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ORIGINAL ARTICLE

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Regulation of pro-inflammatory and anti-inflammatory cytokine responses by Kupffer cells in endotoxin-enhanced reperfusion injury after total hepatic ischemia

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Abstract The effects of Kupffer cells on cytokine responses in endotoxin-enhanced reperfusion injury after total hepatic ischemia were investigated in this study. Male rats pretreated with either normal saline solution (NS group) or gadolinium chloride ($GdCl_3$) to inhibit Kupffer cell function (GC group) were subjected to 60 min of hepatic ischemia. These animals received either normal saline solution or sublethal doses of endotoxin (1 mg/kg) at reperfusion. In the NS group, endotoxin administration induced an enhanced tumor necrosis factor-alpha (TNF-α) and interleukin-10 production 1 h after reperfusion with a subsequent peak of macrophage inflammatory protein-2 (MIP-2) levels, which resulted in a 7-day survival rate of 30%. Despite endotoxin administration, $GdCl_3$ pretreatment significantly suppressed TNF-α and increased interleukin-10 production 1 h after reperfusion, which led to a decline in MIP-2 production and amelioration of functional and structural liver damage with a 7-day survival rate of 80%. Augmented pro-inflammatory and anti-inflammatory cytokine responses by Kupffer cells were associated with endotoxin-enhanced reperfusion injury after hepatic ischemia. Kupffer cell blockade has a potential to attenuate the insult via modulation of cytokine responses.

Keywords Liver · Ischemia-reperfusion injury · Endotoxin · Kupffer cell · Cytokine

Introduction

Shortage of grafts is a serious problem in liver transplantation [22]. Split liver and living related liver transplantation has been performed to address this issue [25, 42]. Grafts from non-heart-beating donors (NHBDs) may have a potential as a resource to compensate for this situation [37]. The most common candidates for liver transplantation are patients with end-stage liver disease with high levels of endotoxin, an integral component of the cell wall of gram-negative bacilli [23, 44]. Endotoxin acts as a potent stimulus for Kupffer cells, the resident macrophages of the liver, to produce various inflammatory mediators and is involved in deterioration in various types of insults [18, 30, 35, 38]. Hepatic ischemia and reperfusion (HIR) injury is inevitable in liver transplantation. The response of the ischemically damaged liver to sub-lethal doses of endotoxin is of great significance when the use of hepatic grafts from NHBDs is considered. However, little information is available on the influence of sub-lethal portal endotoxin for the ischemically damaged liver.

Many studies have been performed to clarify the mechanisms involved in the development of HIR injury [4, 8, 21, 26, 27, 29, 34, 45]. During the initial phase of HIR injury without endotoxemia, Kupffer cells are activated and release reactive oxygen intermediates (ROIs) and pro-inflammatory cytokines, including tumor

necrosis factor-alpha (TNF-α) [4, 27]. The enhanced TNF-α production may play a significant role in a sequence of events causing severe liver injury by polymorphonuclear neutrophils (PMNs) [4, 8, 27, 34]. TNF-α may be associated with upregulation of adhesion molecules and potent chemo-attractant pro-inflammatory cytokines, named CXC chemokines, such as macrophage inflammatory protein-2 (MIP-2) [7, 12]. In the cytokine family, an important point to be noted is that interleukin-10 (IL-10), an anti-inflammatory cytokine, has shown an action contrary to TNF-α with pleiotropic actions in sepsis models [39, 40]. Elimination of Kupffer cells could attenuate endotoxin-induced liver damage or HIR injury by damping the inflammatory signals, thus causing a decline in cytokine and ROIs [8, 29, 34, 36]. However, it remains unknown whether the blockade of Kupffer cell function modulates the production of pro-inflammatory and anti-inflammatory cytokines and chemokine in HIR injury, despite endotoxin administration at the time of reperfusion. The present study was designed to determine the influence of sub-lethal endotoxin administration at the time of reperfusion in the development of HIR injury and the role of Kupffer cells for the regulation of pro-inflammatory and anti-inflammatory cytokine responses in these insults.

Materials and methods

Male Sprague–Dawley rats, weighing 250–280 g, were purchased from Japan SLC (Hamamatsu, Japan). The experiment protocols followed the criteria of our institution and the National Research Council for the care and use of laboratory animals in research. The animals were starved for 12 h before the experiment but were allowed water ad libitum. The surgical procedure was performed under general anesthesia with intra-abdominal injection of sodium pentobarbital (25 mg/kg). The animals were divided into two groups : rats given 0.9% normal saline solution as controls (NS group), and rats treated with intravenous gadolinium chloride ($GdCl_3$: 7 mg/kg) for 2 days before the induction of hepatic ischemia to inhibit the phagocytic function of Kupffer cells (GC group). $GdCl_3$ was purchased from Aldrich Chemical (Milwaukee, Wis., USA). We used a total hepatic ischemic model with an extra-corporeal porto-systemic shunt to produce a severe ischemic insult to the whole liver without splanchnic congestion. After laparotomy, a porto-systemic shunt was placed between a cecal branch of the portal vein and a jugular vein by use of a polyethylene tube of 0.97 mm inner diameter (Natsume Seisakusho Ltd., Tokyo, Japan). After intravenous injection of sodium heparin (100 IU/kg), animals from each group were subjected to 60 min of total hepatic ischemia by cross-clamping of the hepatic artery, the portal vein, and the common bile duct with a vascular microclip. Reperfusion following hepatic ischemia was achieved by removal of the microclip. In the NS and GC groups, animals given either normal saline solution or a sub-lethal dose of lipopolysaccharide (1 mg/kg, *Escherichia coli* 026: B6, Difco, Detroit, Mich., USA) as endotoxin via the portal vein at the beginning of reperfusion were subdivided into NS-N and NS-E groups, and GC-N and GC-E groups (Fig. 1). The extra-corporeal porto-systemic shunt was removed after portal injection of normal saline or endotoxin. For each group, ten animals were utilized for assessment of survival, and five animals were used for each time point considered for laboratory tests. Blood samples were taken from the aorta for measurement of the plasma aspartate aminotransferase (AST), TNF-α, IL-10 and MIP-2 levels at 1, 3, and 6 h after reperfusion. The animals were killed immediately thereafter, and their livers were removed for histological studies.

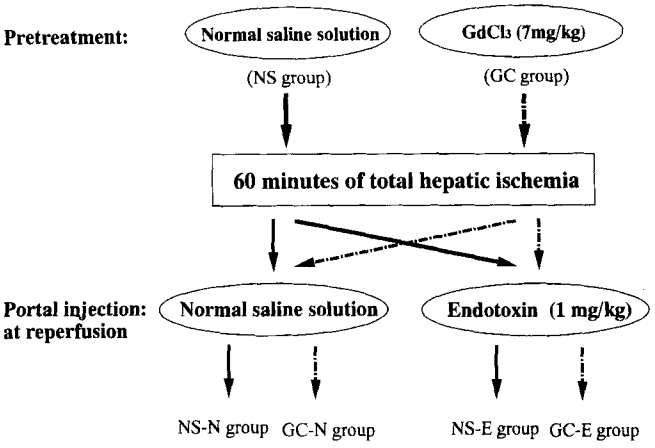

Fig. 1 Grouping pattern in our experiment

Measurement of plasma AST levels

We measured plasma AST levels to assess hepatic parenchymal damage at 1, 3 and 6 h after reperfusion, using a Hitachi 736 autoanalyzer (Hitachi Ltd., Tokyo, Japan).

Plasma TNF-α assay

Blood samples were collected in heparinized sterile tubes. Plasma was separated by centrifugation at 3,000 *g* for 15 min and stored at –84 °C until required for assay. Determinations were done in a 96-well microtiter plate, with a TNF-α test kit (Genzyme, Cambridge, Mass., USA) based on an enzyme-linked immunosorbent assay (ELISA). All samples were tested in duplicate. The plate was read on a microplate reader (EL 340, Biotek Instruments, Winooski, Vt., USA) at 450 nm, and the TNF-α concentrations in experimental samples were calculated from a standard curve.

Plasma MIP-2 assay

Determinations of plasma MIP-2 levels were done in a 96-well microtiter plate, with a rat MIP-2 kit (BioSource International, Camarillo, Calif., USA) based on an ELISA. All samples were tested in duplicate. The plate was read on a microplate reader at 450 nm, and the MIP-2 concentrations in experimental samples were calculated from a standard curve.

Plasma IL-10 assay

Plasma IL-10 activities were measured at 1 and 6 h after reperfusion in the NS-E and GC-E groups. Determinations of these levels were done in a 96-well microtiter plate, with a cytoscreen immunoassay kit (BioSource International) based on an ELISA. All samples were tested in duplicate. The plate was read on a microplate reader at 450 nm, and the IL-10 concentrations in experimental samples were calculated from a standard curve.

Histological studies

Resected liver specimens were fixed in 10% formalin and embedded in paraffin. Sections of 3 μm were made and stained with hematoxylin and eosin (HE) for histological examination. PMNs that had accumulated in the liver were stained by the naphthol AS-D chloroacetate esterase technique [19]. PMNs were identified by positive staining and by their morphology, and were counted under light microscopy at a magnification rate of 450. Results were expressed as the number of PMNs per 30 high-power fields. Blind analysis was done for all histological studies.

Determination of survival rate

After the abdominal incision was closed, the rats were provided with food and water ad libitum. We followed-up the animals for 7 days after surgery to assess survival.

Statistical analysis

All values were expressed as mean ± SD. Statistical evaluation of the biochemical and histological data was done by analysis of variance and the Newman–Keul test for multiple comparisons. Fisher's exact test with Yates' correction was used to determine the significance of differences in the survival rate. $P < 0.05$ was considered significant.

Results

Plasma AST levels

The plasma AST levels after reperfusion increased with the length of reperfusion period in all groups. Portal endotoxin administration at reperfusion caused marked increase in the plasma AST levels. The plasma AST level in the NS-E group was maximal at 6 h after reperfusion (5,469 ± 1,301 IU/l), but pretreatment with $GdCl_3$ significantly suppressed the elevation of the plasma AST levels (3,337 ± 569 IU/l) ($P < 0.05$) (Table 1).

Plasma TNF-α levels

The plasma TNF-α levels reached a peak at 1 h after reperfusion in all groups with significant difference, compared with non-ischemic levels (Fig. 2). Endotoxin administration at the time of reperfusion markedly raised the plasma TNF-α levels and those of the NS-E group 1 h after reperfusion had reached a peak value of 1,268 ± 678 pg/ml. Pretreatment with $GdCl_3$ significantly suppressed the elevation of plasma TNF-α levels throughout the experimental periods when compared with the NS-E group ($P < 0.05$) (Fig. 2B).

Plasma MIP-2 levels

The NS-N group showed significant elevation of plasma MIP-2 levels when compared with the GC-N group at 1 and 3 h after reperfusion ($P < 0.01$) (Fig. 3A). Plasma MIP-2 levels in the NS-E group were markedly increased after reperfusion, but pretreatment with $GdCl_3$ significantly inhibited the increase of plasma MIP-2 levels until 6 h of reperfusion ($P < 0.01$) (Fig. 3B). Unlike the time course of plasma TNF-α levels, the plasma MIP-2 levels in the NS-E and GC-E groups reached peak values at 3 h after reperfusion.

Plasma IL-10 levels

Plasma IL-10 levels under non-ischemic conditions were 2.2 ± 1.0 pg/ml in the NS group and 7.2 ± 3.6 pg/ml in the GC group, respectively. A significant increase in plasma IL-10 levels was registered in the NS-N (63.8 ± 25.9 pg/ml) and GC-N (91.1 ± 14.0 pg/ml) groups at 1 h after reperfusion, compared with the levels of the non-ischemic controls, but no statistical significance was observed between these two groups. With portal endotoxin administration, plasma IL-10 levels were markedly increased in the NS-E and GS-E groups

Table 1 Plasma AST activities and 7-day survival rates. *NS* pretreatment with normal saline solution, *GC* pretreatment with gadolinium chloride, *N* normal saline administration at reperfusion, *E* endotoxin administration at reperfusion

Parameter	Experimental groups			
	NS-N	GC-N	NS-E	GC-E
Plasma AST levels (IU/L)				
Non-ischemic controls	61 ± 4	135 ± 12	61 ± 4	135 ± 12
1 h after reperfusion	2,821 ± 637[a,b]	1,969 ± 265[a]	4,154 ± 864[a,c,d]	2,522 ± 572[a]
3 h after reperfusion	3,300 ± 348[a,b]	2,730 ± 130[a]	5,285 ± 1,474[a,b,e,f]	2,948 ± 343[a]
6 h after reperfusion	3,455 ± 572[a]	3,035 ± 658[a]	5,469 ± 1,301[a,e,e,f]	3,337 ± 569[a]
7-day survival rate (%)	10/10 (100%)	10/10 (100%)	3/10 (30%)[h,i,j]	8/10 (80%)

[a] $P < 0.01$ vs non-ischemic controls in each group
[b] $P < 0.05$ vs GC-N group at the corresponding time
[c] $P < 0.01$ vs GC-N group at the corresponding time
[d] $P < 0.05$ vs GC-E group at the corresponding time
[e] $P < 0.01$ vs GC-E group at the corresponding time
[f] $P < 0.05$ vs NS-N group at the corresponding time
[g] $P < 0.01$ vs NS-N group at the corresponding time
[h] $P < 0.01$ vs NS-N group
[i] $P < 0.01$ vs GC-N group
[j] $P < 0.05$ vs GC-E group

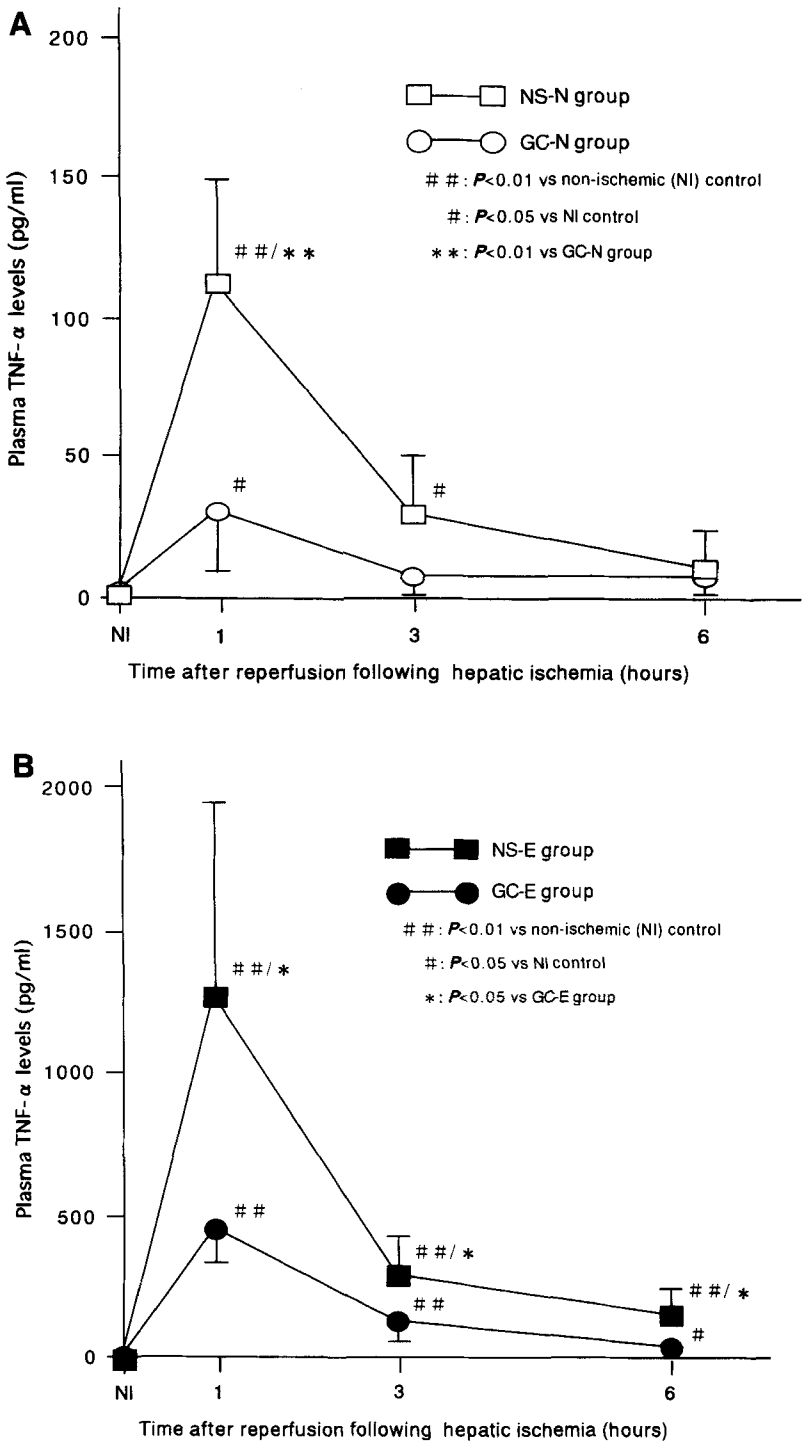

Fig. 2A, B Time course of plasma TNF-α levels after reperfusion following 60 min of total hepatic ischemia. **A** Plasma TNF-α levels in the NS-N and the GC-N groups. **B** Plasma TNF-α levels in the NS-E and the GC-E groups

until 6 h after reperfusion. Plasma IL-10 levels in the GC-E group were significantly higher than those in the NS-E group at 1 h after reperfusion (Fig. 4). The IL-10/TNF-α ratio in the GC-E group was maintained at above 1 throughout the experiment, while those of the NS-E group with most severe functional and structural damages of the liver remained under 1 (Fig. 4).

Histological findings

Focal necrosis was seen in the livers of the NS-N group without endotoxin administration 6 h after reperfusion. Endotoxin administration at reperfusion caused massive liver necrosis with hemorrhage from the midzonal to the pericentral areas and marked PMN accumulation within

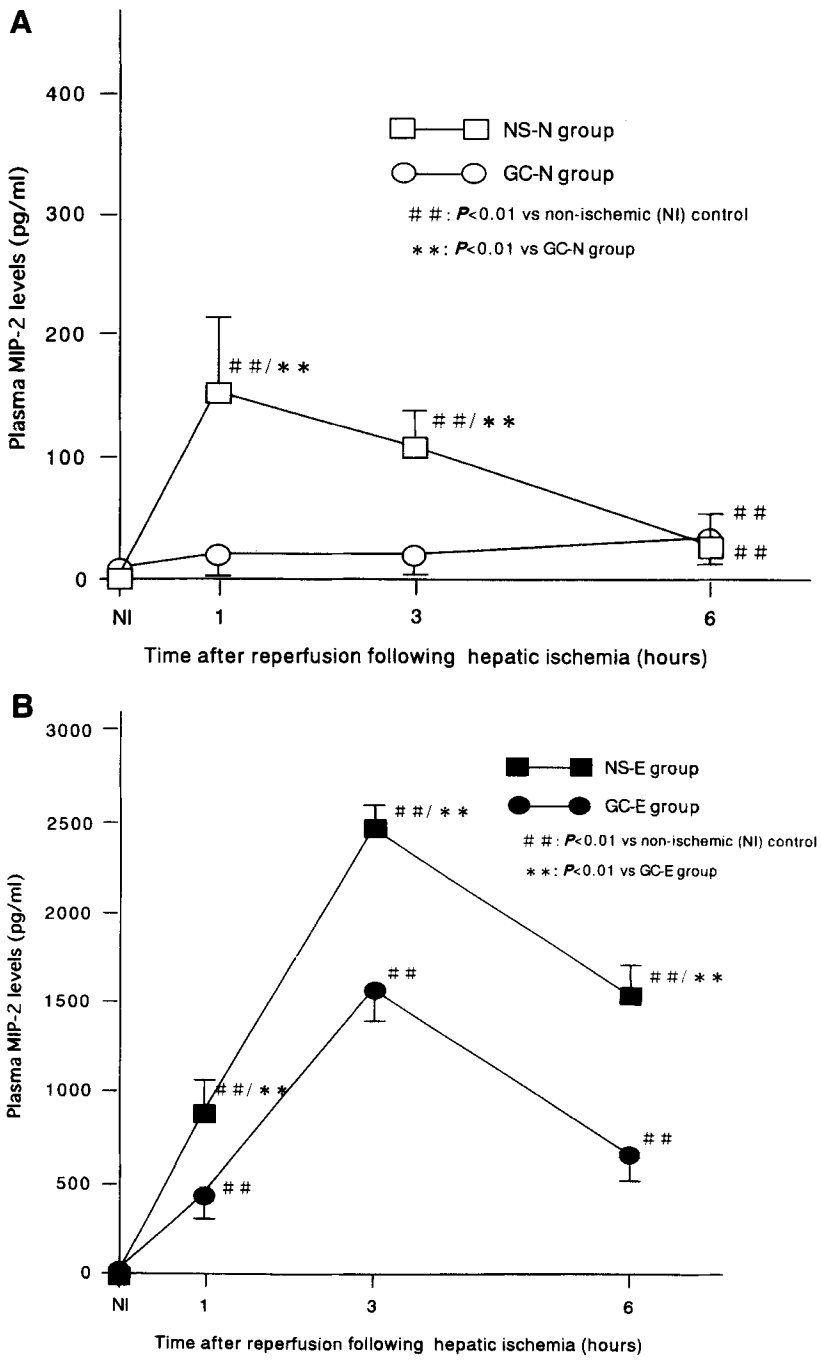

Fig. 3A, B Changes in plasma MIP-2 levels after reperfusion following 60 min of total hepatic ischemia. **A** Plasma MIP-2 levels in the NS-N and the GC-N groups. **B** Plasma MIP-2 levels in the NS-E and GC-E groups

the hepatic sinusoids in the NS-E group (Fig. 5A). Less-severe damage was seen in the livers of the GC-E group (Fig. 5B).

PMN infiltration in the liver

The time course of hepatic PMN infiltration revealed a similar pattern for all groups. Hepatic PMN infiltration in the NS-N group significantly increased from 1 h of reperfusion, relative to that in the GC-N group ($P < 0.05$) (Fig. 6A). Endotoxin administration at reperfusion caused a marked increase in hepatic PMN infiltration in the NS-E and GC-E groups. The GC-E group with $GdCl_3$ pretreatment showed significant inhibition of hepatic PMN infiltration, when compared with the NS-E group throughout the experimental periods ($P < 0.05$) (Fig. 6B). The increase in PMN infiltration was sustained with the length of reperfusion period throughout the experiment.

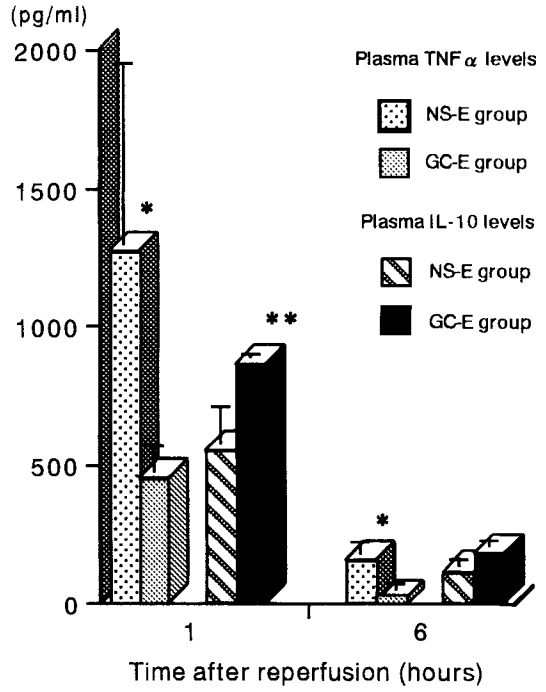

Fig. 4 Comparison of plasma TNF-α and IL-10 levels after reperfusion following 60 min of total hepatic ischemia in the NS-E and GC-E groups. $^{**}P < 0.05$, $^{**}P < 0.01$

Survival

Seven-day survival rates after 60 min of total hepatic ischemia were 100% in the NS-N and GC-N groups. However, endotoxin administration at reperfusion caused a deterioration of the survival rate in the NS-E group (30%), whereas the GC-E group pretreated with $GdCl_3$ had a significantly better survival rate of 80% ($P < 0.05$) (Table 1).

Discussion

NHBDs may have potential as a source of grafts, but the safety and viability of liver grafts harvested from those donors has not been established [37, 43]. There have been many reports on the factors affecting the outcome after liver transplantation [3, 23 24, 44]. Markedly elevated plasma endotoxin levels at the time of induction and after hilar dissection were closely associated with the occurrence of postoperative sepsis in liver transplantation for recipients of end-stage liver disease and cirrhosis [23, 44]. Recipients were susceptible to endotoxin with an increase in mortality in rat liver transplantation [1]. Considering the clinical application of grafts from NHBD, the influence of endotoxin must be determined in reperfusion injury following warm ischemia. Since few studies on the mechanisms of HIR injury have focused on the influence of endotoxin for the ischemically damaged liver, little is known about the effect of sub-lethal doses of endotoxin on the liver subjected to a tolerable ischemic time. The influence of endotoxin for HIR injury has been investigated in pioneer studies by the group of Jaeschke and associates [14, 15]. However, they used endotoxin originating from *Salmonella* in their experiment of HIR injury to clarify the mechanism of multiple organ failure. Thus, the objectives of this study were completely different from those in their investigations. Based on our previous results that a dose of 1 mg/kg was sub-lethal and that warm hepatic ischemia of up to 60 min was tolerable for normal rats, an experiment was designed to determine the influence of endotoxin on the ischemically damaged liver and on the cytokine responses in the development of the insult [17, 36].

The present study demonstrated that IL-10 is produced early, together with TNF-α, in endotoxin-enhanced reperfusion injury after hepatic ischemia, leading to a subsequent increase of MIP-2, and that these cytokine responses are relevant for the development of this insult. IL-10 is a potent anti-inflammatory cytokine that inhibits the production of other cytokines, including IL-1, IL-6, and TNF-α [20]. Although we did not determine the effect of IL-10 by passive immunization in this study, neutralization studies by Standiford and associates indicated that endogenous IL-10 production is instrumental in downregulating the overproduction of TNF-α and MIP-2 during endotoxemia [31]. Involvement of IL-10 in HIR injury has been indicated by Yoshidome and associates [45]. They demonstrated that recombinant murine IL-10 reduced serum TNF-α and MIP-2 levels by suppressing the activation of the transcription factor, nuclear factor kappa B (NFκB), and protected against HIR injury. However, endogenous IL-10 production was not determined in their studies, and the plasma profile of this cytokine remains unknown in HIR injury. In our study, plasma IL-10 levels were rapidly augmented with endotoxin administration after hepatic ischemia, but the GC-E group showed significantly higher IL-10 production than did the NS-E group, unlike the response of TNF-α for endotoxin. From these results, IL-10 may be derived from sources other than Kupffer cells ent study, although IL-10 is released by monocytes, lymphocytes, endothelial cells, and Kupffer cells [10, 20].

An inflammatory pathway during the early phase of HIR injury culminates in hepatic PMN accumulation occurring at the later phase of the injury [8, 9, 34]. Recruited PMNs may directly damage hepatocytes by releasing ROIs and proteases [8, 13]. TNF-α, a primary instigator of HIR injury, may have a feeble function as a neutrophil chemotaxin, and other mediators would be associated with hepatic PMN sequestration at a later phase in HIR injury, since recombinant TNF-α was not directly chemotactic for PMNs [32]. TNF-α has been shown to promote CXC chemokines, including MIP-2

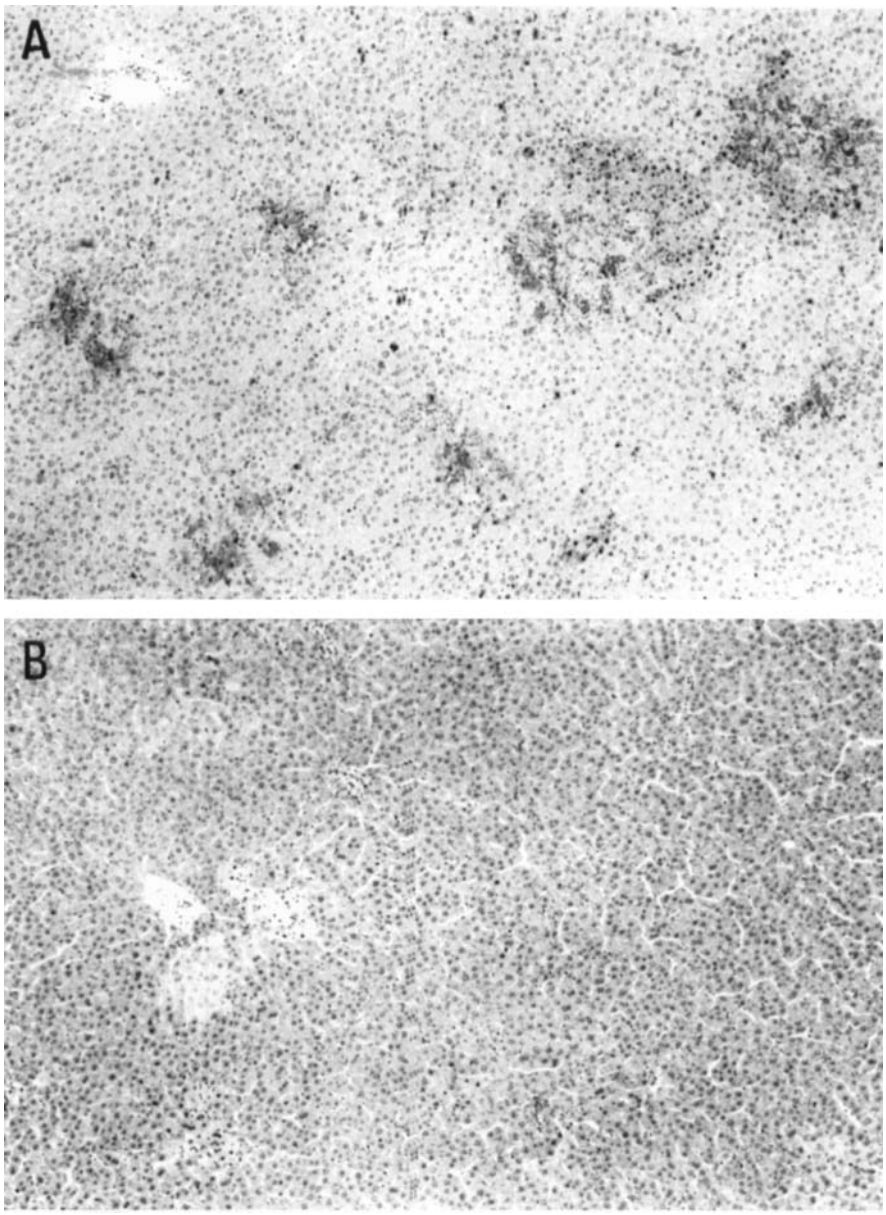

Fig. 5 Histological findings in the liver at 6 h of reperfusion after 60 min of total hepatic ischemia. **A** Massive liver necrosis with marked PMN infiltration was observed from the midzonal to the pericentral areas in the NS-E group. **B** Less severe damage was observed in the liver of the GC-E group. HE stain, original magnification ×68

[5, 6, 12, 27]. MIP-2 was produced by activated Kupffer cells as well as monocytes and macrophages [2, 41] and played a significant role in recruiting PMNs in several experimental models [12, 31, 45]. In the present study, increased MIP-2 production was observed throughout the experimental periods, due to sub-lethal endotoxin administration. Walley and colleagues [41] indicated that systemic MIP-2 levels were dramatically decreased by $GdCl_3$ pretreatment in a cecal ligation and puncture model. In addition to their results, our data showing significant suppression of MIP-2 production by $GdCl_3$ pretreatment demonstrated that this chemokine is mainly derived from Kupffer cells in the pathophysiological condition associated with endotoxemia. Some investigations, including our previous study, indicated that cell adhesion molecules were associated with the development of HIR injury [16, 33]. Besides serving as a potent neutrophil chemoattractant, MIP-2 has been shown to increase β2-integrin expression on PMNs [28]. Moreover, chemokines are linked to upregulation of integrins and intercellular adhesion molecule-1 (ICAM-1) [2]. PMNs in the inflammatory condition are likely to adhere to sinusoidal endothelial cells via the interaction with integrins and ICAM-1, which would lead to deterioration of HIR injury through the derangement of hepatic sinusoids, as shown in Fig. 5.

Much attention has been focused on the role of Kupffer cells in the pathophysiology of HIR injury and

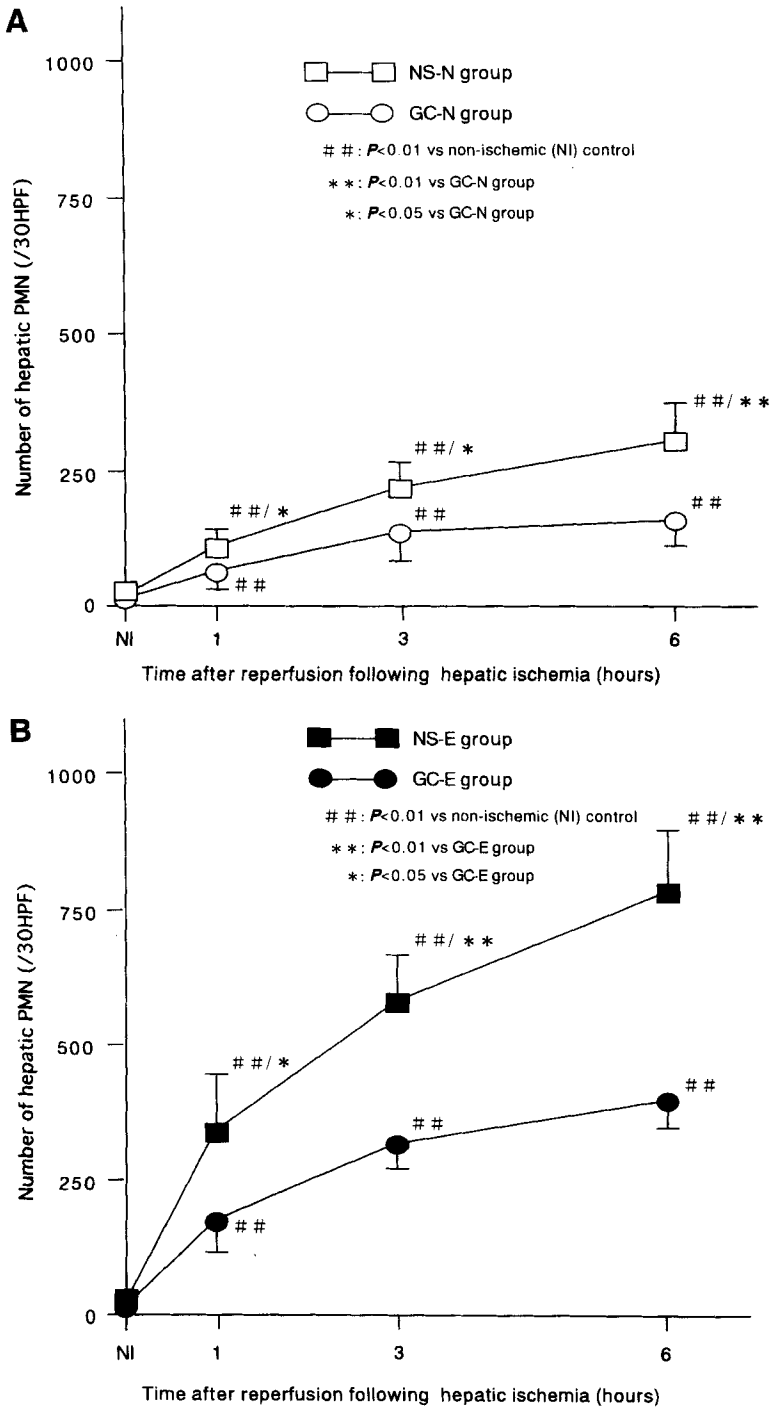

Fig. 6 Time course of PMN infiltration into the liver after reperfusion following 60 min of total hepatic ischemia. **A** Number of hepatic PMNs in the NS-N and GC-N groups. **B** Number of hepatic PMNs in the NS-E and GC-E groups

endotoxemia [8, 9, 15, 29, 34, 35, 38]. Several studies have demonstrated that Kupffer cells yielded deleterious effects in the early phase of these events [8, 9, 15, 29, 34, 35, 38], but there is some controversy surrounding this issue. Vajdova and associates [38] indicated that blockade or elimination of Kupffer cells attenuated preservation-reperfusion injury of rat livers exposed to endotoxin, while their experiment failed to reduce hepatic PMN infiltration. In our study, blockade of Kupffer cell function caused inhibition of MIP-2 production and hepatic PMN accumulation, which would be mediated by interaction between TNF-α and IL-10. The different conditions in each experiment may explain the discrepant results. Regarding the blockade effect of Kupffer cells, it is worthwhile to note that the IL-10/TNF-α values in the GC-E group were maintained at

above 1 throughout the experiment, while those in the NS-E group with most severe liver damage remained under 1. IL-10 inhibits the adhesion of PMNs to activated endothelial cells and downregulates the expression of vascular cell adhesion molecule-1 on these cells and the expression of ICAM-1 [11, 45]. These actions of IL-10 may be indirectly associated with the amelioration of endotoxin-enhanced reperfusion injury after hepatic ischemia. Although the mechanisms of enhanced IL-10 production remain unknown under depletion of Kupffer cells, a rise in the IL-10/TNF-α ratio at the initial phase of injury may be important to attenuate endotoxin-enhanced reperfusion injury after total hepatic ischemia. Thus, modulation of pro-inflammatory and anti-inflammatory cytokine responses would be associated with the improvement of liver damage in HIR injury, irrespective of the exposure of endotoxin to the ischemically damaged liver.

In conclusion, the present study demonstrated that Kupffer cells are associated with the development of endotoxin-enhanced reperfusion injury after hepatic ischemia by enhanced MIP-2 production through the modulation of TNF-α and IL 10. The preferential blockade of Kupffer cells would be beneficial for the prevention of reperfusion injury of ischemically damaged grafts in liver transplantation.

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