ORIGINAL

Protective effect of FK506 and Thromboxane synthase inhibitor on ischemia-reperfusion injury in non-heart-beating donor in rat orthotopic liver transplantation

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Abstract : The study investigated the possibility of pharmacologically modulating hepatic allograft function from non-heart-beating donors (NHBDs) using male Lewis rats. The donors were divided into 4 groups : Group 1 in which the vehicle was administered, Group 2 in which FK506 (tacrolimus;a powerful immunosuppressive agent) was administered, Group 3 in which OKY046 (a specific thromboxane synthetase inhibitor) was administered and Group 4 in which FK506 and OKY046 were administered. The recipients received orthotopic liver transplantation. The survival rates differed significantly between the recipients that had received liver transplantation from Groups 1 and 4. The serum liver enzyme and inflammatory cytokine concentrations of the recipients which had received liver transplantation from Groups 2, 3 and 4 were significantly lower than those of the recipients that had received liver transplantation from Group 1. Although there was no significant difference, all parameters were better in the recipients that had received transplantation from Group 4 than those that had received transplantation from Groups 2 and 3. The action mechanisms of FK 506 and OKY 046 are completely different. Therefore, concomitant use of FK506 and OKY046 might have additive effects on liver transplantation from NHBDs. In conclusion, we demonstrated that pretreatment of NHBDs using FK506 and OKY 046 ameliorated graft viability. J. Med. Invest. 51 : 76-83, February, 2004

Keywords : ischemia-reperfusion injury, non-heart-beating donor, orthotopic liver transplantation, FK 506, OKY046

INTRODUCTION

Liver transplantation (LTx) has become recognized as a standard treatment for end-stage liver disease. However, despite an increase in potential transplant patients, a serious shortage of donor organs has limited its clinical application. The use of non-heart-beating donors (NHBDs) could relieve the donor organ shortage provided that the outcome of the recipient is not jeopardized. Furthermore, recent estimates indicate that an increase of 20-25% in organ donors could be realized if NHBDs were used routinely (1). In NHBDs, the liver inevitably suffers from warm ischemia injury related cardiac arrest before cold perfusion. Thus, new modalities to attenuate these ischemia reperfusion injuries are required to establish the safety and efficacy of LTx from NHBDs.

FK506, a powerful immunosuppressive agent, may enhance hepatocyte regeneration, and may also ameliorate hepatic ischemia-reperfusion injury of the liver. The effect is great interest and is worthy of thorough investigation, because the augmentation of hepatic regeneration and protection from ischemic injury may be crucial not only in liver transplantation, but also

in other types of liver surgery.

Ischemia-reperfusion injury is a complex series of multistep processes. It has been shown that ischemia can activate phospholipase A2, with the consequent release of free arachidonic acid from the cell membranes.

Thromboxane A2 (TXA), the main product of arachidonic acid metabolism in platelets, works as a powerful platelet aggregating agent and a stimulator of contractive activity of smooth muscles including the blood vessels and trachea. Accordingly, TXA can exacerbate the progression of ischemia-induced liver damage (2). OKY046 (sodium(E)-3-[4-(1)-imidazolylmethl phenyl]) is a specific thromboxane synthetase inhibitor which suppresses the production of TXA without affecting other cyclooxygenase pathways. Several reports have suggested the beneficial effect of this drug on warm ischemic damage to the liver after reperfusion and on cold preservation/reperfusion injury of the liver (3).

In the present study, to investigate the possibility of pharmacologic modulation of the hepatic allograft function from NHBDs, the effects of treatment with FK506 and OKY046 were evaluated in rat orthotopic liver transplantation.

MATERIALS AND METHODS

Animals

Male Lewis rats 8 to 9 weeks weighing 190 to 250 g supplied from Charles River Japan (Yokohama, Japan) were used for both donors and recipients. The rats were selected because they have no rejection each other. All rats were allowed free access to water and standard laboratory diet until just before operation.

Liver transplantation

Room temperature during liver transplantation was maintained around 23 . The induction of general anesthesia was Ether inhalation. Five minutes after administration of 5000 IU intravenous heparin in donors, the abdominal aorta were cut and cardiac arrest was induced by phlebotomy as a model from NHBDs. Then, warm ischemia was induced for 60 min at room temperature. At the end of the warm ischemia period, the graft was perfused in situ via the portal vein with 10 ml of chilled lactated Ringer's solution. The liver was harvested and stored cold in a bath of the same solution until transplantation. The graft from the NHBDs was transplanted orthotopically by the technique described by Kamada and Calne (4) with minor modifications. The suprahepatic vena cava was anastomosed with

6-0 Prolene (Ethicon, Somerville, NJ) continuous suture and portal vein, and infrahepatic vena cava reconstruction was performed by the cuff technique. The bile duct connection was made with the use of an intraluminal splint. A 16 G intravenous catheter (TERUMO, Tokyo, Japan) was used for the portal vein cuff and infrahepatic vena cava. And a 22 G intravenous catheter was used for the cuff of the bile duct splint. No attempt was made to anastomose the hepatic artery. After closing the abdomen, the recipients were warmed with a heating lamp for 30 min. All of the recipients for the survival study were transferred to an individual cage after LTx in a room in which the temperature was constantly maintained at 23   and observed daily for two weeks. Food pellet and water were available at all times. The recipients for sample collection were killed 30 min, 60 min and 180 min after reperfusion. Immunosuppressants were not administrated during or after the operation.

The donor groups

The donors were divided into 4 groups before liver transplantation. Group 1, (Control group) ; The rats in Group 1 were treated with vehicle. Group 2, (FK506 group) ; The rats in Group 2 were treated with FK506 (Fujisawa Pharmaceuticals Ltd., Osaka Japan) 1.0 mg/ kg/day intramuscularly for 4 days just prior to warm ischemia. Group 3, (OKY046 group) ; The rats in Group 3 were treated with OKY046 (Ono Pharmaceutical, Osaka Japan) 10 mg/kg intravenously through the penile vein for 60 min, 40 min, 20 min before the induction of warm ischemia. Then, the total volume of OKY046 was 30 mg/kg. Group 4, (FK506 OKY046 group) ; The rats in Group 4 were treated both with FK506 and OKY046 by the same methods. The dose of FK 506 and OKY 046 were determined following the protocol of previous authors (1, 21). The recipients received transplantations from a rat in one of these donor groups using the previously described technique.

Blood chemical analysis

Blood samples were drawn from the abdominal aorta, and the samples were centrifuged immediately at 3000 rpm, at 4   for 5 min for the determination of serum Aspartate aminotransferase (AST), Alanine aminotransferase (ALT) and Lactate dehydrogenase (LDH), which were measured using an autoanalyzer. The remaining plasma was stored in a freezer and used for measuring Interleukin 6(IL-6) and tumor necrosis factor-α (TNF-α), which are inflammatory cytokines. An ELISA kit (TFB, Tokyo, Japan) was used to perform measurements of IL-6 and TNF-α.

Histological examinations

For light microscopy, liver tissue were taken at 30 min, 1 hr, and 3 hr after reperfusion, and they were fixed in 4% formaldehyde, processed routinely, and embedded in paraffin. Thick paraffin sections were stained with hematoxylin and eosin.

Statistical analysis

The results were expressed as mean± SD. Differences in survival were determined using the Kaplan-Meier Survival Analysis. All laboratory data, including AST, ALT, LDH, IL-6, TNF-α relationship with Groups were tested by the repeated measures ANOVA, and the differences of effects between the groups were tested by post hoc test (Fisher's PLSD analysis). All statistical analysis was performed using the computer software package Statview V (Abacus Concepts, Berkeley, California). *P*< 0.05 was considered significant.

RESULTS

There was no significant difference in anhepatic time, cold ischemic time and recipient's operation time time (Table 1).

Table 1.   Operation times between four groups
For each group, anhepatic time, cold ischemia time and operation time are shown. There was no significant difference between the 4 groups.

Group	Time(min) (mean± S.D.)		
	Anhepatic	Cold ischemic	Recipient operation time
Group 1 Control (n=27)	12.8± 0.9	35.4± 3.1	38.8± 1.9
Group 2 FK506 (n=24)	12.7± 0.7	35.6± 2.9	39.0± 2.4
Group 3 OKY (n=27)	12.3± 0.6	34.8± 2.7	37.0± 2.2
Group 4 FK OKY (n=24)	12.5± 0.9	35.2± 3.2	38.4± 2.2

Postoperative survival

In the recipients, postoperative survival was assessed for 14 days (Fig. 1). All of the rats in Group 4 (n=6) survived (6/6, 100%). In Group 2 (n=6) and Group 3 (n=6), 5 out of the 6 rats survived (5/6, 83%). In Group 1, only 2 out of the 6 rats survived for 14 days, and there was a significant difference between Group 1 and Group 4
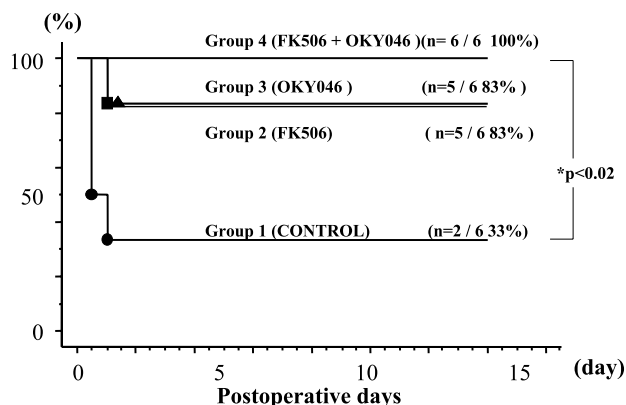

Figure. 1.   14 days survival
The 14-day survival rate of each group is shown. All the deaths occurred within 2 days of the transplantations. There was a significant difference between Group 1 and Group 4.

(P <0.05). In Group 1, the deaths of the 4 rats all occurred within 2 days, and the remaining 2 rats survived for 14 days. In Group 2 and Group 3, the deaths also occurred within 2 days. The cause of all these deaths was hepatic failure because they had no bleeding and no intra-operative or post-operative complications. It was suggested that life-threatening microcirculation disorders occurred a relatively short time after reperfusion. It was also assumed that a concomitant administration of FK505 and OKY046 had a stronger protective effect than an administration of either FK 506 or OKY 046.

Serum liver enzymes and inflammatory cytokine

The serum AST concentration was higher in Group 1 than in the drug-treated Groups (Fig. 2). There was alternans action between the serum AST concentration and Groups (F=2.367, p<0.05). In Group 1, the AST concentrations were high immediately after reperfusion.
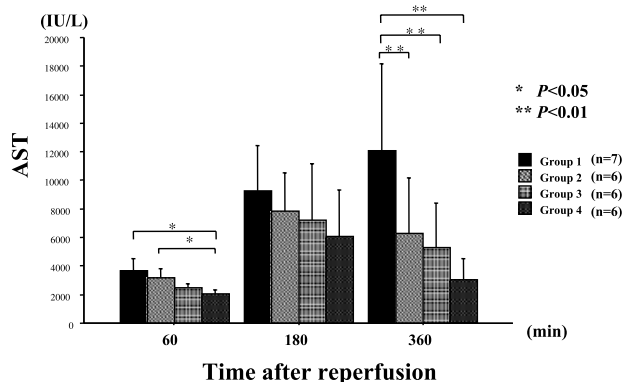

Figure. 2.   Serum AST concentrations after reperfusion
Immediately after reperfusion, the serum AST concentrations in Group 2, Group 3 and Group 4 were lower than in Group 1. 360 min after reperfusion, there were statistically significant differences when Group 1 was compared to Group 2, Group 3 and Group 4.

At 360 min after reperfusion, the AST concentration in Group 1 (12058.6± 6108.2 IU/L) was significantly higher than in Group 2 (4620.0± 1597.7 IU/L, P<0.01), in Group 3 (5268.3± 3114.4 IU/L, P<0.01) and Group 4 (3016.7± 1487.2 IU/L, P<0.01). Especially, the AST concentrations were particularly low in Group 4. The serum ALT concentrations in the drug-treated groups were lower than in the control group after reperfusion. There was alternans action between the serum ALT concentration and Groups (F=2.727, p<0.05). And at 60 min after reperfusion, the serum ALT concentration was significantly lower in Group 4 (1676.7± 133.2 IU/L) than in Group 2 (3600.0± 959.0 IU/L, P <0.05) and Group 3 (2025± 289.9 IU/L, P<0.05). At 360 min after reperfusion, the serum ALT concentration was significantly higher in Group 1 (8672.9± 4373.3 IU/L) than in Group 2 (3615.0± 1662.7 IU/L, P<0.01), Group 3

(2886.7± 1094.3 IU/L, P<0.01) and Group 4 (2511.7± 1614.9, P <0.01) (Fig. 3). The serum LDH at 360 min after reperfusion in Group 1 (80814.3± 40370.9 IU/L) was also higher than in the drug-treated Groups {Group 2 (29923.3± 25702.5 IU/L) (P<0.01), Group 3 (18681.7± 13495.4 IU/L) (P<0.01), Group 4 (15401.7± 21211.3 IU/L) (P<0.01)}. There was alternans action between the serum LDH concentration and Groups (F=3.264, p<0.05). And at 60 min after reperfusion, the serum LDH concentration was significantly lower in Group 4 (20176.7± 3067.2 IU/L) than in Group 2 (43100.0± 13046.1 IU/L, P<0.05) and Group 3 (26485.0± 700.0 IU/L, P<0.05) (Fig. 4). The serum concentrations of interleukin 6 (IL-6), which is an inflammatory cytokine, were considerably higher in the control group than in the drug-treated groups immediately after reperfusion. There was alternans action between the serum
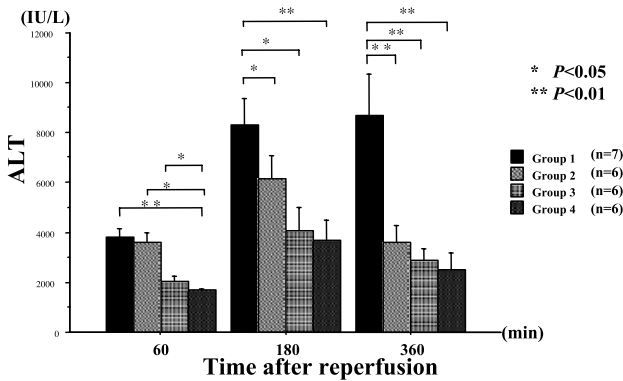

Figure. 3.   Serum ALT concentration after reperfusion
Immediately after reperfusion, the serum ALT concentrations were lower in Group 3 and Group 4 than in Group 1. 360 min after reperfusion, there were statistically significant differences when Group 1 was compared to Group 2, Group 3 and Group 4.
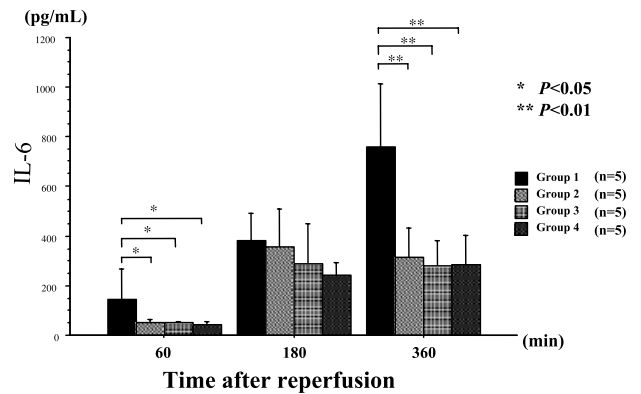

Figure. 5.   Serum IL-6 concentrations after reperfusion
Immediately after reperfusion, the serum IL-6 concentrations were considerably higher in Group 1 than in Group 2, Group 3 and Group 4. 360 min after reperfusion, there were statistically significant differences when Group 1 was compared to Group 2, Group 3 and Group 4.
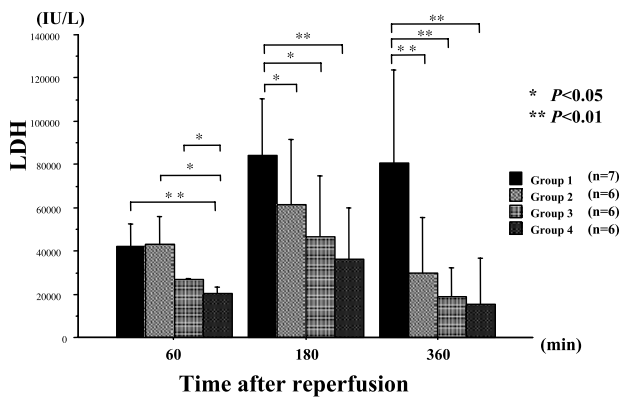

Figure. 4.   Serum LDH concentrations after reperfusion
360 min after reperfusion, there were statistically significant differences in the serum LDH concentrations when Group 1 was compared to Group 2, Group 3 and Group 4.
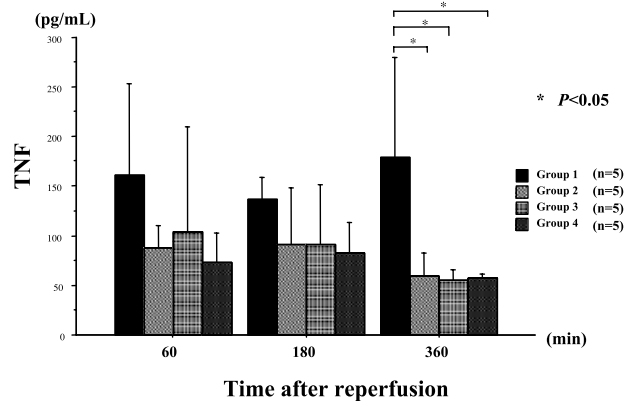

Figure. 6.   Serum TNF-α concentrations after reperfusion
The high serum TNF-α concentrations in Group 1 persisted until 360 min after perfusion. In Group 2, Group 3 and Group 4, these concentrations gradually decreased. 360 min after reperfusion, there were statistically significant differences when Group 1 was compared to Group 2, Group 3 and Group 4.

IL-6 concentration and Groups (F=4.381, p<0.01). And the differences between them 360 min after reperfusion was statistically significant (Group 1 : 757.4± 256.1 pg/ml) (Group 2: 313.2± 117.5 pg/ml, *P*<0.01) (Group 3: 278.0± 102.2 pg/ml, *P* <0.01) (Group 4: 282.0± 121.1 pg/ml, *P*<0.01) (Fig. 5). In Group 1, high concentrations of TNF-α were observed immediately after reperfusion and persisted until 360 min after perfusion. In the drug-treated groups, although high concentrations of TNF-α were also observed immediately after reperfusion, they gradually decreased and, 360 min after reperfusion, were significantly lower than in Group 1 (Group 1 : 179.6± 100.6pg/ml) (Group 2 : 59.8± 22.7 pg/ml, *P* <0.05) (Group 3 : 55.1± 11.1 pg/ml, *P* <0.05) (Group 4: 57.4± 3.6 pg/ml, *P* <0.05). But there was no alternans action between the serum TNF-α concentration and Groups (F=0.670, p=0.674) (Fig. 6).

Histological findings

At 360 min after reperfusion, light microscope findings revealed that, in Group 1, erythrocytes were frequently sludged in the sinusoids, and the hepatocyte vacuolization was prominent, as shown in Fig. 7a. By contrast, in the drug-treated groups, only isolated erythrocytes were found, and the hepatocyte vacuolization was minimal (Fig. 7 b, c, d).

At 360 min after reperfusion, there was no significant difference in the laboratory data and the survival rates, and there was no difference in the histological findings between the group in which the 2 drugs were concomitantly used (Group 4) and the groups in which each drug was used separately (Group 2 and Group 3). However, all the parameters were better in Group 4 than in Group 2 and Group 3, and there was a significant difference of survival rate between only in Group 1 and Group 4.

DISCUSSION

Liver transplantation (LTx) has emerged as highly successful therapy for patients with liver failure. The

A : Group 1

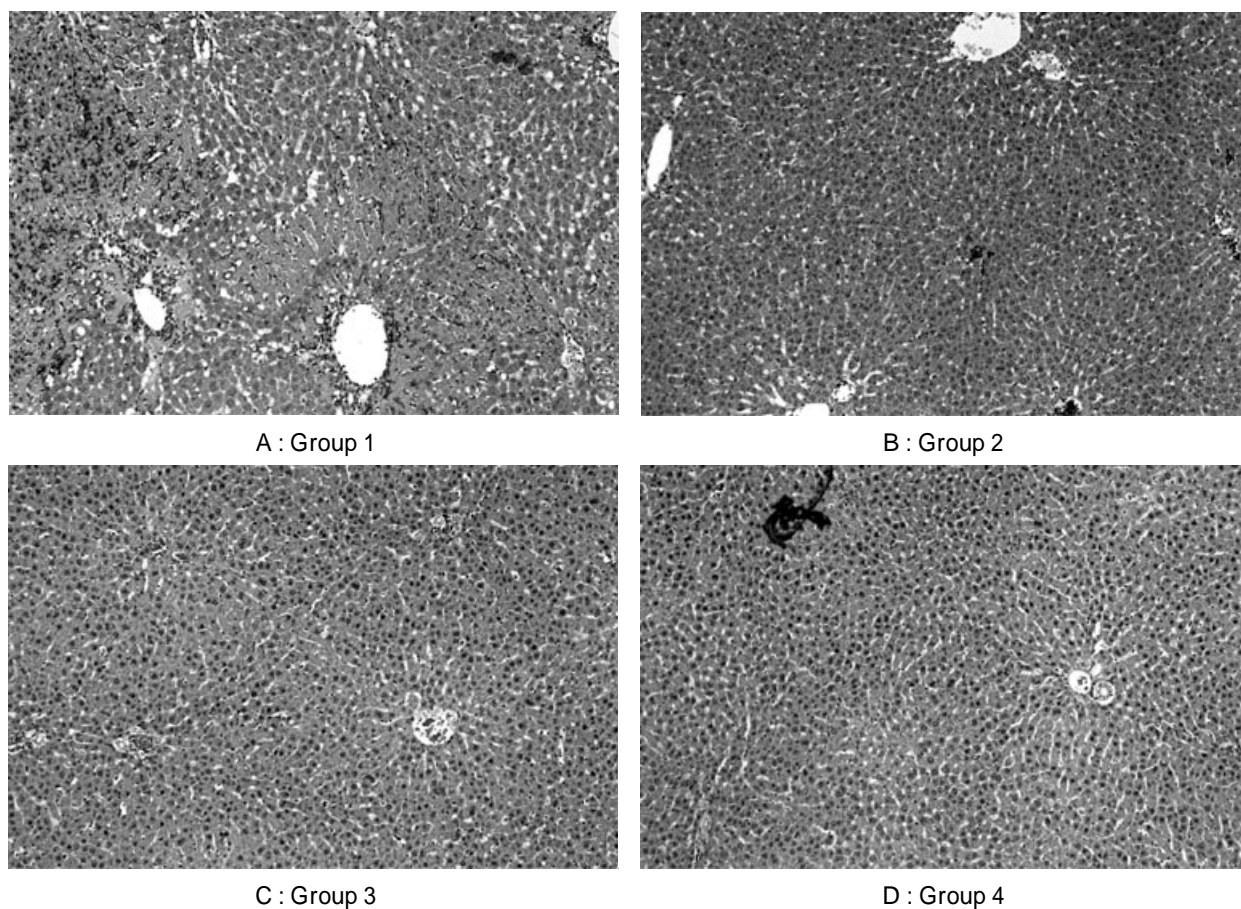B : Group 2

C : Group 3

D : Group 4

Figure. 7.   Histological findings
A) Erythrocytes were frequently sludged in the sinusoids, and the hepatocyte vacuolization was prominent.
B, C, D) Only isolated erythrocytes were found, and the hepatocyte vacuolization was minimal.

critical shortage of transplantable organs has mandated the pursuit of safe transplantation using unconventional donor organs (5). Over the past few years, NHBDs have composed approximately 1% of the total cadaveric donors, but there are estimates that controlled NHBDs have the potential to increase the cadaver donor pool by 25-42%, with at least 1,000 controlled NHBDs each year (6, 7). Transplantation of organs from NHBDs could help to decrease the disparity in organ supply and demand. The main obstacle to the use of livers from NHBDs is warm ischemia to the liver related to cardiac arrest. Liver grafts from NHBDs are unsuitable for LTx because the graft viability is deteriorated by warm ischemia and severe reperfusion injury. Clinical and experimental reports suggest that the liver can tolerate warm ischemia, even for periods up to 60 min and more (8). However, in LTx, the allograft sustains inevitable cold ischemia in addition to re-warming injury during vascular anastomoses. To increase the number of viable hepatic allografts available from NHBDs, new strategies are required.

Recent studies have demonstrated that FK506, in addition to being a powerful immunosuppressive agent, ameliorates the hepatic injury induced by normothermic ischemia and reperfusion (9). There is evidence that FK506 enhances, in the early phase of reperfusion, adenosine triphosphate (ATP) recovery and diminishes peroxidative damage in ischemically injured hepatocytes (10). FK506 has also been shown, in the later phase of the reperfusion period, to inhibit TNF-α, which may activate endothelial cells to express adhesion molecules and to secrete platelet-activating factor (PAF) (11). Thus, the beneficial effect of this immunosuppressant on hepatic ischemia is multifactorial (9).

On the other hand, TXA is a prostaglandin, which potently induces platelet aggregation and vascular contraction (12). This factor is increased by the ischemic injury of organs, host rejection of transplanted organs, trauma, and shock (13, 14). Thus, anti-TXA agents are reported to reduce ischemia-induced organ injury and the degree of rejection (12). OKY046 is a specific thromboxane synthetase inhibitor, which suppresses the production of TXA without affecting other cyclooxygenase pathways. Several reports have suggested the beneficial effect of this drug on warm ischemic damage to the liver after reperfusion and on cold preservation/reperfusion injury of the liver (3).

Consequently, FK506 and OKY046 have a completely different mechanism of preventing ischemia/reperfusion injury. Concomitant use of these 2 drugs may make LTx from NHBDs safer. In this study, the survival rate in the FK506 OKY046 group was significantly higher than in the control group. Until 6 hr after reperfusion, the serum ALT, AST and LDH concentrations increased in the control group, while in the FK506 group, the OKY046 group and the FK506 OKY046 group, these levels peaked 3 hr after reperfusion and thereafter significantly decreased. Especially in the FK506 OKY046 group, these concentrations decreased the most. In the control group, the concentrations of TNF-α significantly increased 60 min after reperfusion and remained high until 6 hr after perfusion. However, 6 hr after reperfusion, this concentration was significantly lower in the other 3 groups than in the control group, and in the FK506 OKY046 group, this level was also significantly lower than in the control group after 3 hr of reperfusion. Until 6 hr after reperfusion, the IL-6 concentration in the control group increased, while this concentration remained low in the other 3 groups. This concentration was low 3 hr after reperfusion only in the FK506 OKY046 group. TNF-α is clearly an important mediator of ischemia/reperfusion injury (15). Specifically, TNF-α has been documented to activate neutrophils to produce local damage during hepatic ischemia/reperfusion injury (16). In addition, the release of TNF-α after injury not only mediates local injury, but participates in distant organ dysfunction as well (17). Also, undefined mechanisms of tissue toxicity by TNF-α exist that are not coupled with neutrophil activity (18). Neutralization of TNF-α has been documented to decrease hepatocellular damage after ischemia/reperfusion injury (18). Investigation of hepatic ischemia has shown that FK506 pretreatment reduces TNF-α levels, as well as neutrophil migration (19). Other studies have shown that FK506 pretreatment results in decreased expression of IL-6 (20).

The donor pretreatment with OKY046 reduces intrahepatic thromboxane production after reperfusion, and pretreatment of NHBDs with OKY046 reduces liver endothelial cell damage and injury to the microcirculation after reperfusion (1). In this study, TNF-α and IL-6 were inhibited by the administration of OKY046 alone, as well as by the administration of FK506. The cause of this is assumed to be that the platelet aggregation inhibiting effect of OKY046 reduced the cellular damage, and the secondary release of inflammatory cytokines, which was induced by the cellular damage, was inhibited (Fig. 8). By concomitantly using these 2 drugs, additive effects are expected during LTx from NHBDs. However, regarding pretreatment of donors, further evaluation is required, including the side effects of drugs.

Ischemia / reperfusion injury

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Inflow of neutrophils, platelets, complements and coagulation factors

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FK506 ——→ **Activation of local T-cells**   **OKY046**

↓   ↓

Activation of Kupffer cells ——→   **TXA2**

↓   ↓

Inflammatory cytokines

↓

Endothelial cells ←—— **Vasoconstriction and platelet aggregation**

↓

Neutrophilic infiltration and expression of adhesion molecules
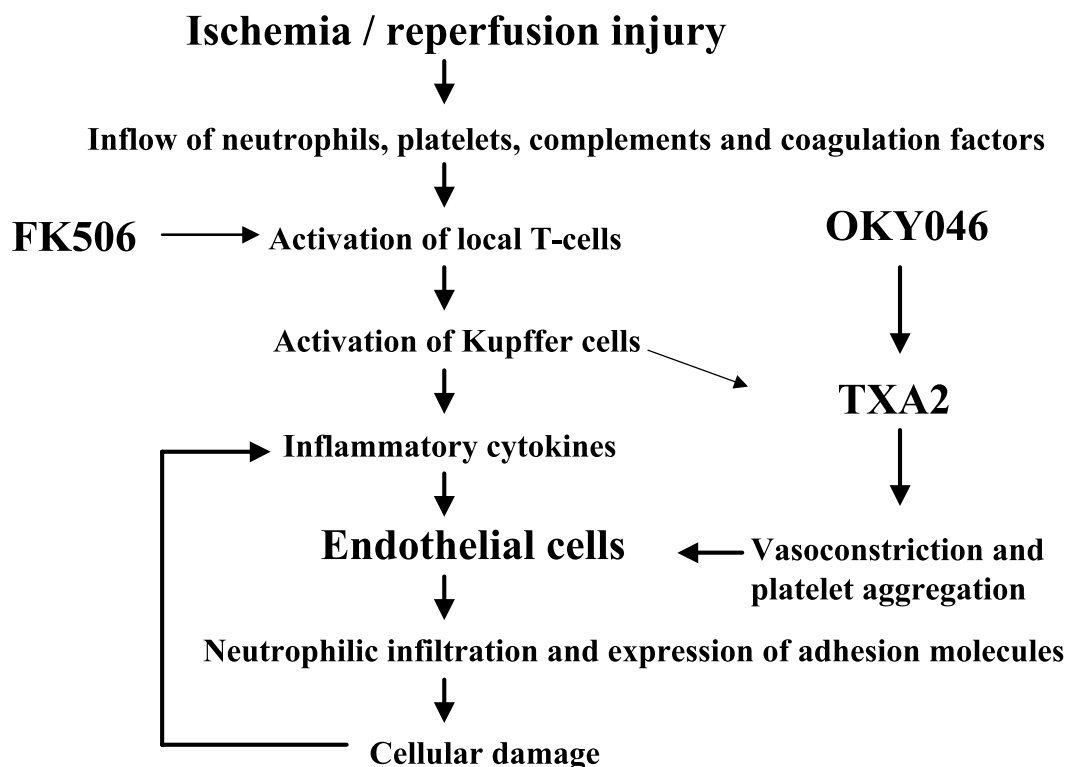
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Cellular damage

Figure. 8. The action mechanisms of FK506 and OKY046 are shown. Administration of OKY046 alone reduced cellular damage by inhibiting platelet aggregation. This is assumed to decrease secondary release of inflammatory cytokines.

CONCLUSION

In conclusion, we demonstrated that pretreatment of NHBDs with pharmacologic modulation of FK 506 and OKY046 ameliorated graft viability.

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