Expression of macrophage inflammatory protein-1α in Kupffer cells following liver ischemia or reperfusion injury in rats

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AIM: To explore the expression of macrophage inflammatory protein-1α (MIP-1α) in Kupffer cells (KCs) following liver ischemia/reperfusion injury IRI in rats.

METHODS: Forty male SD rats were divided randomly into five groups. A model of partial warm ischemia/reperfusion injury in the rat liver was established. KCs were isolated and incubated one hour, six hours, 12 h, and 24 h after the reperfusion. Tumor necrosis factor alpha (TNF-α) and interleukin-1beta (IL-1β) in the supernatants were measured by ELISA. MIP-1α in KCs was detected by immunocytochemical and RT-PCR.

RESULTS: No or few MIP-1α protein and mRNA were expressed in the KCs of the control group. Its expression in the IRI group had a significant increase after the reperfusion (P < 0.05), which was contrary to the control group.

CONCLUSION: The active behavior of the MIP-1α gene in KCs following liver ischemia/reperfusion injury is assumed to be one of the major causes for the hepatic ischemia/reperfusion injury.

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Key words: Liver; Ischemia/reperfusion; Kupffer cell; Macrophage inflammatory protein-1α

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Suigama, Inc. (USA). Triozol was from Invitrogen, Inc, (USA). RNA PCR Kit and DNA Maker were purchased from TaKaRa Biotechnolooy (Dalian) Co., Ltd. (China). Lysozyme, MIP-1α polyclonal antibody and SABC kit were purchased from Wuhan Borsd Biological Engineering Co., Ltd. (China). TNF-α and IL-1β ELISA kit were obtained from Shanghai Sengxiong Biotech Industry Co., Ltd. (China).

**Animals**

Male Sprague Dawley (SD) rats weighing 250-300 g were used in the study. They were obtained from the Experimental Animal Center of the Xi’an Jiaotong University. All animals were housed in a macoion cage in rooms maintained at a temperature of 22-24°C using a 12/12-h light/dark cycle. The animals were given a standard rat chow and fasted overnight before the experiment with water allowed ad libitum. Care was provided in accordance with the procedure outlined in the “Guide for the Care and Use of Laboratory Animals” (NIH publication No.85-23, revised 1996). The study was approved by the subcommittee on research animal care at our institution.

**Hepatic ischemia/reperfusion injury model in rats**

Forty-eight SD rats were divided randomly into six groups: the sham operation group (control group) and the ischemia/reperfusion injury I - V group (IRI I - V group). The trial rats were starved for 12 h but were allowed to drink water. A model of the partial warm hepatic ischemia/reperfusion injury was established at room temperature by the Nauta et al method [13]. In the experiment, the rats were anesthetized with aether. The liver was exposed by a midline incision before operation. The left lateral and median hepatic lobes were occluded with a microvascular clamp for 45 min. After 45 min of ischemia, reperfusion was induced by removing the vascular clamp. 0 h, 1 h, 6 h, 12 h and 24 h after the reperfusion, the rats were killed to isolate the Kupffer cells at the same time. The sham control rats underwent the same treatment, but without vascular occlusion.

**Kupffer cells isolation**

With the reference to the collagenase perfusion technique described by Knittel et al [8], the Kupffer cells were isolated by an improved method. In the experiment, the liver was perfused for 30 min with D-Hank’s containing 0.01% EDTA and removed to be smashed by scissors. The resulted stuff was digested for 45 min in a solution of 0.5 g/L type IV collagenase and was filtered through 200-mesh strainer. The filtrate was washed thoroughly and suspended in 3mL PBS, layered on 3 mL 25% percoll solution and 3 mL 50% percoll solution, centrifuged at 2500 r/min for 25 min to separate the cells. The cells between the layers of 25% percoll solution and 50% percoll solution were carefully extracted. The freshly isolated cells were suspended in DMEM medium supplemented with 25 mM HEPES, 10% fetal bovine serum, penicillin (100 U/mL), streptomycin (100 μg/mL), and L-glutamine (2 mmol/L). Cell suspensions (4 mL, 8 × 10^6 cells) were plated on 60-mm culture dishes and maintained in an incubator at 37°C in a humidified atmosphere of 90% air-100 mL/L CO2 for 1 h. The supernatants were discarded and the adherent cells were incubated for 4 h. The cellular components of adherent cells were examined by Lysozyme immunohistochemical staining to determine the percentage of Kupffer cells. The percentage of Kupffer cells in the adherent cells was over 90% (Figure 1). The cells were counted after the trypan blue exclusion. The viability of the cells was over 90% in the adherent cells. The Kupffer cells and supernatants were collected in all the groups and stored in refrigeration at -70°C for subsequent analysis.

**Expression of TNF-α and IL-1β in supernatants**

TNF-α and IL-1β in the supernatants of primary cultures of rat Kupffer cells were measured by TNF-α and IL-1β ELISA kits. The concentrations of TNF-α and IL-1 were estimated based on the absorbance read by an ELISA reader (EL800) at 450 nm.

**Immunocytochemical detection of MIP-1α protein**

Immunocytochemical staining of Kupffer cells was performed with the strept-avidin-biotin-peroxidase complex (SABC) method [14]. Kupffer cells adhered to glass slides were fixed with aether and ethanol. They were soaked in 30% hydrogen and pure methanol (1:50) for 30 min at room temperature. After incubation in normal goat serum blocking solution, the glass slides were incubated in rabbit anti-rat MIP-1α polyclonal antibody (1:200) at 4°C overnight. PBS takes the place of MIP-1α antibody as the negative staining. Then the glass slides were incubated in biotined goat anti-rabbit IgG for 20 min at 20°C. Finally, the values of the mean optical density were measured under the same magnification using an image analysis system (original magnification × 400).

**Determination of MIP-1α mRNA by semiquantitative RT-PCR**

Total RNA was isolated from rat Kupffer cells using Trizol reagent. One microgram of RNA was reverse-transcribed to complement DNA using RNA PCR Kit according to the manufacturer’s instructions. The PCR primers were synthesized on the basis of Gen-Bank data. The primers were chemically synthesized using DNA
synthesizer (Tiangen Biotech Co., Ltd, Beijing China). Their sequences were 
5'-TTTGAGGAGCAACGCTTTG-G-3', 5'-GAAGAGTCCTGATTGAGGC-3' for MIP-
1α and 5'-CATAGCAAGATGTTGAAGG-3', 5'-
TCCACAGCTTTCTGATGGC -3' for GAPDH. All 
the PCR reactions had an initial denaturation step at 94°C for 
3 min, and a final extension at 72°C for 5 min using 
PTC-100 (MJ Research, Inc. USA). The PCR amplification 
cycling conditions were 94°C 30 s, 52°C 30 s, 72°C 30 s, 
40 cycles for MIP-1α; 94°C 30 s, 53°C 30 s, 72°C 30 s, 35 
cycles for GAPDH. Following RT-PCR, 5 µL samples of 
amplified products were resolved by electrophoresis in 
1.5% agarose gel, and stained with ethidium bromide. 
The intensity of each PCR product was semiquantitatively 
evaluated using the labworks analysis software (UVP, Inc, 
Upland, CA, USA).

Statistical analysis
Results were expressed as mean ± SE. Statistical 
calculations were made using the SPSS10.0 software 
packet. One-factor analysis of variance was applied to 
determine whether the differences among the three groups 
were statistically significant. In all the cases, P values lower 
than 0.05 were considered to be statistically significant.

RESULTS
Measurement of levels of TNF-α, IL-1β in supernatants
The cytokines released from the Kupffer cell were studied by 
ELISA. As shown in Figure 2, the concentration of 
TNF-α in the supernatant of primary cultures of rat 
Kupffer cells in the control group was 13.89 ± 2.04 ng/L, 
and it increased at least ten times in IRI group I (P < 0.01). 
Following reperfusion injury stimulation, the TNF-α level 
was increased with time, and reached the maximum (230.6 
± 26.3 ng/L) 6 h after the stimulation (P < 0.01). Even 
24 h after the reperfusion, the concentration of TNF-α 
(133.68 ± 12.15 ng/L) was still significantly high compared 
with that in the control group (P < 0.01). A comparison of 
IL-1β production in supernatant of Kupffer cells is 
shown in Figure 3. The concentration of IL-1β in 
the control group was 64.65 ± 4.63 ng/L and significantly 
increased with time, reaching its maximum (189.8 ± 13.13 
ng/L) 6 h after the reperfusion (P < 0.01), and slightly 
declined (146.30 ± 11.90 ng/L) 24 h after the following 
reperfusion.

Measurement of the levels of MIP-1α protein in Kupffer 
cells
Figure 4 shows the immunohistochemical staining of the 
Kupffer cells that were isolated 12 h after the reperfusion 
of rat liver. The cytoplasmas of these Kupffer cells were stained brown. On the contrary, in the control group, 
IRI group I and II, the Kupffer cells were not or weakly 
brown stained. We used an image analysis system to 
measure the values of the mean optical density of the 
Kupffer cells in all the groups and found the levels of 
MIP-1α protein in Kupffer cells had a significant increase 
at 6 h, 12 h and 24 h intervals (P < 0.01), which was 
contrary to the control group (Figure 5).

Expression of MIP-1α mRNA in Kupffer cells
PCR products were electrophoresed on agarosegels and 
photographed (Figure 6). The track numbered 1 is for the 
control group and the tracks numbered 2-6 are for the 
ischemia/reperfusion injury I-V group. The track marked 
M is for DNA maker. Quantitative data of MIP-1α mRNA 
levels in Kupffer cells were represented by the ratio of 
relative absorbance and expressed as mean ± SD (Figure 7). 
It showed that the Kupffer cells in the control group had 
low but detectable levels of MIP-1α mRNA. There was no 
statistical significance between the control group and the 
ischemia/reperfusion injury I group (P > 0.05). The MIP-
1α mRNA level in Kupffer cells significantly increased with 
time, reaching its maximum 6 h after the reperfusion injury (P < 0.01), and slightly declined at 24 h interval but 
was still much higher than that in the control group (P < 0.01).

DISCUSSION
Hepatic ischemia/reperfusion injury is one of the major 
complications of liver resection surgery, transplantation, 
and hypovolemic shock. The detailed biochemical 
mechanisms of liver injury caused by ischemia/reperfusion 
are complex and not well known till now. Several animal
Modles were used to establish the pathological process of hepatic ischemia/reperfusion injury, such as the liver transplantation model, the partial warm or cold ischemia/reperfusion injury model and the total hepatic ischemia/reperfusion injury model[16-18]. In this study, the model of rat liver ischemia-reperfusion injury is established by the clamping and unclamping of the vessels to the left lateral and median hepatic lobes, which account for 70% of the rat’s liver mass. This hepatic insult is similar to the clinical situation where the whole liver is rendered ischemic during total vascular exclusion (which is not tolerated in the rat) for liver resections. We determine the outcomes 0 h, 1 h, 6 h, 12 h and 24 h respectively after the onset of reperfusion, when a number of mechanisms began to function, such as the increase in the cytokine production, the enhanced oxidative metabolism, and the increase in phagocytic activity, which contribute to the hepatic injury[1,2]. Therefore, the findings of the present research reveal the pathological process of both ‘early and late’ phase ischemia/reperfusion injury.

Kupffer cells play an important part in mediating ischemia and reperfusion injury. When activated during the ischemia and subsequent reperfusion, they generate excessive inflammatory cytokines and oxygen-derived free radicals, which play a particularly important role in the pathogenesis of hepatic ischemia and reperfusion injury[19]. In this study, low levels of TNF-α and IL-1β were detected in the supernatant of primary cultures of rat Kupffer cells in the control group. At the early phase of the ischemia/reperfusion injury, they significantly increased and nearly reached its maximum. Twenty-four h after the reperfusion, these inflammatory cytokines were still significantly high compared with that in the control group. The mechanism of induction of TNF-α and IL-1β bioactivity after hepatic ischemia/reperfusion is complex. As a survival factor in hepatocytes under certain physiological conditions, for example, liver regeneration, Kupffer cells secrete low levels of TNF-α and IL-1β. During the very early phase of the
hepatic ischemia/reperfusion injury, the activated Kupffer cells produce high concentrations of TNF-α and IL-1β that represents the results obtained 0 h and 1 h after the reperfusion. The secretions of these toxic inflammatory cytokines precedes the activation of adhesion factors, chemotactic agents, and the sequestration of neutrophils in the liver and appear to be a key mediator of the inflammatory response to promote the release of other cytokines.

Kupffer cells are both a target and a source of chemokines. Therefore, we also examine the expression of MIP-1α, an important member of CC chemokine subfamily, in Kupffer cells in an ischemia/reperfusion injury. The MIP-1α proteins are not detected by immunocytochemical staining in the cells of the control group and IRI group I and II. Likewise, the synthesis of MIP-1α mRNA in the cells of the control group and IRI group I are not active. After the reperfusion injury, the expression of MIP-1α protein and mRNA significantly increase. This may indicate that the genes MIP-1α are inactive in normal Kupffer cells. Some inflammatory factors stimulate MIP-1α gene in Kupffer cells during the reperfusion injury. These findings are in agreement with those in the research by Bukara M et al. The role of MIP-1α in the pathological process of hepatic ischemia/reperfusion injury is not well known. However, previous studies have proved that MIP-1α can induce mononuclear macrophage to secrete TNF-α and IL-1β. Therefore, we suppose the active behavior of the MIP-1α gene in the Kupffer cell following liver ischemia/reperfusion injury is assumed to be one of the major causes for the hepatic ischemia/reperfusion injury.

In conclusion, our study focuses on the immediate, innate immune response of the Kupffer cells and demonstrates the prominent expression of MIP-1α in the Kupffer cells in the hepatic ischemia/reperfusion injury. These theories may provide a better understanding of the mechanisms of hepatic ischemia/reperfusion injury and will be useful for the therapies of hepatic ischemia/reperfusion injury in the fields of hepatic surgery, preservation, and rejection.

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REFERENCES

1 Fondevila C, Busuttil RW, Kupiec-Weglinski JW. Hepatic ischemia/reperfusion injury—a fresh look. Exp Mol Pathol 2003; 74: 86-93

2 Lentsch AB, Kato A, Yoshidome H, McMasters KM, Edwards MJ. Inflammatory mechanisms and therapeutic strategies for warm hepatic ischemia/reperfusion injury. Hepatology 2000; 32: 169-173

3 Strieter RM, Standiford TJ, Huffnagle GB, Colletti LM, Lukacs NW, Kunkel SL. “The good, the bad, and the ugly.” The role of chemokines in models of human disease. J Immunol 1996; 156: 3583-3586

4 Baggioni M, Dewald B, Moser B. Interleukin-8 and related chemotactic cytokines—CXCl and CC chemokines. Adv Immunol 1994; 55: 97-179

5 Rollins BJ. Monocyte chemoattractant protein 1: a potential regulator of monocyte recruitment in inflammatory disease. Mol Med Today 1996; 2: 198-204

6 Taub DD. Chemokine-leukocyte interactions. The voodoo that they do so well. Cytokine Growth Factor Rev 1996; 7: 355-376

7 Luster AD. Chemokines—chemotactic cytokines that mediate inflammation. N Engl J Med 1998; 338: 436-445

8 Matsukawa A, Hogaboam CM, Lukacs NW, Kunkel SL. Chemokines and innate immunity. Rev Immunogenet 2000; 2: 339-358

9 Chaisavaneyakorn S, Moore JM, Mirel L, Othoro C, Otieno J, Chaiyaroj SC, Shi YP, Nahlen BL, Lal AA, Udhayakumar V. Levels of macrophage inflammatory protein 1 alpha (MIP-1α) and MIP-1 beta in interstitial blood plasma samples from women with placental malaria and human immunodeficiency virus infection. Clin Diag Lab Immunol 2003; 10: 631-636

10 Rossi D, Zlotnik A. The biology of chemokines and their receptors. Annu Rev Immunol 2000; 18: 217-242

11 Kaplan AP, Kuna P, Reddigari SR. Chemokines and the allergic response. Exp Dermatol 1995; 4: 260-265

12 Naua RJ, Tsimojanni E, Uribe M, Walsh DB, Miller D, Butcherfield A. Oxygen-derived free radicals in hepatic ischemia and reperfusion injury in the rat. Surg Gynecol Obstet 1990; 171: 120-125

13 Knittel T, Fellmer P, Ramadori G. Gene expression and regulation of plasminogen activator inhibitor type I in hepatic stellate cells of rat liver. Gastroenterology 1996; 111: 745-754

14 Meng X, Zhang J. Study on expression and distribution of macrophage inflammatory protein-1α in atherosclerotic tunica intima of artery from type 2 diabetes by using immunohistochemistry. China journal of modern medicine 2005; 15: 20

15 Jaeschke H. Mechanisms of reperfusion injury after warm ischemia of the liver. J Hepatobiliary Pancreat Surg 1998; 5: 402-408

16 Kamada N, Calne RY. A surgical experience with five hundred thirty liver transplants in the rat. Surgery 1983; 93: 64-69

17 Jaeschke H, Smith CV, Mitchell JR. Reactive oxygen species during ischemia-reflow injury in isolated perfused rat liver. J Clin Invest 1988, 81: 1240-1246

18 Kojima Y, Suzuki S, Tsuchiya Y, Konno H, Baba S, Nakamura S. Regulation of pro-inflammatory and anti-inflammatory cytokine responses by Kupffer cells in endotoxin-enhanced reperfusion injury after total hepatic ischemia. Transpl Int 2003; 16: 231-240

19 Zhang JX, Wu HS, Wang H, Zhang JH, Wang Y, Zheng QC. Protection against hepatic ischemia/reperfusion injury via downregulation of toll-like receptor 2 expression by inhibition of Kupffer cell function. World J Gastroenterol 2005; 11: 4423-4426

20 Bukara M, Bautista AP. Acute alcohol intoxication and gadolinium chloride attenuate endotoxin-induced release of CC chemokines in the rat. Alcohol 2000; 20: 197-203

S- Editor Wang J L- Editor Ma JY E- Editor Bai SH