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Interleukin-8 Release during Hypoxia-Reoxygenation by Kupffer Cells in Rats with Obstructive Jaundice

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Abstract Cytokine release during hypoxia/reoxygenation by Kupffer cells in male Wistar rats with obstructive jaundice was studied *in vitro* to investigate the kinetics of interleukin-8 (IL-8) release by Kupffer cells during hypoxia/reoxygenation, and to study the influence of endotoxin during the reoxygenation period. The rats were divided into two groups : one underwent bile duct ligation (Group OJ) ; and the other underwent a sham -operation (Group C). Kupffer cells were isolated by collagenase digestion and centrifugal elutriation. After the cells were exposed to hypoxia (95% nitrogen), they were exposed to reoxygenation (95% oxygen). In addition, they were stimulated with lipopolysaccharide (LPS) (0, 1, 10ng/ml). In both groups, the levels of IL-8 were increased in hypoxia/ reoxygenation period, and reoxygenation after hypoxia further intensified IL-8 production. In the hypoxia period, the IL-8 levels in Group OJ were significantly increased compared with those in Group C. With the LPS challenge, there was no significant difference in IL-8 levels in either group. In conclusion, obstructive jaundice induced Kupffer cells activation with increased IL-8 production during hypoxia/reoxygenation.

Key words : Kupffer cells, Obstructive jaundice, Hypoxia/reoxygenation, Interleukin-8

Introduction

Hepatic injury induced by ischemia/reperfusion is an important clinical problem, when operating patients with hepato-biliary disease. It has been reported that the resident macrophages of the liver (Kupffer cells) release a number of mediators, including a variety of cytokines[1,2], toxic superoxide anions[3,4], nitric oxide[5], and eicosanoids[6], which are involved in ischemia/reperfusion injury. We previously demonstrated that OKY-046, a thromboxane A2 synthetase inhibitor, influenced Kupffer cells in terms of the avoidance of hypoxia, and depressed the concentration of interleukin-8 (IL-8) in liver tissue[7]. *In vivo,* we can not study Kupffer cells function and avoid the influence of the cytokine networks between the Kupffer cells, the sinusoidal endothelial cells, the neutrophils, and the monocytes, nor can we avoid the influence of gut-delivered endotoxin caused by portal congestion. This *in vitro* study was thus designed to determine the kinetics of IL -8 release by Kupffer cells during hypoxia/ reoxygenation, and to further investigate the influence of the endotoxin on Kupffer cells during the reoxygenation period.

Materials and Methods

Experimental Animals

Male Wistar rats (Japan SLC Inc. Hamamatsu, Japan) aged 8 to 10 weeks (weighing

186 to 227g), were given a commercial pellet diet and water ad libitum, and were housed in cages at 22±2℃ under normal lighting (12h light/dark cycle) conditions. The animals were divided into two groups ; the obstructive jaundice model (Group OJ) (n=8), and the sham-operated control (Group C) (n=6). The animals in Group OJ were fasted for a period of 24 hours before surgery, but were allowed access to water. The animals were anesthetized with ether. After shaving and disinfecting the abdomen with 70% ethanol, a midline incision was made. The common bile duct (CBD) was identified, double-clipped with LIGA CLIPS (EMCA ; Ethicon Inc., New Brunswick, NJ, USA), and divided. The abdomen was then closed in two layers. The sham-operated control animals underwent identical procedures except for ligation and division of the CBD.

Kupffer Cell Isolation

Fourteen days after the clipping and division of the CBD, the following experiment was undertaken. The Kupffer cells were isolated by the method of Lepay and Kayano with some modifications.[8,9] Under anesthesia with intraperitoneal injection of pentobarbital sodium (50mg/kg), the rat liver was perfused through the portal vein with calcium and magnesium-free Hanks' balanced salt solution (HBSS) containing 0.01% ethylene diaminetetraacetic acid (EDTA) and 1,000 units of heparin (pH adjusted to 7.4). After 4 minutes, the rat liver was reperfused with HBSS containing 0.015% collagenase (Type S-1 ; Nitta Zelatin Co., Ltd., Osaka, Japan), 0.5mM calcium chloride, and 0.2% bovine serum albumin (BSA) (PESEL+LOREI GmbH & Co., Frankfurt, Germany). The perfusion was performed at a flow rate of 12 ml/min at 37℃. All the perfusates contained 20mM HEPES at pH 7.4. The excised liver was minced with ice-cold HBSS containing 0.2% BSA. After filtration through a nylon mesh, the isolated cells were centrifuged at 45×g for 2minutes and the supernatant, which contained nonparenchymal cells, was collected and used in the next step. Kupffer cells were separated from the whole nonparenchymal cell using centrifugal elutriation (Beckman J2-21 centrifuge and JE6-B rotor, Beckman Instruments, Palo Alto, CA, USA). The suspension of Kupffer cells was centrifuged at 500×g for 6 minutes. The resultant cell pellet was resuspended at a concentration of 2×106cells/well in RPMI 1640 with 10% heat-inactivated fetal calf serum (FCS). Subsequently 5ml were plated in each well of a 6-well dish. After the cells were incubated at 37℃ for 1 hour in an atmosphere of 95% air and 5% carbon dioxide, non adherent cells were removed by washing three times with 5ml of RPMI. After the cells were incubated for 1 hour, the following experiment was undertaken. The purity and viability of the Kupffer cells was more than 90% according to peroxidase staining and the trypan blue exclusion test.

Experimental Design

After the cells were incubated at 37℃ in an atmosphere of hypoxia (95% nitrogen, and 5 % carbon dioxide) for 90 minutes, they were incubated in an atmosphere of reoxygenation (95% oxygen, and 5% carbon dioxide). During reoxygenation, the cells were stimulated with lipopolysaccharide (LPS ; E. coli 026 : B6, DIFCO LABORATORIES Inc. Detroit, MI, USA) for 120minutes at concentrations of 0 (medium), 1, and 10ng/ml. At the end of normoxia, hypoxia, and reoxygenation, the supernatant was removed and immediately frozen at −40℃ to prevent mediator deterioration, and replaced with fresh media (Fig. 1).

Measurement of Serum Samples

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

Measurement of Cytokines in Culture Supernatant

The IL-8 concentrations were measured quantitatively using enzyme-linked immunosorbent assays (ELISA). IL-8 was measured using a ELISA for rat interleukin-8/cytokine-induced neutrophil chemoattractant (CINC) (Institute of Cytosignal Research Inc., Tokyo, Japan,

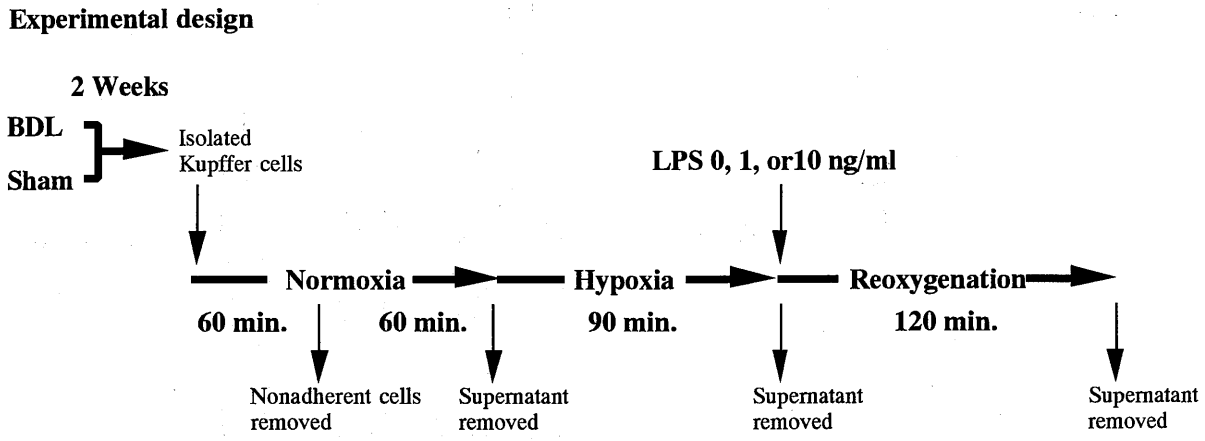

Fig. 1. Experimental design. BDL ; bile duct ligation, LPS ; lipopolysaccharide

and Amersham Life Science Co., Tokyo, Japan).

Statistical Analysis

The data are expressed as the mean ± SE. Statistical comparisons were performed by analysis of variance for repeated measures. The time differences at a time point were assessed using the least significant differences procedure. Statistical significance was defined as $p<0.05$.

Results

Serum Parameters

Fourteen days after the clipping and division of the CBD, jaundice was confirmed by determining serum bilirubin concentrations. The total bilirubin concentration was 18.1± 6.9mg/dl in Group OJ, and 0.4±0.4mg/dl in Group C. The GOT concentration was 554.8 ±120.9 Karmen units in Group OJ, and 42.5±28.9 Karmen units in Group C. The GPT concentration was 101.0±15.4Karmen units in Group OJ, and 4.2 ± 4.6 Karmen units in Group C. All parameters in Group OJ were significantly increased compared with those in Group C (Table).

Changes in IL-8 Concentrations

The IL-8 concentrations in the normoxia period were 0.22±0.03ng/ml in Group OJ, and 0.14±0.04ng/ml in Group C. There was no statistically significant difference between the two groups. The IL-8 concentrations in the hypoxia period were 0.78±0.16ng/ml in Group OJ, and 0.24±0.09ng/ml in Group C. These levels were higher, but not significantly different than the levels in the normoxia period. The concentrations in Group OJ was

Table. Serum biochemistry in the obstructive jaundice and control groups

		Group OJ	Group C	
Total bilirubin	(mg/ml)	18.1±6.9	0.4±0.4	p<0.01
GOT	(Karumen unit)	554.8±120.9	42.5±28.9	p<0.01
GPT	(Karumen unit)	101.0±15.4	4.2±4.6	p<0.01

GOT : glutamic oxaloacetic transaminase
GPT : glutamic pyruvic transaminase
Group OJ : obstructive jaundice
Group C : control

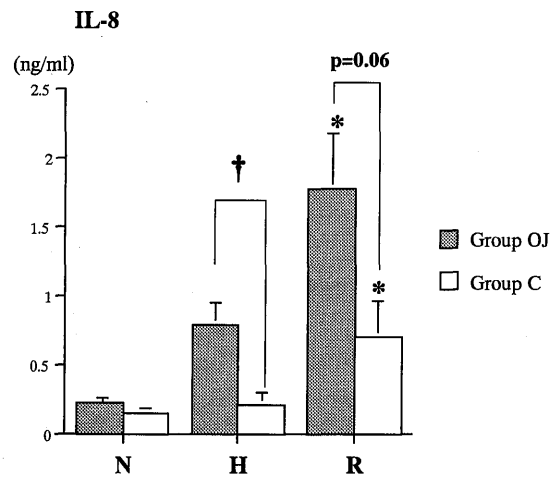

Fig. 2. Changes in interleukin-8 (IL-8) concentrations during hypoxia/reoxygenation. Values are expressed as mean±SEM. * Significant difference ($p<0.05$) from the baseline values (normoxia). † Significant difference ($p<0.05$) between Group OJ and Group C. Group OJ : obstructive jaundice (n=8), Group C : control (n=6), N : normoxia, H : hypoxia, R : reoxygenation

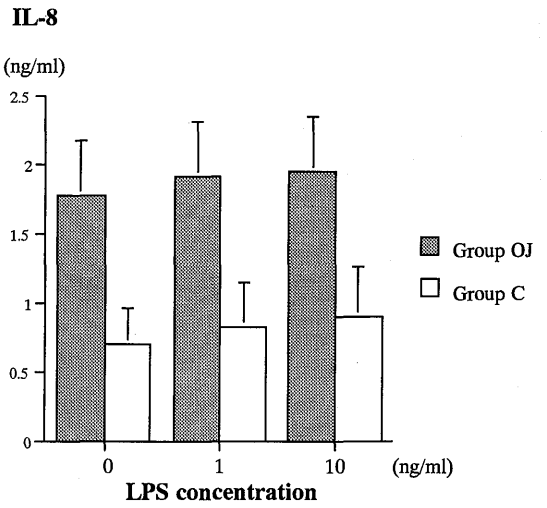

Fig. 3. Interleukin-8 (IL-8) production in Kupffer cells with lipopolysaccharide (LPS) challenge in the reoxygenation period. Group OJ : obstructive jaundice (n=8), Group C : control (n=6)

significantly higher in this period, compared with that in Group C. The IL-8 concentrations in the reoxygenation period were 1.77±0.4ng/ml in Group OJ, and 0.69±0.27ng/ml in Group C. These levels were significantly higher than those in the normoxia and hypoxia periods. The concentrations in Group OJ was higher than that in Group C ($p<0.1$, Fig. 2). LPS challenge in the reoxygenation period resulted in IL-8 concentrations of 1.77±0.4ng/ml (0ng/ml LPS), 1.91±0.4ng/ml (1ng/ml LPS), and 1.94±0.41ng/ml (10 ng/ml LPS) in Group OJ, and 0.69±0.27ng/ml (0ng/ml LPS), 0.82±0.32ng/ml (1ng/ml LPS), and 0.89±0.37ng/ml (10ng/ml LPS) in Group C. There were no statistically significant differences in IL-8 concentrations in Group OJ or Group C after LPS stimulation (Fig. 3).

Discussion

In patients with obstructive jaundice, surgery is associated with increased morbidity and mortality despite recent advances in preoperative and postoperative management.[10,11] Ischemia/reperfusion injury of the liver in the presence of obstructive jaundice is an important clinical factor influencing the outcome of surgery. Many authors have reported that Kupffer cells, which are the largest population of fixed tissue macrophages in the body, play an important role in ischemia/reperfusion injury, releasing a variety of mediators.[1-6] Obstructive jaundice alters the function of Kupffer cells with respect to the phagocytic activity as well as the secretory capability for a number of mediators.[12,13] Previous studies have shown that immunologic function of Kupffer cell is depressed, specifically with respect to bacterial clearance.[14,15] On the other hand, it has been reported that Kupffer cells release a number of mediators, including cytokines, in obstructive jaundice.[7] It is not clear, however, whether Kupffer cells release cytokines after ischemia/reperfusion injury in obstructive jaundice. *In vivo*, we can not study this question and avoid the influence of the cytokine networks between Kupffer cells

and the other cells, including the neutrophils. Furthermore, the influence of gut-delivered endotoxin caused by portal congestion can not be eliminated. Therefore, using an *in vitro* study, we aimed to determine the kinetics of IL-8 release by Kupffer cells during hypoxia/reoxygenation, and to further investigate the influence of endotoxin on Kupffer cells during the reoxygenation period.

The progression of hepatic reperfusion injury is mediated mainly by neutrophils.[16] IL-8 is an important inflammatory mediators which recruit neutrophils from the circulation into the sites of infection or tissue damage. IL-8 induces neutrophil migration and adhesion of cells to sites of injury. The migrated neutrophils release a variety of cytotoxic mediators such as oxygen metabolites and proteases. Our data show that IL-8 concentrations in both groups were increased in hypoxia/reoxygenation period, and reached a maximum in the reoxygenation period. These levels were significantly higher compared with those in the normoxia and hypoxia periods. Hypoxia could induce the release of IL-8 by Kupffer cells. Although the machanisms which hypoxia/reoxygenation induces the release of IL-8 are not clear, the association of this phenomenon with activation of a nuclear factor kB(NF-kB)-related transcription factor may be an important mechamism driving transcription. Reactive oxygen has been shown to induce nuclear translocation of the transcription factor NF-kB.[17] Consistent with the presence of NF-kB and cis-regulatory enhancer binding protein (C/EBP) binding sites in the promoter of the human IL-8 gene, NF-kB, along with a C/EBP-like factor, can drive expression of IL-8.[18] We also revealed that hypoxia-induced IL-8 production was enhanced by reoxygenation. Similarly it has been demonstrated that anoxic preconditioning and oxygen stress augmented the production of monocyte-derived IL-8.[19] Hypoxia followed by reoxygenation stimulates tyrosine kinase enzymes and increases intracellular calcium.[20] Those events are thought to induce IL-8 production in the reoxygenation period. IL-8 concentrations in Group OJ were higher than those in Group C in the hypoxia and reoxygenation periods. The IL-8 production by Kupffer cells is up- regulated in obstructive jaundice.[7] IL-8 is a well-known neutrophil chemoattractment and activating factor, and can be produced by Kupffer cells early after ischemia-reperfusion, recruiting neutrophils and contributing to the reperfusion injury.[21] We thus propose that IL-8 is an important cytokine in ischemia/reperfusion injury.

Another problem in reperfusion injury is the endotoxin delivered from the portal vein due to intestinal congestion. It has been reported that portosystemic bypass prevents the delivery of endotoxin in portal venous blood due to intestinal congestion, and reduces hepatic ischemia-reperfusion injury.[22] Exposure of Kupffer cells to endotoxin results in a prompt increase in the production of inflammatory mediators.[23] It has been reported that IL-8 was induced 4 hours after onset of LPS stimulation.[24] Our data show that there was no significant difference in IL-8 concentrations with LPS stimulation in ether group. In our experiment, the cells were stimulated with LPS for 2 hours. This time of LPS stimulation may be too short to induce IL-8 production.

Multiple organs suffer from the overproduction of these cytokines, despite the fact that activated Kupffer cells play a part in the immunological host defense. These cytokines also have beneficial effects via cytoprotective mediators.[25] Further study is needed to investigate the interaction between Kupffer cells and other cells.

In conclusion, obstructive jaundice induced Kupffer cell activation with increased IL-8 production during hypoxia/reoxygenation. It is considered that IL-8 plays an important role in ischemia/reperfusion injury.

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