Tacrolimus (FK506) Limits Accumulation of Granulocytes and Platelets and Protects against Brain Damage after Transient Focal Cerebral Ischemia in Rat

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We investigated the neuroprotective effect of tacrolimus (FK506) on the ischemia-reperfusion injury caused by transient focal brain ischemia induced by middle cerebral artery (MCA) occlusion for 60 min in rats. Neuronal damage visualized as a decrease of MAP2 immunoreactivity was observed in the cerebral cortex at 9 h after MCA occlusion and further expanded at 24 h. Hypoxic areas visualized with an immunohistochemical reaction for 2-nitroimidazole, a hypoxia marker (hypoxyprobe-1), and accumulation of granulocytes and platelets were also observed at 9 h and 24 h after MCA occlusion. Tacrolimus (1 mg/kg, i.v.), administered immediately after MCA occlusion, attenuated cortical damage and decreased the hypoxyprobe-1 positive area, as well as the number of granulocytes and platelets at 24 h after MCA occlusion. Immunohistochemical analysis showed that tacrolimus reduced the number of blood vessels positively stained for ICAM-1, E-selectin and P-selection. These results suggested that tacrolimus limited attachment of granulocytes and platelets to blood vessels by inhibiting the expression of adhesion molecules and protected neuronal tissue from hypoxic insults.

Key words hypoxia; transient focal ischemia; granulocyte; platelet; adhesion molecules; rat

Cerebral ischemia is one of the most frequent causes of disability and death in later life. The primary aim of therapeutic intervention for cerebral ischemia is to reduce the volume of brain damage and thus to minimize the neurological impairment. Because neuronal degeneration after cerebral ischemia can progress rapidly and is often irreversible, the benefit of effective pharmacological intervention is quite limited at present. The only medication proven to be effective and widely used at the acute stage of human stroke is tissue plasminogen activator (tPA).1

In addition to its immunosuppressive effects, tacrolimus has been widely used to prevent allograft rejection in organ transplantation. Immunosuppression is mediated by inhibition of calcineurin in T-cells, with subsequent failure of NF-AT to translocate into the nucleus and to induce transcription of IL-2.2 In addition to its immunosuppressive effects, tacrolimus has been reported to exert neuroprotective activity in a variety of animal models of cerebral ischemia.8–11 The neuroprotective effect of tacrolimus has been explained by several mechanisms such as an anti-apoptotic effect via inhibition of cytochrome c release from mitochondria, an anti-inflammator effect and so on.12,13 However, the neuroprotective mechanism of tacrolimus has not been fully elucidated.

Tacrolimus has been reported to exert an anti-thrombotic effect14 and inhibit the expression of adhesion molecules in in vitro studies,15,16 however, no studies examining effect on the occurrence of re-occlusion of microvasculature in transient focal cerebral ischemia have been reported.

With recent development of 2-nitroimidazole hypoxia markers (hypoxyprobe-1), it is now possible to detect the distribution of tissue hypoxia (pO2 < 10 mmHg) by using immunohistochemical technique.17 Using this method, we have previously reported that a hypoxic condition exists within cerebral cortex in rats at 3, 9 and 24 h after MCA occlusion for 60 min, and proposed that plugging of microvasculature with granulocytes and platelets would be responsible for the hypoxic condition.18 We also reported that tacrolimus attenuated cortical neuronal damage slightly at 9 h, with a significant effect at 24 h after MCA occlusion.19 In the present study, we therefore investigated the inhibitory effect of tacrolimus on accumulation of granulocytes and platelets and on tissue hypoxia at 9 and 24 h after transient focal cerebral ischemia in rats.

MATERIALS AND METHODS

Surgical Procedure Experiments were carried out using male Wistar rats (8—10 week-old, 300—395 g) purchased from Charles River Japan (Hino, Japan). This study was approved by the Experimental Laboratory Animal Committee of Fujisawa Pharmaceutical Co., Ltd. (now Astellas Pharmaceutical Inc.).

The procedure for transient MCA occlusion was as described by Koizumi et al.19 Briefly, each rat was anesthetized with inhalation of 4.0% halothane in oxygen–nitrogen (30% oxygen and 70% nitrogen). A mixture of 1.5% halothane and oxygen–nitrogen was used to maintain anesthesia during operation. Each animal was placed in the supine position and a midline incision was made in the skin of the neck. The right common carotid artery (CCA) was carefully exposed without
damage to the vagus nerve. The right external carotid artery (ECA), internal carotid artery (ICA) and CCA were carefully isolated and maintained in a “Y” shape using a silk thread. The right ECA and CCA were permanently ligated, and an incision to insert a piece of monofilament was made at the bifurcation. The monofilament was made of 4-0 nylon surgical suture (Nicchou, Tokyo, Japan), 19 mm in length, and was coated with silicone (Xantopren L; Heraeus Kulzer, Dormagen, Germany) mixed with a hardener (Optosil; Heraeus Kulzer) to thicken the distal 5 mm to about 0.4 mm in diameter. The proximal tip of the monofilament was heated, creating a globular stopper for easy removal of the monofilament. The monofilament was inserted into the lumen of the ICA. In this position, the monofilament can pass through the origin of the MCA and thereby occlude it. The silk suture around the ICA was tied to fixate the monofilament. The neck wound was closed and the animal was allowed to recover from anesthesia. One hour after MCA occlusion, each animal was re-anesthetized and the neck wound was re-opened. The monofilament was carefully removed to resume blood flow to the MCA and the neck wound was closed again. Neurological deficits such as forelimb flexion and circling to the left were noted following recovery from anesthesia. Animals with no neurological deficits (approximately 5% of animals) were excluded from the experiment.

Each rat received an intraperitoneal injection of hypoxyprobe-1 in saline (60 mg/kg; Chemicon, Temecula, U.S.A.) 1 h before sacrifice.

**Histopathology** At 9 (n = 5 in each group) and 24 h (n = 6 in each group) after MCA occlusion, each rat was anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused with physiological saline containing 10 U/ml heparin. Each brain was removed, rapidly frozen by immersion in isopentane at −70 °C and sliced into 5 μm sections at the level of the caudate putamen. Endogenous peroxidase activity was suppressed by incubation in 0.3% H2O2 in PBS containing 0.1% sodium azide for 10 min. Background signals were blocked by incubating tissue sections in PBS containing 10% normal donkey serum for 10 min. Tissue sections were incubated for 1 h at room temperature (23–25 °C) with anti-MAP2 (1:1000 dilution; Chemicon), anti-hypoxyprobe-1 (1:500 dilution; Chemicon), anti-CD61 (1:50 dilution; Pharmingen, San Diego, U.S.A.), anti-granulocyte (1:1000 dilution; Pharmingen), anti-ICAM-1 (1:500 dilution; Seikagaku Kogyo, Tokyo, Japan), anti-CD62P (P-selectin, 1:25 dilution; Pharmingen), or anti-E-selectin (1:500 dilution; Pharmingen). After washing, tissue sections were incubated for 30 min at room temperature with HRP-conjugated anti-mouse IgG (1:150 dilution; Jackson laboratories, Bar Harbor, U.S.A.). The presence of each antibody was detected with 0.05% diaminobenzidine tetrahydrochloride (DAB) and tissue sections were counter-stained with hematoxylin.

**Drug** Tacrolimus was synthesized at Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan). Injection formulation of tacrolimus dissolved in 20 mg/ml polyoxyethylene hydrogenated castor oil 60 (HCO-60) as a solubilizer and 5% ethanol was diluted 20 folds with physiological saline and administered intravenously. The placebo ampoule of tacrolimus containing the solvent alone, was similarly diluted with physiological saline and administered to rats in the control group. Tacrolimus was administered intravenously at a dose of 1 mg/kg immediately after MCA occlusion. Injection volume was adjusted to 2 ml/kg.

**Statistical Analysis** The ratio of the area with no or faint MAP2 immunoreactivity and the area with positive hypoxyprobe-1 immunoreactivity in the ipsilateral cortex was calculated using imaging software (Macscape, Mitani Corporation, Fukushima, Japan). Briefly, stained sections were captured as digital images (HC-2500, Fujifilm, Tokyo, Japan). The area of lowest (no or faint) intensity of MAP2 immunoreactivity and the area with highest (positive) intensity of hypoxyprobe-1 immunoreactivity compared with those of the contralateral cortex in the same section were selected. The number of CD61 positive spots corresponding to platelets, granulocyte positive cells and ICAM-1, P-selectin or E-selectin positive vessels were counted in the ipsilateral cortex and expressed as number/mm². The results were expressed as mean±S.E. Statistical analysis was performed using Student’s t test. Significant values were those with p<0.05 (*) and p<0.01 (**).

**RESULTS**

**Topographic Changes of Degenerated Tissue and Hypoxic Areas** The protective effect of tacrolimus against cerebral neuronal damage was studied using immunohistochemical staining for MAP2 (Figs. 1, 2). Representative photographs of MAP2 and hypoxyprobe-1 immunostaining from the cerebral cortex at 24 h after MCA occlusion are shown in Fig. 1. The decrease of MAP2 immunoreactivity was detected in the cerebral cortex of the placebo group except for faint staining in layer IV to VI. In contrast, in the tacrolimus group, the MAP2 immunoreactivity was still retained in the cortex. The hypoxyprobe-1 immunoreactivity was clearly visible mainly in layer IV to VI in the placebo group, whilst the hypoxyprobe-1 immunoreactivity was hardly detectable in the tacrolimus group. Neuronal degeneration represented by a decrease of the MAP2 immunoreactivity was present at 9 h after MCA occlusion and expanded further at 24 h in placebo group (Fig. 2A). In the tacrolimus group, loss of the MAP2 immunoreactivity was hardly detected at 9 h after MCA occlusion, and the area with loss of the MAP2 immunoreactivity was significantly reduced as compared to that in the placebo group at 24 h (p<0.01) (Fig. 2A). The hypoxic region expressed with positive hypoxyprobe-1 immunoreactivity was observed at 9 h and more at 24 h in the placebo group (Fig. 2B). Tacrolimus significantly reduced the hypoxic area at 24 h after MCA occlusion (p<0.01) (Fig. 2B).

**Accumulation of Granulocytes and Platelets** To characterize the participation of granulocytes and platelets in the development of neuronal damage, accumulation of these cell types was investigated immunohistochemically (Figs. 3, 4). Representative photographs with immunostaining for granulocytes and platelets at 24 h after MCA occlusion are shown in Fig. 3. Accumulation of granulocytes and platelets was decreased in the cortex in the tacrolimus group as compared to the placebo group. In the placebo group, accumulation of granulocytes was still limited at 9 h but increased at 24 h after MCA occlusion. On the one hand, platelets were constantly visible at 9 h and 24 h after MCA occlusion. Tacrolimus decreased the number of granulocytes (p<0.01) and platelets (p<0.05) in the cortex at 24 h after MCA occlu-
To elucidate the mechanism of accumulation of granulocytes and platelets, we counted the number of blood vessels immunoreactive for ICAM-1, P-selectin, and E-selectin in the cortex (Figs. 5, 6). Representative photographs with immunostaining for ICAM-1, P-selectin, and E-selectin at 24 h after MCA occlusion are shown in Fig. 5. Those adhesion molecules were expressed on the endothelium at 9 h after MCA occlusion and the number of immunoreactive blood vessels were increased at 24 h in the placebo group. Treatment with tacrolimus decreased the number of ICAM-1 positive vessels at 24 h (p<0.05), P-selectin positive vessels at 24 h (p<0.01), and E-selectin positive vessels at 9 h (p<0.05) and 24 h (p<0.05) after MCA occlusion (Fig. 6).

DISCUSSION

In the present study, we showed that tacrolimus administered immediately after MCA occlusion ameliorated the development of brain damage in the cortical tissue, confirming
our previous observations on the neuroprotective action of the drug in the same transient focal ischemia model. Since tacrolimus has been reported to exert an anti-thrombotic effect, we thought it is important to clarify the involvement of anti-thrombotic effect of tacrolimus in its protective action on brain injury associated with ischemia-reperfusion. Expanding on our previous study, the present investigation showed that tacrolimus significantly attenuated the expansion of hypoxic tissue in the cortical area at 24 h after MCA occlusion, as assessed by hypoxyprobe-1 immunostaining. We also observed the accumulation of granulocytes and platelets at 24 h after MCA occlusion, suggesting that the re-occlusion of microvasculature with granulocytes and platelets would be responsible for the hypoxic condition.

del Zoppo et al. found accumulation of granulocytes and platelets in a baboon model, concluding that the resulting hemagglutination was the cause of re-occlusion. In human stroke, as in animal models, re-occlusion after re-canalization has been reported and considered to be a potentially serious problem. Re-occlusion after thrombolytic therapy with tPA has been reported to occur at a high frequency, and has been considered to be the cause of insufficient outcome and mortality for this therapy. Recently, a Phase I clinical study for argatroban, a direct thrombin inhibitor, co-administrated with tPA was reported. An argatroban/tPA combination therapy has shown a tendency to improve the rate of re-canalization compared to tPA monotherapy, however, the rate of re-occlusion was not altered. Thus, the development of a drug to prevent re-occlusion is highly desirable for treatment of acute stroke in humans. Our present study suggest that tacrolimus has promising potential for prevention of re-occlusion after thrombolytic therapy.

Tacrolimus has been shown to inhibit granulocytes infiltration in various ischemia-reperfusion injury models,
however, its inhibitory effect on the attachment of platelets to endothelium is less well known. In our present study, accumulation of platelets was clearly suppressed by tacrolimus at 24 h after MCA occlusion. Adhesion of granulocytes and platelets to endothelium is accompanied by expression of adhesion molecules on the endothelium. Our results clearly demonstrated the inhibitory effects of tacrolimus on the expression of ICAM-1, P-selectin and E-selectin after transient focal cerebral ischemia. Up-regulation of adhesion molecules on the endothelial cell surface is thought to be induced by cytokine stimulation. It is reasonable to assume that tacrolimus might suppress the expression of adhesion molecules by inhibiting cytokine production in the infarcted tissue. Further studies are necessary to fully elucidate the mechanism for an inhibitory action of tacrolimus on the up-regulation of adhesion molecules after transient cerebral ischemia.

Adverse effects such as nephropathy and hyperglycemia in the allograft recipients have been revealed after tacrolimus treatment. However, these adverse effects were thought to be caused by long-term use. The minimal lethal dose of tacrolimus in rats was reported at 18 mg/kg for intravenous dosing (18-fold higher dose than that used in this study), and no unusual clinical sign or gross pathological abnormality was observed at 2 weeks after MCA occlusion in our previous study. Therefore, the risk of serious adverse effects associated with dosing regimen adopted in this study would be extremely low.

In conclusion, the present results taken together suggest that tacrolimus limited attachment of granulocytes and platelets to blood vessels by inhibiting the expression of adhesion molecules and protected neuronal tissue from hypoxic insults following focal brain ischemia, suggesting tacrolimus could have promising potential for prevention of re-occlusion after thrombolytic therapy.

REFERENCES