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TNF-α and IL-8 Release by Sinusoidal Endothelial Cells during Hypoxia-reoxygenation in Rats with Obstructive Jaundice

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Abstract The release of cytokines by sinusoidal endothelial cells (SECs) during hypoxia reoxygenation was studied in vitro in male Wistar rats with obstructive jaundice to investigate the kinetics of tumor necrosis factor-α (TNF-α) and interleukin-8 (IL-8) release by SECs during hypoxia-reoxygenation. The rats were divided into two groups according to the procedure performed. An obstructive jaundice group (Group OJ) underwent bile duct ligation and a control group (Group C) underwent a sham-operation. SECs were isolated by collagenase digestion and centrifugal elutriation. After exposing the cells to hypoxia in 95% nitrogen, they were reoxygenated in 95% oxygen. The levels of TNF-α and IL-8 were significantly decreased in group OJ compared with those in group C, indicating that obstructive jaundice induced functional damage to SECs with decreased cytokine production during hypoxia-reoxygenation.

Introduction

Hepatic ischemia-reperfusion injury is a major clinical dilemma for patients undergoing surgery for hepato-biliary disease. It has been reported that the sinusoidal liver cells, comprised of Kupffer cells and SECs, release a number of mediators, including a variety of cytokines that are involved in ischemia-reperfusion injury.[1-3] In a previous study using an obstructive jaundice rat model, we demonstrated that the IL-8 released by Kupffer cells plays a major role in hepatic ischemia-reperfusion injury in vivo and in vitro study.[4,5] SECs, which are known to participate in hepatic ischemia-reperfusion injury, release cytokines similarly to Kupffer cells 6, 7 and express adhesion molecules.[1] This in vitro study was thus designed to determine the kinetics of TNF-α and IL-8 released by SECs in obstructive jaundice during hypoxia-reoxygenation.

Methods

Experimental Animals

Male Wistar Rats (Japan SLC Inc. Hamamatsu, Japan) aged 7 weeks old and weighing 148 to 171 g, were given a commercial pellet diet and allowed water ad libitum. They were housed in cages at 22℃ under normal lighting with a 12h light and dark cycle. The animals were divided into two

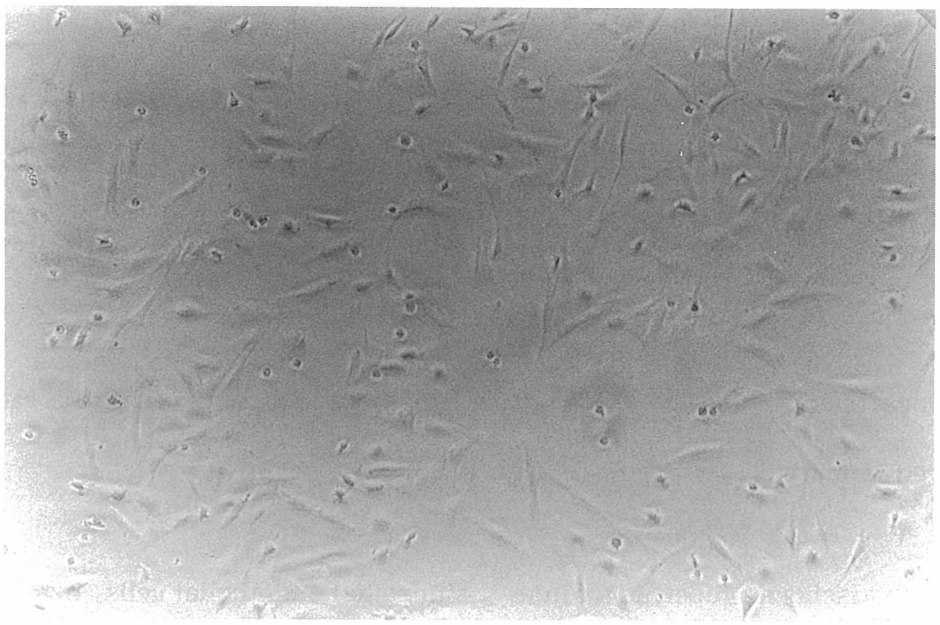

Fig 1. The "blanching tubular network pattern" peculiar to sinusoidal endothelial cells (SECs).

groups, namely : an obstructive jaundice group (group OJ)(n=7), and a sham control group (group C)(n=7). The animals were anesthetized with ether, then after shaving and disinfecting the abdomen with 70% ethanol, a midline incision was made. The common bile duct (CBD) was identified, doubleclipped with LIGA CLIPS (EMCA ; Ethicon Inc., New Brunswick. NJ, USA), and divided. Finally, the abdomen was closed in two layers. The sham-operation was an identical procedure except that the CBD was not ligated and divided.

Isolation of the SECs

The following experiment was carried out 14 days after the ligation and division of the CBD. First, SECs were isolated by the method of Kayano and Okamura with some modifications.[5,8] 8 Briefly, under anesthesia with an intraperitoneal injection of pentobarbital sodium (50 mg/kg), the rat liver was perfused through the portal vein with calcium- and magnesium- free Hanks' balanced salt solution (HBSS) containing 0.015% collagenase (type S-1 ; Nitta Zelatine Co., Ltd., Osaka, Japan), 0.5mmol calcium chloride, and 0.2% bovine serum albumin (BSA) (PESSEL+LOREI GmbH & Co., Frankfurt, Germany). Perfusion was performed at a flow rate of 12 ml/min at 37℃, all the perfusates containing 20mM HEPES at pH 7.4. The excised liver was minced with ice-cold HBSS containing 0.2% BSA. After filtration through nylon mesh, the isolated cells were centrifuged at 45×g for 2 min, and supernatant, which contained nonparenchymal cells, was collected and used in the next step. SECs were separated from the nonparenchymal cells using centrifugal elutriation (Beckman J2-21 centrifuge and JE 6-B rotor, Beckman Instruments, Palo Alto, CA, USA). The cell suspension of SECs was centrifuged at 500×g for 6 min. The resultant cell pellet was resuspended at a concentration of 2.0×10^6 cells/well in RPMI1640 with 10% heat inactivated fetal calf serum (FCS). Subsequently 5ml of the suspension was plated in each well of a 6-well dish. After the cells had been incubated at 37℃ for 1 h in an atmosphere of 95% air and 5% carbon dioxide, non-adherent cells were removed by washing three times with 5ml RPMI 1640 with 10% FCS. After the cells had been incubated for 90 min, the following experiment was undertaken. The purity and viability of the SECs were greater than 90% in any time at any case according to morphological observation and the trypan blue exclusion test. (Fig 1.)

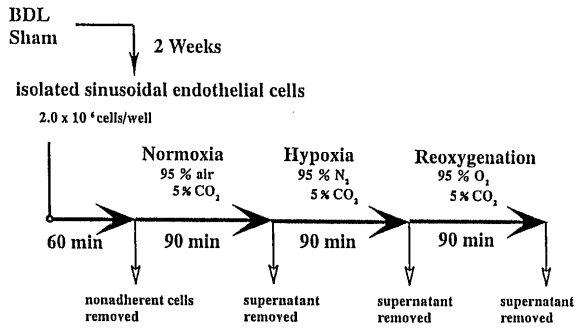

Fig 2. Experimental design. BDL ; bile duct ligation

Experimental Design

After the cells had been incubated at 37°C in an atmosphere of hypoxia created by 95% nitrogen and 5% carbon dioxide for 90 min, they were then incubated in an atmosphere of reoxygenation with 95% oxygen and 5% carbon dioxide. At the end of each session of normoxia, hypoxia, and reoxygenation, the supernatant was removed and replaced with fresh RPMI 1640 with 10% FCS (Fig 2.).

Measurement of Serum Samples

Total bilirubin, glutamic-oxaloacetic transaminase (GOT), and glutamic-pyruvic transaminase (GPT) concentrations were measured to assess damage to the hepatic parenchyma, using a Monocard chemistry system (AMCO Inc., Tokyo, Japan).

Measurement of Cytokines in the Culture Supernatant

Concentrations of the cytokines TNF-α and IL-8 were measured quantitatively using enzyme-linked immunosorbent assays (ELISA). TNF-α was measured using an ELISA for rat TNF-α (BioSource International, CA, USA) and IL-8 was measured using a ELISA for rat interleukin-8 (CINC/cytokine induced neutrophil chemoattractant ; Institute of Cytosignal Research Inc., Tokyo, Japan, and Amasham Life Science Co., Tokyo, Japan).

Statistical Analysis

Data are expressed as means±SEM. Statistical analysis within groups was performed using an analysis of variance for repeated measures. Time differences from the base line (time 0) for each group were assessed by a two-way analysis of variance. Statistical significance was accepted at $p<0.05$.

Results

Serum Parameters

Jaundice was confirmed by determining the serum bilirubin concentrations 14 days after clipping and division of the CBD. The total bilirubin concentration was 19.6±5.1 mg/dl in Group OJ and 0.6±0.3 mg/dl in Group C ; the GOT concentration was 578.7± 102.8 Karmen units in Group OJ and 33.4± 18.6 Karmen units in Group C; and the GPT concentration was 101.0±29.5 Karmen units in Group OJ and 6.8±4.1 Karmen units in Group C. All parameters in Group OJ were significantly increased compared with those in Group C ($p<0.01$) (Table 1).

Changes in TNF-α Concentration

The TNF-α concentrations in the normoxia

Table 1. Serum biochemistry in the obstrucitive jaundice and control groups

		Group OJ	Group C	
Yotal bilirubin	(mg / ml)	19.6± 5.1	0.6± 0.3	p <0.01
GOT	(Karumen unit)	578.7±102.8	33.4±18.6	p <0.01
GPT	(Karumen unit)	101.0± 29.5	6.8± 4.1	p <0.01

GOT, glutamic-oxaloacetic transaminase ; GPT, glutamic-pyruvis transaminase ; Group OJ, obstructive group ; Group, sham control group

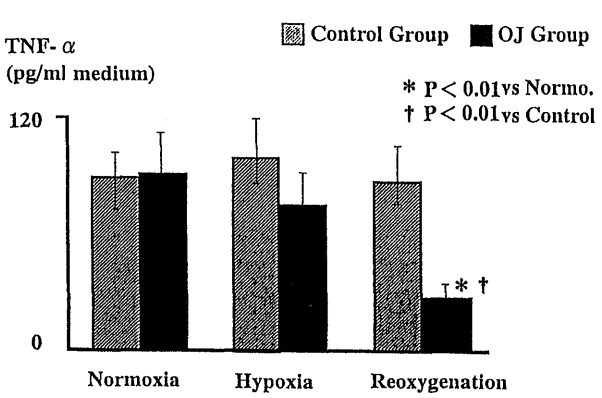

Fig 3. Changes in tumor necrosis factor-α concentration during hypoxia-reoxygenation. Values are expressed as means ± SE * Significant difference ($p < 0.01$) from the base line values (normoxia) † Significant difference ($p < 0.01$) between Group OJ and Group C. Group OJ, obstructive jaundice group (n =7) ; Group C, sham control group (n =7)

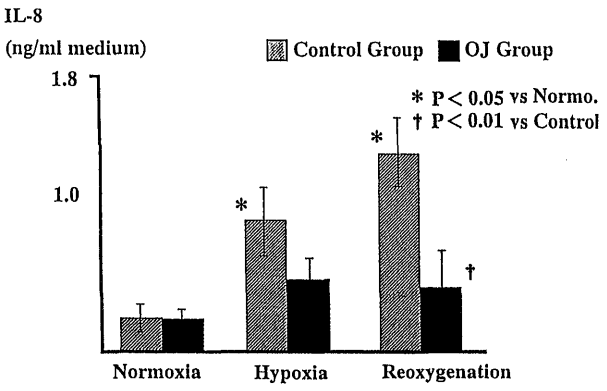

Fig 4. Changes in interleukin-8 (IL-8) during hypoxia-reoxygenation. Values are expressed as means ± SE * Significant difference ($p < 0.05$) from the base line values (normoxia) † Significant difference ($p < 0.01$) between Group OJ and Group C. Group OJ : obstructive jaundice group (n =7) Group C : sham control group (n =7)

period were 88.4±17.8 pg/ml in Group OJ and 89.1±20.8 pg/ml in Group C, whereas those in the hypoxia period were 73.1±18.2 pg/ml in Group OJ, and 100.3±12.7 pg/ml in Group C. These differences were not statistically significant. However, the TNF-α concentrations in the reoxygenation period were 20.7±5.5 in Group OJ and 87.4±18.3 in Group C, this difference being statistically significant ($p<0.01$). Moreover, the TNF-α concentration in Group OJ was significantly lower in the reoxygenation period than that in the normoxia period ($p<0.01$) (Fig 3.). Changes in IL-8 Concentration The IL-8 concentrations in the normoxia period were 0.20± 0.7ng/ml in Group OJ, and 0.20±0.057ng/ml in Group C, and the IL-8 concentration in the hypoxia period was 0.45±0.15ng/ml in Group OJ and 0.86±0.23ng/ml in Group C. These differences were not statistically significant. However, in the hypoxia period, the concentrations in Group C were significantly higher than those in the normoxia period ($p<0.05$). The concentrations in the reoxygenation period were 0.42±0.12ng/ml in Group OJ and 1.31±0.37ng/ml in Group C. The IL-8 level in Group OJ during the reoxygenation period was significant lower than that in Group C ($p<0.01$). Moreover, the IL-8 level in Group C was significantly higher in the reoxygenation period than that in normoxia period ($p<0.05$) (Fig 4).

Discussion

Previous studies have found that hepatic ischemia-reperfusion injury was induced by leukocytes and other inflammatory cells in damaged tissues.[9,10] Cytokines, such as TNF-α and IL-8, play a key role in mediating the host inflammatory response, and are likely to draw leukocytes to loci of hypoxic vascular injury. On the other hand, biliary obstruction is known to induce inflammatory cytokines.[11] In patients with obstructive jaundice, surgery is associated with increased morbidity and mortality, despite recent advances in preoperative and postoperative management.[12,13] Ischemia reperfusion injury of the liver in the presence of obstructive jaundice is an important clinical factor influencing the outcome of surgery. In a previous study using a rat model of obstructive jaundice, Orita et al. reported that Kupffer cells release a significant amount of IL-8 and play an important role in hepatic ischemia-reperfusion injury.4 However, in

that study, it could not be ascertained whether the Kupffer cells originally reacted to ischemia-reperfusion stress because of neutrophils or gut-delivered endotoxin. Excluding this problem, Okamura et al. proved by an in vitro study that isolated Kupffer cells derived from obstructive jaundice liver release a significant amount of IL-8 and play an important role in hepatic ischemia-reperfusion injury.[5] SECs are also known to release cytokines similarly to Kupffer cells. The main purpose of this in vitro study was therefore to evaluate the influence of hypoxia-reoxygenation stress on SECs in rats with obstructive jaundice, focusing particular attention on production of the cytokines TNF-α and IL-8.

At first, we evaluated the level of TNF-α and IL-8 from SEC derived from normal liver for hypoxia and reoxygenation stress. The levels of TNF-α reached to the highest at 60 minutes and IL-8 reached at 120 minutes after stimulation,[14–16] then we chose 90 minutes of hypoxia period, because we measured the TNF-α and IL-8 simultaneously. Furthermore, in a previous study, Orita et al[4]. reported that maximal safety time of hepatic warm ischemia is 30 minutes in vivo, so that 90 minutes is a enough time that hypoxia stimulate SECs in our vitro study. In addition, congestion of the small intestine was not observed during operation, so that our experimental models might not be affected by microbial translocation. TNF-α is considered to be an initial mediator of the cytokine cascade, and appears to be the first cytokine into the blood stream.[17,18] Furthermore, it has been reported that only IL-1 and TNF-α can induce IL-8 which plays a major role in ischemia-reperfusion injury[19]. Interestingly, in our study, hypoxia-reoxygenation stress did not increase the level of TNF-α from the SECs. But the IL-8 concentration in the control group increased in a time dependent manner during hypoxia and reoxygenation period. Karakurum et al.[7] reported that IL-8 was released into the culture medium in a time dependent manner when endothelial cells were exposed in an hypoxic atmosphere, and after reoxygenation, the level continued to be elaborated for up to 4 h. This data supported our result. It has been reported that the high concentration of IL-8 in the reoxygenation period induces the migration of neutrophils, which then release a variety of cytokines, causing hepatic reperfusion injury.[7,20] In normal liver, IL-8 release during hypoxia-reoxygenation period from SECs probably contribute to hepatic ischemia-reperfusion injury.

In obstructive jaundice liver, it is well known that ischemia-reperfusion injury occurs more severely than in normal liver. Yoshidome et al. 21 reported that obstructive jaundice impairs SECs and that SECs damage in association with sinusoidal deterioration during obstructive jaundice renders liver susceptible to ischemia-reperfusion relative to normal liver. Okamura et al.7 reported that Kupffer cells delivered from the obstructive jaundice liver produced a large amount of IL-8 during hypoxia-reoxygenation. Thus, they concluded that Kupffer cells are up regulated by obstructive jaundice and release much IL-8 following the hypoxia-reoxygenation period. Ohtsuka et al. reported that the marker of SEC injury showed no significant differences between OJ and normal rat liver.22 In our study, TNF-α and IL-8 levels in group OJ were not statistically lower than those in group C after the normoxia period, because SECs were not damaged in obstructive jaundice. And the concentrations of TNF-α and IL-8 released from SECs decreased by obstructive jaundice stimulation in vivo and following hypoxia-reoxygenation stress in vitro, although the viability of SECs were more than 90% in all periods. We ascertained that SEC is probably down regulated by obstructive jaundice in contrast to Kupffer cells, and that the release of TNF-α and IL-8 from SECs is inhibited following the hypoxia and reoxygenation periods. Deaciuc et al. reported that activated Kupffer cells caused SECs f unctional damage.23 Sarphie et al. reported that the activation of Kupffer cells modulated hepatic SECs functional properties, suggesting disturbance of hepatic SEC's function.[24] Furthermore, Niwano et al. reported that blockade of activation of Kupffer cells would be effective for the presentation of damage t o SECs.[25] One possibility is that SECs are damaged by activated Kupffer cells in obstructive jaundice, and that the release

of TNF-α and IL-8 from damaged SECs are inhibited following the hypoxia and reoxygenation periods. In conclusion, SECs are down regulated by obstructive jaundice in contrast with Kupffer cells and cytokine release from SECs are inhibited by following the hypoxia-reoxygenation stress.

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