4.0×10^4 cells/ml) were cultured on polycarbonate membrane (3.0 μm pore size) Transwell™ clusters. After reaching confluency, cells were treated with various concentrations of cyclosporin A or tacrolimus for 24 h. To initiate the transport experiments, cells were washed three times with phosphate-buffered saline (PBS; 141 mM NaCl, 4 mM KCl, 2.8 mM $CaCl_2$, 1 mM $MgSO_4$, 10 mM D-glucose, 10 mM HEPES for pH 7.4, 37°C); PBS was put on the receiver side and 10 nM [^{3}H]sucrose was loaded on the donor side of the cell insert. The cells were incubated at 37°C for 3, 6, 9, 15 and 20 min. The transport of [^{3}H]sucrose was quantified by adding 4 ml of a scintillation fluid (Clear-sol; Nacarai Tesque, Kyoto, Japan) and counting radioactivity in a liquid scintillation counter (LS6500, Beckman Instrument, CA, USA). The cellular protein was measured by the method of Lowry et al. (1951), using bovine serum albumin as a standard. The real permeability coefficient (P_{trans}) of [^{3}H]sucrose was calculated by the following equation:

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

Where P_{app} and P_{filter} were the appearant permeability coefficient estimated in the transport study in the presence and absence of MBEC4 cells, respectively.

2.4. Quantification of the activities of the marker enzymes

MBEC4 (6.3×10^4 cells/ml) were cultured on 6-well Collagen Type 1 plates and treated with various concentrations of cyclosporin A or tacrolimus for 24 h. Quantification of the activities of lactate dehydrogenase (LDH), alkaline phosphatase and γ-glutamyltranspeptidase in the culture medium was performed as described previously

(Bessey et al., 1946; Wrobewaki and La Due, 1955; Del Pino et al., 1992). The cellular protein was measured by the method of Lowry et al. (1951).

2.5. MTT assay and trypan blue exclusion test

MBEC4 (6.7×10^4 cells/ml) were cultured on 96-well plates and treated with various concentrations of cyclosporin A or tacrolimus for 24 h. To initiate MTT assay, the drug solution was removed and 1.0 mg/ml MTT solution (50 μl) was added to each well. The cells were incubated for 3 h at 37°C. At the end of the incubation, MTT solution was removed and 50 μl of propanol was added. The plate was then vigorously shaken using a Micro Mixer (TAITEC; Saitama, Japan) in order to ensure solubilization of the blue formazan. The absorbance of each well was measured, using a microplate reader (model 450: BIORAD; Tokyo, Japan) with a 595-nm test wavelength and a 620-nm reference wavelength (Denizot and Lang, 1986). Results are expressed as a percentage of the absorbance in vehicle-treated cells. Trypan blue (0.1% w/v) was added to vehicle- or drug-treated cells and incubated for 10 min at 37°C. After trypsinization, viable cells were counted in a hemacytometer. The viable cells were calculated as a percentage of the total cells counted. Cell viability was then expressed as a percentage of that of the vehicle-treated cells.

2.6. Cellular uptake of [^{3}H]vincristine by MBEC4 cells

MBEC4 cells (4.0×10^4 cells/ml) were cultured on 24-well Collagen Type 1 plates and treated with 0.1, 1, and 10 μM cyclosporin A or tacrolimus for 24 h. To initiate the uptake experiment, [^{3}H]vincristine was added to each well (final concentration of [^{3}H]vincristine; 30 nM), and cells were incubated for 1 h at 37°C. The same experiment using cells treated with the corresponding amount of drugs for 1 h was performed to compare the acute effect of drugs on *P*-glycoprotein. To terminate the uptake, the test solution was removed by suction and the cells were washed three times with 1 ml of ice-cold PBS. For quantification of the radioactivity associated with the cells, the cells were solubilized with 200 μl of 3 N NaOH. After neutralization with 100 μl of 6 N HCl, the samples were placed in a plastic vial and 4 ml of a scintillation fluid (clear-sol; Nacarai Tesque, Kyoto, Japan) was added to determinate the radioactivity using a LS6500 (Beckman Instrument). The cellular protein was measured by the method of Lowry et al. (1951). The uptake of [^{3}H]vincristine was expressed as the cell to medium ratio obtained by dividing uptake by the drug concentration and correcting for the amount of cellular protein (μl/mg protein).

2.7. Quantification of P-glycoprotein by ELISA

MBEC4 cells (6.0×10^2 cells/ml) were cultured on 96-well plates and treated with cyclosporin A or tacrolimus for 24 h. The quantification of *P*-glycoprotein was performed as described previously (Tatsuta et al., 1994). In brief, the cells were washed three times with serum-free medium and fixed with 100 μl of 10% formalin neutral buffer solution (pH 7.4) for 4 h. The cells were incubated with 200 μl of RPMI 1640 containing 100 mM glycine, 1% bovine serum albumin and 0.05% NaN_3 for 12 h at 4°C. After the blocking solution was aspirated, 60 μl of the first antibody solution (PBS containing 3 μg/ml of anti-*mdr* serum and 3% bovine serum albumin) was added to each well and incubated for 2 h at room temperature. The cells were then treated with 100 μl of the second antibody solution (alkaline phosphatase-conjugated goat anti-rabbit IgG at 1:12500 dilution in PBS containing 3% bovine serum albumin) for 1 h at room temperature and 100 μl of 4 mg/ml *p*-nitrophenyl phosphate solution was added. After incubation at room temperature for 60 min, absorbance at 405 nm in each well was measured with a microplate reader (model 450: BIORAD). The absorbance of the wells without the first antibody in each group was subtracted as background, and the amount of *P*-glycoprotein expressed in the treated cells is expressed relative to the amount expressed in the vehicle-treated cells.

2.8. Northern blot analysis

Total RNA fractions were isolated from drug-treated cells using TRIZOL reagents (Life Technologies, MD, USA) and Northern blot analysis was performed as described previously (Tatsuta et al., 1994). Total RNA (30 μg) was subjected to electrophoresis on 1.2% agarose gel containing 2.1 M formaldehyde, 0.02 M 3-(*N*-morpholino) propanesulfonic acid (pH 7.0), 8 mM sodium acetate and 1 mM EDTA (pH 8.0). The RNA was then transferred to a nylon membrane filter (Hybond-N, Amersham, Buckinghamshire, UK) in 20 × standard saline citrate (SSC) overnight, and the filter was fixed by drying for 2 h at 80°C. The blotted filter was hybridized using a ^{32}P-labeled (BcaBEST™ Labeling Kit, Takara Shuzo, Shiga, Japan) probe specific to mouse *mdr1b* at 65°C under high-stringency conditions. We used the 166-bp fragment corresponding to 2034 bp-2199 bp for the *mdr1b* probe (Gros et al., 1986; Hsu et al., 1989). The filter was then washed twice with 2 × SSC/0.1% Sodium dodecyl sulfate for 5 min at room temperature.

2.9. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Quantitative RT-PCR for the *mdr1b* gene was performed according to the method previously described (Narasaki et al., 1997). Firstly, to assess the measurable range of this method, total RNA was isolated from MBEC4 and serially diluted. The measurable range was determined as the range showing a clear linear correlation between the density of band and amount of total RNA (data not shown). For quantitation, total RNA fractions were isolated from

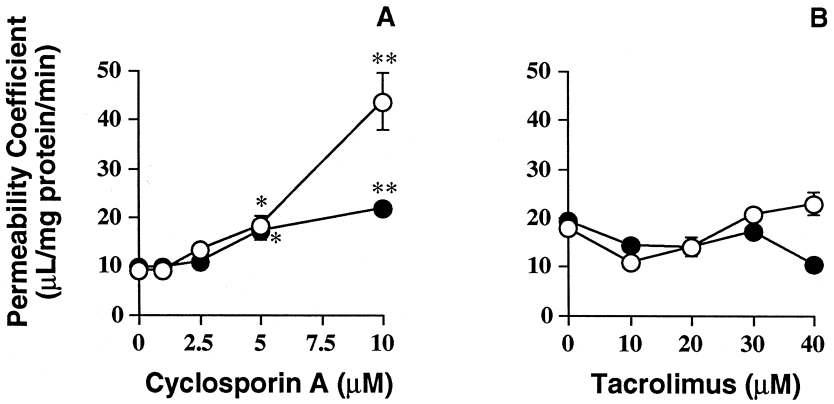

Fig. 1. Concentration-dependent effect of cyclosporin A or tacrolimus on the transcellular transport of 10 nM [^{3}H]sucrose across MBEC4 cells. Cells were exposed for 24 h to the indicated concentrations of cyclosporin A (A) or tacrolimus (B). The transport of 10 nM [^{3}H]sucrose from the luminal to the abluminal side (open symbol) and from the abluminal to the luminal side (closed symbol) was determined as described in Section 2. Data are means ± S.E. ($n = 3$). Significant differences from control; $^*P < 0.05$, $^{**}P < 0.005$.

drug-treated cells using TRIZOL reagents (Life Technologies). cDNA was prepared from 15 ng total RNA and 1.67 μM random primer (Takara Shuzo), in a total volume of 10 μl with 50 U reverse transcriptase (Life Technologies). PCR was performed in a final volume of 20 μl containing an amount of cDNA equivalent to 3 ng of total RNA, 0.2 μM each of sense and antisense primers, and 0.025 U of Ex Taq DNA polymerase (Takara Shuzo). The PCR primers were 5′-GATCCCAGAGTGACACTGAT-3′ and 5′-CAAAAGGAAACCAGAGGCAC-3′ for mouse *mdr1b*. Amplification was performed in a DNA thermal cycler (Techne, Cambridge, UK) according to the following protocol: initial denaturation for 5 min at 98°C; five cycles of denaturation for 1 min at 95°C, primer annealing for 1 min at 37°C, and polymerization for 1 min at 72°C; 30 cycles of denaturation for 45 s at 95°C, primer annealing for 45 s at 50°C, and polymerization for 45 s at 72°C; and final extension for 5 min at 72°C. Each 8 μl of PCR product was analyzed by electrophoresis on 5% acrylamide gels with ethidium bromide staining. The gels were exposed to Polaroid 667 films under UV light. The density of each band was analyzed using NIH Image software and normalized to GAPDH gene expression.

2.10. Statistical analysis

All of the data are expressed as means ± S.E. Statistical analysis was performed by using Student's *t*-test. The differences between means were considered to be significant when P values were less than 0.05.

3. Results

3.1. Effect of cyclosporin A or tacrolimus on the transport of [^{3}H]sucrose across MBEC4 cells

Firstly, to study the effect of cyclosporin A or tacrolimus on the permeability of MBEC4 cells, we measured the transcellular transport of [^{3}H]sucrose in MBEC4 cells ex-

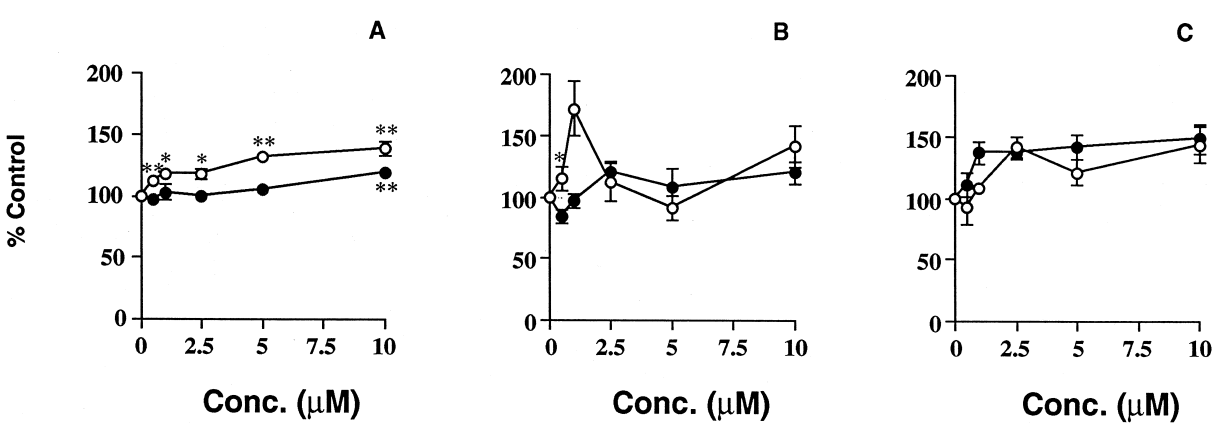

Fig. 2. The activities of lactate dehydrogenase (A), alkaline phosphatase (B) and γ-glutamyltranspeptidase (C) in the culture medium of MBEC4 cells. Cultures were exposed for 24 h to the indicated concentrations of cyclosporin A (open symbol) or tacrolimus (closed symbol). Data are means ± S.E. ($n = 6$). Significant differences from control; $^*P < 0.05$, $^{**}P < 0.005$.

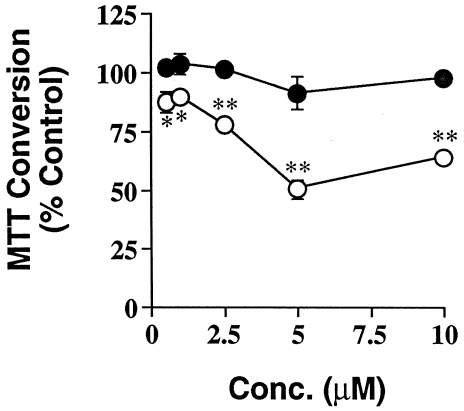

Fig. 3. Effect of cyclosporin A or tacrolimus on mitochondrial MTT reduction in MBEC4 cells. Cultures were exposed for 24 h to the indicated concentrations of cyclosporin A (open symbol) or tacrolimus (closed symbol). Data are means ± S.E. ($n = 4$). Significant differences from control; $^{*}P < 0.05$, $^{**}P < 0.005$.

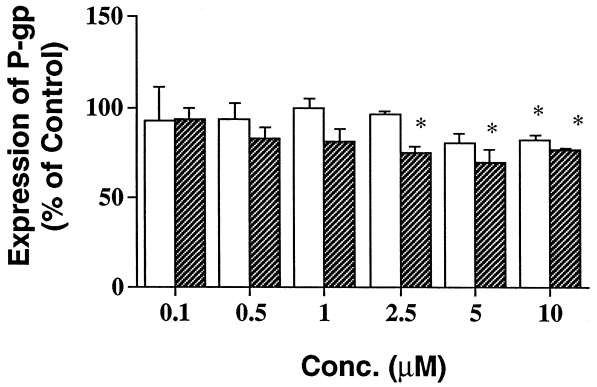

Fig. 5. Effect of cyclosporin A or tacrolimus on the expression of P-gp in MBEC4 cells. Cultures were exposed for 24 h to the indicated concentrations of cyclosporin A (□) or tacrolimus (shaded square with diagonal lines). Data are means ± S.E. ($n = 4$). Significant differences from control; $^{*}P < 0.05$, $^{**}P < 0.005$.

posed to cyclosporin or tacrolimus for 24 h. The transcellular transport of [^{3}H]sucrose both from the luminal to the abluminal side and from the abluminal to the luminal side was significantly increased by the exposure of MBEC4 cells to cyclosporin A at a concentration of 5–10 μM, while tacrolimus showed no effect in the range of 1–40 μM (Fig. 1).

3.2. Effect of cyclosporin A or tacrolimus on the activities of marker enzymes in the culture medium

Fig. 2 shows the activities of LDH, alkaline phosphatase and γ-glutamyltranspeptidase in the culture medium of vehicle-, cyclosporin A-, and tacrolimus-treated MBEC4 cells. The activities of alkaline phosphatase and γ-glutamyltranspeptidase, membrane-bound enzymes, were not affected by cyclosporin A or tacrolimus (0.5–10 μM) (Fig. 2B, C), while cyclosporin A or tacrolimus increased the release of LDH, a cytosolic enzyme, in a dose-dependent manner (Fig. 2A). Cyclosporin A induced the release of LDH at 0.5–10 μM, while tacrolimus induced it at only 10 μM.

3.3. Effect of cyclosporin A or tacrolimus on the cell viability

MBEC4 cells were exposed to various concentrations of cyclosporin A or tacrolimus or vehicle alone for 24 h, and cell viability was assessed by MTT assay. MTT conversion was decreased by approximately 50% compared with that of the control by the treatment with cyclosporin A (0.5–10 μM), whereas cytotoxicity was not observed with tacrolimus (0.5–10 μM) (Fig. 3). We performed the trypan

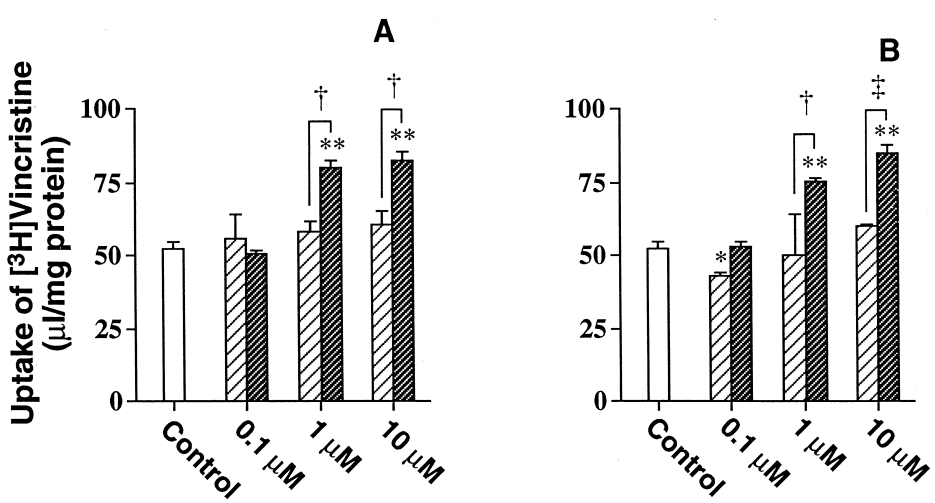

Fig. 4. Effect of cyclosporin A or tacrolimus on the uptake of 30 nM [^{3}H]vincristine by MBEC4 cells. Cultures were exposed for 1 h (square with diagonal lines) or 24 h (shaded square with diagonal lines) to the indicated concentrations of cyclosporin A (A) or tacrolimus (B). Data are means ± S.E. ($n = 3$). Significant differences from control; $^{*}P < 0.05$, $^{**}P < 0.005$. Significant differences between (square with diagonal lines) and (shaded square with diagonal lines) $^{\dagger} < 0.05$, $^{\ddagger} < 0.005$.

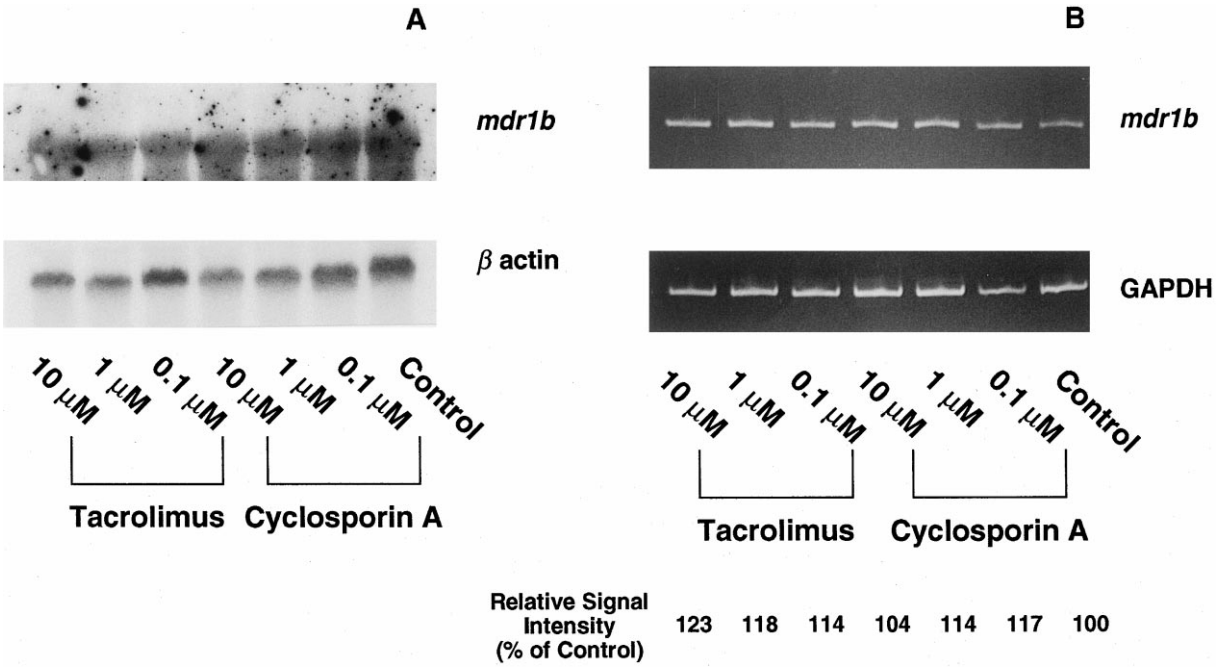

Fig. 6. Northern blot analysis (A) and RT-PCR analysis (B) of total RNA extracted from MBEC4 cells cultured in the presence or absence of 0.1, 1, 10 μM cyclosporin A or tacrolimus. Extraction of total RNA from MBEC4 cells 24 h after cyclosporin A or tacrolimus treatment and the procedure for the northern blot analysis and the RT-PCR analysis of total RNA were as described in Section 2.

blue exclusion assay to confirm this finding and obtained similar results. The number of viable cells was decreased by the treatment with cyclosporin A or tacrolimus. The number of viable cells (25%) in the presence of 1 μM cyclosporin A was smaller than that (50%) in the presence of 1 μM tacrolimus (data not shown)

3.4. Effect of cyclosporin A or tacrolimus on the uptake of [^{3}H]vincristine by MBEC4 cells

We examined the effect of cyclosporin A and tacrolimus on the uptake of [^{3}H]vincristine, a typical substrate of *P*-glycoprotein, by MBEC4 cells. The cells were exposed to 0.1, 1 and 10 μM cyclosporin A or tacrolimus for 1 or 24 h to evaluate the dose- and time-dependent effect of the drugs on the function of *P*-glycoprotein. In treated with drug for 1 h, the uptake of 30 nM [^{3}H]vincristine was not different from that of vehicle-treated control cells, while in cells treated with drug for 24 h, the uptake of 30 nM [^{3}H]vincristine was significantly increased by the two drugs at the concentrations of 1 μM and 10 μM (Fig. 4A, B).

3.5. Effect of cyclosporin A or tacrolimus on the expression of P-glycoprotein in MBEC4 cells

To investigate whether the increase in the uptake of [^{3}H]vincristine by the drug-treated MBEC4 cells was due to the inhibition of *P*-glycoprotein function or to a decrease in *P*-glycoprotein expression, we measured the expression of *P*-glycoprotein in MBEC4 cells. The expression of *P*-glycoprotein in MBEC4 cells was decreased by the treatment with 10 μM cyclosporin A or 2.5–10 μM tacrolimus for 24 h (Fig. 5), while there was no change in the cellular protein level (data not shown). There was discrepancy in the concentration dependence between the effect of the drugs on the uptake of [^{3}H]vincristine (Fig. 4) and that on the expression of *P*-glycoprotein (Fig. 5).

3.6. Effect of cyclosporin A or tacrolimus on the content of mdr1b mRNA in MBEC4 cells

P-glycoprotein expressed on MBEC4 cells is mainly coded by the *mdr1b* gene (Tatsuta et al., 1994). We then carried out Northern blot analysis by using a probe specific to the *mdr1b* gene. The content of the *mdr1b* gene was not affected by the treatment with 0.1, 1 and 10 μM cyclosporin A or tacrolimus (Fig. 6A). To confirm this phenomenon, we performed quantitative RT-PCR analysis for *mdr1b* gene and obtained similar results (Fig. 6B).

4. Discussion

Recently, it has been reported that cyclosporin A or tacrolimus induce serious neurotoxicity, encephalopathy, and that several factors may participate in the occurrence of encephalopathy (De Groen et al., 1987; Brat et al., 1992; Worthmann et al., 1994; Hinchey et al., 1996). Because the transport of both drugs across the blood–brain barrier is restricted by *P*-glycoprotein (Tsuji et al., 1993;

Shirai et al., 1994), these drugs rarely have side effects on the central nervous system. Cyclosporin A is reported to inhibit cell growth and to induce apoptosis in rat C6 glioma cells (Mosieniak et al., 1997). Moreover, the involvement of direct cytotoxic effect of cyclosporin A on neurons in the occurrence of encephalopathy has been reported (Mosieniak et al., 1997). In this study, we hypothesized that the occurrence of encephalopathy was due to the alteration of the function or the permeability of the blood–brain barrier. We then investigated the mechanism of the immunosuppressant-induced encephalopathy based on an in vitro study using MBEC4 cells.

To examine the effect of the drugs on the permeability of the cells, the transcellular passive transport of [^{3}H]sucrose across MBEC4 cells was measured (Fig. 1). The permeability coefficient of [^{3}H]sucrose in both directions was increased by treatment with 5 μM cyclosporin A, while there was no effect of the treatment with tacrolimus within the range of 10–40 μM. These results suggested that the effect of cyclosporin A on the permeability of the cells was greater than that of tacrolimus and that cyclosporin A could compromise the barrier property of brain capillary endothelial cells. Cyclosporin A induced the release of LDH within the range of 0.5–10 μM in a dose-dependent manner, while tacrolimus induced it only at 10 μM (Fig. 2A), suggesting that both drugs injured the cell membrane and that cyclosporin A was more toxic than tacrolimus. This injury of the tight junction and the cell membrane might lead to an increase in permeability, and the exposure of nerve cells to drugs that enter the brain might induce encephalopathy. The activities of alkaline phosphatase and γ-glutamyltranspeptidase, membrane-bound enzymes, in the culture medium were not affected (Fig. 2B, C), suggesting that there was no serious injury to the membrane. Moreover, we examined the effect of the drugs on cell viability by MTT assay and the trypan blue exclusion test. There was little effect of the treatment with tacrolimus within the range of 0.5–10 μM, whereas cyclosporin A significantly decreased cell viability. These findings suggested that cyclosporin A was more toxic to MBEC4 cells than tacrolimus in the same concentration range and that the decreased cell viability was involved in the increased permeability shown in Fig. 1. Similar observations were reported by Loreal et al., 1991 and Massicot et al., 1997 in hepatocytes and a renal epithelial cell line, respectively. We considered the possible differences in toxicity between cyclosporin A and tacrolimus as follows. (1) Since the lipophilicity of cyclosporin A is higher than that of tacrolimus (Tanaka et al., 1996; Lauerma et al., 1997), cyclosporin A may enter the cell more easily. (2) These two drugs may induce cytotoxicity in an individual manner. There are almost same number of case reports of cyclosporin A- or tacrolimus-induced encephalopathies. Our data that cyclosporin A was more toxic than tacrolimus seems to be contradictory. However, the alteration of the blood–brain barrier permeability induced by these two drugs was due to a change in the paracellular route and transcellular transport system, in addition to cytotoxicity. So, the discrepancy between our cytotoxicity data and the frequency of encephalopathy may indicate that the contribution of cytotoxicity, which induces an alteration of blood–brain barrier permeability, is different between cyclosporin A and tacrolimus. However, further studies are necessary to examine the effect of the drug in a low concentration range, and longer-lasting experiments must be carried out with the drugs, because the drug concentrations in this study were approximately 10-fold higher than the concentrations used in the clinical field (cyclosporin A 80–200 nM; tacrolimus 5–20 nM).

Cyclosporin A and tacrolimus are well-known as substrates and inhibitors of *P*-glycoprotein (Takeguchi et al., 1993; Tanaka et al., 1996), and the distribution of both drugs to the brain is restricted by *P*-glycoprotein (Tsuji et al., 1993; Shirai et al., 1994). Therefore, we examined the effect of cyclosporin A or tacrolimus on the function of *P*-glycoprotein at the blood–brain barrier. As shown in Fig. 4, the uptake of [^{3}H]vincristine, a substrate of *P*-glycoprotein, by MBEC4 cells was significantly increased by the treatment with cyclosporin A or tacrolimus for 24 h compared with that of vehicle-treated control cells or that of cells treated with the drugs for 1 h. These results suggested that both drugs affected the activity of *P*-glycoprotein under the conditions of drug treatment for 24 h. Moreover, there was no difference in the potency cyclosporin A and tacrolimus. Previous studies demonstrated that the level of *P*-glycoprotein expression was altered by *P*-glycoprotein antagonists such as Ca^{2+} channel blockers (Muller et al., 1994), cyclosporin A (Herzog et al., 1993; Jette et al., 1996; Hauser et al., 1998) and that the accumulation of drugs transported by *P*-glycoprotein was modulated. To investigate whether the increase in vincristine uptake resulted from an alteration of *P*-glycoprotein expression or not, we measured the content of *P*-glycoprotein in MBEC4 cells treated with cyclosporin A or tacrolimus for 24 h. The expression of *P*-glycoprotein was decreased in the presence of a high concentration of both drugs (Fig. 5). Cyclosporin A and tacrolimus are inhibitors of *P*-glycoprotein. However, the increased uptake of vincristine by MBEC4 cells may be due to the decreased expression of *P*-glycoprotein rather than to interference with its function. This finding may explain the discrepancy in concentration dependence between the uptake of vincristine and the expression of *P*-glycoprotein. Moreover, the analysis of the level of *mdr1b* mRNA in MBEC4 cells treated with cyclosporin A or tacrolimus for 24 h was carried out to evaluate the process involved in the decreased expression of *P*-glycoprotein by these two drugs (Fig. 6A, B). The content of *mdr1b* mRNA was not changed by the treatment with 0.1, 1, 10 μM cyclosporin A or tacrolimus. These results suggest that the decreased expression of *P*-glycoprotein may be due to alteration of the metabolic turnover of *P*-glycoprotein, a process in-

volving translation. However, further studies are needed to test this hypothesis directly.

Our data indicated that the two drugs increased permeability, were cytotoxic to the cell membrane and decreased cell viability in brain capillary endothelial cells. Cytotoxicitiy was greater with cyclosporin A than with tacrolimus at the same concentration range. Moreover, the expression of *P*-glycoprotein was decreased in the presence of both drugs, and the intensity of the effect on *P*-glycoprotein was not different between cyclosporin A and tacrolimus. The decreased expression of *P*-glycoprotein induced by these two drugs may be due to alteration of the metabolic turnover of *P*-glycoprotein (inhibition of the translation process from mRNA to protein and/or accelerated degradation process, down-regulation of *P*-glycoprotein). However, in this study, we used high concentrations of the drugs compared to those used in the clinic. In the future, it is necessary to investigate the effect of these two drugs at a concentration similar to that used in clinical practice.

In conclusion, our findings suggest that a direct cytotoxic effect on brain capillary endothelial cells and an enhancement of permeability across the blood–brain barrier, based on inhibition of the efflux process, may be partly involved in the mechanism of immunosuppressant-induced encephalopathy.

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