FK506 Itself Does Not Demonstrate Neurotoxicity in the Mouse Brain

Hisahiro Sakai¹, Yoshihiro Takeuchi¹, Hisashi Kawano¹, Hiroko Matsushita¹, Ichiro Yamazoe¹ and Toru Sugimoto¹

¹Department of Pediatrics, Kyoto Prefectural University of Medicine, Kawaramachi-Hirokoji, Kamigyō-ku, Kyoto 602–8566
and ²Department of Pediatrics, Shiga University of Medical Science, Setatsukinowacho, Otsu 520–2192

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We examined histochemical changes in Nissl’s staining, glial fibrillary acidic protein (GFAP)- and myelin basic protein (MBP)-immunoreactive cell bodies and fibers in mouse brains after intraperitoneal or intraventricular injection of FK506 (tacrolimus, Prograf®). After intraperitoneal injection of FK506, there were no marked changes in either GFAP- and MBP-immunohistochemical staining or Nissl’s staining. After intraventricular injection, there were no marked changes in either MBP-immunohistochemical staining or Nissl’s staining, but extensive increases in GFAP-immunoreactive cell bodies and the densities of GFAP-immunoreactive fibers were detected in the olfactory tubercle, caudate putamen, hippocampus and neo-cortex. In this study, there were no statistically significant differences in GFAP-immunoreactive cell bodies, or the densities of GFAP-immunoreactive fibers between the FK506 injected group and placebo group. These results suggest that FK506 itself does not demonstrate neurotoxicity. However, various factors such as vasoconstriction appear under various conditions, such as GVHD or infection in vivo, especially after organ transplantation. Therefore, it can be said that not only FK506, but also various other factors are involved in neurotoxicity and FK506-related leukoencephalopathy.

Key words: FK506, FK506-related leukoencephalopathy, FK binding protein, Intraventricular injection, Glial fibrillary acidic protein

I. Introduction

FK506 (tacrolimus, Prograf®) has recently been approved for immunosuppression in organ transplantation, although its use is accompanied by a wide spectrum of neurotoxic side effects [5, 11, 15–17, 19, 22, 25]. A syndrome of immunosuppressant-related leukoencephalopathy was proposed as an uncommon neurological syndrome occurring in patients with organ transplants involving demyelination, in particular in the parieto-occipital region and centrum semiovale. The syndrome is not associated with a particular absolute serum level of FK506. However, it resolves spontaneously after decreasing the dose of FK506. The FK506-related syndrome has a similar radiographic and pathologic appearance to an analogous syndrome that occurs in patients taking cyclosporine-A (CsA) [3]. FK506 and CsA each bind their own immunophilins, and drug-immunophilin binding results in the inhibition of calcium-calmodulin-activated phosphatase calcineurin. Calcineurin is widely distributed throughout the nervous system, and controls neural cell death [3, 4, 23, 26]. A number of reports have revealed the neuroprotective effect of FK506 [2, 18, 28, 29]. However, central nervous system side effects, such as seizures, tremors, blindness and leukoencephalopathy, have been reported in 40% of patients receiving FK506 [8]. In a minority of transplanted patients, severe and sometimes life-treating toxicity occurs. Although the mechanism of this neurotoxicity remains unknown, few reports have examined its cause [3, 8, 24] or the occurrence of FK506-related leukoencephalopathy in animals. Therefore, we investigated the neurotoxicity of FK506 using experimental animals.

II. Materials and Methods

Intraperitoneal injection

Eight-week-old male JCL; ICR mice (CLEA Japan), weighing 28 g–32 g, were housed under standard conditions
at constant temperature (22–23°C) and humidity under 12-hr light/dark cycles. The mice were randomly divided into three groups (n=26): a low dose FK506 injection group; group L (n=4), a middle dose FK506 injection group; group M (n=15), and a high dose injection group; group H (n=7). The mice in group L had intraperitoneal injections of low dose FK506 (=0.1 mg/Kg/day) for two weeks (group L1, n=1) or four weeks (group L2, n=3), while the mice in group M had intraperitoneal injections of middle dose FK506 (=1.0 mg/Kg/day) for two weeks (group M1, n=13) or four weeks (group M2, n=2), and the mice in group H had intraperitoneal injections of high dose FK506 (=10.0 mg/Kg/day) for two weeks (group H1, n=5) or four weeks (group H2, n=2). We compared histopathological brain changes among groups H, L and M.

Intraventricular injection

Eight-week-old JCL-ICR male mice were divided into three groups (n=23). One was a no-operation group; group C (n=5), another received a placebo (5.0 μl) via intraventricular injections; group P (n=7), and the other received FK506 (0.001 mg/kg=5.0 μl) injections; group F (n=11). We gave injections via a right-lateral ventriculopuncture (Bregma from 1.0 mm to right, 0.5 mm to back, depth 1.5 mm) with a 27 gauge needle and a micro-syringe (Hamilton). After one week (group P; n=5, group F; n=9) or four weeks (group P; n=2, group F; n=2), we compared histopathological brain changes among groups F, P and C with an optical microscope.

Histochemical procedure

Under anesthesia following peritoneal injections of pentobarbital, the animals were subjected to transcardial perfusion with cold phosphate-buffered saline (PBS, 0.9% NaCl in 0.1 M phosphate buffer) followed by perfusion-fixation with 4% paraformaldehyde. The brains were removed, postfixed for 2 days, placed in 20% sucrose at 4°C for 2 days, and stored at ~80°C. Brains were cut into a series of 20-μm sections using a cryostat. The sections were collected in PBS, pH 7.4, containing 0.3% Triton X-100 (PBST). Free-floating sections in PBST were incubated in glial fibrillary acidic protein (GFAP) (DAKO) and myelin basic protein (MBP) (Ultra Clone) antisera at a dilution of 1:10 (GFAP) and 1:500 (MBP) for 48 hr at 4°C. Then, they were washed with PBST. As a second antibody and peroxidase conjugate, a Vectastain ABC Kit (Vector Laboratories) was used. After rinsing in PBS, serial sections were developed for peroxidase activity with 3’,3’-diaminobenzidine, and mounted on slides. The slides were dried overnight in air, dehydrated in ethanol and mounted with Entellan. To identify the cell groups, other alternate section series were used for Nissl’s staining using cresyl violet [13, 14].

Statistical analysis

Using morphological analysis, we mapped GFAP- and MBP-immunoreactive fibers based on an atlas [6]. A semi-quantitative method was used. The variation in densities was evaluated subjectively as follows: 3+: high density, 2+: medium, 1+: low density. We assessed 3+ or 2+ as positive areas and 1+ as negative areas. To estimate changes in the number densities of staining fibers, each section was viewed with a microscope and we measured the positive area with an NIH IMAGE (Wayne Rasband, National Institutes of Health) [12]. The Student’s t-test was used on these data and a value of p<0.05 was taken to represent a statistically significant change.

III. Results

Intraperitoneal injection

In each of the FK506 intraperitoneal injection groups, no marked changes were detected (group L1, L2, M1, M2, H1, H2) using Nissl’s staining, GFAP-immunohistochemistry or MBP-immunohistochemistry compared to results for control mice. With Nissl’s staining, no necrosis and another findings were detected in any tissue slices, irrespective of the amount or period of injection. No changes in GFAP- or MBP-immunoreactive cell bodies and fibers were detected in any slices, irrespective of the amount or period. Also, there was no difference in GFAP- and MBP-immunoreactivity in any tissue slices among all intraperitoneal injection groups and control mice. In particular, the mice in group H (highest dose and longest term) did not show changes in GFAP- and MBP-immunoreactivity in their brains.

Intraventricular injection

MBP immunohistochemistry

In the intraperitoneal injection placebo group (Group P) and FK506 group (Group F), no marked histochemical changes were detected with Nissl’s staining or MBP-immunohistochemistry compared to results for control mice. Changes in MBP-immunoreactive cell bodies and fibers were not detected in any tissue slices. Also, there was no difference in MBP-immunoreactivity in any tissue slices among all intraventricular injection groups and control mice.

GFAP immunohistochemistry

As for mice that received intraventricular injections one week earlier, 5 cases in group P showed marked increases in GFAP-immunoreactive cell bodies, with fibers were detected in the olfactory tubercle, caudate putamen, hippocampus and neo-cortex, compared to the results for group C (Fig. 1A–C). As for mice that received intraventricular injections one week earlier, all cases (9 cases) in group F showed marked increases in GFAP-immunoreactive cell bodies, with fibers were detected in the olfactory tubercle, caudate putamen, hippocampus and neo-cortex compared to the results for group C (Fig. 1D–F). The distribution schema of GFAP-immunoreactive cell bodies and fibers are shown (Fig. 2). We measured the GFAP-immunoreactive area of each slice. A table of the mean % of the GFAP-positive area is shown (Table 1). Using statistical analysis (Student’s t-test) with these data, there was no significant difference in
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The average GFAP-positive area between groups F and P (P<0.05). As for mice that received intraventricular injections four weeks earlier, the findings in each slice were better than groups F and P (Fig. 1H–J). In the olfactory tubercle, caudate putamen, hippocampus, and neo-cortex, the number of GFAP-immunoreactive cell bodies and fibers decreased as compared with mice that received intraventricular-injections one week earlier in groups F and P.

IV. Discussion

In this study, after intraperitoneal injection of FK506, there was no marked change in either GFAP- or MBP-immunohistochemical staining, or Nissl’s staining after

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**Fig. 1.** A–C: GFAP immunoreactivity in the caudate putamen (A), hippocampus (B) and neo-cortex (C) of a mouse that received intraventricular-injection of a placebo one week earlier. D–F: GFAP immunoreactivity in the caudate putamen (D), hippocampus (E) and neo-cortex (F) of a mouse that received intraventricular-injection of FK506 one week earlier. All pictures (A to F) show increases in GFAP-immunoreactive cell bodies and fibers. G–I: GFAP immunoreactivity in the caudate putamen (G), hippocampus (H) and neo-cortex (I) of a mouse that received intraventricular-injection of FK506 four weeks earlier. Increases in GFAP immunoreactivity disappear. Bar=20 μm.
Fig. 2. A, B: Distribution of GFAP-immunoreactivity in a mouse that received intraventricular-injection of the placebo (A) and FK506 (B) one week earlier. The variation in densities was estimated subjectively: 3+, high; 2+, medium; 1+, low. Slice 1 was 1.94 mm in front of the bregma. Slice 2 was 0.86 mm in front of the bregma. Slice 3 was 0.22 mm behind the bregma. Slice 4 was 1.82 mm behind the bregma. Slice 5 was 3.08 mm behind the bregma. Slice 6 was 4.16 mm behind the bregma.
intraventricular injection, and no statistically significant differences in GFAP-immunoreactivity between the FK506-injected group and the placebo group. An insufficient amount of FK506 did not lead to the present results, because Ohra found interstitial edema and fibrosis in rabbit hearts after intravenous injection of FK506 (0.4 mg/kg/day) for 4 weeks, and suppression of spontaneous motion in mice after intraperitoneal injection of FK506 (3.2 mg/kg) [20]. It is thought that this suppression is induced by FK506 acting on the nervous system, but it remains unclear whether this suppression is induced by only FK506; we did not find this suppression in the mice in this study. FK506 at 0.1 mg/kg/day is usually injected into a vein in organ transplantation in humans. Therefore, it could be said that the mice in this research received intraperitoneal injections of enough FK506 to cause neurotoxicity. As for FK506 injected into the veins, about 2% distributed in the brain [7], and intraventricular-injection with FK506 at 50 μl (=16.0 mg/kg) corresponds to injection into a vein of about 1.0 g/kg. This amount is much higher than the amount of FK506 used in human organ transplantation. Therefore, it could be said that the mice in this research received sufficient intraventricular-injection of FK506 to cause neurotoxicity. However, at intraperitoneal-injection there were no significant histochemical findings, and at intraventricular injection there were no statistical histochemical changes, in this study. Therefore, it is possible that not FK506 itself, but another factor is involved in neurotoxicity.

The present results also suggested that not FK506, but another factor, caused the GFAP-positive area after intraventricular-injection. The infiltration pressure of the dissolution liquid, or intraventricular injection procedure, is a possible factor other than FK506 itself. The infiltration pressure of the FK506 dissolution liquid is very high, so that the dissolution liquid can injure brain tissue. However, because the GFAP-immunoreactive area in this study was chiefly distributed in the olfactory tubercle, caudate putamen, hippocampus and neo-cortex without extending to the concentric circle, it cannot be concluded that only the infiltration pressure theory is correct. According to Anderson, intraventricular-injection treatment increased the astroglial population [1]. In Guo-Ross’s study, the GFAP-immunoreactive area was found in the frontal cortex and hippocampus of rats that received intraventricular-injections of saline [9]. Guo-Ross used rats and killed them 1, 3, or 7 days after injection, but in this study the mice used were killed 7 days after injection. The GFAP-reactive area was mainly distributed in the hippocampus and cortex in both this and Guo-Ross’s study. In Guo-Ross’s study, the olfactory tubercle and caudate putamen were not observed. It is possible that the GFAP-reactive area in Guo-Ross’s study was induced by the intraventricular injection procedure. Therefore, it seems that the GFAP-reactive area in this study was related to the intraventricular injection procedure. In addition, in the present study, the increase in the GFAP-immunoreactive cell bodies and fibers were thought to be induced by the intraventricular injection procedure and also to be transient, because there was only a small GFAP-immunoreactive area in brains from mice killed four weeks after intraventricular injection.

If another factor besides FK506 itself is related to the neurotoxicity of FK506, the most likely candidate is calcineurin. Hashimoto reported reactive astrocytes and calcineurin coexistence [10]. Calcineurin is the target molecule of FK506 and the FK506 binding protein (FKBP) complex. In addition, Dawson reported that FKBP distributed mainly at the olfactory tubercle, caudate putamen, hippocampus and neo-cortex [4]. The GFAP-immunoreactive area in this study may have been related to FKBP and calcineurin. However, it is unclear from our results whether or not the GFAP-immunoreactive area is related to FKBP and calcineurin. According to Yardin’s research, there was no difference between neuronal cultures with FK506 and normal neuronal cultures in terms of neuronal cell apoptosis [29]. This suggested that FK506 itself did not injure neural cells in vitro. In addition, the results of this study showed that FK506 itself did not injure neural cells in vivo. In contrast, the FK506-FKBP complex inhibits calcineurin, thereby leading to a neuroprotective effect [2, 18, 28, 29]. Recent studies suggested that FK506 could reduce infarct size in a model of focal cerebral ischemia, and prevent hippocampal neuronal damage in transient global ischemia. This neuroprotective effect is thought to be related to evidence that FK506 protects neurons by inhibiting calcineurin, thereby decreasing the formation of nitric oxide produced by enzyme nitric oxide synthetase [28], while low concentrations of FK506 block N-methyl-D-aspartate neurotoxicity [26]. Therefore, the mechanism of FK506’s neuroprotective function is almost clear, but it is unclear how calcineurin relates to FK506 neurotoxicity.

The second candidate is blood vessels. FK506 also contracts blood vessels. Thus, vasoconstriction is a more likely hypothesis for FK506-related leukoencephalopathy. FK506 causes ischemic disturbances in the brain via endothelial damage and vasoconstriction [8]. However, this hypothesis implies not that FK506 itself has neurotoxicity, but that a third factor, that is, the blood endothelium, is closely involved in the appearance of FK506-related leukoencephalopathy.

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### Table 1. The mean % (area) of the GFAP-positive area in each whole slice

<table>
<thead>
<tr>
<th>Slice</th>
<th>F</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>53.3±2.6</td>
<td>51.8±1.9</td>
</tr>
<tr>
<td>2</td>
<td>37.8±1.6</td>
<td>37.8±0.84</td>
</tr>
<tr>
<td>3</td>
<td>36.1±1.2</td>
<td>37.2±1.9</td>
</tr>
<tr>
<td>4</td>
<td>42.2±1.2</td>
<td>41.4±2.3</td>
</tr>
<tr>
<td>5</td>
<td>46.3±0.87</td>
<td>45.6±1.5</td>
</tr>
<tr>
<td>6</td>
<td>36.0±1.2</td>
<td>37.4±1.1</td>
</tr>
</tbody>
</table>

Using statistical analysis (Student’s t-test) with these data, there was no significant difference between groups F and P (P>0.05). 1: About 1.94 mm in front of the bregma. 2: About 0.86 mm ahead of the bregma. 3: About 0.22 mm in front of the bregma. 4: About 1.82 mm behind the bregma. 5: About 3.08 mm behind the bregma. 6: About 4.16 mm behind the bregma.
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It seems that FK506 is more susceptible to be influenced by other factors when using intraperitoneal than intraventricular injections. However, in this study there were no changes in Nissl’s staining, GFAP-immunohistochemistry or MBP-immunohistochemistry in mice that received intraperitoneal injections of sufficient FK506, irrespective of the amount or period. Therefore, it seems that there are several factors related to FK506 neurotoxicity. At organ transplantation, it is possible that not only FK506, but also various factors such as infection, nephrotoxicity, graft versus host disease (GVHD), and damage to endothelial cells, alter the balance of vasodilators and vasoconstrictors and cause similar vasocostriction and vasospasm in cerebral vessels.

It is unknown whether FK506 and the FKBP complex are associated with injury to endothelial cells and vasocostriction. However, it has been reported that the level of FKBP is 10–40 times higher in the brain than in immune tissues [27]. FK506 toxicity has frequently been observed in organs with high FKBP contents, such as the brain and kidneys [15]. Therefore, its complex may be associated with almost all FK506 complications [21].

In conclusion, in this study neurotoxicity was not induced by FK506 alone, and the mechanism of FK506-related leukoencephalopathy was not clarified. We believe that various factors, such as vasoconstriction, cause FK506-related leukoencephalopathy under various conditions, such as GVHD or infection in vivo, especially after organ transplantation, and further study with animals regarding FK506-related leukoencephalopathy under these various factors and conditions is required.

V. Acknowledgments

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VI. References

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