Reduction of FK-506 requirements by combination with polyethylene glycol superoxide dismutase in orthotopic rat liver transplantation

Prakash N. Rao, PhD,[a,f] Xin Cai, MD,[a,f] Raman Venkataramanan, PhD,[b] Jeffrey L. Platt, MD,[d] Anthony Demetris, MD,[a,c] Allen Thunberg, MD,[e] Connie Faltynek, PhD,[e] Thomas Starzl, MD, PhD,[a] and Prem Kumar, MD[f]

Pittsburgh, Pa., Durham, N.C., Malvern, Pa., and New Orleans, La.

Reperfusion after ischemia results in endothelial cell injury and Kupffer cell activation. Inflammatory cytokines thus released can induce major histocompatibility complex antigens and increase the immunogenecity of the graft. An orthotopic rat liver allotransplant model was used to test the hypothesis that prevention of reperfusion injury by infusion of polyethylene glycol superoxide dismutase (PEG-SOD) would result in long-term allograft survival in the presence of subthreshold immunosuppressive dosages. ACI rats were used as donors, and Lewis strain rats as recipients. Orthotopic liver transplantation was initially performed to identify a subthreshold dose of the immunosuppressant FK-506, which would be unable to extend survival longer than control untreated rats with this strain combination. After testing three intramuscular FK-506 doses of 0.04, 0.08, and 0.16 mg/kg, it was observed that an FK-506 dose of 0.04 mg/kg/day for 14 days was unable to extend survival longer than in untreated recipients. This dose of FK-506 was used in combination with PEG-SOD at doses of 1000, 3000, 10,000, or 30,000 units. Recipient animals were treated intravenously with PEG-SOD as a loading dose to facilitate tissue penetration on day 1, and beginning on the day of transplantation, every 2 days for the duration of the study. Results of histologic studies and mean survival time were compared in untreated recipients and in rats treated with PEG-SOD plus 0.04 mg/kg/day FK-506. Mean survival time was increased significantly in these animals ($p < 0.007$) to 40.6 ± 25.6 days as compared with either untreated rats (10.0 ± 2.7 days) or rats treated with 0.04 mg/kg FK-506 alone (13.7 ± 4.2 days). Histologic examination demonstrated a significant reduction in the cellular infiltrate in rats treated with PEG-SOD plus FK-506, as compared with recipients treated with either agent alone or left untreated. Our results therefore suggest a potential approach to reducing immunosuppression in transplantation. (J ALLERGY CLIN IMMUNOL 1995;95:1276-81.)

Key words: *FK-506, PEG-SOD, immunosuppression, rat liver transplantation*

Organ procurement, preservation, and subsequent implantation involve a period of ischemia followed by reperfusion. Reperfusion injury after ischemia is thought to be caused by toxic oxygen radicals released from endothelial cells. Although generation of oxygen radicals is localized to the microvascular endothelial cell,[1,2] the deleterious biochemical and immunologic consequences are manifest in the entire organ.[3,4]

Free radical scavengers have been used with limited success,[5,6] unless they were delivered intracellulary by liposomes or immunoliposomes.[7-9] It

From the Departments of [a]Surgery, [b]Pharmaceutical Sciences, and [c]Pathology, University of Pittsburgh; [d]the Department of Surgery, Duke University, Durham; [e]The Sterling Research Group Inc., Malvern; and [f]the Department of Medicine, Louisiana State University Medical Center, New Orleans.

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Reprint requests: Prakash N. Rao, PhD, Department of Medicine, Section of Allergy and Clinical Immunology, Louisiana State University Medical Center, 1542 Tulane Ave., New Orleans, LA 70112-2822.

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Abbreviations used

MST: Mean survival time
OLTX: Orthotopic liver transplantation
PEG-SOD: Polyethylene glycol
SOD: Superoxide dismutase

has recently been observed that the conjugation of polyethylene glycol (PEG) to antioxidants such as superoxide dismutase (SOD) and catalase significantly increases their half-life from 6 minutes to 40 hours in the rat. Treatment of endothelial cells with these scavengers results in increased endothelial enzyme activity and oxidant resistance.[10, 11] PEG-SOD and PEG-catalase have been successfully used in the treatment of experimental cerebral and myocardial ischemia and reperfusion injury.[12-14]

We and others have demonstrated that reperfusion after cold preservation results in endothelial cell injury and Kupffer cell activation.[15, 16] Inflammatory cytokines, such as tumor necrosis factor, thus released can induce production of major histocompatibility complex antigens[17] and increase the immunogenecity of the donor organ.

We hypothesized that the prevention of ischemia and reperfusion injury by PEG-SOD would result in lowering the immunogenecity of the graft and could therefore result in significantly lowering immunosuppressive requirements.

METHODS

An orthotopic liver transplant (OLTX) model was used. Inbred male rats were used in all experiments. ACI (RT1) rats, weighing approximately 225 to 250 gm, were used as donors; and Lewis rats (RT1) rats, weighing 275 to 300 gm, were used as recipients. The procedure for OLTX was that of Kamada and Calne.[18] In our experience, this procedure exposes the donor liver to a warm ischemia period of approximately 45 minutes. OLTXs were performed in a randomized fashion. FK-506 was given for immunosuppression in intramuscular doses of 0.04 (n = 6), 0.08 (n = 8), and 0.16 (n = 6) mg/kg/day for 14 days. All three doses were lower than the 0.64 to 1.28 mg/kg/day range used conventionally in this model and strain combination.[19] An intramuscular FK-506 dose of 0.04 mg/kg/day for 14 days did not extend survival longer than in untreated recipients (Table I) and was used in further experiments with PEG-SOD. PEG-SOD at a dose of 1000, 3000, 10,000, or 30,000 U/kg was given alone or in combination with FK-506, 0.04 mg/kg. Recipient animals were treated intravenously with the appropriate PEG-SOD dose on day 1 (a loading dose to facilitate tissue penetration), and beginning on day 0 (day of OLTX), every 2 days for the duration of the study.

TABLE I. MST in FK-506–treated rats

No.	Experimental group	MST (days)
1	Control—no FK-506 (n = 8)	10.0 ± 2.7
2	FK-506 (0.04 mg/kg) (n = 12)	13.7 ± 4.2*
3	FK-506 (0.08 mg/kg) (n = 8)	83.5 ± 29.9
4	FK-506 (0.16 mg/kg) (n = 6)	92.0 ± 11.2

*Group 2 versus group 1: p = not significant.

Separate OLTXs were carried out when FK-506 was combined with PEG-albumin at a dose of 30,000 U/kg.

Effect of PEG-SOD on FK-506 pharmacokinetics

The effect of PEG-SOD (30,000 U/kg) on FK-506 pharmacokinetics was studied in a separate experiment in which rats were treated intramuscularly with 1.0 mg/kg/day of FK-506 for 14 days in the presence (n = 3) or absence (n = 3) of PEG-SOD (30,000 U/kg). A dose of 1.0 mg/kg/day was chosen to ensure that plasma FK levels would be measurable. A dose of 0.04 mg/kg/day would result in FK-506 levels lower than the minimal detection limit of the assay (0.1 ng/ml). Blood samples were drawn by means of tail vein puncture on days 0, 3, 6, 7, 9, 14, 15, 16, 17, 18, 19, and 20 and were analyzed for FK-506 levels.[20]

Statistical analysis

Statistical comparisons of mean survival time (MST) in the experimental groups were performed with both the Savage (Mantel-Cox) and the Wilcoxon signed rank tests. A p value of less than 0.05 was considered significant. Differences in FK-506 levels between the PEG-SOD–treated animals and those not treated with PEG-SOD were analyzed by a one-way analysis of variance.

RESULTS

Survival

Tables I and II show the relationship between FK dosage and graft survival. MST in rats treated with 0.04 mg/kg/day of FK-506 was 13.7 ± 4.2 days and was not significantly different from that of control (untreated) animals (10.00 ± 2.7 days). Treatment with PEG-SOD alone at a dose of 1000, 3000, 10,000, or 30,000 U/kg also did not result in significantly prolonged survival, although some improvement was observed. No improvement in survival was observed when PEG-albumin was used alone. When rats were treated with a combination of 30,000 U/kg of PEG-SOD and 0.04 mg/kg of FK-506, the MST was significantly increased ($p < 0.007$) to 40.6 ± 25.6 days. No significant improvement in survival was observed at PEG-SOD doses of 1000, 3000, or 10,000 U/kg when used in combination with FK-506.

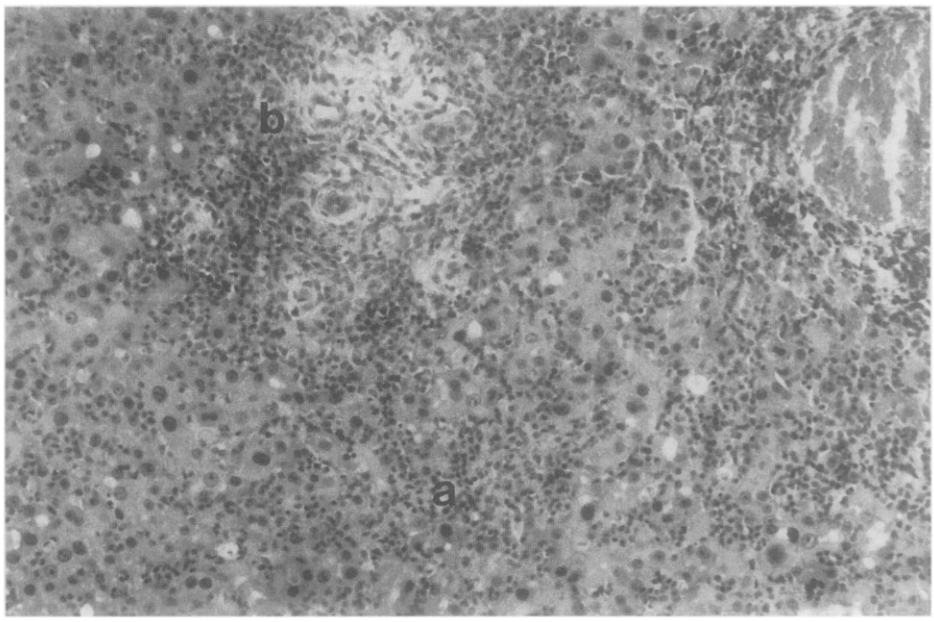

FIG. 1. Photomicrograph of rat liver undergoing acute cellular rejection. There is a significant infiltration of inflammatory cells within the portal triads and sinusoids (*a*). Some tunneling of the infiltrate beneath the portal vein endothelium is also observed (*b*). (Hematoxylin and eosin; ×64).

TABLE II. MST in experimental groups

No.	Treatment	Survival time	Mean ± SD	*p* Value*
1	FK-506 0.04 mg/kg (n = 6)	10, 10, 12, 13, 16, 21	13.7 ± 4.2	—
2	PEG-SOD 1,000 U/kg (n = 6)	16, 9, 8, 8, 9, 5	8.8 ±3.9	0.60
3	PEG-SOD 3,000 U/kg (n = 6)	6, 10, 11, 11, 12, 12	10.2 ± 2.2	0.10
4	PEG-SOD 10,000 U/kg (n = 6)	13, 10, 12, 12, 19, 10	12.0 ± 1.9	0.31
5	PEG-SOD 30,000 U/kg (n = 7)	8, 8, 11, 12, 13, 51, 60	23.3 ± 22.2	0.65
6	FK-506 0.04 mg/kg + PEG-SOD (n = 8) 1000 U/kg	10, 13, 14, 17, 24, 26, >100†	27.5 ± 29.8	0.08
7	FK-506 0.04 mg/kg + PEG-SOD 3,000 U/kg (n = 6)	6, 8, 9, 13, 43, 48	21.2 ± 19.0	0.68
8	FK-506 0.04 mg/kg + PEG-SOD 10,000 U/kg (n = 6)	7, 9, 13, 19, 66†, 68†	30.3 ± 28.7	0.34
9	FK-506 0.04 mg/kg + PEG-SOD 30,000 U/kg (n = 12)	14, 14, 16, 17, 19, 35, 59†, 70†, 72†, 60†, 60†	40.64 ± 25.63*	0.006
10	FK-506 0.04 mg/kg + PEG-ALB 30,000 U/kg (n = 6)	10, 12, 14, 8, 11, 12	11.16 ± 2.04	0.33

ALB, Albumin.
*Survival compared with group 1.
†Killed.

Histology

Fig. 1 is a photomicrograph of a control untreated liver undergoing acute cellular rejection. Treatment with FK-506 (0.04 mg/kg) alone was unable to prevent acute rejection, as demonstrated by the presence of a cellular infiltrate in the sinusoids (Fig. 2). Treatment with PEG-SOD alone (Fig. 3) also did not prevent acute cellular rejection. When the animals were treated with a combination of 0.04 mg/kg/day of FK-506 and PEG-SOD at a dose of 30,000 U/kg, a significant reduction in cellular infiltration was observed with a resultant arrest of acute rejection and prolongation of survival (Fig. 4).

Effect of PEG-SOD treatment on FK-506 pharmacokinetics

There was no significant difference in FK-506 concentrations between the two groups except

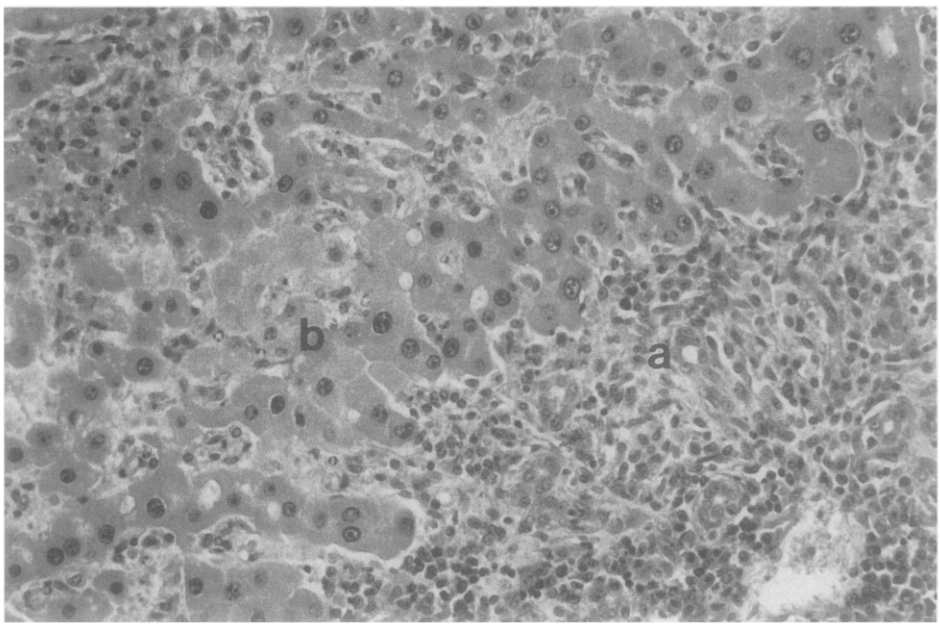

FIG. 2. Photomicrograph of rat liver treated with subthreshold doses of FK-506. Treatment with a suboptimal dose of FK-506 is unable to prevent acute cellular rejection. As in Fig. 1, a significant inflammatory infiltrate is present within the portal triads and sinusoids *(a)*. Some degree of hepatocellular necrosis is also evident *(b)*. (Hematoxylin and eosin; ×64).

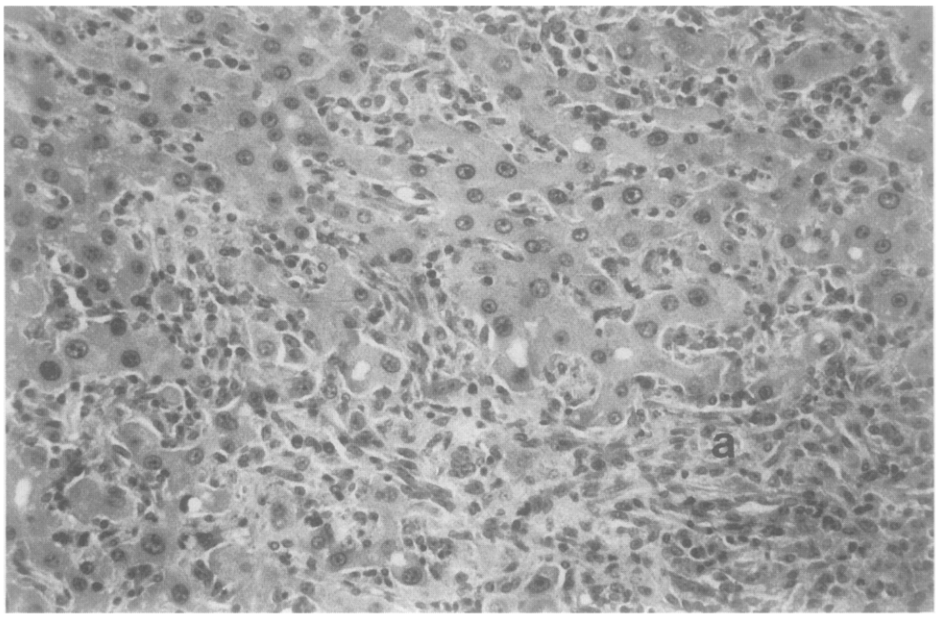

FIG. 3. Photomicrograph of rat liver treated with 30,000 U/kg of PEG-SOD. Treatment with 30,000 U/kg of PEG-SOD alone resulted in a reduction in the cellular infiltration into the sinusoids as compared with Figs. 1 and 2. However, treatment with PEG-SOD alone was unable to completely prevent acute cellular rejection *(a)*.

on days 17, 18, 19, and 20 when significantly elevated levels of FK-506 were observed in animals treated with 30,000 U/kg PEG-SOD (Table III).

DISCUSSION

The results suggest that the addition of 30,000 U/kg of PEG-SOD to subthreshold doses of FK-506 causes a significant amelioration of orthotopic

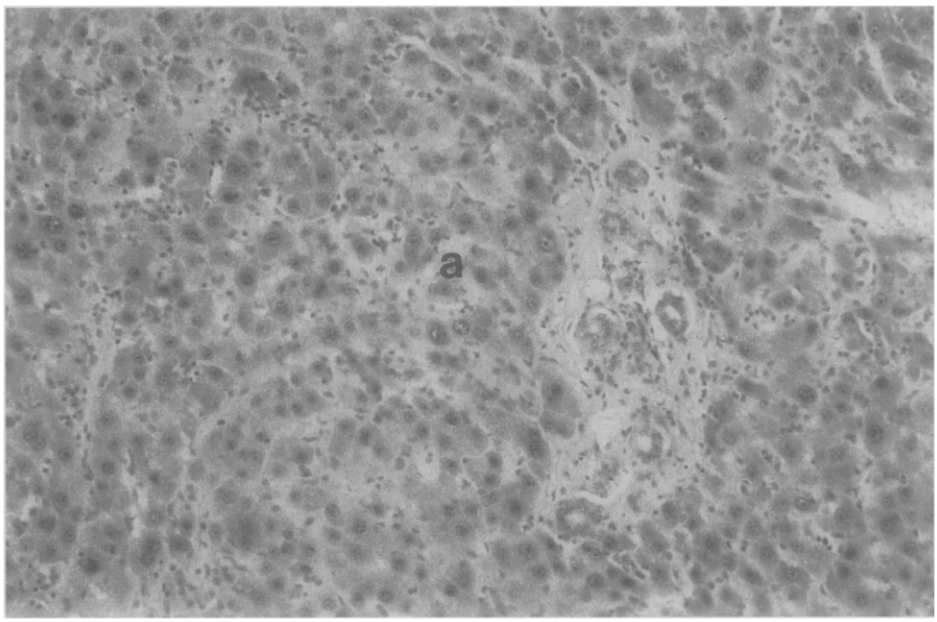

FIG. 4. Micrograph of rat liver treated with FK 0.04 mg/kg and 30,000 U/kg of PEG-SOD. Treatment with a combination of PEG-SOD and suboptimal doses of FK-506 results in a significant reduction of cellular infiltration into the portal triads and sinusoids *(a)*. Hepatocellular architecture is better preserved. (Hematoxylin and eosin; × 64).

TABLE III. Circulating FK-506 levels in the presence and absence of of PEG-SOD

No.	Experiment day	FK level (ng/ml)*	FK level (ng/ml)†	*p* Value‡
1	0	—	1.06 ± 0.25	—
2	3	17.8 ± 3.75	17.46 ± 1.76	NS
3	6	22.57 ± 7.07	14.4 ± 0.78	NS
4	7	18.70 ± 4.37	21.0 ± 1.02	NS
5	9	14.90 ± 2.19	16.56 ± 1.60	NS
6	14	19.07 ± 5.06	16.96 ± 4.92	NS
7	15	24.01 ± 8.13	18.60 ± 2.12	NS
8	16	14.85 ± 7.70	15.0 ± 5.37	NS
9	17	15.95 ± 1.77	9.65 ± 0.49	<0.05
10	18	13.90 ± 2.26	9.0 ± 0.85	<0.05
11	19	11.50 ± 0.14	7.55 ± 0.78	<0.05
12	20	11.15 ± 1.06	7.60 ± 2.68	<0.05

NS, Not significant.
*In the presence of PEG-SOD.
†In the absence of PEG-SOD.
‡Levels in the presence of PEG-SOD versus levels in the absence of PEG-SOD.

rat liver rejection. A nonsignificant prolongation of survival was observed with a PEG-SOD dose of 30,000 U/kg used alone. No such prolongation was observed with PEG-albumin either alone or in combination with FK-506 (0.04 mg/kg). We hypothesize that this benefit is caused by a decrease in reperfusion injury caused by PEG-SOD.

We and others have demonstrated that reperfusion injury to the endothelial cell can activate the expression of inflammatory cytokines such as interleukin-1, interleukin-6, and tumor necrosis factor.[15, 16] These inflammatory cytokines further induce the expression of major histocompatibility complex-class I and class II antigens,[17] which are thought to mediate acute cellular rejection.[21] Although subthreshold doses of FK-506 are unable to prevent acute rejection, it is possible that the addition of PEG-SOD to the therapeutic regimen, by protecting the endothelial cell, significantly inhibits the inflammatory cascade and thus reduces

the immunogenecity of the graft. Conversely, it is also possible that PEG-SOD prevents free radical–mediated allograft destruction. Our hypothesis is supported by results of a recent study.[21]

Another possible explanation of the results is that PEG-SOD, by preventing reperfusion injury, alters the breakdown of cytochrome P-450[22] and consequently changes the metabolism of FK-506. This is supported by the pharmacokinetic data, which suggest that treatment with PEG-SOD appears to result in significantly higher levels of FK-506. In the animals that received transplants the PEG-SOD treatment may have resulted in FK levels sufficient to prevent cellular rejection. Interestingly, the addition of PEG-SOD did not markedly alter the half-life of FK-506 (4 to 7 days) as compared with that in control animals (5 to 6 days).

We conclude that immunosuppressive therapy including PEG-SOD may inhibit acute cellular rejection in orthotopic liver transplantation, even in the presence of subthreshold doses of immunosuppressants. Further investigation directed toward the mechanism of this beneficial response may contribute to the design of new strategies for immunosuppression.

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