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Effect of Pretreatment with FTY720 and Cyclosporin on Ischaemia–Reperfusion Injury of the Liver in Rats

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Abstract

The effect of pretreatment with FTY720 (2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol hydrochloride) or cyclosporin, or both, on neutrophil-mediated injury has been examined by use of a rat model of transient clamping of hepatic flow.

Pretreatment with FTY720 alone or with cyclosporin induced a marked reduction of circulatory lymphocytes, whereas the use of these drugs in combination was very effective at suppressing the elevation of the number of peripheral polymorphonuclear neutrophils (PMN) after reperfusion. Pretreatment with cyclosporin, with or without FTY720, significantly reduced hepatic damage, whereas FTY720 alone tended to prolong hepatic damage. Pretreatment of cyclosporin alone, but not in combination with FTY720, significantly reduced the accumulation of PMN and led to lower myeloperoxidase levels in the damaged liver.

In conclusion, pretreatment with cyclosporin, with or without FTY720, reduced hepatic damage after warm ischaemia–reperfusion, whereas pretreatment with FTY720 alone tended to prolong this damage.

Immunosuppressants affect mainly immunological cells, resulting in prevention of rejection reaction and autoimmunity. To reduce adverse effects the combined use of these drugs with different actions has been recommended. Pretreatment with some immunosuppressive drugs, for example cyclosporin (Hayashi et al 1988; Suzuki et al 1993), tacrolimus (Kawano et al 1991; Garcia-Criado et al 1997) and anti-T lymphocyte antibody (Zwacka et al 1997) has been shown to protect against neutrophil-mediated reperfusion injury, although the mechanism is not fully understood (Ohmori et al 1998).

FTY720 (2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol hydrochloride), a potent immunosuppressive compound, is a chemical derived by modification of ISP-1 produced by *Izaria sinclairii* (Fujita et al 1994). This agent substantially reduces the number of circulatory lymphocytes (Suzuki et al 1996; Mitsusada et al 1997), preventing graft rejection in animal organ transplant models. Recent studies have revealed the synergistic effect of FTY720 with cyclosporin in the renal allograft of non-human primates (Troncoso et al 1999) and in the proliferative in-vitro response of peripheral lymphocytes in man (Wang et al 1998). It has also been reported that FTY720 is sequestered by the lymph nodes as a result of acceleration of lymphocyte homing (Chiba et al 1998) and suppresses T cell infiltration into the allograft (Yanagawa et al 1998).

In this study we have investigated the effect on ischaemia–reperfusion injury of the liver of pretreatment with FTY720 both alone and in combination with cyclosporin; these compounds have completely different actions on immune cells.

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Materials and Methods

Animals

Experiments were performed on adult male Wistar rats, 15–16 weeks, purchased from Japan SLC (Shizuoka, Japan). They were maintained under

specific pathogen-free conditions and had free access to standard rat chow and tap water.

Chemicals

FTY720 powder was supplied by Yoshitomi Pharmaceutical Industries (Osaka, Japan). The agent was dissolved in distilled water (0·5 $mg\,mL^{-1}$) for oral administration. Cyclosporin (Sandimmune; Sandoz, Tokyo, Japan) was dissolved in olive oil at a concentration of $5\,mg\,mL^{-1}$. ABTS (2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid)) tablets and buffer solution were obtained from Boehringer Mannheim (Penzberg, Germany). Naphthol AS-D chloroacetate esterase, superoxide anion dismutase, cytochrome-C and phorbol myristate acetate were purchased from Sigma (St Louis, MO).

Experimental protocol

The first experiment was performed to examine whether pretreatment with FTY720 or cyclosporin alone or in combination protects against ischaemia–reperfusion liver injury. Rats, 300–340 g, were randomly assigned to four groups ($n = 7$ per group): group 1, pretreatment with FTY720 alone; group 2, pretreatment with cyclosporin alone; group 3, pretreatment with FTY720 and cyclosporin; group 4, pretreatment with vehicle. Group 1 received oral FTY720 at a dose of $1{\cdot}0\,mg\,kg^{-1}$ once daily for 3 days. Group 2 received oral cyclosporin at a dose of $10\,mg\,kg^{-1}$ once daily for 3 days. Group 3 received a combination of $1{\cdot}0\,mg\,kg^{-1}$ FTY720 and $10\,mg\,kg^{-1}$ cyclosporin once daily for 3 days. Group 4 were given distilled water as a control. On day 3, normothermic ischaemia of the liver was induced as described elsewhere (Ohmori et al 1998). Briefly, a midline incision was made under ether anaesthesia and both portal vein and hepatic artery were clamped for 20 min by use of microclip. The clip was then released and the abdominal wall was closed by means of a continuous running suture. Whole blood (approx. 0·5 mL) was sampled 0, 1, 3 and 6 h after reperfusion for hepatic enzyme measurement and total and differential leukocyte count. Six hours after reperfusion the animals were killed and liver tissue was sampled for measurement of tissue myeloperoxidase levels, and histological assessment of peripheral polymorphonuclear neutrophil (PMN) infiltration.

The second experiment was designed to study serum tumour necrosis factor-α (TNF-α) levels and the functional change of peripheral PMN of the rat treated with FTY720 after ischaemia–reperfusion. Other sets of rats were divided into FTY720 or vehicle groups ($n = 6$ in each). These animals were prepared identically to those in the first experiment and underwent total hepatic ischaemia for 20 min. Whole blood (approx. 0·3 mL) was sampled 0, 1, 3 and 6 h after reperfusion for measurement of serum TNF-α. Six hours after reperfusion whole blood (approx. 7 mL) was collected from the abdominal aorta for measurement of circulatory PMN.

Serum transaminase level and peripheral leukocyte count

Serum aspartate aminotransferase and alanine aminotransferase were measured by means of ultraviolet absorption with an automatic analyser (Hitachi 7170, Tokyo, Japan). Total leukocyte count was measured by autoanalysis (Sysmex, Toyo Medical Electronics, Tokyo, Japan). Differentiation of leukocytes was determined by a skilled technician.

Liver tissue myeloperoxidase assay

Tissue myeloperoxidase levels were used as an index of PMN infiltration of the liver. Liver tissue ($1\,cm^3$) obtained 6 h after reperfusion was frozen in liquid nitrogen and stored at −70°C until measurement. Tissue samples were then homogenized in phosphate-buffered saline (10 mL) and centrifuged at 100 000 *g* and 4°C for 30 min. The resulting supernatant was assayed for myeloperoxidase activity by use of a modification of the method of Shindler et al (1976). Briefly, reaction mixture containing the supernatant (10 μL) and ABTS buffer (100 μM, 200 μL) with H_2O_2 was incubated for 60 min at 25°C in a 96-well plate. The optical density at 414 nm was determined by means of a plate reader (Spectramax 340; Molecular Devices, Sunnyvale, CA). Myeloperoxidase activity was calculated by use of an extinction coefficient of $3{\cdot}6 \times 10^4\,mol^{-1}\,cm^{-1}$ for ABTS and normalized to protein levels ($\mu mol\,(mg\,protein)^{-1}$). The protein concentration was determined by the method of Lowry et al (1951).

Histological assessment of PMN infiltration

Hepatic specimens were also evaluated by histological examination for PMN infiltration of the liver after reperfusion. At least 2 sections were prepared from each sample and were stained with naphthol AS-D chloroacetate esterase as a specific marker for PMN (Moloney et al 1960). PMN were identified by positive staining and morphology and counted by light microscopy under 400 ×

magnification. All fields were examined blind by two expert pathologists (M. Mori and T. Kanno). The data were expressed as PMN/50 high-power field (HPF).

TNF-α assay

Serum TNF-α levels was measured by means of an enzyme-linked immunoassay kit (Biosource International, Camarillo, CA) according to a modification of the method of Sheehan et al (1989). Standard curves were obtained by analysis of rat recombinant TNF-α at concentrations of 1·95–250 pg mL^{-1}. Assay plates were read at 450 nm with a microplate reader (Spectramax 340).

Function of circulatory PMN

The function of circulatory PMN was assessed by superoxide anion production, by use of a minor modification of the method reported by Yuo et al (1989). Mixed leukocyte suspension was obtained after sedimentation in the presence of 3% dextran in saline and gradient centrifugation with lympholyte-M (Cosmo Bio, Tokyo, Japan). Purified PMN suspension was obtained by hypotonic lysis at 4°C and washing twice in phosphate buffer. PMN was resuspended in Hanks balanced salt solution (Nissui Pharmaceutical, Tokyo, Japan) at a concentration of 3×10^6 cells mL^{-1}. PMN O_2^- production was determined by a modification of the superoxide anion dismutase-inhibitible cytochrome C reduction method, with phorbol myristate acetate as stimulant (Yuo et al 1989). PMN suspension containing $1{\cdot}5 \times 10^5$ PMN, phorbol myristate acetate (10 μM) or its control buffer, superoxide anion dismutase (200 units mL^{-1}) or its vehicle, and cytochrome C (12 μg mL^{-1}) were placed on FCS-coated wells in a total volume of 200 μL/well. After incubation for 60 min at 37°C, O_2^- production (nmol/$1{\cdot}5 \times 10^5$ cells h^{-1}) was determined by use of an extinction coefficient of $2{\cdot}1 \times 10^4$ mol^{-1} cm^{-1} for cytochrome C, by measuring the absorbance at 540 and 550 nm in a spectrophotometer (Spectramax 340).

Statistical analysis

Results are presented as means ± s.e.m. or ± s.d. Statistical analysis was performed by one way analysis of variance or by use of Student's unpaired *t*-test, as appropriate. A value of $P < 0{\cdot}05$ was considered to be indicative of significance.

Results

Changes in the number of circulatory lymphocytes and PMN after ischaemia–reperfusion

Pretreatment with FTY720 alone or with cyclosporin induced a marked decrease of circulatory lymphocytes before and after ischaemic damage (Figure 1A). Before ischaemia there was no difference between the number of PMN measured for the groups treated with FTY720, either alone or with cyclosporin, and the number measured for the vehicle-treated group. After reperfusion, however, numbers were lower for groups treated with FTY720 than for the vehicle-treated group. In particular, pretreatment with FTY720 in combination with cyclosporin resulted in significant sup-

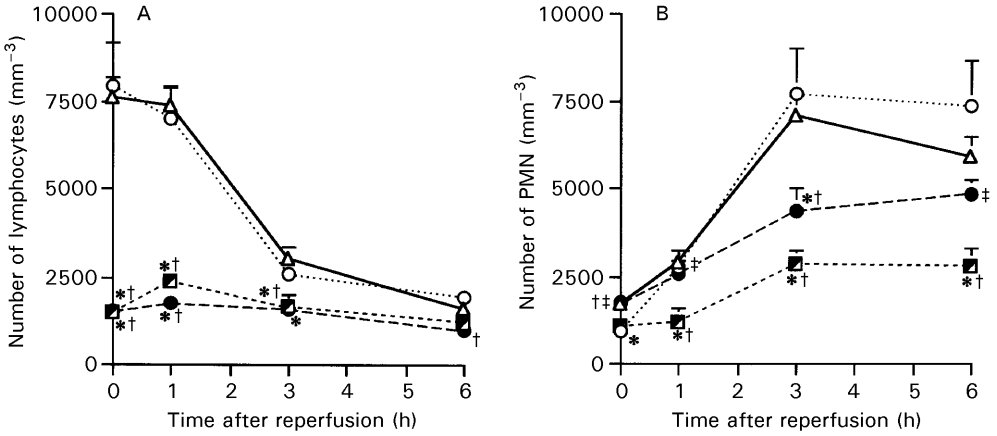

Figure 1. Changes in the number of A. peripheral lymphocytes and B. PMN after liver ischaemia–reperfusion for rats treated with FTY720 (●) and cyclosporin (○) either alone or in combination (◩) (△ = vehicle). Results are means ± s.e.m. *$P < 0{\cdot}05$ compared with vehicle group; †$P < 0{\cdot}05$ compared with cyclosporin group; ‡$P < 0{\cdot}05$ compared with combined FTY720 and cyclosporin group.

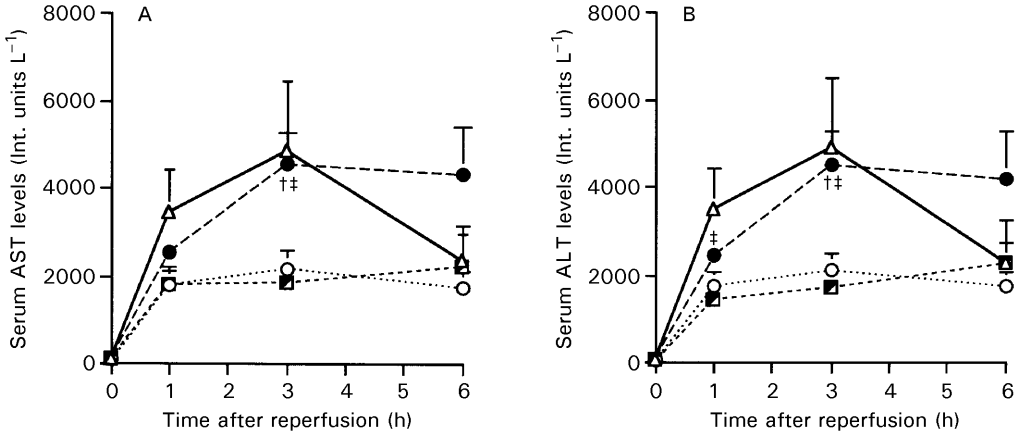

Figure 2. Changes in A. serum aspartate aminotransferase and B. alanine aminotransferase levels after liver ischaemia–reperfusion for rats treated with FTY720 (●) and cyclosporin (○) either alone or in combination (◩) (△ = vehicle). Results expressed as means ± s.e.m. †$P < 0{\cdot}05$ compared with cyclosporin group; ‡$P < 0{\cdot}05$ compared with combined FTY720 and cyclosporin group.

pression of the elevation of the number of PMN after reperfusion (Figure 1B). There were no significant differences between the numbers of lymphocytes and PMN for the group treated with cyclosporin alone and for that treated with vehicle.

Changes in serum transaminase levels after ischaemia–reperfusion

Pretreatment with cyclosporin alone or with FTY720 significantly reduced the hepatic damage after reperfusion, compared with pretreatment with FTY720 alone (Figures 2A, B). In the group treated with FTY720 alone there was a tendency for hepatic damage to be prolonged compared with the vehicle-treated group (Figures 2A, B).

Myeloperoxidase level and the number of PMN in the liver after ischaemia–reperfusion

Tissue myeloperoxidase levels and the numbers of PMN infiltrating the liver after reperfusion are summarized in Table 1. Pretreatment with cyclosporin alone significantly reduced the accumulation of PMN and reduced myeloperoxidase levels in the damaged liver, compared the group receiving FTY720 alone. Accumulation of PMN in the FTY720-alone group tended to increase compared with that in the vehicle-treated group; tissue myeloperoxidase levels in these groups were similar. Myeloperoxidase levels and the number of PMN for the group treated with FTY720 and cyclosporin in combination were between those of the groups treated with FTY720 alone and with cyclosporin alone.

Changes in serum TNF-α levels of rats after ischaemia–reperfusion

Serum TNF-α levels of the vehicle group increased from $< 1{\cdot}5\,\text{pg mL}^{-1}$ before hepatic clamping to $7{\cdot}2 \pm 4{\cdot}3\,\text{pg mL}^{-1}$ 1 h after reperfusion, then decreased. There was no significant difference between serum TNF-α levels of the vehicle- and the FTY 720-treated groups.

Comparison of PMN activation after ischaemia–reperfusion

In-vitro circulatory PMN function for the FTY720-treated rats 6 h after ischaemia–reperfusion was not significantly different from that of vehicle-treated rats ($1{\cdot}79 \pm 1{\cdot}14$ and $1{\cdot}44 \pm 0{\cdot}88\,\text{nmol}/1{\cdot}5 \times 10^5$ cells h^{-1}, respectively).

Table 1. Comparison of tissue myeloperoxidase levels and the number of peripheral polymorphonuclear neutrophils infiltrating the liver after liver ischaemia–reperfusion for rats treated with FTY720 either alone or with cyclosporin.

	Vehicle	FTY720	FTY720 + cyclosporin	Cyclosporin
Myeloperoxidase levels in the liver (μmol (mg protein)$^{-1}$)	0·89 ± 1·06	0·86 ± 0·96	0·68 ± 0·38	0·57 ± 0·69
Number of peripheral polymorphonuclear neutrophils[a]	1·38 ± 0·77	2·19 ± 0·92	1·42 ± 0·95	0·99 ± 0·36*

Results are means ± s.d. (n = 7). *$P < 0{\cdot}05$ compared with FTY720 group. [a]/50 high-power field.

Discussion

Ischaemia–reperfusion injury induces activation of several inflammatory pathways and results in free radical- (Bulkley 1987; Clavien et al 1992), cytokine- (Colletti et al 1996; Garcia-Criado et al 1997), complement- (Jaeschke et al 1993) and neutrophil-mediated tissue damage (Hernandez et al 1987; Jaeschke et al 1990; Langdale et al 1993). This ischaemia–reperfusion injury occurs in a biphasic pattern—acute oxygen free radical- and subacute neutrophil-mediated damage; subacute neutrophil-mediated damage is suppressed by depletion of T cells from the host (Zwacka et al 1997). It has been reported that conventional immunosuppressants, such as cyclosporin and tacrolimus, reduce both tissue free-radical levels and neutrophil infiltration after ischaemia–reperfusion (Garcia-Criado et al 1997).

FTY720, a novel immunosuppressant, induces a marked reduction both of circulatory lymphocyte numbers (Chiba et al 1998) and infiltration of T cells into the allograft (Yanagawa et al 1998). It is reported that the number of mature lymphocytes, especially $CD4^+$ T cells, is depleted in animals receiving FTY720 (Enosawa et al 1996), but that the animals do not suffer from viral infection (Mitsusada et al 1997). These data prompted us to test whether ischaemia–reperfusion liver damage could be reduced by pretreatment with FTY720.

Our results showed that ischaemia–reperfusion liver damage could not be reduced by pretreatment with FTY720 alone, even though this agent caused a marked decrease in the number of circulatory lymphocytes. The number of peripheral PMN was not influenced by pretreatment either with FTY720 alone or in combination with cyclosporin, but PMN migration was delayed after ischaemia–reperfusion. Protection against ischaemia–reperfusion liver damage as a result of pretreatment with immunosuppressants was observed after treatment with cyclosporin either alone or with FTY720. However, liver damage was prolonged by use of FTY720 alone. Because of the possible effect of FTY720 on PMN function, we examined both local and systemic PMN function after liver ischaemia–reperfusion.

These results show that tissue myeloperoxidase levels and the number of PMN infiltrating the damaged liver were higher in the group treated with FTY720 alone than in that treated with cyclosporin alone. Circulatory PMN function in the FTY720-treated rats after ischaemia–reperfusion was not significantly different from that in the vehicle-treated rats. Systemic TNF-α response was not affected by pretreatment with FTY720, as shown previously (Yanagawa et al 1998). These results suggest that treatment with FTY720 does not significantly affect systemic cytokine production or function of circulating PMN. Further studies will be focused on adhesion and rolling of PMN in the liver (Garcia-Criado et al 1998).

In conclusion, pretreatment with cyclosporin with or without FTY720 reduced hepatic damage after warm ischaemia–reperfusion whereas FTY720 alone tended to prolong this damage.

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