J. Tokyo Med. Univ., **66**(3) : 419-428, 2008

The neuroprotective effects of long-term, low dose administration of FK506 (tacrolimus) on hypoxic-ischemic encephalopathy in the neonatal rat

Makiko MIYATA, Yukito TAKEI, Akinori HOSHIKA

Department of Pediatrics, Tokyo Medical University

Abstract

FK506 (tacrolimus) is an immunosuppressant agent used in organ transplantation. We previously reported that single doses of FK506 were ineffective after hypoxia-ischemia (HI). We investigated whether low dose (1 mg/kg) FK506 shows protective effects, if given repeatedly over a longer period of time after insult. We also studied the brain protection mechanism of FK506 by studying the dynamics of activated microglia, myelin, and temporal changes of the brain derived neurotrophic factor (BDNF) level, and whether the above procedure produces less side effects, by examining the mortality and body weight increment rates. In 7-day-old Wistar rat pups ($n=69$), HI was induced by transient occlusion of the left carotid artery and 8% O_2 for 90 min. The rats were then resuscitated by releasing the carotid artery occlusion and reoxygenated in room air. After the HI/reperfusion insult, rats were randomized to a group receiving intraperitoneally 1.0 mg/kg FK506 dissolved in 0.1 ml/kg normal saline or the same amount of vehicle (0.1 ml/kg normal saline) for one time on the first day (FK506-1d, $n=13$; vehicle-1d, $n=10$), or once daily for 3 days (FK506-3d, $n=13$; vehicle-3d, $n=13$), or once daily for 7 days (FK506-7d, $n=7$; vehicle-7d, $n=13$). At 8 days after the HI/reperfusion insult, the ratio of the left (with lesion) to the right (without lesion) cerebral hemispheric weight in the FK506-3d and FK506-7d rats were significantly higher than those in the vehicle-3d and vehicle-7d ($p=0.002$, $p=0.02$, respectively). The histological findings of Iba-1 and MBP stains demonstrated that low dose FK506 administrations for 3 (FK506-3d rats) or 7 days (FK506-7d rats) inhibited the activation of microglia and myelin sheath damage in the damaged brain, compared with the vehicle rats. BDNF levels in the FK506-3d and FK506-7d rats were significantly higher than those in the vehicle-3d and vehicle-7d ($p=0.05$, $p=0.04$, respectively). There was no significant difference between these parameters in the FK506-1d rats, compared with those in the vehicle-1d group. The weight gains during the experiment for 7 days in the FK506-3d and the FK506-7d rats were significantly lower than vehicle-7d. The mortality rates in the FK506-7d rats were significantly higher than the vehicle-7d rats. These results demonstrated that the low dose FK506, single dose of which was not effective, was neuroprotective for brain damage, when administered for longer-terms in neonatal HIE animal models. The inhibition of activated microglia and the elevation of neurotrophic factors have been subjected to be involved in the mechanism of the neuroprotective effects of long-term low dose FK506 administration on the neonatal HIE. Even at low doses, FK506 had more influence on the general condition of neonatal animals, when it was given for longer periods than when given in single doses.

Received January 21, 2008, Accepted February 19, 2008

Key words : Hypoxic-ischemic encephalopathy (HIE), FK506, Activated microglia, White matter injury, Neurotrophic factor BDNF

Corresponding author : Makiko MIYATA, Department of Pediatrics, Tokyo Medical University, 6-7-1 Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan

Tel : +81-3-3342-6111 Fax : +81-3-3344-0643 E-mail : km.miyata@nifty.com

Introduction

Hypoxic-ischemic encephalopathy (HIE), caused by neonatal asphyxia can have serious sequalae such as neonatal death, cerebral palsy, and mental retardation to name a few[1)2)]. Much research and treatment studies have been aimed at the conquest of this problem[3-5)], but so far no established solution has been found. In 1992, some neuroprotective effects of an immunosuppressant, cyclosporine A (CsA) in a localized brain ischemic model in adult animals were reported for the first time[6)]. This was followed by another study, which reported that another immunosuppressant, FK506 also possessed similar neuroprotective effects[7)]. FK506 has been clinically used as an immunosuppressant frequently after many surgical transplant procedures, including neonatal cases[8-10)]. It also can be used to protect the brain through peripheral intravenous injection because of its high osmosis through the blood-brain barrier[11-13)]. These two features would make FK506 clinically useful for human neonates, should its brain protection effects be confound. To attain this goal, our research group first studied neuroprotective effects by FK506, using a neonatal hypoxia-ischemia (HI)/reperfusion brain injury model in the rat[14)], and Sunohara et al showed dose dependent therapeutic effects[15)]. They demonstrated that while a higher dose (2 mg/kg) had protective effects, a lower dose (1 mg/kg) did not. Likewise, using a similar model, Nakada et al found neuroprotective effects of combined administration of low dose FK506 and 7NI for 7 days[14)]. They, however, also reported some FK506-related side effects such as retarded body weight increase, and deaths during the treatment. These findings suggest a reciprocal dose dependency tendency of FK506 on the neonatal HIE, i.e., higher dosage would produce greater brain protection effects through suppressed immunity, but at the same time, more severe side effects. We noted that not only necrosis but also apoptosis, in other words, delayed neuronal cell death, were involved in the brain injury due to HIE in the neonate. Therefore, we hypothesized that neuroprotective effects without such side effects might be provided by long-term administration of a lower dose of FK506, single doses of which are not effective, for the prevention of the further progression of delayed neuronal cell death which would be seen several days after the HI insult. The beneficial mechanism of the FK506 is attributed to its neuronal protection effects by inhibitory action on the calcineurine, which is done by making a conjugation with its binding protein immunophilin, FKBP12[7)16)17)]. Recently, several investigators speculated that FK506 with FKBP52, another immunophilin, would facilitate nerve regeneration by stimulating the production of neural nutrition factors[18)]. Furthermore, the increased number of the activated microglial cells also accounted for the death of neurons as well as the white matter lesion[19-21)]. Namely, these microglial cells facilitate production of neurotoxic factors such as cytokines which in turn cause neuronal death and destruction of the oligodendrocytes, which eventually induces white matter lesions[22-25)]. On the other hand, these activated microglias protect the brain by producing many neuroprotective substances such as neurotrophic factors[26)27)]. Therefore, the activated microglias have a double-edged sword effect by possessing neuroprotective and neuroinjurious features. However, although some brain protective effects of FK506 have been demonstrated, its mechanisms, especially on the microglia, and on neurotrophic factors are not well understood.

The purposes of the present paper are to investigate using HI/reperfusion neonatal rat models: (1) whether a low dose (mg/kg) FK506 (no protective effects have been reported with a single dose after HI exposure) shows some protective effects if given repeatedly over a longer period of time, (2) whether it is possible to elucidate the operating brain protection mechanism of FK506 used with the above procedure by studying the dynamics of activated microglias, myelin, and temporal changes of the BDNF level, (3) whether the above procedure produces less side effects by examining the mortality and body weight increment rates.

Materials and methods

Subjects

Wistar dams and their litters were purchased from CLEA Japan Inc (Tokyo, Japan) and maintained on a 12-h cycle of light and dark with food and water freely available. Seven-day-old rat pups of either sex (n= 69), weighing between 12 and 18 grams, were removed from the litters for preparation and study, and returned to be suckled by their dams at all other times during the experimental procedures.

Experimental procedure

All experimental procedures were carried out in strict accordance with the guidelines of the Animal Ethical Committee of Tokyo Medical University. In neonatal asphyxia, temporal hypoxia-ischemia is always followed by reperfusion. Therefore, we adopted a brain insult of not only HI but also reperfusion for the neonatal HIE animal models. Under 5% isoflurane inhalation anesthesia, the left carotid artery was exposed, and temporarily occluded by clipping with the Sugita Aneurysm Clip (Mizuno Co. Ltd, Tokyo, Japan) for 90 min and the neck incision was closed. The entire surgical procedure lasted no longer than 10 min. The rats were then placed in an airtight 500 ml plastic chamber containing humidified 8% oxygen/92% nitrogen gas, creating the

desired HI condition for 90 min. The rats were then resuscitated by releasing the carotid occlusion and reoxygenated with room air. Rectal temperatures of all animals in the chamber were continuously maintained at 36.5 to 37.0°C automatically by means of a temperature controller system (ATC-101B, Unique Medical Co, Tokyo, Japan) to avoid hypothermia throughout the experiment.

Chemicals

FK506 was purchased from Fujisawa Pharmaceutical (Osaka, Japan).

Experimental protocol

After the HI/reperfusion insult, rats were divided into three groups, according to the periods of drug administration. In the first group, rats were intraperitoneally randomly given either 1.0 mg/kg FK506 (dissolved in 0.1 ml/kg normal saline, $n=13$) or the same amount of vehicle (0.1 ml/kg normal saline, $n=10$) after the insult for one time on the first day (one dose). In the second group, either 1.0 mg/kg FK506 ($n=13$) or vehicle ($n=13$) was randomly given every 24 hrs for three days (total 3 doses). In the third group either 1.0 mg/kg FK506 ($n=7$) or vehicle ($n=13$) was randomly given every 24 hrs for seven days (total 7 doses). After drug administration, all animals were then returned to their dams to be cared for and suckled for seven days.

Brain weight measurement and histological assessment

On the eighth day after the HI insult (pups were then 15 days old), circulating blood was taken transcardially under deep anesthesia with isoflurane inhalation. The brains were immediately removed intact and then cut to divide them into left and right hemispheres and each weight was measured. In order to assess the severity of the whole brain damage, we measured the hemispheric brain weight to obtain the ratio of left (with lesion) and right (without lesion) hemispheric brain weight. Then the cerebral hemispheres were placed into 4% paraformaldehyde in 0.1 M phosphatase buffer, pH 7.4 for one week. The brains were then embedded in paraffin and cut coronally into 4 μm thick slices. All coronal sections were stained with ionized calcium-binding adapter molecule 1 (Iba-1), and myelin basic protein (MBP).

Iba-1 staining

After deparaffination, the sections were place in the autoclave (105°C, 10 min) with citric acid buffer (PH=6.0, 0.01 mol). In order to block endogenous peroxidase activity, rehydrated sections were treated with 0.3% H_2O_2 in absolute ethanol for 10 min, then washed twice in PBS for 5 min. Then polyclonal Anti Iba-I, Rabbit (Wako Pure Chemicals, Osaka, Japan) was added and the sections were incubated overnight at 4°C. After the incubation, the staining took place using the LSAB kit (Dako Cytomation Co. Ltd, Denmark) as a secondary antibody. The method indicated by the instructions of the manufacture was followed completely. For contrast, standard hematoxylin staining was used. In order to assess the influence of FK506 on activated microglia after HI/reperfusion brain injury, both the left (with lesion) and the right (without lesion) hemispheres of all coronal sections at the level of the arcuate nucleus were photographed by a CCD camera (Olympus Co., Tokyo, Japan) to measure the areas of the Iba-1 positive portion using an image analyzer system (Scion image, Scion Corporation, Washington, D.C., U.S.A.). The ratio of the left and the right Iba-1-positive areas in the hemispheres were assessed.

Myelin Basic Protein staining (MBP)

Sections were deparaffinized and rehydrated, followed by washing twice in distilled water and in PBS for 5 min. To block endogenous peroxidase activity, rehydrated sections were treated with 0.3% H_2O_2 in absolute ethanol for 10 min, then washed twice in PBS for 5 min. Then polyclonal antibody (Nichirei Biosciences Inc, Tokyo, Japan) was added to each section, which was incubated overnight at 4°C. After washing with PBS, sections were processed by the Histofine Simple Stain Max PO(M) (Nichirei Biosciences Inc). The manufacturer's instructions were followed exactly. Counterstaining was performed with the standard hematoxylin method. In order to assess the influences of FK506 on the myelin sheath damages after HI/reperfusion brain injury, both hemispheres with (left) and without (right) lesions of all coronal sections at the level of the arcuate nucleus were photographed by a CCD camera (Olympus Co., Tokyo, Japan) to measure the areas of the MBP-positive portion using an image analyzer system (Scion image). The ratios of the left and the right Iba1-positive hemispheric areas were assessed.

Measurement of the Brain derived neurotrophic factor (BDNF)

The plasma was separated by centrifugation with EDTA for 10 min at 800 G, then preserved at −50°C, then BDNF were extracted by an enzyme-linked immunosorbent assay kit (ChemiKine BDNF Sandwich ELISA Kit, Chemicon International Co, USA) later.

Body weight gain and mortality

We also evaluated nutritional status and mortality rates during the 8-day experiment. The body weight just before the HI procedure (age 7 days) and euthanization (15-days old), were measured and the ratio between these two measurements was calculated for every pup.

5. Statistical analysis

Comparisons between values in the two groups were made by means of Student's unpaired t-test, when the data was normally distributed; otherwise the Mann-Whitney U-test was used. Fisher's exact test was used to compare the mortality rates. A value of $p<0.05$ was

considered to indicate a statistically significant difference. The results were expressed as means±SD.

Results

There was no significant difference in body weight between the FK506 and vehicle treated rats before HI insult.

Cerebral hemispheric weight

There was no significant difference between the ratio of the left (with lesion) to right (without lesion) cerebral hemispheres in the FK506-1d group and that in the vehicle-1d group. The ratios in the FK506-3d and FK506-7d groups were significantly higher than those in the vehicle-3d and vehicle-7d ($p=0.002$, $p=0.02$, respectively), which means that FK506 administration for 3 or 7 days significantly lowered the severity of the brain damages compared with the vehicle (Fig. 1).

Iba-1 staining

Fig. 2 shows typical histological examples of anti-Iba-1 antibody staining of the cerebral hemisphere in the vehicle (Fig. 2a), and the FK506 (Fig. 2b) rats. There were some degrees of increases in the intensity of staining as well as in the number of stained microglia, which means activated microglia, in the left (with lesion) hemisphere of all rats, especially in the vehicle rat (Fig. 2a).

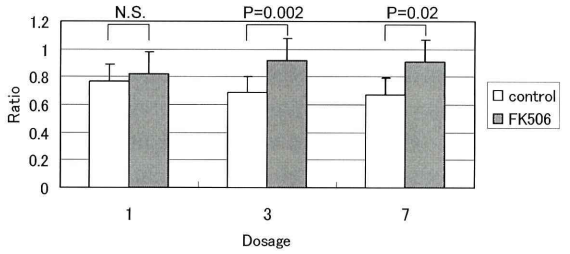

Fig. 1 The ratio of the left to right cerebral hemispheric weight. There was no significant difference in FK506-1d and vehicle groups.
The ratio in the FK506-3d ($p=0.002$), the FK506-7d ($p=0.02$) were significantly higher than in the vehicle-only animals. It is assumed that the higher the value of ratio, the lesser the brain damage.

We assessed the ratio of the left (with lesion) to right (without lesion) hemispheric Iba-1-positive areas in order to evaluate the activation of microglia due to brain damage. There was no significant difference between the ratio in the FK506-1d group and that in the vehicle-1d group (Fig. 3). However, the ratios in the FK506-3d and FK506-7d rats were significantly lower than those in the vehicle-3d and vehicle-7d ($p=0.001$, $p=0.03$, respectively) (Fig. 3), which means the FK506 administrations for 3 or 7 days showed to inhibit the activation of microglia in the brain, compared with the vehicle.

MBP staining

Fig. 4 shows typical histological examples of MBP staining of cerebral hemisphere in the vehicle (Fig. 4a, c, e), and the FK506 (Fig. 4b, d, f) rats. There were some degrees of decreases in MBP staining, which means myelin sheath damage after HI/reperfusion brain injury, in the left (with lesion) hemispheres of all rats, especially in the vehicle rats (Fig. 4a, c, e).

We assessed the ratio of left (with lesion) to right (without lesion) hemispheric MBP-positive area in order to evaluate myelin sheath damage. There was no significant difference between the ratio of the FK506-1d and the vehicle-1d rats (Fig. 5). However, in the FK506-3d and FK506-7d, the ratios were significantly higher than those in the vehicle-3d and vehicle-7d ($p=0.04$, $p=0.02$, respectively)(Fig. 5), which means that FK506 administration for 3 or 7 days caused less myelin sheath damages in the brain than the vehicle alone.

Brain derived neurotrophic factor (BDNF)

The BDNF levels in the FK506-3d and FK506-7d rats were significantly higher than those in the vehicle-3d and vehicle-7d ($p=0.05$, $p=0.04$, respectively) (Fig. 6), whereas there was no significant difference between

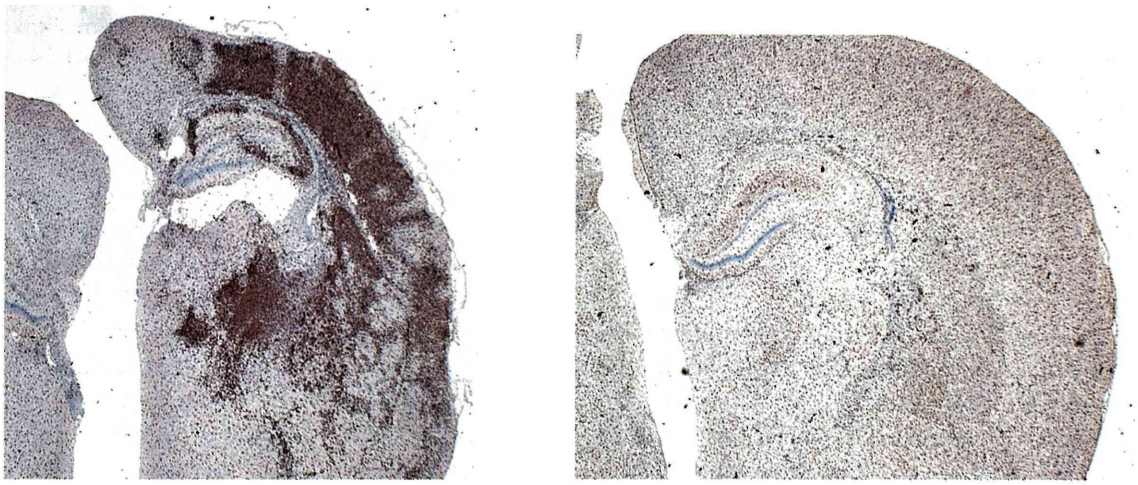

Fig. 2 Typical examples of Iba-1 staining of the left hemisphere (with lesion) in the vehicle-7 (a), and the FK506-7 (b).

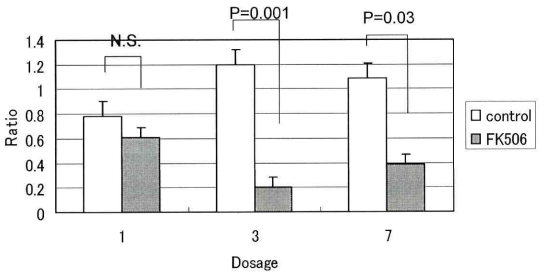

Fig. 3 The ratio of the left to right hemispheric Iba-1-positive area.
There was no significant difference in the FK506-1d and the vehicle groups. The ratio in the FK506-3d ($p=0.001$), the FK506-7d ($p=0.03$) were significantly lower than the vehicles. It is assumed that the lower the ratio, the lesser the activated microglia.

the ratio in the FK506-1d and that in the vehicle-1d (Fig. 6).

Body weight gain and mortality rates

There was no significant difference between the weight gains in the FK506-1d and the vehicle-1d (Fig. 7), though in the FK506-3d and FK506-7d, these were significantly higher than those in the vehicle-3d and vehicle-7d ($p=0.005$, $p=0.002$, respectively) (Fig. 7), which means deterioration in the nutritional status in the rats of FK506 administrations for 3 or 7 days.

No control or FK506-1d animal died at any stage of the experiment. The mortality for FK506-3d was 19% (3/16), whereas for FK506-7d, it was 36% (4/11). The difference between FK506-3d and vehicle-3d was not significant ($p>0.01$), but there was a significant

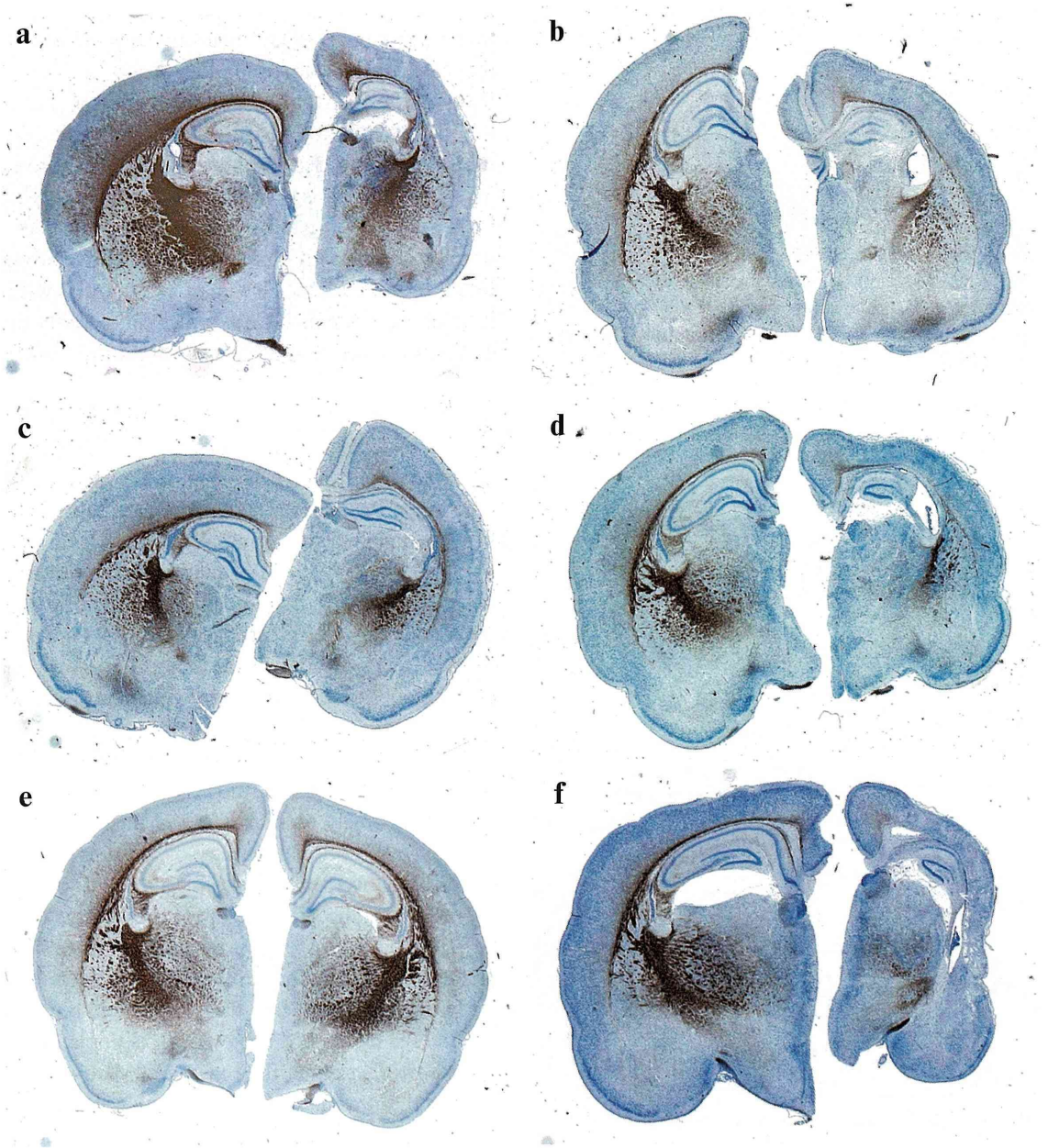

Fig. 4 Typical examples of MBP staining in the FK506-1d (a), -3d (c), -7d (e), and vehicle-1d (b), -3d (d), -7d (f). The left (with lesion) and right (without lesion) hemispheres are shown together.

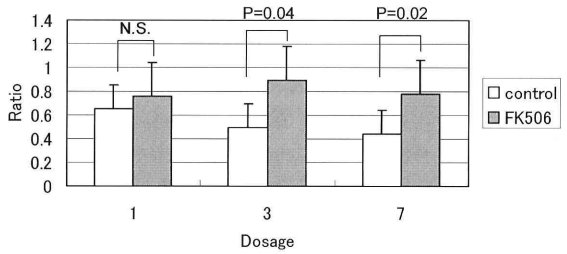

Fig. 5 The ratio of the left to the right hemispheric MBP staining-positive area.
There was no significant difference in the FK506-1d and that in the vehicle. The ratio in the FK506-3d ($p=0.001$), and the FK506-7d ($p=0.03$) were significantly higher than the vehicles. It is assumed that the higher the ratio, the lesser the damage to the myelin sheath.

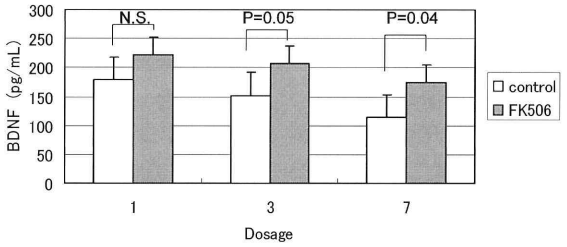

Fig. 6 The blood levels of BDNF.
There was no significant difference in the FK506-1d and vehicle groups. The values in the FK506-3d ($p=0.05$) and FK506-7d ($p=0.04$) were significantly higher than the vehicles.

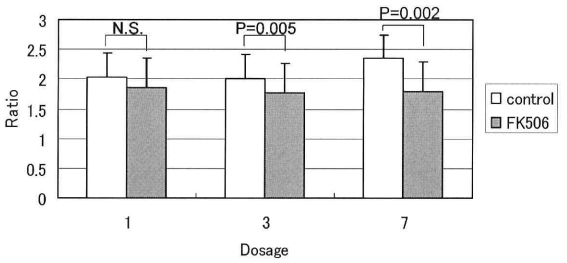

Fig. 7 The body weight gains.
There was no significant difference between the weight gains in the FK506-1d and the vehicle-1d. In FK506-3d and FK506-7d, the ratios were significantly lower than in the vehicle group ($p=0.005$, $p=0.002$, respectively).

Table 1 Mortality rates.

	FK506-1d	FK506-3d	FK506-7d
Survived *n*, pts (%)	13, (100%)	13, (81%)	7, (64%)
Died *n*, pts (%)	0, (0%)	3, (19%)	4, (36%)

In the FK506-1d, all 13 animals survived. The difference between FK506-3d and vehicle-3d was not statistically significant ($p>0.1$), there was a difference between FK506-7d and vehicle-7d ($p=0.02$).

difference between FK506-7d and vehicle-7d ($p<0.02$) (Table 1)

Discussion

The present investigation revealed that a single low dose (1 mg/kg/day) FK506 did not have brain protective effects after HI/reperfusion insult, but when the same dosage was given repeatedly for a longer period, i.e., 3 or 7 days, brain protection effects were obtained. Calcineurin, which is a phosphatase dependent on Ca^{2+} and calmodulin, is activated by excitatory agents such as the glutamic acid secreted in the cell when the brain is subjected to a severe injury such as HI. Among these mechanisms, dephosphorylation of various enzymes induced by calcineurin activation is considered to be involved in neuronal damage[28]. It has been reported that calcineurin induces neurotoxicity by activation of the NMDA receptor and the release of NO resulting from nNOS activation[29][30]. In addition, it has been found that apoptosis is induced directly by calcineurin itself through its dephosphorylation of Bad protein[31–33]. Thus, calcineurin is both directly and indirectly involved in the onset of neuronal cell death. FK506 as an immunosuppressant has high osmosis through the brain-blood barrier. It makes a conpound with one of its binding protein, FKBP, which supposedly manifests a neuroprotective effect by inhibiting calcineurin-mediated events[7][16][17]. However, the molecular mechanisms on the action of FK506 is not clear but is considered to be expressed at different time points during the process of neuronal cell death. We believe that this time range was important to obtain the protective effects. It takes time before a cerebral neuronal injury is caused by apoptosis[34][35]. The primary neuronal death within the core of infarct that occurs within a short time after the brain damage, is probably due to necrosis, while delayed neuronal death occurring over days bears features of apoptotic process involving energy supply, switching on the gene transcription, and protein synthesis[36–38]. The occurrence of delayed cell death creates favorable conditions for a therapeutic window, giving a chance for timely pharmacological intervention in order to salvage the normal activity of neurons. One of the reasons for the effectiveness of the low- and long term-regimen after the HI/reperfusion insult was thought to be the suppression of the delayed nerve cell death by maintaining the blood level of the FK506 high enough for a certain length of time, apparently providing a cumulative effect, not achieved by a single dose.

On the other hand, the Iba-1 and MBP stains indicated that the myelin sheath lesions as well as multiplication of the activated microglial cells were inhibited by the low- and long-term dose regimens. There are few studies in the neonatal HIE animal model literature on

white matter lesions or their prevention, although neonatal white matter lesions, such as in periventricular leukomalacia causes cerebral palsy, have close relationships with neurological sequalae[39)40)].

Recently, some investigators reported that FK506 exhibits its protective effects by directly controlling the activation and population of the microglia which leads to the inhibition of the cytokines such as IL-1β, and TNFα production[41)]. The multiplication of the activated microglia is connected with the injury of the oligodendrocytes, which induces the myelin sheath damage, which in turn causes lower white matter volume[40)42)]. Therefore, the FK506 is likely to reduce white matter damages associated with the volume decrement through its inhibitory action on the activation of the microglia.

The production of neurotrophic factor was increased to protect nerve cells on brain injury. The increment of neurotrophic factors in the CSF and/or plasma has been also reported in the cases of the neonatal HI[43)]. One possible explanation is that some immunosuppressant inhibit calcineurin, which in turn indirectly inhibits the dephosphoration of the cyclic AMP-response element-binding protein (CREB), one of the cAMPs of the neurotrophic factor BDNF[44)]. This series of actions promotes production of the BDNF. Also, there is a report in which the FK506, together with its binding protein immunophilin, FKBP52, acts directly on this neurotrophic factor in an adult Parkinson's animal model[18)]. Compared with the controls, the experimental groups which received low- and long-term doses of FK506 showed higher BDNF levels, which suggest that the desired brain protection effects were obtained by facilitating production of BDNF, a neurotrophic factor.

The systemic influence of this medication must be considered. Compared to the 1-day group, higher numbers of nutritional disturbances and deaths were found in the 3-day and 7-day groups, particularly in the latter. FK506 has both dose dependent neuroprotective and the immunosuppressant effects. Thus, it is expected that extended FK506 usage would lead to more desirable neuroprotective effects but at the same time, more undesirable immunosuppressant disturbances as side effects. This expectation was borne out in the present study. Among the three groups, the 3-day group showed neuroprotective effects and relatively mild side effects. Observations of cerebral energy metabolism in the newborn piglet's HIE models using magnetic resonance spectroscopy demonstrated that they had not only an acute ("primary") depleted brain energy metabolism during HI insult but also delayed ("secondary") energy failure within a few days after the insult[45)]. Perhaps, the administration of FK506 continued for three days might be long enough to maintain its blood level to somewhat prevent this delayed ("secondary") energy failure. Finally, considering the long-term nerve damaging processes such as apoptosis, more investigations for using FK506 for a longer time are desirable.

Conclusion

In the present study, neuroprotective effects of FK506, an immunosuppressant drug were demonstrated on a neonatal HI/reperfusion model. When this agent was administered in a long-term low dose mode (1 mg/kg/day for 3 or 7 days), it was more effective than when it was given in a single low dose mode (1 mg/kg/day for 1 time). The study also suggested that the brain protective effects of FK506 seen after HI/reperfusion injury, involved the inhibition of activated microglial cells and myelin sheath injury, as well as the facilitation of production of the neurotrophic factor BDNF. However, it was found that even such low dose administration still caused some side effects, such as elevated mortality rate and decelerated body weight increment rate due to the growth, when it was used for certain extended periods. For these reasons, it is important to find an optimal mode of administration, which maintains sufficient therapeutic brain protection effects, while minimizing adverse reactions.

Acknowledgments

The authors thank Prof. Kudo, Mr. Ishikawa, and Mr. Fujita, Department of Pathology, Tokyo Medical University, for useful technical advices for histology. The authors are indebted to Prof. J. Patrick. Barron of the International Medical Communications Center of Tokyo Medical University for his review of this manuscript.

This study was supported by MEXT, Kakenhi (13670840).

References

1) Delivoria PM, Mishra OP : Mechanisms of cerebral injury in perinatal asphyxia and strategies for prevention. J Pediatr **132S** : 30-34, 1998
2) Vannucci RC, Perlman JM : Interventions for perinatal hypoxic-ischemic encephalopathy. Pediatrics **100** : 1004-1014, 1997
3) Feng Y, Fratkins JD, LeBlanc MH : Estrogen attenuates hypoxic-ischemic brain injury in neonatal rats. Eur J Pharmacol **507** : 77-86, 2005
4) Bona E, Hagberg H, Laberg EM, Bagenholm R, Thoresen M : Protective effects of moderate hypothermia after neonatal hypoxia-ischemia : short- and long-term outcome. Pediatr Res **43** : 738-745, 1998
5) Hagberg H, Gilland E, Diemer NH : Hypoxia-ischemia in the neonatal rat brain : histopathology after post-treatment with NMDA and non-NMDA

receptor antagonists. Biol Neonate **66**: 206-213, 1994
6) Shiga Y, Onodera H, matsuo Y, Kogure K: Cyclosporin A protects against ischemia-reperfusion injury in the brain. Brain Res **595**: 145-148, 1992
7) Shakey J, Butcher SP: Immunophilins mediate the neuroprotective effect of FK506 in focal cerebral ischemia. Nature **371**: 336-339, 1994
8) Starzl TE, Todo S, Fung J, Demetris AJ, Venkataramman R, Jain A: FK506 for liver, kidney, and pancreas transplantation. Lancet **2**: 1000-1004, 1989
9) Markus PM, Cai X, Ming W, Demetris AJ, Fung J, Starzl TE: Prevention of graft-versus-host disease following allogeneic bone marrow transplantation in rats using FK506. Transplantation **52**: 590-594, 1991
10) European FK506 Multicentre Liver Study Group: Randomised trial comparing tacrolimus(FK506) and cyclosporine in prevention of liver allograft rejection. Lancet **344**: 423-428, 1994
11) Dawson TM, Steiner JP, Dawson WL, Dinerman JL, Uhl GR, Snyder SH: Immunosuppressant FK506 enhances phosphsrylation of nitric oxide synthase and protects against glutamate toxicity. Proc Natl Acad Sci **90**: 9808-9812, 1993
12) Bochelen D, Rudin M, Sauter A: Calcineurin inhibitors FK506 and SDZ ASM 981 alleviate the outcome of focal cerebral ischemic/reperfusion injury. J Pharmacol Exp Ther **288**: 653-659, 1999
13) Uchino H, Minamikawa-Tachino R, Kristian T, Perkins G, Narazaki M, Sjesjo BK, Shibasaki F: Differential neuroprotection by cyclosporine A and FK506 following ischemia corresponds with differing abilities to inhibit calcineurin and the mitochondrial permeability transition. Neurobiol Dis **10**: 219-233, 2002
14) Nakata C, Takei Y, Hoshika A: Combination therapy with FK506 and 7-nitorindazol inhibits brain damage due to hypoxia-ischemia/reperfusion in the newborn rat. J Tokyo Med Univ **64**: 368-379, 2006
15) Sunohara D, Takei Y, Hoshika A: FK506 (tacrolimus) inhibits hypoxic ischemic encephalopathy (HIE) in the newborn rat: Evaluation of dose dependency and therapeutic time window. J Tokyo Med Univ **65**: 159-169, 2007
16) Liu J, Farmer JD Jr, Lane WS, Friedman J, Weissman I, Schreiber SL: Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell **66**: 807-815, 1991
17) Steiner JP, Dawson TM, Fotuhi M, Glatt CE, Snowman AM, Cohen N, Snyder SH: High brain densities of the immunophilin FKBP colocalized with calcineurin. Nature **358**: 584-587, 1992
18) Nitta A, Murai R, Maruyama K, Furukawa S: FK506 protects dopaminergic degeneration in rodent brain, in Mapping the Progress of Alzheimer's and Parkinson's Disease. Advances In Behavioral Bio **51**: 463-468, 2002
19) Stoll G, Jander S: The role of microglia and macrophages in the pathophysiology of the CNS. Prog Neurobiol **58**: 233-247, 1999
20) Kato H, Walz W: The initiation of the microglial response. Brain Pathol **10**: 137-143, 2000
21) Biran V, Joly LM, Heron A, Vega C, Mariani J, Renolleau S, Marlangue CC: Glial activation in white matter following ischemia in the neonatal P7 rat brain. Exp Neurology **199**: 103-112, 2006
22) Rothwell NJ: Cytokines-killers in the brain? J Physiol **514**: 3-17, 1999
23) Bona E, Andersson A, Blomgren K, Gilland E, Puka-Sundrali M, Gustafson K, Hagberg H: Chemokine and inflammatory cell response to hypoxia-ischemia in immature rats. Pediatr Res **45**: 500-509, 1999
24) Boutin H, Lefeuvre RA, Hora R, Asano M, Iwakura Y, Rothwell NJ: Role of Il-1alpha and Il-1beta in ischemic brain damage. J Neurosci **21**: 5528-5534, 2001
25) Merrill JE, et al: Microglial cell cytotoxicity of oligodendrocytes is mediated through nitric oxide. J Immunol **151**: 2132-2141, 1993
26) Ghrmann J, Matsumoto Y, Kreutzberg GW: Microglia: intrinsic immunoeffector cell of the brain. Brain Res **20**: 269-287, 1995
27) Sawada M, Suzumura A, Marunouchi T: Cytokine network in the central nervous system and its roles in growth and differentiation of glial and neuronal cells. Int J dev Neurosci **13**: 253-264, 1995
28) Cousin MA, Robinson PJ: The dephosphins: dephosphorylation by calcineurin triggers synaptic vesicle endocytosis. Trends Neurosci **24**: 659-665, 2001
29) Kaminska B, Gaweda-Walerych K, Zawadzka M: Molecular mechanisms of neuroprotective action of immunosuppressants; facts and hypotheses. J Cell Mol Med Jan-Mar **8**: 45-58, 2004
30) Dawson VL, Kizushi VM, Huang PL, Snyder SH, Dawson TM: Resistance to neurotoxicity in cortical cultures from neuronal nitric oxide synthase-deficient mice. J Neurosci **16**: 2479-2487, 1996
31) Wang HG, Pathan N, Ethell IM, Krajewski S, Yamaguchi Y, Shibasaki F, McKeon F, Bobo T, Franke TF, Reed JC: Ca^{2+}-induced apoptosis through calcineurin dephosphorylation of Bad. Science **284**: 339-343, 1999
32) Li JY, Furuichi Y, Matsuoka N, Mutoh S, Yanagihara T: Tacrolimus (FK506) attenuates biphasic cytochrome c release and Bad phosphorylation following transient cerebral ischemia in mice. Neuroscience **142**: 789-797, 2006
33) Sharkey J, Jones PA, McCarter JF, Kelly JS: Calcinurin inhibitors as neuroprotectants. Cns

Drugs **13** : 1-13, 2000
34) Pulsinelli W, Brierley JB, Plum F : Temporal profile of neuronal damage in a model of transient forebrain ischemia. Ann Neurol **11** : 491-498, 1982
35) Beilharz EJ, Williams CE, Dragunaw M, Sirimanne ES, Gluckman PD : Mechanisms of delayed cell death following hypoxic-ischemic injury in the immature rat ; evidence for apoptosis during selective neuronal loss. Molecular Brain Res **29** : 1-14, 1995
36) Charriaut-Marlanque C, Margaill I, Represa I, Popovici T, Plotkine M, Ben-Ari Y : Apoptosis and necrosis after reversible focal ischemia ; an in situ DNA fragmentation analysis. J Cereb Bllod Flow Metab **16** : 186-194, 1996
37) MacManus JP, Hill IE, Huang ZG, Rasquinha I, Xue D, Buchan AM : DNA damage consistent with apoptosis in transient focal ischaemic neocortex. Neuroreport **5** : 493-496, 1994
38) Linnik MD, Zobrist RH, Hatfield MD : Evidence supporting a role for programmed cell death in focal cerebral ischemia in rats. Stroke **24** : 2002-2008, 1993
39) Folkerth RD : Periventricular leukomalacia ; Overview and Recent Findings. Pediatric Dev Pathol **9** : 3-13, 2006
40) Billiards S, Haynes RL, Folkerth RD, Trachtenberg FL, Liu LG, Volpe JJ, Kinney HC : Development of microglia in the cerebral white matter of the human fetus and infant. J Comparative Neurology **497** : 199-208, 2006
41) Zawadzka M, Kaminska B : A novel mechanism of FK506-mediated neuroprotection ; downregulation of cytokine expression in glial cells. Glia **49** : 36-51, 2004
42) Haynes RL, Folkerth RD, Keefe RJ, Sung I, Swzeda LI, Rosenberg PA, Volpe JJ, Kinney HC : Nitrosative and oxidative injury to premyelinating oligodendrocytes in periventricular leukomalacia. J Neuropathol Exp Neurol **62** : 441-450, 2003
43) Nikolaou KE, Malamitsi-Puchner A, Boutsikou T, Economou E, Boutsikou M, Puchner KP, Baka S, Hassiakos D : The varying patterns of neurotrophon changes in the perinatal period. Ann N.Y. Acad Sci **1092** : 426-433, 2006
44) Miyata K, Omori H, Uchino H, Yamaguchi T, Isshiki A, Shibasaki F : Involvement of the brain-derived neurotrophic factor/TrkB pathway in neuroprotective effect of cyclosporine A in forebrain ischemia. Neuroscience **105** : 571-578, 2001
45) Lorek A, Takei Y, Cady EB, Wyatt JS, Penrice J, Edwards AD, Peebles D, Wylezinska M, Owen-Reece H, Kirkbride V, Cooper CE, Aldridge RF, Roth SC, Brown G, Delpy DT, Reynolds EOR : Delayed ("secondary") cerebral energy failure after acute hypoxia-ischemia in the newborn piglet : continuous 48-hour studies by phosphorus magnetic resonance spectroscopy. Pediatr Res **36** : 699-706, 1994

新生仔ラット低酸素虚血/再灌流脳障害モデルに対する免疫抑制剤 FK506 低用量長期間投与の神経保護効果

宮田 真貴子　武井 章人　星加 明徳

東京医科大学小児科学講座

今回我々は、新生仔ラット低酸素虚血/再灌流脳障害モデルを用いて、低酸素性虚血性脳症 (HIE) に対する免疫抑制剤 FK506 の神経保護効果について検討した。本研究の目的は、(1) 単独では神経保護効果を示さない低用量 (1 mg/kg) FK506 を長期間投与することで神経保護効果を得ることができるかどうか、(2) ミクログリアの活性化、ミエリン鞘および神経栄養因子 BDNF に及ぼす影響について検討することで FK506 低用量長期間投与の神経保護作用機序を説明できるかどうか、(3) 体重増加率と死亡率を検討し、FK506 低用量長期間投与がより副作用の少ない治療法かどうかを検討することである。対象は、日齢 7 の Wistar 系ラット (n=69)、左総頸動脈をクリップで一過性に閉塞し、チャンバー内を 8% O_2 に保ち 90 分の低酸素虚血 (HI) 負荷とした。90 分後に閉塞を解除し、room air で蘇生した。FK506 単日投与群は負荷解除直後に FK506 (1 mg/kg) を生食に溶解し 1 回腹腔内投与した。3 日投与群は同様の操作を 24 時間毎に計 3 回投与し、7 日投与群は 24 時間毎に計 7 回投与した。それぞれの群で同量の生食を同様回数腹腔内投与し、各群の対照とした。HI 負荷後 8 日目に血液採取、脳摘出、脳組織の免疫染色および体重測定を行った。左右大脳半球重量比の比較では、FK506 の 3 日、7 日投与群は生食群に比べて有意に患側脳重量の減少を抑制した。Iba-1 染色と MBP 染色の検討では、FK506 の 3 日、7 日投与群は生食群に比べて、ミクログリアの活性化及びミエリン鞘障害を有意に抑制した。また神経栄養因子 BDNF の血中濃度は、FK506 の 3、7 日投与群は生食群に比べて有意に上昇していた。1 日投与群ではこれらについて FK506 群と生食群の間に有意な差を認めなかった。一方、体重増加率は FK506 の 3 日、7 日投与が生食群に比べ有意に低く、また FK506 の 7 日投与群は死亡率が有意に高かった。これらの結果より新生児 HIE モデルにおいて低用量 FK506 は単日投与では神経保護効果を得られないが、長期間投与することで神経保護効果を得られることがわかった。また FK506 低用量長期間投与による神経保護作用機序には、活性化ミクログリアとミエリン鞘障害の抑制、および BDNF 産生促進が関与することが推測された．しかし FK506 は副作用の観点から低用量であっても長期間投与することで全身に与える影響も強いことが示された。

〈キーワード〉 低酸素性虚血性脳症 (HIE)、FK506、活性化ミクログリア、白質障害、神経栄養因子 BDNF