FK-506: A New Immunosuppressive Agent, Failed to Reduce Cerebral Vasospasm after Experimental Subarachnoid Hemorrhage

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(Received 20 January 1993/Accepted 2 April 1993)

ABSTRACT. To define the relationship between the immunologic reaction and the pathogenesis of cerebral vasospasm (VS) following experimental subarachnoid hemorrhage (SAH), we examined the effect of a cell mediated immunosuppressive agent, FK-506, isolated from *Streptomyces tsukubaensis*, by using the canine SAH model. There was a significant vasoconstriction in the basilar artery in the control group after SAH. This constriction, however was not successfully prevented by FK-506 or combination of FK-506 and steroid, since there was no significant difference in the vessel caliber size among these groups. The pathologic approach, accompanied by immunohistochemistry, could not discriminate the differences in the nature of the lesion between the untreated group and FK-506 treated groups, except for slight lymphocytic infiltrations present around the basilar artery of untreated group. Histopathologically, inflammatory reactions consisting of neutrophils, that were not suppressed by FK-506 treatment, were clearly seen around the spastic vessels in the subarachnoid space. Furthermore, several constrictive changes or degenerative alterations were also observed in the spastic vascular wall. Immunohistochemically, the deposition of IgG, IgM and C₃ was present in the intima and the luminal side of the smooth muscle layer, and capillary vessels of the brain stem. It is considered that this deposition was caused by increased vascular permeability in VS. On the basis of the above findings that the cell mediated immunosuppressive agent, FK-506 failed to prevent vasoconstriction or pathologic lesions but lymphocytic infiltrations, it is considered that the cell mediated immunopathogenesis may play little role in producing VS following SAH.—**KEY WORDS:** canine, cerebral vasospasm, FK-506, subarachnoid hemorrhage.

J. Vet. Med. Sci. 55(4): 581-586, 1993

Cerebral vasospasm (VS) following subarachnoid hemorrhage (SAH) shows a biphasic pattern including early and delayed phases. Chronic ischemia caused by delayed VS is considered to be responsible for a significant proportion of the functional deficiency and mortality [18, 19]. Therefore, studies on pathogenesis of VS and development of a potent remedy for the condition is considered to be the most important investigation in this field.

The focus of the cerebrovascular research shifted toward a hypothesis that involvement of immunologic responses may be an important factor for the pathogenesis of VS following SAH since the appearance of clinical reports describing elevated serum levels of immune complex in patients suffering from VS [12, 14, 15, 24]. In 1990, Peterson *et al.* reported that an immunosuppressant, cyclosporine A (CsA) may be clinically applicable as a prophylactic agent for delayed VS after experimental SAH in dogs [13]. It is still controversial, however, whether what immunologic reactions play some roles in its pathogenesis, although it is generally accepted that VS initially occurs in contact with autologous blood clot in the subarachnoid space. In spite of the general thought that CsA suppresses production of interleukin-2 (IL-2) to affect cellular immunity and thus interferes with the pathogenesis of VS, our preliminary investigation on

human VS patients revealed low levels of IL-2 in the cerebrospinal fluid (unpublished data). Thus, the effect of CsA as a prophylactic agent against VS through inhibition of IL-2 production is questionable.

The aim of the present study is to investigate the effect of a potent immunosuppressant FK-506 on VS using a canine SAH model since its suppressive effect is more potent and specific to cytotoxic T-cell generation than that of CsA. FK-506 was extracted from *Streptomyces tsukubaensis* strain No. 9993 in 1984, and has been well adapted to organ transplantation as a substitute for CsA [6, 10, 11]. The immunosuppressive effects of both drugs are known to be extremely similar, although each compound differs in chemical structure, i.e., CsA is grouped in a cyclic peptide family and FK-506 belongs to macrolides [1, 10, 17]. By comparing the reported effects of CsA and the results of this study with FK-506, it is also aimed to extend discussions on the immunopathogenesis of VS.

MATERIALS AND METHODS

Animal model and cerebral angiography: The experimental protocol followed "double hemorrhage canine model" of chronic VS, which was comparable to the human counterpart in the angiographic appearance as well as in the time course [8, 16, 25].

Twenty-six beagle dogs of either sex (weighing 9–12 kg) were allocated randomly to the treated and untreated groups. Anesthesia was induced with an intravenous injection of 30 mg/kg sodium pentobarbital with spontaneous breathing, and the blood gas was frequently monitored during the course of the procedure. Thereafter, animals were placed in the prone position with the neck flexed 30 degrees down. After surgical exposure of the vertebral artery, an angiographic catheter was advanced. A baseline angiogram of the basilar artery was obtained by using 5 ml of meglutamine diatrizoate as 200 mg iodine/ml. The cisterna magna was punctured aseptically with a spinal needle (23 gage), and 0.25 ml/kg of cerebrospinal fluid was withdrawn, which was replaced with 0.5 ml/kg of autologous blood over a period of 2 min. The animals were maintained in the prone position with the neck flexed 30 degrees down for 30 min to allow the injected blood to coagulate around the basilar artery. After 48 hrs, they were anesthetized again and the same amount of autologous blood was injected in the cisterna magna with the same manner. Angiography of the basilar artery was simultaneously performed by the above mentioned protocol so as to demonstrate the time course of the caliber change, with follow-up on days 2 and 7.

The care of the animals and procedures in the present protocols complied with the "Principle of Laboratory Animal Care" and had been approved by the Animal Care Committee of the Institution.

Treatment schedule and experimental groups: FK-506 was kindly provided by Fujisawa Pharmaceutical Co. (Osaka, Japan). Dosage and formula followed the preliminary reports; that is, an immunosuppressive dose of 0.16 mg/kg/day in dogs was given as an i.m. formula [10]. The i.m. formula solution was freshly prepared every time by dissolving the drug in saline to make a final concentration suitable for administering 0.2 ml/kg. Experimental groups were prepared as follows; Group A (n=11) was untreated (control), Group B (n=6) was treated by i.m. formula of FK-506 at 0.16 mg/kg/day, and Group C (n=9) was treated methyl prednisolone as an adjunct low-dose steroid at 0.5 mg/kg/bid i.v. in addition to the identical administration of FK-506. Each treatment was carried out immediately after the injection to cisterna magna, and was pursued for 7 days until the animals were sacrificed.

Pathologic study: After the last angiographic procedure, the animals were killed with an intravenous dose of sodium pentobarbital (30 mg/kg) followed by exsanguination. The exposed part of basilar artery including the brain stem was quickly fixed in 10% neutral buffered formalin solution and routinely embedded in paraffin. Sections (3 μ m) were stained with hematoxylin and eosin (HE). Other tissue blocks were also immediately frozen in dry ice-acetone, and cryosectioned at 5 μ m for immunohistochemical study by means of avidin biotin complex (ABC) procedure. The sections were fixed in periodate-lysine-paraformaldehyde (PLP) solution, pH 6.2, at 4°C for 10 min. The sections were incubated in 3% H₂O₂ in 0.01 M phosphate buffered saline (PBS), pH 7.4, for 20

min to eliminate endogenous peroxidase. After rinsing twice in PBS for 15 min, the sections were placed in a moist chamber and covered first with 5% normal goat serum for 20 min to minimize non-specific staining and then with either of three primary antibodies, rabbit anti-dog IgG, IgM or C₃ (each at a dilution of 1:500, at room temperature for 2 hrs, Bethyl Laboratories, Montgomery, TX, U.S.A.). The rabbit antiserum as the primary antibody was diluted with 0.5% Tween-20 (Sigma, St. Louis, MO, U.S.A.) in PBS. After rinsing again in PBS for 15 min twice, each section was incubated with biotinylated goat anti-rabbit IgG for 30 min and reacted with ABC reagent (Vector Laboratories Inc., Burlingame, CA, U.S.A.) for 30 min. After rinsing again for 15 min three times, each section was reacted with a mixture of 0.05% 3,3'-diaminobenzidine tetrahydrochloride in 0.05 M Tris-HCl buffer, pH 7.6, with 0.01% H₂O₂ for 10 min to obtain a chromogenic reaction. Finally, the sections were counterstained with Hanzen's hematoxylin.

Negative control slides were treated with the identical procedure in which 0.5% Tween-20 in PBS or normal rabbit serum was substituted for the primary antibody and also incubated without biotinylated antibody or ABC reagent. Simultaneously, positive control slide was prepared from the mesenteric lymph node in the same individual.

Data analysis: The calibers of the basilar artery were measured at five corresponding locations along vessel on the angiograms using a magnifier, and the changes were expressed as a percentage to the identical baseline calibers in the same animals. The accumulated angiographical data in respective groups were expressed as mean $\pm$ SEM. Difference between the calculated data of untreated group and of FK-506 treated groups was evaluated by using Student's unpaired *t* test. Groups were considered to be significantly different if the probability of error was less than 0.05.

RESULTS

Caliber of the basilar artery: The present protocol yielded reproducible constriction of the basilar artery (Fig. 1 A-C). In the untreated Group A (n=11), the caliber of the basilar artery constricted to $81.0 \pm 4.0\%$ (mean $\pm$ SEM) on day 2, and $63.8 \pm 3.5\%$ on day 7 of that measured before experimental SAH in each animal. In the FK-506-treated Group B (n=6), the caliber also decreased to $78.8 \pm 2.4\%$ (mean $\pm$ SEM) on day 2, and $67.9 \pm 3.2\%$ on day 7 of the initial measurement. In the steroid-FK-506-treated Group C (n=9), the caliber were calculated as $77.9 \pm 3.4\%$ (mean $\pm$ SEM) on day 2, and $59.6 \pm 4.1\%$ on day 7 of the initial measurement. The constriction as determined by the caliber size resembled among untreated and FK-506 treated groups, which showed a progressive decreasing tendency during the time course over 7 days. There was no statistically significant difference between any two groups in this experiment (Fig. 2).

Pathologic findings: In the ventral surface of the brain

Fig. 1. Angiogram of the basilar artery of a FK-506 treated dog before SAH (A), on day 2 following SAH (B), and on day 7 following SAH (C). Note severe angiographic narrowing of the vessel diameter.

Fig. 2. Changes in the basilar artery diameter in FK-506 treated group, FK-506+PSL treated group, and untreated group. Each value represents % mean $\pm$ SEM to the original diameter.

stem, almost all basilar arteries were seen under a surgical microscope exhibiting moderate to severe SAH with extensive blood clots in the vessel's cistern, and considerable narrowing was also observed (Fig. 3).

Histologic appearances were almost analogous between untreated and FK-506 treated groups, except for slight lymphocytic infiltrations present around the basilar artery of untreated group. The subarachnoid space was moderately expanded due to edema and numerous red blood cells (Fig. 4). Slight to mild inflammatory reactions consisting of neutrophils were present around the basilar artery and adjacent smaller cerebral vessels. No lymphocytic infiltrations were present in FK-506 treated

Fig. 3. Ventral surface of the brain of a double canine SAH model. The basilar artery is observed embedded in injected autologous blood clots encasing the vessels' cistern.

groups. The spastic vessels showed a severe constrictive change accompanied by folding and corrugation of elastic lamina, several desquamated endothelial cells, and cytoplasmic vacuolation in the smooth muscle layer. No subintimal proliferation and myonecrosis of tunica media

were observed in any groups. In addition, erythrophagocytosis of the autologous blood by macrophages were frequently observed in the subarachnoid space.

Immunohistochemical findings: Deposition of IgG, IgM and C₃ was detected in all groups with similar intensities, with moderately positive reactions in the intima and the

luminal side of the smooth muscle layer (Figs 5, 6 A-C). Furthermore, in the brain stem, capillary vessels consisting of one or two layers also showed moderately positive reaction. In addition, all negative control slides showed no reaction, while all positive control slides were stained as expected.

DISCUSSION

The present study demonstrates that FK-506 does not show a definite prophylactic effect on basilar arterial constriction in our protocol. The administration protocol of FK-506 referred to a well-established method reportedly effective in achieving immunosuppression in canine renal allotransplantation [10]. Furthermore, the drug was given immediately after the initial blood injection, as effects of FK-506 is dependent on the timing of administration that is within 2 to 3 hrs after antigen stimulus [17, 22]. Therefore, the current dosing protocol apparently provided a sufficient effect if any in the experimental animals. Furthermore, another group of animals were given FK-506 plus low dose steroid to potentiate the effect by interfering inflammatory reactions.

In T cells, both FK-506 [7, 22] and CsA [1, 23] disrupt an unknown step in the transmission of signals from the T cell antigen receptor to cytokine genes, especially IL-2, resulting in inhibition of cell mediated immune response. Furthermore, FK-506 is known to inhibit cytotoxic T cell generation at about 100 times lower concentration than CsA *in vitro* [7]. Hence, our results indicate that cell mediated immune response caused by IL-2 may play little role in the pathogenesis of VS. However, if the condition is not suppressed by a potent inhibitor of T cell functions, the reason for the apparent suppressive effect of CsA

Fig. 4. Subarachnoid space of the FK-506 treated dog. There are moderate expansion due to edema and numerous red blood cells around the spastic basilar artery in the subarachnoid space. HE $\times 80$.

Fig. 5. Spastic basilar artery of the untreated dog. Deposits of IgG (A), IgM (B) and C₃ (C) are seen in the intima and the luminal side of the smooth muscle layer. Immunohistochemical stain, hematoxylin counter stain. $\times 400$.

Fig. 6. Spastic basilar artery of the FK-506 treated dog. Deposition of IgG (A), IgM (B) and C_3 (C) are similar to those of the untreated dog (Fig. 5). Immunohistochemical stain, hematoxylin counter stain. $\times 400$.

remains to be clarified. The non-immunopathologic etiology of VS is further supported by pathologic and immunohistochemical investigations in this study. Pathologic features were not different between the FK-506 treated and untreated group, except that slight lymphocytic infiltrations were present around the basilar artery of untreated group. Severe constrictive changes or degenerative alterations, which have been reported to be specific for SAH [2, 3–5, 16, 21] were observed in the spastic vascular wall, and these changes were not inhibited by FK-506 treatment. Although lymphocytic infiltrations were suppressed by FK-506 treatment, a prominent inflammatory reaction consisting of neutrophils was demonstrated around the spastic vessels in the subarachnoid space. This inflammation may not be initiated by T-cell mediated immunologic reactions if it is not suppressed by FK-506 treatment.

Immunohistochemical detection of immunoglobulins and complements was not different between untreated and FK-506 groups. Deposition of IgG, IgM and C_3 was observed even in the FK-506 treated groups. Hoshi *et al.* reported in human vasospasm patients that the immunoglobulins and complements were deposited in tunica media of the spastic vascular wall and that the deposition was not specific to VS [4]. It is considered that deposition of IgG, IgM and C_3 is not local immune response, but that they may be percolated to the muscular walls due to increased vascular permeability secondary to desquamation of endothelial cells and injury of lamina elastica in VS. Indeed, according to our previous study with vascular permeability in VS following SAH by using horse radish peroxidase (HRP) as a tracer, HRP permeated up to muscular layer through the subintima, and elevated

vascular permeability continued for several days [9, 20]. Whether these deposits of immunoglobulins and complement protein may caused by a tissue injury, a condition so-called myonecrosis, is unclear at present. It is suggested, however, that this increased vascular permeability could further cause continuous vasoconstriction by facilitating more direct action of secreted spasmogen to the media muscular layer.

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