Transpl Int (1994) 7 [Suppl 1]: S507–S511

Takahiro Ishii
Yang Il Kim
Toshiro Tatsuma
Katsunori Kawano
Tetsuji Kai
Michio Kobayashi

Immunodepressants ameliorate normothermic ischemia injury to the rat liver by down-regulating tumor necrosis factor, not by alleviation of lipid peroxidative injury

Takahiro Ishii (✉) · Yang Il Kim
Toshiro Tatsuma · Katsunori Kawano
Tetsuji Kai · Michio Kobayashi
Department of Surgery I,
Oita Medical University, Hasama-cho,
Oita 879-55, Japan

Abstract Mechanisms by which immunodepressants (Cyclosporine, CsA; FK 506, FK; Azanthioprine, AZA) ameliorate warm ischemic injury of the liver were examined. Female Sprague-Dawley rats were subjected to 60-min of normothermic liver ischemia. Animals were assigned to one of four groups: group I, control with vehicle treatment; groups II, III, and IV, treatment with CsA (10 mg/kg), FK (1 mg/kg), and AZA (1 mg/kg), respectively. The immunosuppressive agents were given per os for 4 consecutive days prior to the induction of hepatic ischemia. In addition to a survival study, plasma levels of endotoxin, serum activities of tumor necrosis factor-α (TNF), plasma levels of phosphatidylcholine hydroperoxide (PCOOH) as a lipid peroxide, and serum alanine aminotransferase (ALT) were investigated in blood samples collected from the suprahepatic vena cava. A 7-day survival period was significantly higher in the immunosuppressed animals. Serum TNF levels were elevated and peaked at 3 h following reperfusion. When, the peak values were compared, the animals given immunodepressants had significantly lower levels of TNF (217.0 ± 40.6 pg/ml for group I, 67.6 ± 13.7 for group II, 87.9 ± 28.3 for group III and 89.1 ± 19.9 for group IV; Mean ± SEM). Plasma PCOOH levels were also elevated following reperfusion, but with no statistical difference among the groups. Our data suggest that immunodepressants ameliorate warm ischemia/reperfusion injury through modulation of TNF production and not through a diminution of lipid peroxidative injury.

Key words Liver ischemia
Immunodepressants · Cyclosporine
FK 506 · Azathioprine
Tumor necrosis factor
Lipid peroxidative · Rat

Introduction

Ischemia and reperfusion injury of the liver remains a problem both in liver surgery and liver transplantation. Consequently, the precise etiology for ischemia/reperfusion injury and protecting this organ with pharmacological interventions have received much focus of attention. Here is increasing evidence showing that tumor necrosis factor-α (TNF), a macrophage cytokine, mediates diverse biological responses. Moreover, a causal role for TNF in the pathogenesis of ischemia/reperfusion injury of the liver was confirmed [5, 9]. Recent studies revealed that reperfusion injury of the liver supervenes after revascularization as a result of production of the oxygen-

derived free radicals which lead to lipid peroxidation [21, 25].

We reported that cyclosporine (CsA), azathioprine (AZA) and FK 506 (FK) ameliorate ischemic injury of the liver in rats and pigs [8, 12, 13, 14, 15, 16]. The objective of the present study was to examine mechanisms by which immunodepressants exert beneficial effects on warm ischemia in the rat liver, with special reference ot TNF production and lipid peroxidative injury.

Materials and methods

Female Sprague-Dawley rats weighing 200–300 g were used throughout this study. A temporary normothermic liver ischemia was induced as previously described elsewhere [12]. Briefly, the abdomen was opened through a midline incision, following anesthesia with ether. The portal vein and the hepatic artery to the left lateral and median lobes were occluded with a small vascular clip. After 60-min of ischemia, the rats were re-anesthetized and the clip was released. The right lateral and caudate lobes were excised on reperfusion, leaving only the ischemic hepatic ischemia and mesenteric venous congestion will not occur. The antibiotic, cefamandole sodium (100 mg/kg), was administered intramuscularly just prior to laparotomy. The animals were not deprived of food or water prior to the experiments. Guidelines for animal experimentation of Oita Medical University were strictly followed.

The rats were assigned to one of four groups. In the control group (group I), the animals underwent warm liver ischemia with vehicle pretherapy. Group II rats received CsA (10 mg/kg/day p.o.) for 4 days prior to the induction of liver ischemia. Group III and Group IV rats were given FK (1 mg/kg) and AZA (1 mg/kg) by the same protocol as animals in group II. CsA (Sandoz Ltd., Basel, Switzerland) was dissolved in olive oil at 10 mg/ml. FK (Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan) was suspended in saline at 1 mg/3 ml, and AZA (Wellcome Foundation Ltd., London, U.K.) was suspended in olive oil at 1 mg/ml.

For the survival study, 150 rats were subjected to 60-min of liver ischemia. Autopsy was done in all animals that died during the observation period, and survivors were killed 7 days after the surgery. Another set of animals was prepared in the same manner described above, for biochemical studies. From four to ten rats were killed to take blood samples, before and at the end of ischemic period, and 1, 3, 6, 12 and 24 h after reperfusion. Blood samples were drawn from the suprahepatic vena cava. The plasma or serum were stored at −70 °C until assayed, as described below.

Plasma endotoxin concentrations were measured by a chromogenic endotoxin-specific method using the recombined limulus coagulation enzymes described elsewhere [23]. Endotoxin Specific Test (Seikagaku Kogyo, Tokyo, Japan) was used; the lower limit of endotoxin detection in this assay was 3 pg/ml. Serum activities of TNF were determined using an ELISA kit (Otsuka, Tokyo, Japan). Concentrations were calculated based on the standard curve obtained with a serially diluted recombinant human TNF-α. In this assay, the lower limit of detectable TNF was 10 pg/ml. Lipid peroxide in the plasma was estimated as levels of phosphatidylcholine hydroperoxide (PCOOH) using a chemiluminescence (CL)-high performance liquid chromatography (HPLC) assay [25]. Serum levels of alanine aminotransferase (ATL) were determined by an ultraviolet method using an autoanalyzer (JEOL JCA-MS24, Tokyo, Japan).

The results were expressed as the mean and the standard error of the mean. Mean values were compared using Student's *t*-test. The chi-square test was used to test for a difference in proportions. Statistical significance was defined as a P value less than 0.05.

Results

The comparative survival rates after 60-min of warm ischemia of the liver are shown in Table 1. The 7-day survival of the animals treated with CsA (85.2%) was significantly higher than that of the olive oil-treated group (56.7%) ($P<0.05$). FK and AZA pretherapy also substantially improved the survival rates (80.0% and 76.7%, respectively) compared with those in the saline-treated group (50.0%) ($P<0.05$). However, no significant difference was found among the groups pretreated with these immunosuppressants. All of the rats died between 12 and 72 h post-surgery. At auto-

Table 1 Survival rates in the rat after 60-min warm ischemia of the liver

Pretreatment	7-Day survival rate (%)
Olive oli	17/30 (56.7)
Saline	19/38 (50.0)
CsA	23/27 (85.2)[a]
FK 506	24/30 (80.0)[b]
AZA	23/30 (76.7)[b]

[a] $P < 0.05$, vs Olive oil
[b] $P < 0.05$, vs Saline

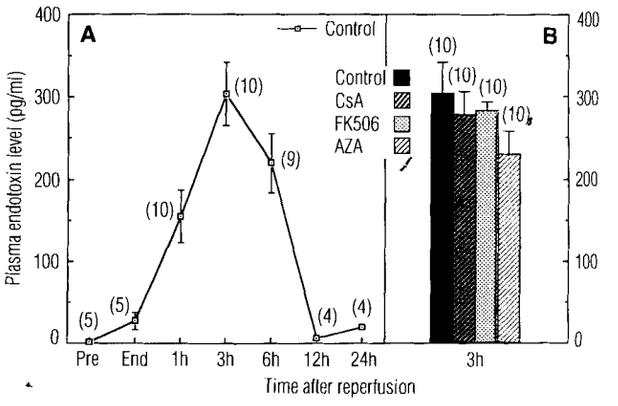

Fig. 1 **A** Serial changes of plasma endotoxin levels following 60-min warm ischemia in the control group (Mean ± SEM). The number in parenthesis means the number of animals studied, for all Figures. **B** Comparison of the plasma peak levels of endotoxin (3 h) following reperfusion after treatment with various immunodepressants. No significant difference was found among the groups (Mean ± SEM)

psy, severe necrosis of the liver was manifest in all cases.

Figure 1A presents serial changes in plasma endotoxin levels in the control animals. Marked elevation in endotoxin activities was detected between 1 and 6 h, peaking at 3 h following reperfusion (303.6 ± 38.2 pg/ml). However, when the peak values were compared, there was no significant difference among the groups (278.2 ± 28.0 pg/ml for group II, 283.2 ± 10.7 for group III and 229.5 ± 29.7 for group IV) (Fig. 1B).

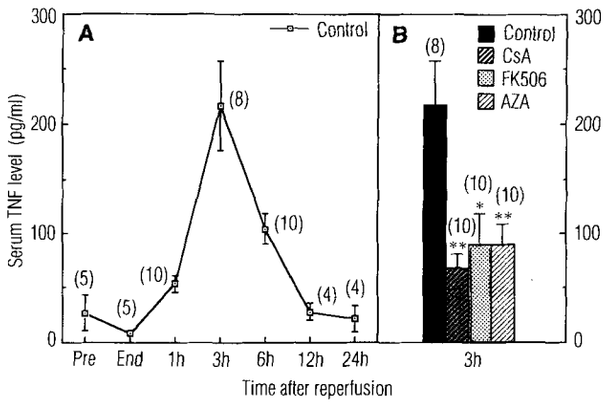

Fig. 2 A Serial changes of serum TNF levels following 60-min warm ischemia in the control group (Mean ± SEM), **B** Comparison of the serum TNF levels at 3 h following reperfusion after treatment with various immunodepressants (Mean ± SEM). $*P < 0.05$ for FK and $**P < 0.01$ for CsA and AZA, vs control group, respectively

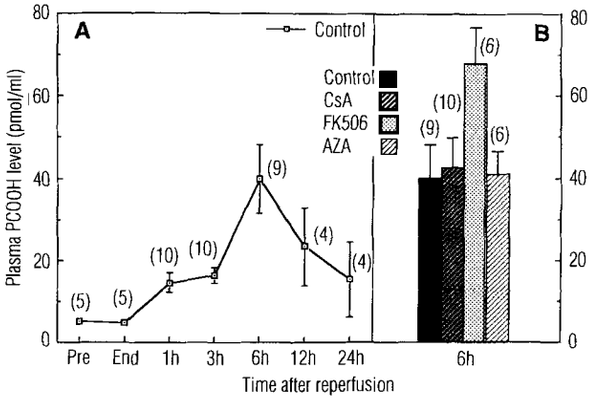

Fig. 3 A Serial changes of plasma PCOOH levels following 60-min warm ischemia in the control group (Mean ± SEM). **B** Comparison of the plasma peak levels of PCOOH (6 h) following reperfusion after treatment with various immunodepressants (Mean ± SEM)

Serial changes of serum TNF levels in the control group are illustrated in Fig. 2A. The peak value was observed at 3 h after reperfusion, a time when the plasma endotoxin reached the maximum (217.0 ± 40.6 pg/ml). Treatment with immunodepressants, however, significantly suppressed the TNF levels, compared with those in group I at a peak time (3 h) following reperfusion (67 ± 13.7 pg/ml for groups II, 89.7 ± 28.3 for group III and 89.1 ± 19.9 for group IV, $P < 0.01$ for group II and IV vs. group I, $P < 0.05$ for group III vs group I) (Fig. 2B).

As shown in Fig. 3A, plasma PCOOH levels in the control reached a maximum at 6 h after reperfusion (39.8 ± 8.4 pmol/ml). However, no difference was found compared with the immunosuppressed animals (42.5 ± 7.4 pmol/ml for group II, 67.8 ± 9.0 for group III and 40.8 ± 5.7 for group IV) (Fig. 3B).

There was no significant difference in serum levels of ALT at 3 h following reperfusion among the groups (4890 ± 591 IU/l for group I, 4385 ± 653 for group II, 3386 ± 388 for group III and 3623 ± 393 for group IV).

Discussion

We obtained evidence that TNF and PCOOH were elevated in suprahepatic blood following 60 min of normothermic hepatic ischemia in rats. In addition, pretherapy with immunodepressants resulted in a significant improvement in the survival rate. This was reflected by a suppression of TNF production, yet the immunodepressants did not affect elevation of plasma PCOOH. Although these observations indicate that TNF and PCOOH are involved in pathogenesis of ischemia-induced hepatic injury, they also suggest that suppression of TNF synthesis is one of the major mechanisms by which immunodepressants ameliorate ischemia/reperfusion injury of the liver, but not via alleviation of lipid peroxidative injury.

TNF is a critical mediator of ischemic injury of the liver [6]. Excessive stimulation of TNF-secreting cells results in a high systemic TNF concentration, presumably pathophysiological aspects of various diseases, including septic shock [1], allograft rejection [11, 20], and ischemic injury of the liver [6, 9, 13]. Since TNF is produced primarily by the monocyte/macrophage stimulated by endotoxin [7], it has been suggested these cells exhibit deleterious effects attributed to the host's inflammatory response to endotoxemia [2]. In the present study, we observed an increase in serum TNF accociated with endotoxemia following ischemia/reperfusion of the liver.

Considering the kinetics of TNF and endotoxin, endotoxemia seems to be one of the most potent trigger of TNF synthesis. Moreover, hepatic macrophages are likely to be exposed to higher concentrations of gut-derived toxins and these cells are the largest fixed macrophage population; Kupffer cells are probably the main source of TNF, in this model. There is evidence that Kupffer cells are activated to produce toxic mediators following cold storage of the liver [3].

The precise mechanism by which immunodepressants suppress the TNF production is unknown. One possible explanation is that immunodepressants might directly inhibit monocytes/macrophages from producing this cytokine, as it is known that cyclosporine blocks lipopolysaccharide-induced TNF secretion in vitro [22]. Immunodepressants probably do not suppress TNF production by down-regulating endotoxin, since plasma endotoxin levels were not suppressed by three agents used in our study. It is possible that immunodepressants suppress TNF activities by modulating generation of oxygen-derived free radicals that are substantially produced on recirculation [19, 21, 24]: The reactive oxygen intermediates can stimulate release of cytokines from monocytes in vitro [17]. However, this mechanism is less likely, since free radical injury did not differ after recirculation, regardless of whether or not immunodepressants were used, as far as lipid peroxidation (PCOOH) of cell membranes are concerned.

Usually, thiobarbituric acid reactants are measured to indicate lipid peroxidation associated with cellular damage caused by free radicals [29]. However, this assay may also measure aldehydes from processes other than hydroperoxide degeneration, and therefore lacks specificity [27]. PCOOH has recently gained attention as a primary peroxidative product of phosphatidylcholine (PC), a most important functional lipid in the cell membrane [28]. Takayama et al. reported that increased levels of PCOOH in the tissue of the liver can reflect hepatic ischemia/reperfusion injury, and confirmed that those levels are an useful index of hepatocellular damage caused by oxidative stress [25]. Furthermore, they demonstrated that the plasma PCOOH levels increased and paralleled those in the liver after hepatic ischemia/reperfusion [26]. Therefore, we determined the levels of the plasma PCOOH in order to examine liver injury caused by oxygen-radicals. In the present study, the PCOOH levels increased during the period of reperfusion and peaked at 6 h. Although there are several reports suggesting that the protective properties of CsA in ischemic injury could be due to a stabilizing effect on lysosomal and mitochondrial membranes [10, 18], our results showed that immunodepressants did not reduce the PCOOH levels after reperfusion. We suggest that immunodepressants may have little or not effect on lipid peroxidative injury of cell membranes. Coincidently, the serum levels of ALT were not decreased in the immunodepressant-treated animals, when compared to findings in the control following reperfusion.

Although the question remains as to why the survival of the animals treated with immunodepressants was significantly improved over the controls, without decline of serum ALT, the improved survival may be due to amelioration of later hepatic damage and also to attenuation of systemic injury mediated though cytokine release. There are reports of pathogenic role for TNF in cardiopulmonary dysfunction following ischemic tissue injury of remote organs [4, 6, 9].

In conclusion, it was demonstrated in our hepatic ischemia model that preoperative treatment of animals with immunodepressants resulted in an improved survival, reflected by a suppressed TNF production in response to endotoxemia, but not via alleviation of lipid peroxidative injury. Based on these observations, the modulation of cytokine generation with immunodepressants may account for the protective effects against ischemia/reperfusion injury in the liver.

Acknowledgements This work was supported by a grant for Scientific Research (A-01440054) from the Ministry of Education, Science and Culture of Japan. The autors thank Prof. M. Ito and Y. Magari from the Department of Laboratory Medicine, Oita Medical University for biochemical analyses, T. Egashira and F. Takayama for helpful comments and M. Ono for technical assistance.

References

1. Beutler B, Milsark IW, Cerami AC (1985) Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. Science 229:869–871
2. Border (1988) Hypothesis: sepsis, multiple systems organ failure, and the macrophage [editorial]. Arch Surg 123:285–286
3. Cardwell-Kenkel JC, Currin RT, Tanaka Y, Thurman RG, Lemasters JJ (1991) Kupffer cell activation and endothelial cell damage after storage of rat livers: effects of reperfusion. Hepatology 13:83–95

4. Caty MG, Guice KS, Oldham KT, Remick DG, Kunkel SI (1990) Evidence for tumor necrosis factor-induced pulmonary microvascular injury after intestinal ischemia-reperfusion injury. Ann Surg 212:694–700
5. Colletti LM, Burtch GD, Remick DG, Kunkel SL, Strieter RM, Guice KS, Oldham KT, Campbell DA (1990) The production of tumor necrosis factor alpha and the development of a pulmonary capillary injury following hepatic ischemia/reperfusion. Transplantation 49:268–272
6. Colletti LM, Remick DG, Burtch GD, Kunkel SL, Strieter RM, Campbell DA (1990) Role of tumor necrosis factor in the pathophysiologic alterations after hepatic ischemia/reperfusion injury in the rat. J Clin Invest 85:1936–1943
7. Decker T, Lohmann-Matthes ML, Karck U, Peters T, Decker K (1989) Comparative study of cytotoxicity, tumor necrosis factor, and prostaglandin release after stimulation of rat Kupffer cells, murine Kupffer cells, and murine inflammatory liver macrophages. J Leukoc Biol 45:139–146
8. Goto S, Kim YI, Kamada N, Kawano K, Kobayashi M (1990) The beneficial effect of pretransplant cyclosporine therapy on recipient rats grafted with a 12-h cold stored liver. Transplantation 49:1003–1004
9. Goto M, Takei Y, Kawano S, Tsuji S, Fukui H, Nishimura Y, Kashiwagi T, Fusamoto H, Kamada T (1992) Tumor necrosis factor and endotoxin in the pathogenesis of liver and pulmonary injuries after orthotopic liver transplantation in the rat. Hepatology 16:487–493
10. Hayashi T, Nagasue N, Kohno H, Chang YC, Galizia G, Nakamura T (1989) Evidence that cyclosporine pretreatment protects lysosomal membrane in liver ischemia in dogs. Transplantation 47:924–926
11. Hoffmann MW, Wonigeit K, Steinhoff G, Herzbeck H, Flad HD, Pichlmayr R (1993) Production of cytokines (TNF-α, IL-1-β) and endothelial cell activation in human liver allograft rejection. Transplantation 55:329–335
12. Kawano K, Kim YI, Kaketani K, Kobayashi M (1989) The beneficial effect of cyclosporine on liver ischemia in rats. Transplantation 48:759–764
13. Kawano K, Kim YI, Goto S, Kai T, Shimada T, Kamada N, Kobayashi M (1992) FK 506 ameliorates normothermic liver ischemia in rats by suppressing production of tumor necorsis factor. Transplant Int 5 [Suppl 1]:S665–S669
14. Kawano K, Kim YI, Ono M, Goto S, Kai T, Kobayashi M (1993) Evidence that both ciclosporin and azathioprine prevent warm ischemia/reperfusion injury to the rat liver. Transplant Int (in press)
15. Kawano K, Kim YI, Kai T, Ishii T, Tatsuma T, Morimoto A, Tamura Y, Kobayashi M (1993) Evidence that FK 506 alleviates ischemia/reperfusion injury to the rat liver: In vivo demonstration for suppression of TNF-α production in response to endotoxemia. Eur Surg Res (in press)
16. Kim YI, Kawano K, Goto S, Iwao Y, Kai T, Ono M, Okada K, Kobayashi M (1991) Beneficial effect of pretreatment with azathioprine on warm and cold ischemia of the swine liver. Transplant Proc 23:2201–2203
17. Koga S, Ogawa S, Kuwabara K, Brett J, Leavy JA, Ryan J, Koga Y, Plocinski J, Benjamin W, Burns DK, Stern D (1992) Synthesis and release of interleukin-1 by reoxygenated human mononuclear phagocytes. J Clin Invest 90:1007–1015
18. Kurokawa T, Kobayashi H, Nonami T, Harada A, Nakao A, Sugiyama S, Ozawa T, Takagi H (1992) Beneficial effects of cyclosporine on postischemic liver injury in rats. Transplantation 53:308–311
19. Marubayashi S, Dohi K, Ochi K, Kawasaki T (1986) Role of free radicals in ischemic rat liver cell injury: prevention of damage by α-tocopherol administration. Surgery 99:184–192
20. Maury CPJ, Teppo AM (1987) Raised serum levels of cachectin/tumor necrosis factor a in renal allograft rejection. J Exp Med 166:1132–1137
21. McCord JM (1985) Oxygen derived free radicals in post-ischemic tissue injury. N Engl J Med 312:159–163
22. Nguyen DT, Eskandai MK, DeForge LE, Raiford CL, Strieter RM, Kunkel SL, Remick DG (1990) Cyclosporin A modulation of tumor necrosis factor gene expression and effects in vitro and in vivo. J Immunol 144:3822–3828
23. Obayashi T, Tamura H, Tanaka S, Ohki M, Takahashi S, Arai M, Masuda M, Kawai T (1985) A new chromogenic endotoxin-specific assay using recombined limulus coagulation enzymes and its clinical applications. Clin Chim Acta 149:55–65
24. Southard JH, Marsh DC, McAnulty JF, Belzer FO (1987) Oxygen-derived free radical damage in organ preservation: activity of superoxide dismutase and xanthine oxidase. Surgery 101:566–570
25. Takayama F, Egashira T, Kudo Y, Yamanaka Y (1992) Chemiluminescence-HPLC assay of phosphatidylcholine hydroperoxide generated by ischemia-reperfusion in the liver of rats. Biochem Pharmacol 44:2412–2414
26. Takayama F, Egashira T, Kudo Y, Yamanaka Y (1993) Effect of anti-free-radical interventions on the increased phosphatidylcholine hydroperoxide in plasma after ischemia-reperfusion in the liver of rats. Biochem Pharmacol (in press)
27. Yamamoto Y, Brodsky MH, Baker JC, Ames BN (1987) Detection and characterization of lipid hydroperoxides at picomole levels by high-performance liquid chromatography. Anal Biochem 160:7–13
28. Yamamoto Y, Niki E, Kamiya Y, Eguchi J, Shimasaki H (1985) Oxidation of biological membranes and its inhibition. Free radical chain oxidation of erythrocyte ghost membranes by oxygen. Biochim Biophys Acta 819:29–36
29. Yoshikawa T, Oyamada H, Ichikawa H, Naito Y, Ueda S, Tainaka K, Takemura T, Tanigawa T, Sugino S, Kondo M (1990) Role of active oxygen species and lipid peroxidation in liver injury induced by ischemia-reperfusion. Nippon Shokakibyo Gakkai Zasshi 87:199–205 (Eng. Abstr)