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FK 506 ameliorates normothermic liver ischemia in rats by suppressing production of tumor necrosis factor

Katsunori Kawano[1], Yang Il Kim[1], Shigeru Goto[1], Tetsuji Kai[1], Tatsuo Shimada[2], Naoshi Kamada[3], and Michio Kobayashi[1]

Departments of [1] Surgery I and [2] Anatomy, Oita Medical College, Oita, Japan
[3] Department of Experimental Surgery, National Children's Medical Research Center, Tokyo, 154, Japan

Abstract. In recent years, there has been growing evidence that tumor necrosis factor-α (TNF) plays an important role in the development of hepatic injury after ischemia-reperfusion. We have previously demonstrated that the immunosuppressants, cyclosporine, azathioprine and FK506 (FK), have a protective effect on warm ischemic injury of the rat liver. In the present study, we attempted to elucidate the mechanism for the beneficial effect of FK on liver ischemia, with special reference to the suppression of TNF production. After 60 min and 90 min of warm liver ischemia, the survival rates were significantly improved by FK pretherapy. This was associated with amelioration of hepatic injury, as assessed by histological examinations and determinations of serum AST and lipid peroxide levels in the liver. After 60 min of liver ischemia, TNF was measurable during the reperfusion period in the sera of the control animals, peaking of 6 h after reperfusion (123 ± 15.8 pg/ml, mean SEM). In contrast, pretreatment with FK significantly suppressed the elevation of serum TNF levels at the same time point (75.8 ± 13.1 pg/ml, $P < 0.05$). The present data showed that liver ischemia-reperfusion resulted in TNF production, and that FK could protect the liver from reperfusion injury by suppressing this production of TNF.

Key words: Liver ischemia – FK 506 – Lipid peroxidation – Tumor necrosis factor

Primary graft nonfunction, which has been reported to be associated with ischemic injury, is a major indication for retransplantation following liver transplantation [1]. Furthermore, there is evidence that severe preservation injury to endothelial cells results in an increased incidence of allograft rejection [2]. Recently, there has been great interest in methods for protecting the liver from ischemia-reperfusion injury. Although the precise mechanism has not been elucidated, we have recently reported that cyclosporine (CsA) and azathioprine (AZA) ameliorate both warm and cold ischemic injury of the liver in rats and pigs [3–6]. More recently, FK506 (FK), a potent new immunosuppressive agent, has been shown to possess a similar protective effect [7, 8], suggesting a possible linkage between the immune system and ischemic injury of the liver.

The purpose of this study was to clarify the mechanism by which FK exerts its beneficial effect on warm ischemia in the rat liver, with special reference to the suppression of lipid peroxides and of tumor necrosis factor-α (TNF) production.

Materials and methods

Female Sprague-Dawley rats weighing 200–300 g were used throughout this study. A temporary normothermic liver ischemia was induced as decribed by us in previous study [3]. Briefly, the abdomen was opened through a midline incision under light ether anesthesia. Liver ischemia was produced by occluding the hepatic artery and the portal vein to the left lateral and median lobes with a small vascular clip. The remaining hepatic lobes were excised at reperfusion, leaving only the ischemic lobes behind. The antibiotic, cefamandole sodium (100 mg/kg), was administered intramuscularly just prior to laparotomy.

The rats were assigned to two groups. In the control group (group I), the animals underwent warm liver ischemia with saline vehicle pretherapy. Group II rats received FK (1 mg/kg/day p.o.) for 4 days prior to the induction of liver ischemia. The FK506 (Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan) was suspended in a physiologic saline solution.

For the survival study, a total of 96 rats were subjected to 60 min or 90 min of liver ischemia. Autopsy was performed in all animals that died during the observation period, and survivors were sacrificed 7 days after surgery.

In a second experiment, four to nine rats were sacrificed before, during and 1, 6 and 12 h after reperfusion. Immediately before sacrifice, blood samples were taken from the inferior vena cava for measuring serum levels of aspartate aminotransferase (S-AST). They were determined by an ultraviolet method using an autoanalyzer (Jeol JCA-MS24, Japan). Serum activities of TNF were measured using an ELISA kit (Otsuka, Tokyo, Japan). At the same time, a portion of the ischemic median hepatic lobe was taken for determination of lipid perioxide content and for histological examination. Lipid peroxide was estimated as levels of malondialdehyde (MDA), using a colorimetric reaction with thiobarbituric acid [9]. Protein concentration was determined according to the method described by Lowry et al. [10]. Sections of the liver were examined with both a light and a transmission electron microscope.

The results were expressed as the mean and the standard error of the mean, and were statistically compared using the generalized Wilcoxon's test or the Student's t-test. Statistical significance was defined as a P value less than 0.05.

Offprint requests to: Katsunori Kawano, M.D., Department of Surgery I, Oita Medical Collge, Oita, 879-55, Japan

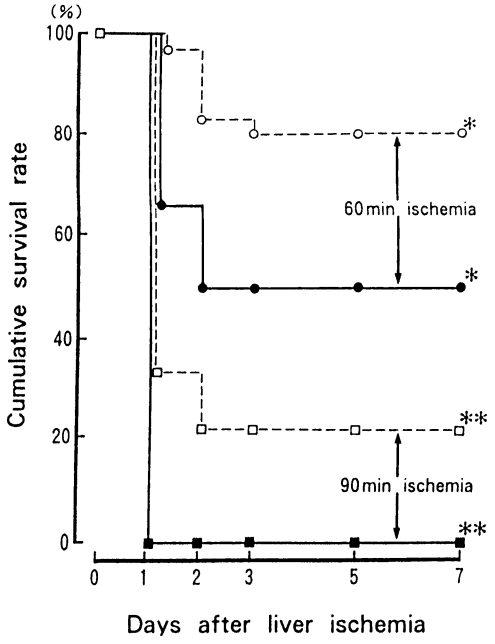

Fig. 1. Cumulative survival rates of the rats after 60 min and 90 min of liver ischemia. The differences between groups I and II were significant at * $P < 0.05$ for 60 min ischemia and * $P < 0.005$ for 90 min ischemia. (Control at 60 min –●– and at 90 min –■–; FK506 at 60 min, --○-- and at 90 min --□--)

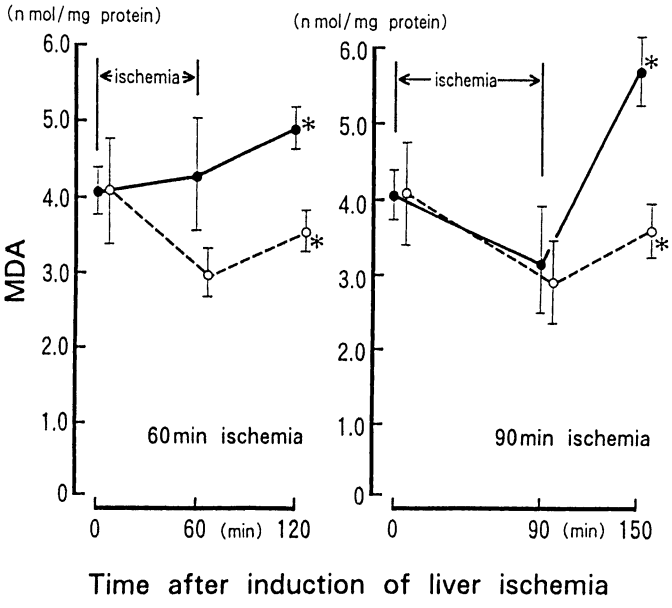

Fig. 2. The effect of FK506 on MDA levels in an ischemic liver. MDA levels were measured before, during and 1 h after 60 min and 90 min of liver ischemia. Each point shows the mean ± SEM. The differences between groups I and II were significant at *$P < 0.01$. (control, –●–; FK506, --○--)

Results

Cumulative survival curves after 60 min and 90 min of liver ischemia are shown in Fig. 1. Survival rates at day 7, calculated using the Kaplan-Meier method, were 50.0% (19/38) and 80.0% (24/30) for groups I and II respectively, after 60 min of ischemia. Those after 90 min of liver ischemia were 0% (0/10) and 22.2% (4/18) for the rats not-treated and treated with FK, respectively. The differences between the two groups after both 60 min and 90 min of liver ischemia were statistically significant ($P < 0.05$ for 60 min ischemia and $P < 0.005$ for 90 min ischemia).

Serial changes in MDA levels are depicted in Fig. 2. After 60 min of liver ischemia, the MDA levels in the control group remained steady during the ischemic period but tended to increase 1 h after reperfusion. In contrast, the MDA levels in the FK-treated group were reduced to 73.2% of the initial value at the and of the ischemic period. At 1 h after reflow of blood, the levels in group II were significantly suppressed compared with those in group I (4.95 ± 0.30 nmol/mg protein, $n = 9$, for group I; 3.59 ± 0.29, $n = 6$, for group II; $P < 0.01$). A similar result was observed in the 90 min of liver ischemia model. At 60 min after reperfusion, the difference in the MDA levels in the two groups was statistically significant (5.72 ± 0.46, $n = 8$, for group I; 3.68 ± 0.28, $n = 5$, for group II; $P < 0.01$).

Histological alterations in representative livers taken at 1 h after reperfusion of ischemic livers are shown in Figs. 3 and 4. On light microscopic examination, parenchymal cells with eosinophilic changes and spotty necrosis of the hepatocytes were seen in the control group (Fig. 3a). In contrast, in the FK-treated group some eosinophilic changes were seen in the hepatocytes associated with swelling, but to a lesser degree (Fig. 3b). In group I electron microscopy showed detachment of endothelial cells from hepatocytes, thus allowing blood cells to infiltrate into Disse's space (Fig. 4a). In contrast, the structures of the hepatic sinusoid and Disse's space were relatively well preserved in the liveer treated with FK (Fig. 4b).

Serial changes of serum TNF levels are illustrated in Fig. 5. In the control group, the levels rose sharply at 6 h following reperfusion and declined thereafter. A similar pattern was observed in group II. When the peak values were compared, however, FK-treated animals (75.8 ± 13.1 pg/ml, $n = 7$) had significantly lower levels than those in the control group (123.2 ± 15.8, $n = 7$; $P < 0.05$). Serum levels of AST showed a parallel change with that of TNF (Fig. 6). A substantial difference between the two groups was seen at 6 h after reperfusion (10942 ± 802 IU/l, $n = 8$, for group I; $7637 P < 807$, $n = 8$, for group II, $P < 0.02$).

Discussion

As has been reported previously for CsA and AZA [3–5], we demonstrated in the present study that pretherapy with FK506 improved the survival of rats following warm liver ischemia. This was reflected by amelioration of hepatic injury which was estimated by histological examination and quantified by measuring serum AST. We hypothesize that suppression of lipid perixodative damage and of TNF production might be the main mechanisms by which FK protects the liver from an ischemic insult.

Deleterious chemical reactions involving oxygen-derived free radicals have been shown to be the causes of

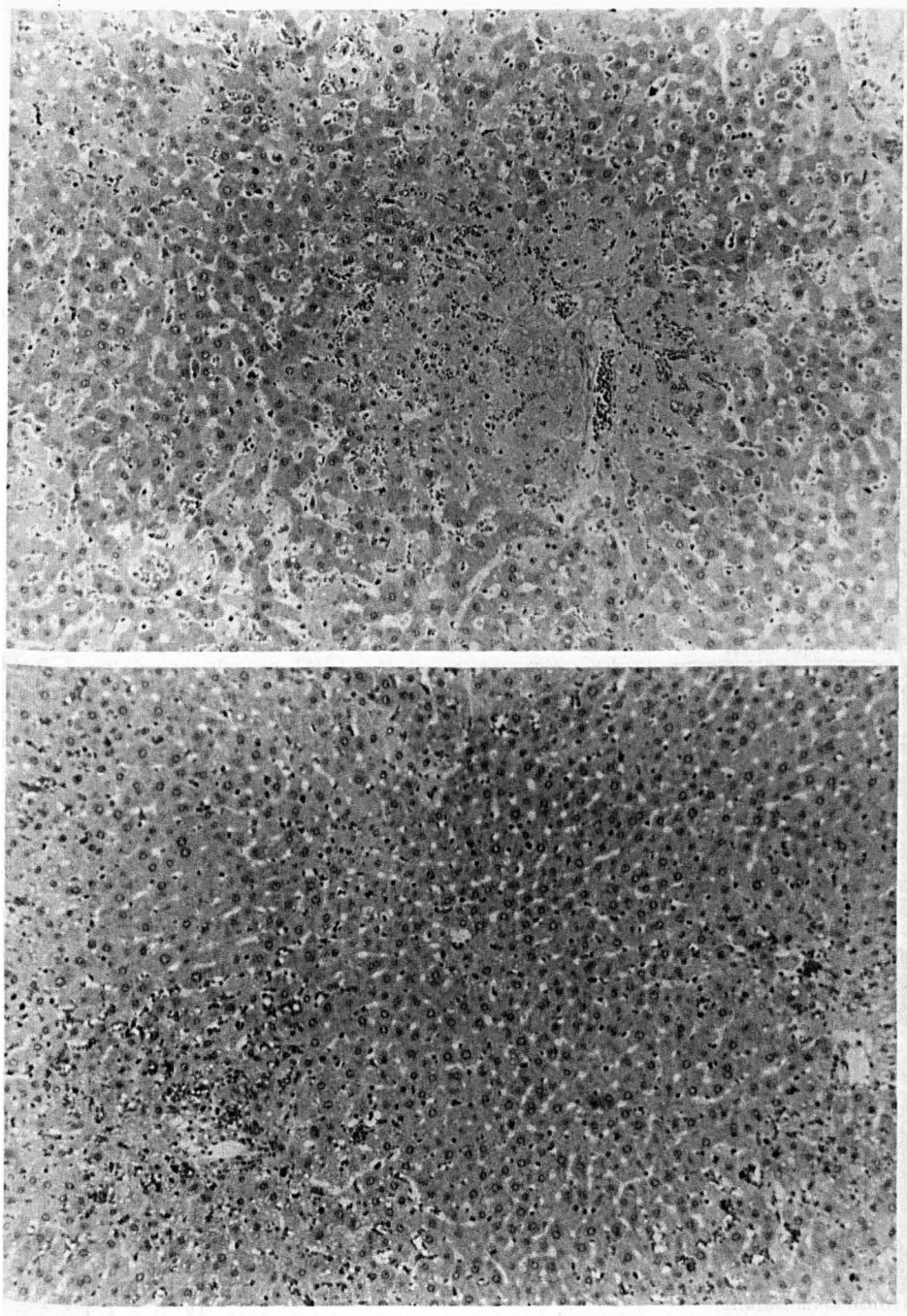

Fig. 3 a, b. Light microscopic examination of the liver **a** not treated and **b** treated with FK506. Specimens were taken 1 h after reperfusion following 60 min of liver ischemia, H&E (× 190)

ischemia-reperfusion injury which occur immediately following reperfusion [11–13]. In the present study, it was observed that pretherapy with FK suppressed the tissue levels of MDA which is one of the stable end-products of lipid peroxides. Although there are some possible sources for the production of the free radicals, it has been suggested that reactive oxygen intermediates are released by activated hepatic macrophages [14, 15] and by circulating neutrophils [16]. In this regard, it has been reported that CsA reduces the capacity of macrophages to produce hydrogen peroxide (H_2O_2) and the superoxide anion ($O_{sup}-_2$ [17], although whether FK suppresses such production of oxygen radicals is unknown. Thus, we postulated that FK suppressed lipid peroxide reactions, which might initiate hepatic reperfusion injury, by inhibiting the activation of macrophages and/or neutrophils.

Cytokines are increasingly being recognized as critical mediators of ischemic injury to the liver [18]. Among the inflammatory cytokines, TNF, which is produced primarily by cells of the monocyte/macrophage lineage including the liver Kupffer cells, has been shown to exert profound effects on both neutrophils and endothelial cells. It has been reported that TNF stimulates neutrophil adhesion to rat liver sinusoidal endothelial cells and increases respiratory burst activity [16, 19]. Moreover, TNF may activate endothelial cells to initiate coagulation [20, 21], and render these cells more susceptible to neutrophil-mediated damage [22]. Taking into consideration the fact that FK

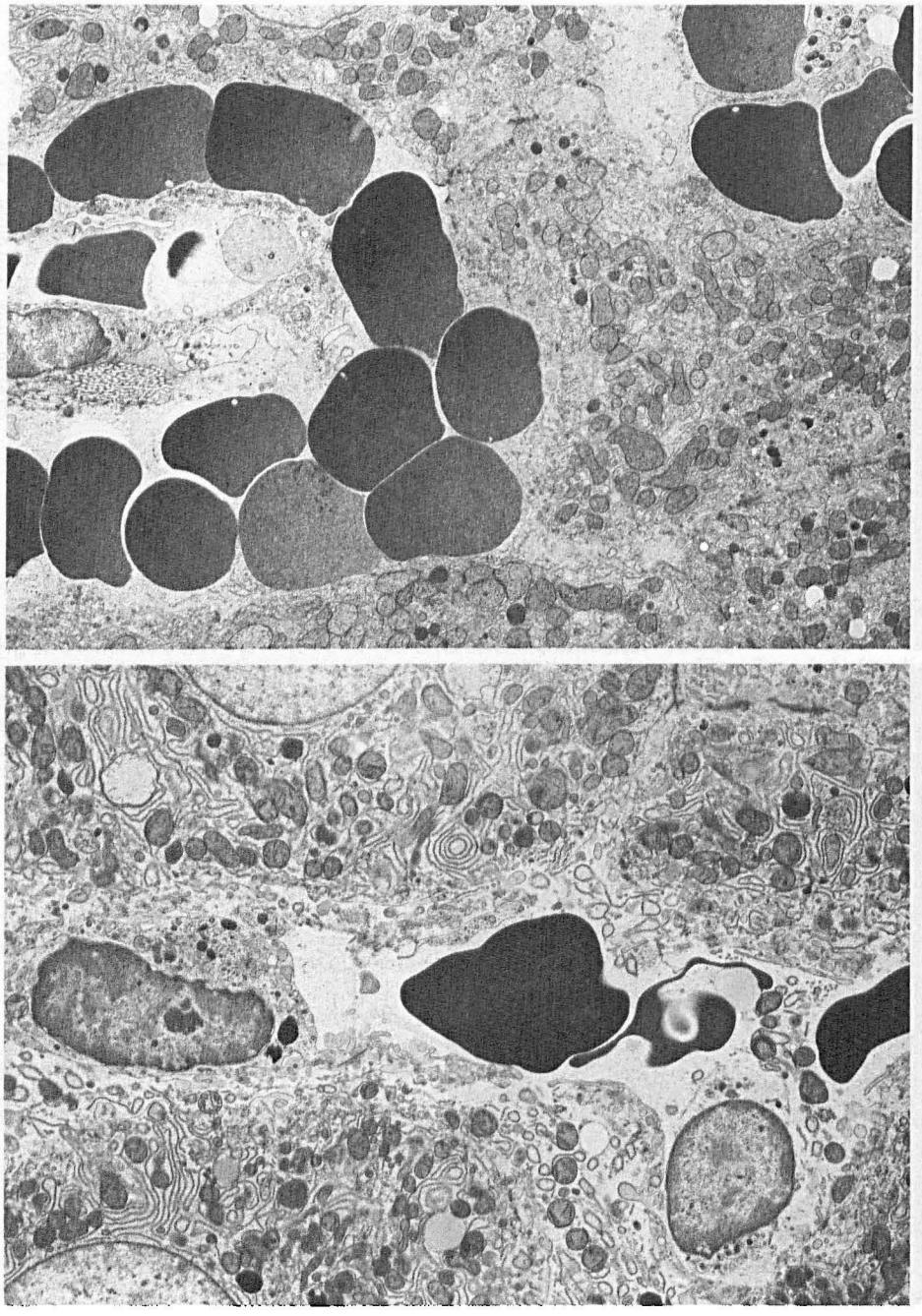

Fig. 4 a, b. Electron microscopic examination of the liver taken 1 h after reperfusion following 60 min of liver ischemia. **a** Liver of the control rats; **b** liver of the animals treated with FK 506 (× 5200)

inhibits the expression of TNF genes [23] we propose that FK exerts its protective effect against liver ischemia by suppressing the production of TNF which might accelerate hepatic injury after reperfusion.

Whether the hepatotrophic effect of FK is dependent on or independent of its immunosuppressive properties remains controversial [24]. However, it is equally conceivable that the hepatotrophic quality of this fungal agent affects the ability to recover from the ischemic injury [25]. We are testing the effect of the pretreatment of the donor or the liver graft liver FK or other immunosuppressants for a possible use in liver transplantation. Moreover, we are pursuing further experiments to observe whether this agent ameliorates pulmonary pathologic changes which are often associated with hepatic injury.

In conclusion, we confirmed the protective effect of FK 506 on warm liver ischemia in the rat. We suggested that suppressed production of lipid peroxides and TNF might account for its beneficial effect on liver ischemia.

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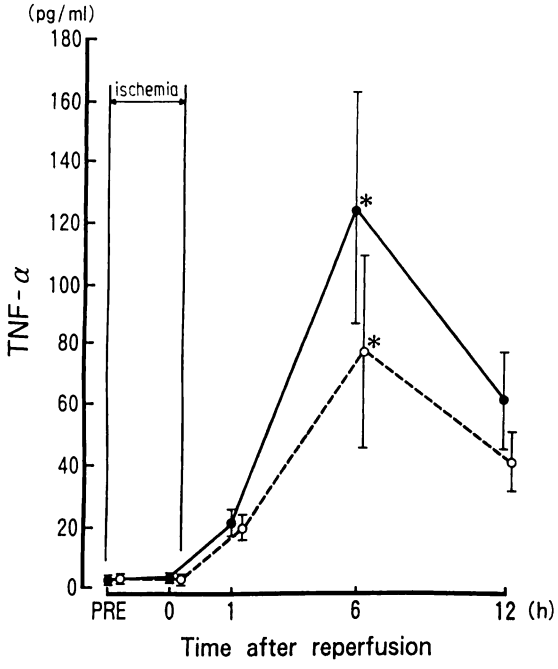

Fig. 5. Serial changes of serum TNF levels after 60 min of liver ischemia in the rats treated or not treated with FK 506. Each point shows the mean ± SEM. The differences between groups I and II were significant $*P < 0.05$ 6 h after reperfusion (control, –●–; FK 506, --○--)

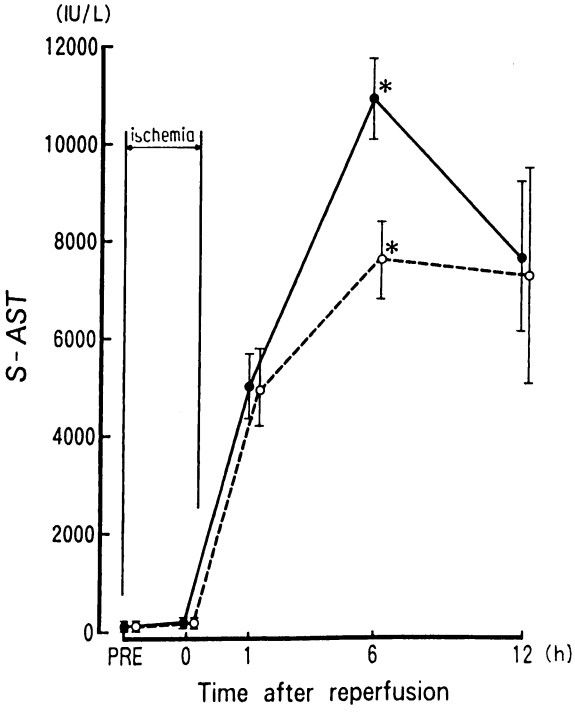

Fig. 6. Serum levels of AST following 60 min of warm liver ischemia. Each point shows the mean ± SEM. The difference between groups I and II 6 h after reperfusion was $*P < 0.02$. (control, –●–; FK 506, --○--)

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