incubated with DAF-2 DA at a final concentration of 10 μM for 1 h at 37 °C and then rinsed three times with serum-free medium. Cells containing DAF2 DA were exposed to cyclosporine (1–5 μM) for 12 h and they were washed three times with assay buffer (143 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 10 mM HEPES, and 11 mM D-glucose, pH 7.4). The fluorescence was measured using a fluorescence multiwell plate reader (Ex(λ) 485 nm; Em(λ) 530 nm, CytoFluor Series 4000). Then, cells were solubilized with 250 μl of 1 M NaOH. Aliquots of the cell solution were removed for protein assay according to the method of Bradford using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA) (Bradford, 1976). Data for each experiment were normalized to the cellular protein.

2.10. Statistical analysis

The values are expressed as the means±S.E.M. Statistical analysis was performed using Student's *t*-test. One way and two way analyses of variance (ANOVAs) followed by Tukey–Kramer's tests were applied to multiple comparisons. The differences between means were considered to be significant when P values were less than 0.05.

3. Results

3.1. Permeability and P-glycoprotein function of MBEC4 cells in MBEC4 monolayer, C6 coculture and rat astrocyte coculture systems

After MBEC4 cells were cultured for 3 days, the basal permeability and P-glycoprotein efflux pump of MBEC4 cells were evaluated in three in vitro blood–brain barrier models (Fig. 1). The permeability coefficient of Na-F for MBEC4 cells was increased by 57.6% in the C6 coculture and reduced by 45.6% in the rat astrocyte coculture, when compared to that in the MBEC4 monolayer (Fig. 1A). The accumulation of rhodamine 123 in MBEC4 cells was significantly increased by 16.8% in the C6 coculture and significantly reduced by 17.9% in the rat astrocyte coculture relative to the MBEC4 monolayer (Fig. 1B).

3.2. Effect of cyclosporine on permeability and P-glycoprotein function of MBEC4 cells in MBEC4 monolayer, C6 coculture and rat astrocyte coculture systems

As shown in Figs. 2 and 3, the exposure to cyclosporine (1–5 μM) for 12 h dose-dependently increased the permeability of Na-F and the cellular accumulation of rhodamine 123 in the MBEC4 monolayer and C6 coculture. The Na-F permeability and the rhodamine 123 accumulation of MBEC4 cells in the rat astrocyte coculture were increased to 127.7±7.2% and 126.7±3.0% of vehicle by 5 μM cyclosporine. These adverse effects of cyclosporine in the

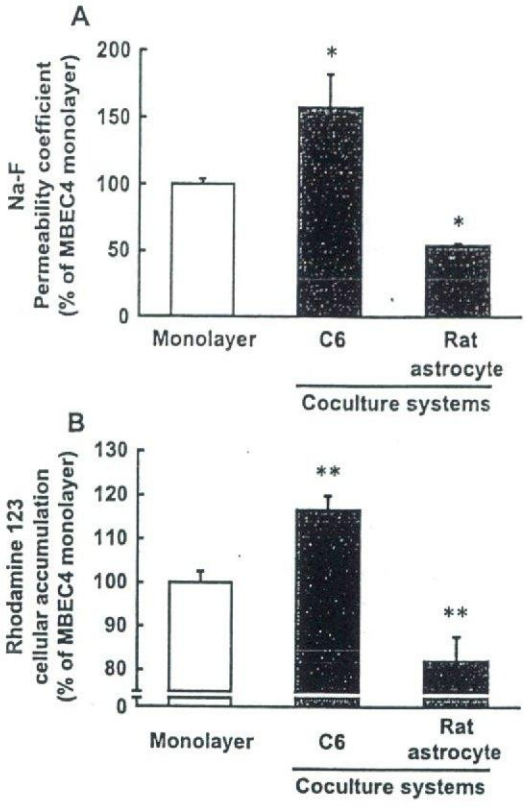

Fig. 1. Permeability and P-glycoprotein function of MBEC4 cells in MBEC4 monolayer, C6 coculture and rat astrocyte coculture systems. (A) MBEC4 permeability coefficients of Na-F. Results are expressed as % of the MBEC4 monolayer ($1.88\pm0.10\times10^{-4}$ cm/min). Values are the means± S.E.M. (n=3–7). *P<0.05, significant difference from MBEC4 monolayer. (B) Rhodamine 123 accumulation in MBEC4 cells. Results are expressed as % of the MBEC4 monolayer (2.19±0.16 nmol/mg protein). Values are the means±S.E.M. (n=4–17). **P<0.01, significant difference from MBEC4 monolayer.

presence of C6 cells and astrocytes were two- to threefold more potent than the effects in the MBEC4 monolayer.

The WST-8 assay showed that cyclosporine at the highest concentration tested (5 μM) had no effect on cell viability in any of the three culture systems (MBEC4 monolayer: 98.2±1.00, C6 coculture: 101.2±1.79, rat astrocyte coculture: 103.9±7.5% of the corresponding vehicle).

3.3. Effect of cyclosporine on stimulation-evoked NO production and spontaneous NO production in MBEC4 cells, rat astrocytes and C6 cells

Cyclosporine alone at concentrations less than 5 μM failed to stimulate NO production to a detectable level (Fig. 4). In the absence of cyclosporine, histamine (100 μM) and phenylephrine (1 μM) produced small amounts of NO in MBEC4 cells and rat astrocytes (9.27±0.69 and 0.32±0.036 μM), respectively (Fig. 4). Cyclosporine (1, 2 and 5 μM) dose-dependently increased histamine (100 μM)- and phenylephrine (1 μM)-evoked NO production in MBEC4 cells and astrocytes, respectively (Fig. 4). The increases induced by 5 μM cyclosporine reached

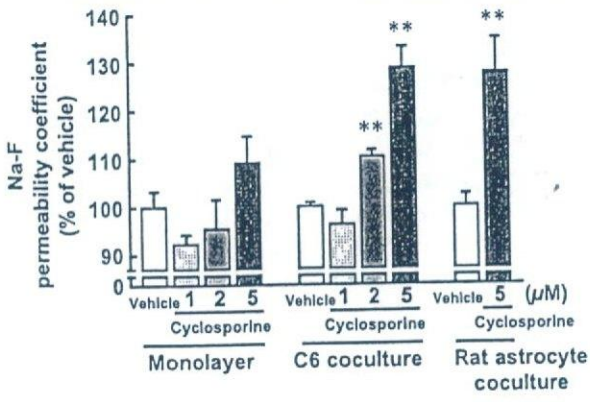

Fig. 2. Effect of cyclosporine on MBEC4 permeability of Na-F in MBEC4 monolayer, C6 coculture and rat astrocyte coculture systems. Transport experiments were performed after 12 h of exposure to cyclosporine. Results are expressed as % of each corresponding vehicle (monolayer; $2.35\pm0.24\times10^{-4}$ cm/min, C6 coculture; $2.34\pm0.25\times10^{-4}$ cm/min, rat astrocyte coculture; $1.16\pm0.03\times10^{-4}$ cm/min). Values are the means± S.E.M. (n=3–24). **P<0.01, significant difference from each corresponding vehicle.

174.1±7.7% and 198.2±15.0% of vehicle in MBEC4 cells and astrocytes, respectively.

When total amounts of NO production during a 12 h period was measured using a NO-specific fluorescent dye, an exposure of cyclosporine (5 μM) for 12 h significantly increased spontaneous (basal) NO production by 70 and 190% in MBEC4 and C6 cells, respectively (Fig. 5).

3.4. Effect of NO synthase inhibitor on cyclosporine-increased permeability of Na-F and accumulation of rhodamine 123 in MBEC4 monolayer and rat astrocyte coculture systems

L-NMMA (1 mM) (a NOS inhibitor) alone had no effect on the permeability of Na-F and the accumulation of rhodamine 123 in the MBEC4 monolayer and C6 coculture.

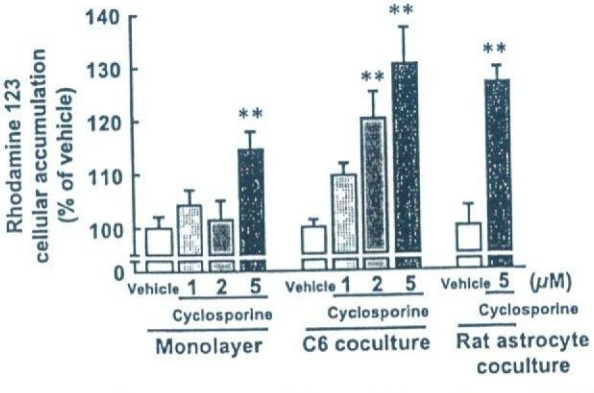

Fig. 3. Effect of cyclosporine on rhodamine 123 accumulation in MBEC4 cells of the MBEC4 monolayer, C6 coculture and rat astrocyte coculture systems. P-glycoprotein function was evaluated after 12 h of exposure to cyclosporine. Results are expressed as % of each corresponding vehicle (monolayer; 3.50±0.56 nmol/mg protein, C6 coculture; 3.79±0.47 nmol/mg protein, rat astrocyte coculture; 0.85±0.04 nmol/mg protein). Values are the means±S.E.M. (n=4–20). **P<0.01, significant difference from each corresponding vehicle.

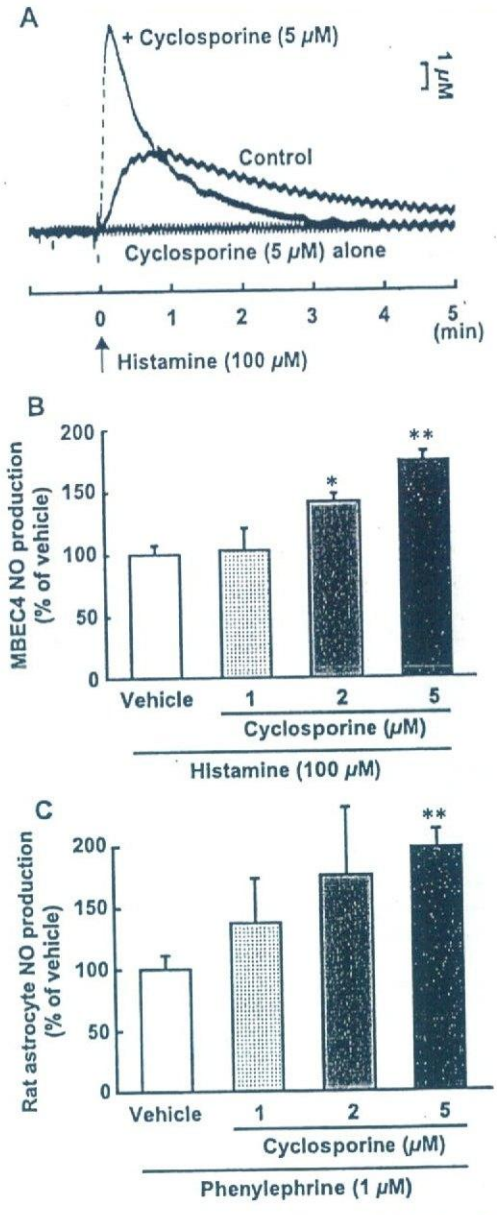

Fig. 4. Effect of cyclosporine on stimulations-evoked NO production. (A) Representative differential pulse amperogram obtained using NO biosensor shows NO production evoked by histamine (100 μM) in the absence (Control, middle trace) and presence of cyclosporine (5 μM) (+cyclosporine, top trace) in MBEC4 cells. The effect of cyclosporine (5 μM) alone on NO production was indicated in the bottom trace. (B) Concentration-dependent facilitatory effect of cyclosporine on histamine-evoked NO production in MBEC4 monolayer. Results are expressed as % of histamine (100 μM)-evoked NO production (vehicle; 9.27±0.69 μM). Values are the means± S.E.M. (n=3). *P<0.05 and **P<0.01, significant differences from vehicle. (C) Concentration-dependent facilitatory effect of cyclosporine on phenylephrine-evoked NO production in rat astrocyte coculture system. Results are expressed as % of phenylephrine (1 μM)-evoked NO production (vehicle; 0.32±0.036 μM). Values are the means±S.E.M. (n=4–7). *P<0.05 and **P<0.01, significant differences from vehicle.

Cyclosporine (5 μM)-increased permeability of Na-F and accumulation of rhodamine 123 in MBEC4 cells were significantly decreased to 114.3±5.9% and 112.6±3.7% of

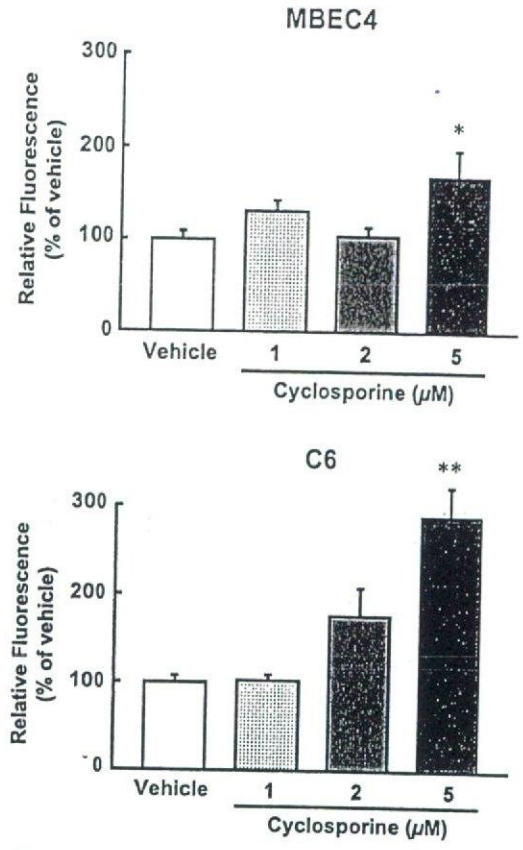

Fig. 5. Effect of cyclosporine on spontaneous NO production in MBEC4 and C6 cells. Cells were loaded with a NO-specific fluorescent dye (DAF-2 DA) and then treated with 1–5 μM cyclosporine for 12 h. The fluorescence intensity of DAF-2 DA was increased by NO produced by cells and this was normalized to the cellular protein. Relative fluorescence is expressed as the ratio (%) to the control value obtained in the absence of cyclosporine (vehicle). Values are the means±S.E.M. (n=8). *P<0.05, significant differences from vehicle.

vehicle, respectively, by 1 mM L-NMMA in the C6 coculture (Fig. 6). In contrast, L-NMMA (1 mM) produced a slight inhibition of the permeability of Na-F and the accumulation of rhodamine 123 induced by Cyclosporine (5 μM) in the MBEC4 monolayer (Fig. 6).

4. Discussion

We made three types of the in vitro blood–brain barrier models; MBEC4 monolayer, C6 coculture, and rat astrocyte coculture systems. MBEC4 cells alone show the highly specialized characteristics of brain microvascular endothelial cells including the integration of tight junctions and expression of P-glycoprotein (Tatsuta et al., 1992, 1994). Astrocytes, a cellular component of the blood–brain barrier, induce and maintain the functioning of the blood–brain barrier through cell-to-cell contact and the secretion of soluble factors (Rubin and Staddon, 1999). The barrier function in these models was evaluated based on the permeability of Na-F and the P-glycoprotein efflux pump of MBEC4 cells. Na-F was used as a marker of permeability through the paracellular route. The permeability and the accumulation of rhodamine 123 in MBEC4 cells were markedly decreased in the presence of rat astrocytes (Fig. 1). These findings suggest that astrocytes participate in tightening the intercellular junctions and facilitating P-glycoprotein function of brain endothelial cells. A positive role for astrocytes in the expression and maintenance of endothelial tight junctions has been documented (Dehouck et al., 1992; Gaillard et al., 2001; Isobe et al., 1996; Rauh et al., 1992; Hayashi et al., 1997), although there are few reports concerning astrocyte-enhanced P-glycoprotein function. The C6 cell line, which originated from rat glioma cells, is commonly used as an experimental model of astrocytes due to convenient handling (Zhang et al., 2004). The barrier function of MBEC4 cells was speculated to be

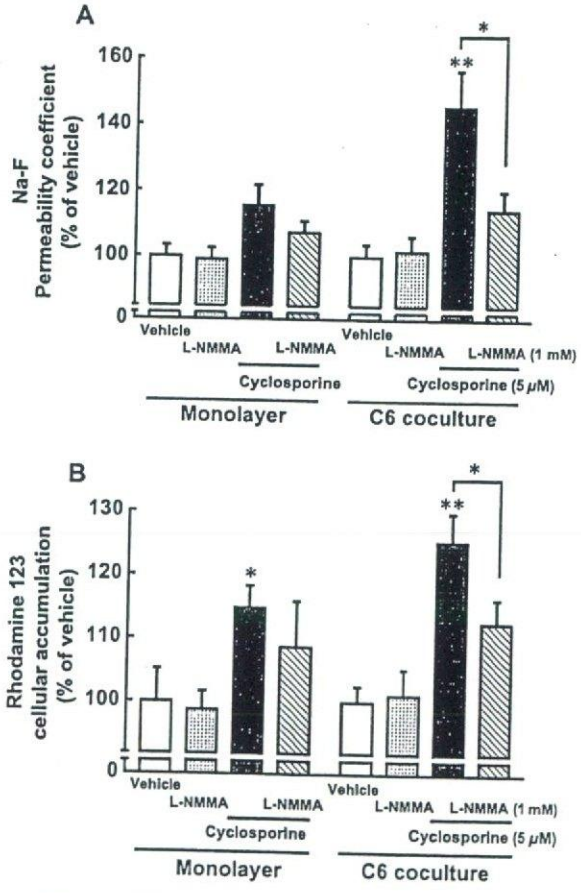

Fig. 6. Effect of NO synthase inhibitor (L-NMMA) on cyclosporine-increased MBEC4 permeability of Na-F (A), and rhodamine 123 accumulation in MBEC4 cells (B) in MBEC4 monolayer and C6 coculture system. (A) Results are expressed as % of each corresponding vehicle (monolayer; $1.73\pm0.10\times10^{-4}$ cm/min, C6 coculture; $2.95\pm0.55\times10^{-4}$ cm/min). Values are the means±S.E.M. (n=7–11). *P<0.05, **P<0.01, significant difference from each corresponding vehicle. (B) Results are expressed as % of each corresponding vehicle (monolayer; 14.2±3.94 nmol/mg protein, C6 coculture; 9.05±2.00 nmol/mg protein). Values are the means±S.E.M. (n=7–12). *P<0.05, **P<0.01, significant differences from each corresponding vehicle.

enhanced by the C6 cells similar the rat astrocyte coculture. However, the present findings indicated hyperpermeability of Na-F and reduced P-glycoprotein function in the C6 coculture. These results may be due to neoplastic changes in the glial characteristics. The precise mechanism by which C6 cells affect the barrier function of MBEC4 cells is now under investigation. There were no apparent differences in the endothelial cell response to cyclosporine between the C6 coculture and rat astrocyte coculture (Figs. 2 and 3). This confirms that the C6 coculture model is suitable for pharmacological research.

Cyclosporine-induced neurotoxicity including tremors, convulsions and encephalopathy occurred frequently in patients with high blood concentrations of cyclosporine, although these concentrations were within the therapeutic range (Gijtenbeek et al., 1999). The maximal blood concentrations of cyclosporine in patients with renal or liver transplantation are known to be in the range varying from 1 to 1.5 μM (Kahan et al., 1995; Keown and Niese, 1998; Grant et al., 1999). Therefore, the concentrations (1–5 μM) of cyclosporine used in the present study are less than three- to fivefold level of the maximal concentrations in patients. Cyclosporine (5 μM) significantly increased the permeability of Na-F and the accumulation of rhodamine 123 in the MBEC4 monolayer. These effects were potentiated markedly in the coculture with C6 cells or rat astrocytes (Figs. 2 and 3). Then, we substituted C6 cells for the primary culture of rat astrocytes to save the experimental animals, costs and labor in the subsequent pharmacological study. The presence of C6 cells increased by two- to threefold the facilitatory and inhibitory effects of cyclosporine (1–5 μM) on the paracellular permeability and P-glycoprotein activity of the MBEC4 monolayer, respectively. These effects were not due to the direct cytotoxicity of cyclosporine. These findings indicated that cyclosporine reduces the barrier function of brain endothelial cells to penetrate into the brain, leading to further aggravation of the blood–brain barrier function by interacting with astrocytes.

A 12-h exposure of cyclosporine (5 μM) in the same schedule as the blood–brain barrier function test significantly increased the accumulation of spontaneous NO production during a 12-h period, when measured using a NO-specific fluorescent dye (Fig. 5). A direct electrochemical NO monitoring failed to detect cyclosporine-increased basal NO production (Fig. 4). This monitoring method is capable of detecting a rapid NO production in response to the stimulation as a sharp current–time curve, even if amounts of NO production are small. However, NO biosensor is relatively difficult to detect a slow NO production with a low peak and a long-lasting plateau phase, even if total amounts during a long period is large. Cyclosporine dose-dependently enhanced histamine- and phenylephrine-evoked NO production in MBEC4 cells and rat astrocytes, respectively (Fig. 4). Cyclosporine exerts pharmacological effects by binding to cyclophilin (peptidyl-propyl isomerase), a highly basic and abundant cytosolic protein (Marks, 1996). This cyclosporine/cyclophilin complex inhibits calcineurin, a serine–threonine phosphatase 2B, thereby blocking its phosphatase activity (Marks, 1996; Yakel, 1997). Calcineurin regulates the activity of ion channels and neurotransmitter release. Calcineurin anchored to the inositol 1,4,5-triphosphate (IP_3) receptor via FKBP12, a FK506-binding protein, regulates the phosphorylation status of the receptor, resulting in a dynamic Ca^{2+}-sensitive regulation of IP_3-mediated Ca^{2+} flux (Cameron et al., 1997). In contrast with FKBP12, cyclophilin does not bind to the IP_3 receptor (Cameron et al., 1995). However, when rat cerebellar microsomes were treated with cyclosporine and cyclophilin, protein kinase C-induced IP_3 receptor phosphorylation and IP_3-stimulated Ca^{2+} flux were markedly increased (Cameron et al., 1995). This suggests that cyclosporine inhibits the dephosphorylation of the IP_3 receptor to maintain a leaky IP_3 receptor channel that was phosphorylated by serine–threonine protein kinases such as protein kinase C. Although the IP_3 receptor becomes leaky, cyclosporine alone appears incapable of elevating $[Ca^{2+}]_{IN}$ over the threshold for activation of the constitutive NO synthase. This may explain the present findings that cyclosporine alone failed to raise NO production above the detection limit of the NO biosensor, while the histamine and phenylephrine-evoked NO production in MBEC4 and C6 cells, respectively, was markedly facilitated by cyclosporine. Histamine and phenylephrine activate phospholipase C through the H_1 receptor and α_1-adrenoceptor, respectively, to generate IP_3 (Lum and Malik, 1994; Daum et al., 1983) and stimulate the leaky Ca^{2+} channel (phosphorylation status of IP_3 receptor) maintained by cyclosporine. These events probably lead to the markedly higher level of $[Ca^{2+}]_{IN}$ than that induced by histamine and phenylephrine in MBEC4 cells and rat astrocytes, respectively. We previously reported that cyclosporine also enhanced α_1-adrenoceptor-mediated NO production in C6 cells (Ikesue et al., 2000). In the brain, various biological substances including noradrenaline, glutamate, histamine and endothelin stimulate G protein-coupled receptors that have a common intracellular signaling pathway (IP_3/diacylglycerol) in astrocytes (Verkhratsky and Kettenmann, 1996). This endogenous stimulator-evoked NO production is highly likely to be augmented by cyclosporine via a mechanism similar to that proposed here. In fact, our in vivo microdialysis experiment showed that an intraperitoneal injection of cyclosporine significantly increased NO production in the rat dorsal hippocampus (Fujisaki et al., 2002).

The present study demonstrated that cyclosporine impaired the barrier function of brain endothelial cells and this effect was remarkably potentiated by co-culturing MBEC4 cells with C6 cells or rat astrocytes. A NO synthase inhibitor, L-NMMA at a concentration of 1 mM showed no effect on the Na-F permeability and the rhodamine 123 accumulation of MBEC4 cells in both culture systems (Fig. 6), suggesting that L-NMMA (1 mM)

has not nonspecific effect on the basal blood–brain barrier functions of MBEC4 cells. L-NMMA (1 mM) significantly blocked the cyclosporine-induced increase in the permeability of Na-F and accumulation of rhodamine 123 in the C6 coculture. This protective effect was moderate in the MBEC4 monolayer (Fig. 6). These findings suggest that cyclosporine-enhanced NO production in astrocytes largely contributes to an impairment of the blood–brain barrier. This notion is supported by our previous findings that NO lowered the function of tight junctions and P-glycoprotein at the blood–brain barrier (Yamauchi et al., in press). The mechanisms by which NO donors increased vascular endothelial permeability involved an increase in the level of cGMP (Gimeno et al., 1998) or the formation of peroxynitrite (Menconi et al., 1998). These substances conceivably influence intrinsic tight junction proteins and the associated actin cytoskeleton through a direct or second signaling pathway (Burgstahler and Nathanson, 1995; Liu and Sundqvist, 1997). It is, therefore, likely that cyclosporine passes through the slightly impaired barrier of brain endothelial cells and then acts on astrocytes to enhance NO production, leading to further aggravation of the blood–brain barrier impairment.

In conclusion, cyclosporine accelerated stimulation-evoked NO production in brain endothelial and astroglial cells. This enhanced production of NO that interacts with each cellular component of the blood–brain barrier is involved in the sequential process of blood–brain barrier functional impairment induced by cyclosporine.

Acknowledgements

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Tacrolimus-Induced Neurotoxicity and Nephrotoxicity Is Ameliorated by Administration in the Dark Phase in Rats

Atsushi Yamauchi,[1,3] Ryozo Oishi,[2] and Yasufumi Kataoka[1]

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SUMMARY

1. Tacrolimus, a potent immunosuppressant, induces impaired renal function and neurological complications. We investigated the influence of dosing time on the neurotoxicity, nephrotoxicity, and immunosuppressive effect of tacrolimus in rats.

2. The repeated injection of tacrolimus in the light phase (8:00) produced a significantly greater increase than that in the dark phase (20:00) in the duration of harmine-induced tremors and in the blood urea nitrogen (BUN) concentration in rats. An immunosuppressive effect of tacrolimus on the xenotransplantation of mouse-to-rat skin grafts was apparent in the dark phase but not in the light phase.

3. The dosing time-dependent pharmacokinetic results were not observed when tacrolimus concentrations in rat whole blood were measured after a single or repeated injection in the light or dark phase.

4. These findings suggest that treatment in the active phase of the diurnal cycle ameliorates neurotoxicity and nephrotoxicity while maintaining the immunosuppressive effect of tacrolimus. The present findings have important implications for therapeutic approaches to avoid tacrolimus-induced neurotoxicity and nephrotoxicity.

KEY WORDS: tacrolimus; neurotoxicity; nephrotoxicity; circadian rhythm; rats.

INTRODUCTION

Tacrolimus is a potent immunosuppressant that blocks calcineurin-mediated T cell activation by binding to immunophilin (FKBP12). This compound is used to prevent allograft rejection in solid organ transplantation and in fatal graft-versus-host diseses after bone marrow transplantation. Multicenter, randomized trials in the USA and Europe demonstrated that tacrolimus induced impaired renal function and neurological complications with a relatively high frequency (20–40%) (European FK506 Multicentre Liver Study Group, 1994; The U.S. Multicenter FK506 Liver Study

[1] Department of Pharmaceutical Care and Health Sciences, Faculty of Pharmaceutical Sciences, Fukuoka University, Nanakuma, Jonan-ku, Fukuoka, Japan.

[2] Department of Hospital Pharmacy, Faculty of Medicine, Kyushu University, Maidashi, Higashi-ku, Fukuoka, Japan.

[3] To whom correspondence should be addressed at Department of Pharmaceutical Care and Health Sciences, Faculty of Pharmaceutical Sciences, Fukuoka University, 8-19-1 Nanakuma, Jonan-ku, Fukuoka, 814-0180, Japan; e-mail: atyama@cis.fukuoka-u.ac.jp.

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Group, 1994). Neurotoxicity including tremors, convulsions, and encephalopathy occurred frequently in patients with high blood concentrations of tacrolimus, although these concentrations were within the therapeutic range. The efficacy or toxicity of drugs such as anticancer drugs has been shown to depend on the dosing time (Levi *et al.*, 1997; Ohdo *et al.*, 2001). Various physiological rhythms in living organisms are considered to be responsible for such chronopharmacological reactions to drugs. Chronotherapy including an optimization of the dosing time is capable of producing more efficient and safer prescriptions for patients than conventional therapy.

The mechanisms of neurotoxicity and nephrotoxicity of tacrolimus are not fully understood. On the basis of our findings plus several reports concerning cyclosporine, an immunosuppressive agents, we present the notion that the immunophilin ligands exert neurotoxicity because of an inhibition of γ-aminobutyric acid neurotransmission and an activation of serotonergic neural activity and nitric oxide production (Fujisaki *et al.*, 2002; Ikesue *et al.*, 2000; Shuto *et al.*, 1998, 1999; Snyder *et al.*, 1998; Steiner *et al.*, 1996; Tominaga *et al.*, 2001;). Several bioactive substances including renine, endothelins, transforming growth factor-β, and nitric oxide are involved in the nephrotoxicity induced by cyclosporin and tacrolimus (Bobadilla *et al.*, 1998; Isram *et al.*, 2001; Kupferman *et al.*, 1994; Lanese *et al.*, 1994; Lanese and Conger, 1993). The physiological activities of these various substances in the brain or kidney are known to indicate circadian rhythms (Brandenberger *et al.*, 1994; Cardinali *et al.*, 1998; Hutson *et al.*, 1984; Hwang *et al.*, 1998). It is, therefore, likely that the incidence of adverse reactions to tacrolimus shows dosing time-dependent variations.

In the present study, we investigated the influence of subchronic treatment with tacrolimus in the light or dark phase of a day for 1–2 weeks on the occurrence of harmine-induced tremors and renal dysfunction in rats. The dosing time-dependent immunosuppressive effect of tacrolimus was also examined using a mouse-to-rat xenotransplantation model. To test whether pharmacokinetic factors are involved in the chronopharmacological action of tacrolimus, the amounts of tacrolimus in rat whole blood were assessed at various periods after a single or subchronic injection in the light or dark phase.

MATERIALS AND METHODS

Animals

Male Wistar rats (7 weeks old) were purchased from Kyudo (Saga, Japan). The animals were maintained on a 12 h light/dark schedule (lights on 7:00 A.M.) at a temperature of 23 ± 2°C with free access to food and water. They were adapted to the light/dark cycle for 2 weeks before the experiments. All the procedures involving experimental animals adhered to the law (No. 105) and notification (No. 6) of the Japanese Government, and were approved by the Laboratory Animal Care and Use Committee of Fukuoka University.

Drugs

Tacrolimus (prograf® injection: Fujisawa Pharmaceutical, Osaka, Japan) was diluted with saline immediately before use. The vehicle solution for the control consisted of polyoxyethylene castor oil (Cremophor EL®, Sigma Chemical CO., St. Louis, MO), ethanol (Wako Pure Chemical Industries Ltd., Osaka, Japan), and saline. This composition was the same as that for the Prograf® injection. Harmine hydrochloride (Sigma Chemical CO., St. Louis, MO) was dissolved in saline. Tacrolimus, vehicle, and harmine were injected intraperitoneally (i.p.) in a volume of 0.5, 0.5, and 0.1 mL/100 g body weight, respectively.

Influence of the Dosing Time of Tacrolimus on Harmine-Induced Tremors and the Concentrations of Serum BUN and Creatinine

Effects of tacrolimus on harmine-induced tremors were evaluated as previously described (Shuto *et al.*, 1998). Tacrolimus (2 mg/kg) or vehicle was injected i.p. once a day for 7 days at 8:00 for the light group or 20:00 for the dark group. Each rat was placed in a screened cage (21.5 × 32× 14 cm) immediately after the 7th injection of tacrolimus to allow adaptation to the new environment and then was introduced to the tremor test. Harmine (10 mg/kg i.p.) was injected 55 min after the final tacrolimus injection. The summed duration of harmine-induced tremors was measured during the 15-min period from 5 to 20 min after harmine administration by the same observer blinded to the pretreatment with tacrolimus or vehicle.

To determine the serum creatinine and BUN concentrations with a Vision analyzer (Abbot Laboratories, Abbot Park, III), blood samples were collected from the rat tail vein 24 h after the final chronic treatment (once a day for 14 days) with tacrolimus (2 mg/kg i.p.) or vehicle at 8:00 or 20:00.

Influence of the Dosing Time of Tacrolimus on Xenograft Survival in Rats Following Xenotransplantation of a Mouse-To-Rat Skin Graft

To make the xenotransplantation model of the mouse-to-rat skin graft, Wistar rats and C57BL/6J mice (15–20 g) were employed as recipients and donors, respectively. The animals were operated on under sodium pentobarbital anesthesia. A square piece (1 cm^2) of skin obtained from the back of the donor mouse was transplanted to where the skin (1 cm^2) was removed from the back of the recipient rat. The skin grafts were protected by gauze with gentacin ointment and bandages for 6 days and rats were housed individually (Shapira *et al.*, 1999).

Xenotransplantation was performed immediately after the first injection of tacrolimus (2 mg/kg i.p.) or vehicle at 8:00 (light group) or 20:00 (dark group). The duration of xenograft survival was determined in each rat with the repeated injection of tacrolimus or vehicle (once a day at 8:00 or 20:00). Rejection was defined as a complete separation of the graft (Shapira *et al.*, 1999).

Pharmacokinetic Influence of the Dosing Time of Tacrolimus

Tacrolimus (2 mg/kg i.p.) was injected once a day at 8:00 (light group) or 20:00 (dark group). Blood samples were obtained from the abdominal aorta at 0.5, 1, 2, 4, 8, 12, and 24 h after a single injection or 24 h after the 7th injection. Tacrolimus concentrations in whole blood were measured with an enzyme immunoassay (IMx; Abbot Laboratories, Abbot Park, III).

Statistical Analysis

Data are expressed as means ± S.E.M. A one-way analysis of variance (ANOVA) followed by the post hoc Sheffe's F-test was used to analyze the concentrations of BUN, creatinine, and tacrolimus. The duration of harmine-induced tremors and that of skin graft survival were evaluated using the Mann–Whitney U-test and log-rank test, respectively. Statistical significance was defined as $P < 0.05$.

RESULTS

Influence of the Dosing Time of Tacrolimus on Harmine-Induced Tremors and the Serum BUN and Creatinine Concentrations

On the basis of the results of preliminary experiments, the dose of harmine was selected to induce a minimal tremor response (the duration of tremors ranged from 0 to 100 s in the light and dark group). Tacrolimus (2 mg/kg i.p., once a day for 7 days) significantly prolonged harmine-induced tremors 2.7-fold compared to the vehicle in the light group ($P < 0.05$), but not in the dark group (Fig. 1). The facilitatory action of tacrolimus on harmine-induced tremors was greater in the light group than dark group (Fig. 1; $P < 0.05$).

In the light group, tacrolimus (2 mg/kg i.p., once a day for 14 days) increased significantly BUN concentrations by 74.7% (Fig. 2(A), $P < 0.01$) and moderately raised creatinine concentrations by 39.5% (Fig. 2(C)). Tacrolimus-affected BUN levels were significantly higher in the light group than dark group ($P < 0.05$). In the dark group, there were only slight differences in the serum BUN and creatinine concentrations between tacrolimus and vehicle treatment (Fig. 2(B) and 2(D)).

Influence of the Dosing Time of Tacrolimus on Xenograft Survival

Figure 3 shows survival curves of the skin xenograft for the light (A) and dark (B) group. The mouse-to-rat skin graft transplanted in the light phase (vehicle; 14.8 ± 0.6 days) survived for a longer period than that transplanted in the dark phase (vehicle; 12.3 ± 0.5 days). In the dark group, tacrolimus (2 mg/kg i.p.) significantly increased the mean survival time by 20% ($P < 0.05$), leading to almost the same levels as those in the light group treated with vehicle or tacrolimus. In the light group, no significant differences were observed between vehicle and tacrolimus treatment.

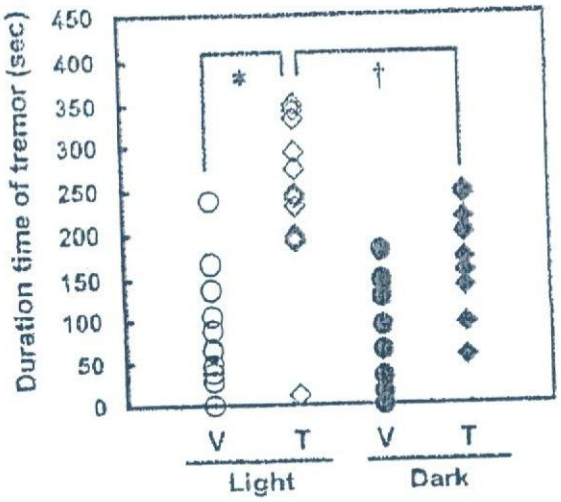

Fig. 1. Influence of the dosing time (8:00 (light) or 20:00 (dark)) of tacrolimus on harmine-induced tremors in rats. The animals were subjected to subchronic treatment with tacrolimus (T) (2 mg/kg i.p.) or vehicle (V) once a day for 7 days. Rats were injected with harmine (10 mg/kg i.p.) 55 min after the final injection of vehicle or tacrolimus. The summed duration of tremors was measured during a 15 min period from 5 to 20 min after harmine injection ($n = 10$–12 per group). $^{*}P < 0.05$; significantly different from the vehicle-treated group. $^{\dagger}P < 0.05$; significantly different from the tacrolimus-treated group in the dark phase.

Pharmacokinetics Influence of the Dosing Time of Tacrolimus After Single or Repeated Injection

The curves for mean tacrolimus concentration versus time after single injections are shown in Fig. 4(A). Tacrolimus levels in whole blood at 1, 4, and 8 h after injection were slightly, although not significantly, higher in the light group than dark group. As shown in Fig. 4(B), the dosing time (light or dark phase) for the repeated injection of tacrolimus had no significant influence on trough levels.

DISCUSSION

The present study demonstrated that the repeated injection of tacrolimus in the light phase (8:00) produced a significantly greater increase than that in the dark phase (20:00) in the duration of harmine-induced tremors and in BUN concentrations in rats. An immunosuppressive effect of tacrolimus on the xenotransplantation of a mouse-to-rat skin graft was apparent in the dark phase but not in the light phase. These findings suggest that treatment in the dark phase (an active phase in the rat diurnal rhythm) ameliorates the neurotoxicity and nephrotoxicity while maintaining the immunosuppressive effect of tacrolimus in rats.

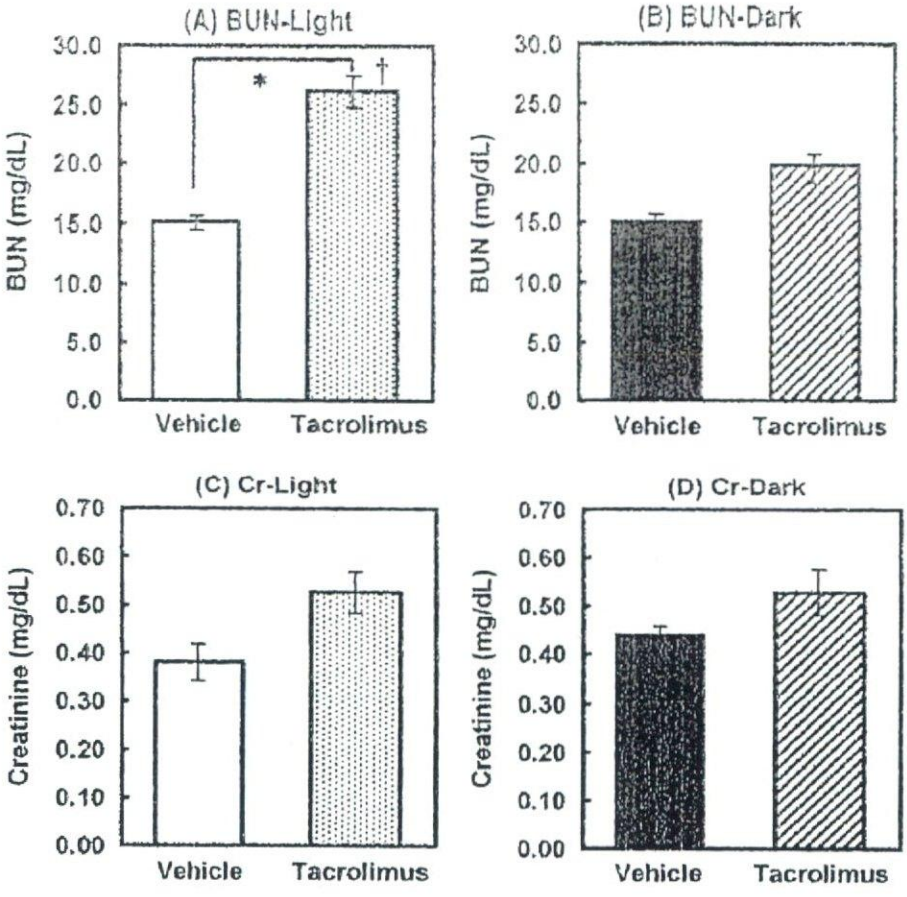

Fig. 2. Influence of the dosing time (8:00 or 20:00) of tacrolimus on the BUN and creatinine concentrations in rats. The animals were subjected to subchronic treatment with tacrolimus (2 mg/kg i.p.) or vehicle once a day for 14 days. Blood samples were collected from the tail vein 24 h after the final tacrolimus injection. Values represent the means ± S.E.M. for 5–7 rats. (A) The BUN concentrations in the light group (BUN-light); (B) The BUN concentrations in the dark group (BUN-dark); (C) The creatinine concentrations in the light group (Cr-light); (D) The creatinine concentrations in the dark group (Cr-dark). $^{**}P < 0.01$; significantly different from the vehicle-treated group. $^{\dagger}P < 0.05$; significantly different from the tacrolimus-treated group in the dark phase.

No chronopharmacological research has been published concerning immunosuppressant-induced neurotoxicity. The present study is the first to provide evidence that a facilitatory action of tacrolimus on harmine-induced tremors shows a diurnal rhythm with a potent effect in the light phase in rats. Harmine is reported to induce tremors by activating serotonergic neurons and inhibiting dopaminergic neurons (Kawanishi *et al.*, 1981). Shuto *et al.* (1998) demonstrated that cyclosporine facilitates serotonergic neural activity to accelerate harmine-induced tremors. This acceleration appeared to be involved in an inhibition of γ-aminobutyric acid neural activity and receptors (Shuto *et al.*, 1999; Tominaga *et al.*, 2001). The precise mechanisms by which tacrolimus neurotoxicity exhibits a diurnal rhythm remains to be determined.

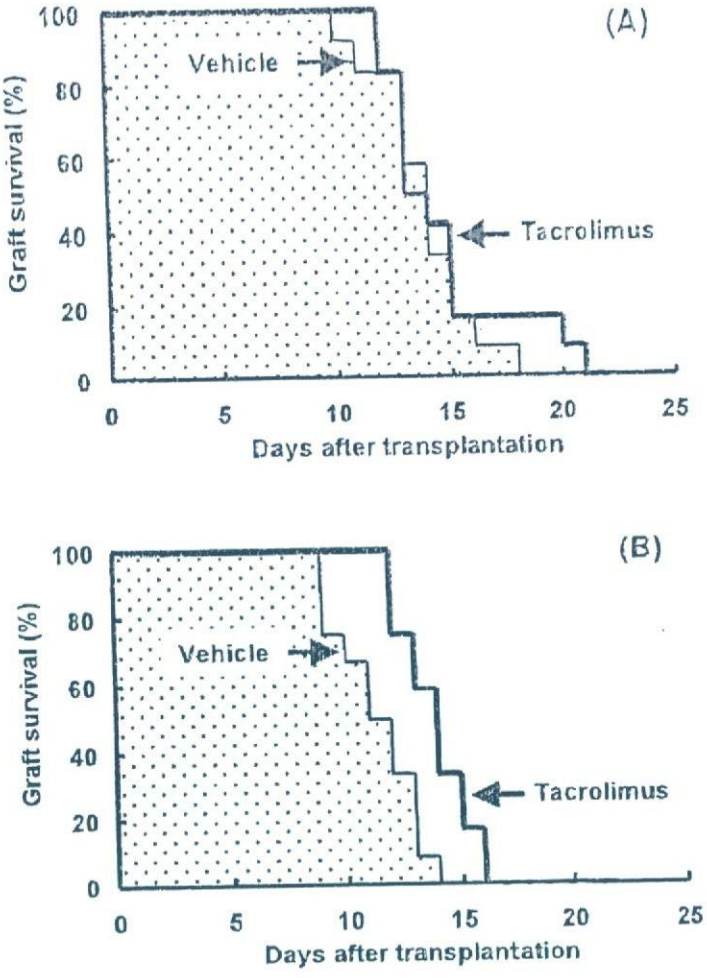

Fig. 3. Influence of the dosing time (8:00 (A) and 20:00 (B)) of tacrolimus on the survival of skin xenografts in a mouse-to-rat skin xenotransplantation model. The animals were subjected to chronic treatment with tacrolimus (2 mg/kg i.p., thick line) or vehicle (thin line and dotted area) once a day until rejection of the graft occurred. Rejection was defined as a complete separation of the graft in each rat. Data are expressed as the percentage of rats in which the graft survived in each group (12 rats each). $^{*}P < 0.05$: significantly different from the vehicle-treated group.

The dosing time-dependent nephrotoxicity of tacrolimus in rats was previously reported by Fujimura and Ebihara (1994). Contrary to our findings, they showed that the tacrolimus-induced elevation in the BUN and creatinine levels was more prominent in the dark phase than in the light phase in rats. Their pharmacokinetic results suggested that an aggravation of renal function induced by tacrolimus in the dark phase is closely associated with the high concentrations of tacrolimus in whole blood in the dark phase. This diurnal rhythm was not observed in the present pharmacokinetic study. The discrepancy in these findings may be due to the different route of administration (an intraperitoneal route in our study versus an oral route

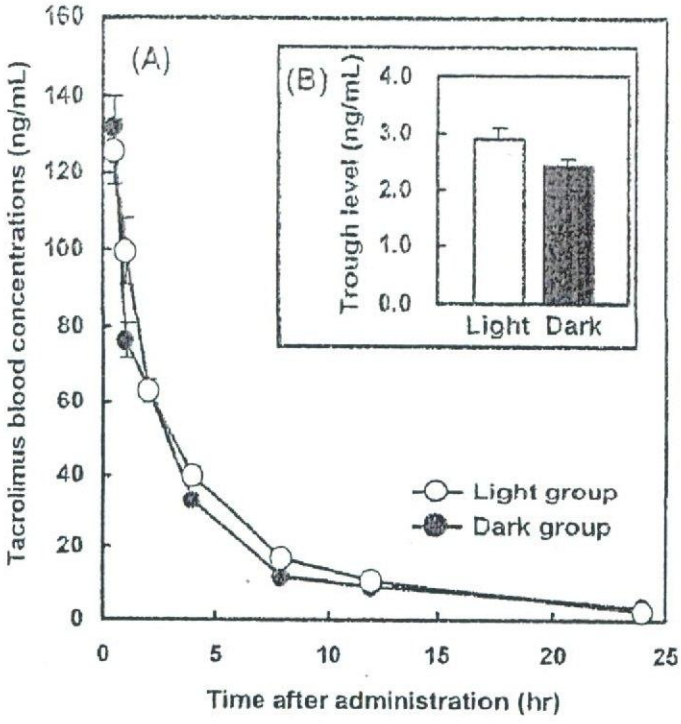

Fig. 4. The pharmacokinetic influence of the dosing time (8:00 (light) or 20:00 (dark)) of tacrolimus (2 mg/kg i.p.) after a single (A) or repeated (B) injection. Blood samples were obtained from the abdominal aorta at 0.5, 1, 2, 4, 8, 12, and 24 h after a single injection or 24 h after repeated injection (once a day for 7 days). Values represent means ± S.E.M. for 5–6 rats.

in Fujimura's study). In the oral administration of tacrolimus, the maximum concentration or area under the concentration–time curve (AUC) of tacrolimus was significantly increased when tacrolimus was given in the morning in humans or in the dark phase in nocturnal rodents (Fujimura *et al.*, 1993, Min *et al.*, 1996, Uchida *et al.*, 1999). These phenomena seem to be caused by several diurnal rhythm-related variables including food ingestion, gastric emptying time, gastric motility, secretion of bile acid, blood flow to the gastrointestinal tract, and gastrointestinal perfusion. In the present study, the dosing time-dependent influences are not involved in tacrolimus absorption, since tacrolimus was intraperitoneally administered. Interestingly, the AUC of tacrolimus on continuous intravenous administration in humans showed no differences between daytime and nighttime (Satoh *et al.*, 2001). This is consistent with our results suggesting that the pharmacokinetic features of tacrolimus following parenteral administration probably indicate no circadian variation. Therefore, the pharmacokinetic factors may be excluded from the mechanisms of diurnal changes in tacrolimus-induced toxicity and efficacy in the present study. The possibility that the distribution of tacrolimus in the kidney and brain has diurnal variations remains to be examined.

Calcineurin inhibitors including tacrolimus and cyclosporine exert an immunosuppressive action due to the inhibition of interleukin (IL)-2 transcription, leading

to T cell inactivation (Liu, 1993; Wiederrecht *et al.*, 2000). Since a diurnal rhythm exists in the secretion of IL-2 or the number of T cells (Born *et al.*, 1997; Palm *et al.*, 1996), the immunosuppressive effects of calcineurin inhibitors are speculated to depend on the dosing time during the day. In the present study, the administration of tacrolimus in the dark phase significantly prolonged the survival time of skin grafts in the xenograft transplantation model. These findings were supported by a report concerning the effect of cyclosporine on a murine heart allograft model (Cavallini *et al.*, 1983). The efficacy of tacrolimus or cyclosporine is likely to increase when the agent is administered in the dark phase in the rodents.

In conclusion, we provided evidence that treatment in the active phase ameliorates the neurotoxicity and nephrotoxicity while maintaining the immunosuppressive effect of tacrolimus in rats. The present findings have important implications for therapeutic approaches to avoid tacrolimus-induced neurotoxicity and nephrotoxicity.

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Rapid Communication

Transforming Growth Factor-β1 Upregulates the Tight Junction and P-glycoprotein of Brain Microvascular Endothelial Cells

Shinya Dohgu,[1,2] Atsushi Yamauchi,[2] Fuyuko Takata,[2] Mikihiko Naito,[3] Takashi Tsuruo,[3] Shun Higuchi,[1] Yasufumi Sawada,[1] and Yasufumi Kataoka[2,4]

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SUMMARY

1. The present study was aimed at elucidating effects of transforming growth factor-β (TGF-β) on blood–brain barrier (BBB) functions with mouse brain capillary endothelial (MBEC4) cells.

2. The permeability coefficients of sodium fluorescein and Evans blue albumin for MBEC4 cells and the cellular accumulation of rhodamine 123 in MBEC4 cells were dose-dependently decreased after a 12-h exposure to TGF-β1 (0.01–10 ng/mL).

3. The present study demonstrates that TGF-β lowers the endothelial permeability and enhances the functional activity of P-gp, suggesting that cellular constituents producing TGF-β in the brain may keep the BBB functioning.

KEY WORDS: transforming growth factor-β; blood–brain barrier; permeability; P-glycoprotein; mouse brain endothelial cells.

INTRODUCTION

The Blood–brain barrier (BBB) is highly restrictive of the transport of substances between blood and the central nervous system. The BBB is a complex system of different cellular component consisting brain microvascular endothelial cells, pericytes

[1] Department of Medico-Pharmaceutical Sciences, Graduate School of Pharmaceutical Sciences, Kyushu University, Higashi-ku, Fukuoka, Japan.
[2] Department of Pharmaceutical Care and Health Sciences, Faculty of Pharmaceutical Sciences, Fukuoka University, Jonan-ku, Fukuoka, Japan.
[3] Institute of Molecular and Cellular Biosciences, University of Tokyo, Bunkyo-ku, Tokyo, Japan.
[4] To whom correspondence should be addressed at Department of Pharmaceutical Care and Health Sciences, Faculty of Pharmaceutical Sciences, Fukuoka University, 8-19-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan; e-mail: ykataoka@cis.fukuoka-u.ac.jp.

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and astrocytes. Astrocytes induce and maintain the properties of the BBB including the integration of tight junctions and expression of P-glycoprotein (P-gp) through cell-to-cell contact and secretion of soluble factors (Rubin and Staddon, 1999). Brain pericytes are important for the control of growth and migration of endothelial cells and the integrity of microvascular capillaries (Ramsauer *et al.*, 2002). These functions are mediated, at least in part, by transforming growth factor-β (TGF-β), a family of multifunctional peptide growth factors (Orlidge and D'Amore, 1987; Sato and Rifkin, 1989). TGF-β isoforms (TGF-β1, 2, 3, 4, and 5) share the same structure (65–80% homology) and display similar biological activity in vitro (Flanders *et al.*, 1998). TGF-β is listed as a compound protecting against neurodegeneration (Flanders *et al.*, 1998). Several cytokines and growth factors influence on the permeability of the BBB, such as vascular endothelial growth factor (Wang *et al.*, 1996) and tumor necrosis factor-α (Deli *et al.*, 1995). However, little is known about the role of TGF-β in the maintenance of BBB function. In the present study, effects of TGF-β1 were examined on the permeability of brain endothelial cells and the functional activity of P-gp. We used mouse brain endothelial (MBEC4) cells showing the highly specialized characteristics of brain microvascular endothelial cells including the expression of P-gp (Tatsuta *et al.*, 1992, 1994).

MATERIALS AND METHODS

MBEC4 cells, which were isolated from BALB/c mouse brain cortices and immortalized by SV40-transformation (Tatsuta *et al.*, 1992), were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO BRL, Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum, 100 units/mL of penicillin, and 100 μg/mL of streptomycin. They were grown in 12-well Transwells^{TM} (Costar, MA) and 24-well plates (FALCON; Becton Dickinson Labware, Lincolin Park, NJ) in a humidified atmosphere of 5% CO_2/95% air at 37°C.

MBEC4 cells (42,000 cells/cm^2) were cultured on the collagen-coated polycarbonate membrane (3.0 μm pore size) of the Transwell^{TM} insert (12-well type). After culture for 3 days, they were washed three times with serum-free medium. Cells were exposed to 0.01–10 ng/mL of TGF-β1 (Sigma, St. Louis) injected into the inside of the insert (luminal side) for 12 h. To initiate the transport experiments, the medium was removed and cells were washed three times with Krebs–Ringer buffer (118 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 11 mM D-glucose, pH 7.4). Krebs–Ringer buffer (1.5 mL) was added outside of the insert (abluminal side). Krebs–Ringer buffer (0.5 mL) containing 100 μg/mL of sodium fluorescein (Na-F) (MW 376, Sigma, St. Louis) and 4% bovine serum albumin (Sigma, St. Louis) mixed with 0.67 mg/mL of Evans blue dye (EBA) (MW 67,000, Wako, Osaka, Japan) was loaded on the luminal side of the insert. Samples (0.5 mL) were removed from the abluminal chamber at 10, 20, 30, and 60 min and immediately replaced with fresh Krebs–Ringer buffer. Aliquots (5 μL) from the abluminal chamber samples were mixed with 200 μL of Krebs–Ringer buffer and then the concentration of Na-F was determined using a multiwell fluorometer (Ex(λ) 485 nm; Em(λ) 530 nm) (CytoFluor Series 4000, PerSeptive Biosystems, Framingham, MA).

The EBA concentration in the abluminal chamber was measured by determining the absorbance of aliquots (150 μL) at 630 nm with a microplate reader (Opsys MR, DYNEX technologies, Chantilly, VA). The permeability coefficient and clearance were calculated according to the method described by Dehouck *et al.* (1992). Clearance was expressed as microliter (μL) of tracer diffusing from the luminal to abluminal chamber and was calculated from the initial concentration of tracer in the luminal and final concentration in the abluminal chamber: Clearance (μL) = $[C]_A \times V_A/[C]_L$ where $[C]_L$ is the initial luminal tracer concentration, $[C]_A$ is the abluminal tracer concentration, and V_A is the volume of the abluminal chamber. During a 60-min period of the experiment, the clearance volume increased linearly with time. The average volume cleared was plotted versus time, and the slope was estimated by linear regression analysis. The slope of clearance curves for the MBEC4 monolayer was denoted PS_{app}, where PS is the permeability × surface area product (in μL/min). The slope of the clearance curve with a control membrane was denoted $PS_{membrane}$. The real PS value for the MBEC4 monolayer (PS_{trans}) was calculated from $1/PS_{app} = 1/PS_{membrane} + 1/PS_{trans}$. The PS_{trans} values were divided by the surface area of the Transwell inserts to generate the permeability coefficient (P_{trans}, in cm/min).

The functional activity of P-gp was determined by measuring the cellular accumulation of rhodamine 123 (Sigma, St. Louis) according to the method of Fontaine *et al.* (1996). MBEC4 cells (21,000 cells/cm^2) were cultured on the collagen-coated 24-well plates. Three days after seeding cells, they were washed three times with serum-free medium and then exposed to 0.01–10 ng/mL of TGF-β1 for 12 h. The medium was removed and cells were washed three times with assay buffer (143 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 10 mM HEPES, and 11 mM D-glucose, pH 7.4). Cells were incubated with 0.5 mL of assay buffer containing 5 μM rhodamine 123 for 1 h. Then, the solution was removed and cells were washed three times with ice-cold phosphate-buffered saline and solubilized in 0.2 N NaOH (0.5 mL). The rhodamine 123 content was determined using a multiwell fluorometer (Ex(λ) 485 nm; Em(λ) 530 nm) (CytoFluor Series 4000, PerSeptive Biosystems, Framingham, MA). The cellular protein was measured by the method of Bradford (1976).

The values are expressed as means ± SEM. Statistical analysis was performed using Student's unpaired *t* test. A single-factor analysis of variance (ANOVA) followed by the Dunnett test was applied to multiple comparisons. The differences between means were considered to be significant when *P* values were less than 0.05.

RESULTS

TGF-β1 at a concentration of 10 ng/mL significantly decreased the permeability of the cells to Na-F and EBA at 3 h by 71.9 and 55.5% of control, respectively, during a period of 6–12 h (Fig. 1(A) and (B)). The permeability coefficients of Na-F and EBA for MBEC4 cells were dose-dependently reduced by 1.7–38.8% and 8.4–42.1%, respectively, after a 12-h exposure to TGF-β1 (0.01–10 ng/mL) (Fig. 1(A) and (B)). As shown in Fig. 2, the cellular accumulation of rhodamine 123 into MBEC4 cells was dose-dependently reduced by 69.0–100.7%

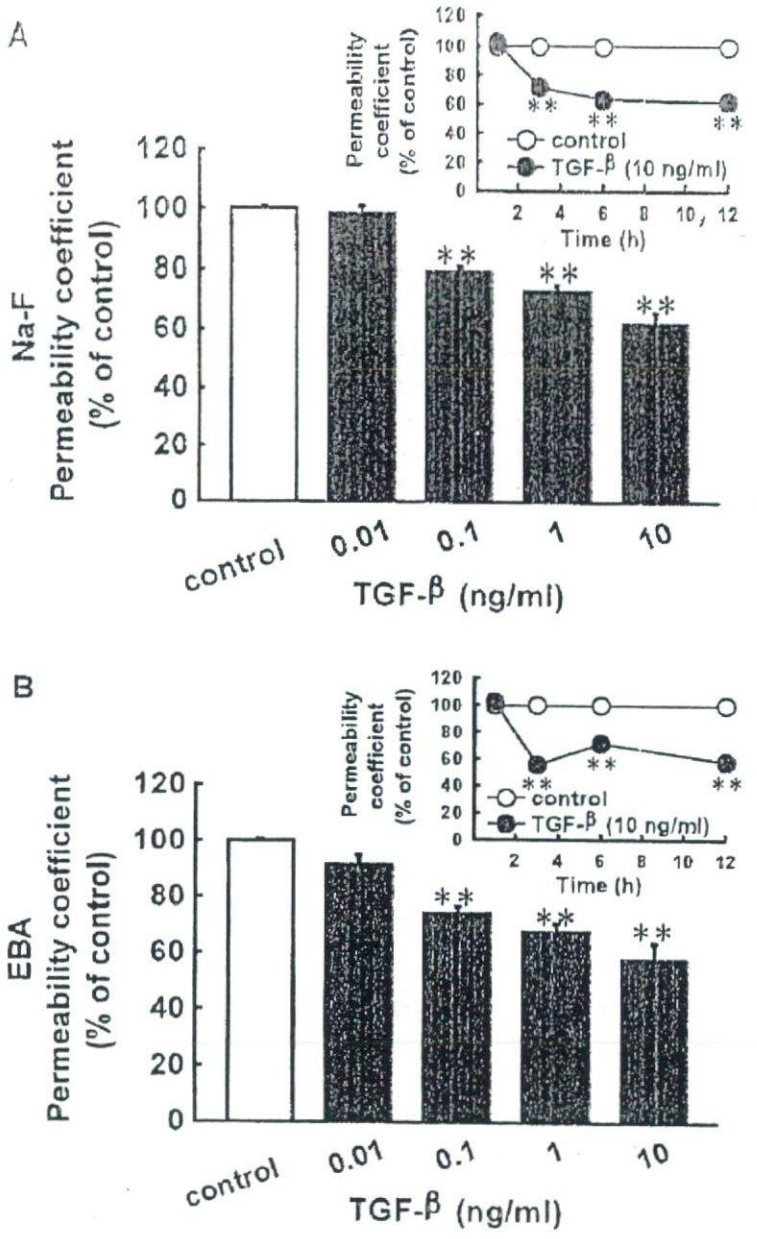

Fig. 1. Changes in the permeability coefficient of Na-F (A) and EBA (B) in MBEC4 cell monolayers after exposure to various concentrations of TGF-β1 for 12 h. The permeability coefficients of Na-F and EBA for the control in panel A and B were 2.71 ± 0.09 × 10^{-4} and 0.85 ± 0.05 × 10^{-4} cm/min, respectively. The inset in each panel shows the time-course of the permeability coefficient of Na-F and EBA after exposure to 10 ng/mL of TGF-β1. The permeability coefficients of Na-F and EBA for the control were 1.34 ± 0.02, 2.10 ± 0.05, 2.17 ± 0.01, and 3.01 ± 0.13 × 10^{-4} cm/min in the inset of panel A (Na-F) for 1, 3, 6, and 12 h, respectively, and 0.46 ± 0.02, 0.97 ± 0.02, 0.66 ± 0.03, and 1.02 ± 0.08 × 10^{-4} cm/min in the inset of panel B (EBA) for 1, 3, 6, and 12 h, respectively. Data are expressed as percentages of control. Values are shown as means ± SEM (n = 4–24). $^{**}P < 0.01$: significant difference from control.