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Macrophage inflammatory protein-1α plays a crucial role in concanavalin A-induced liver injury through induction of proinflammatory cytokines in mice

Satoshi Okamoto[1], Shiro Yokohama[1], Masashi Yoneda[2], Masakazu Haneda[1] and Kimihide Nakamura[1].

[1]Second Department of Medicine, Asahikawa Medical College, Asahikawa, Japan.

[2]Department of Gastroenterology, Dokkyo University School of Medicine, Mibu, Japan.

Address for correspondence:

Shiro Yokohama, MD

Second Department of Medicine, Asahikawa Medical College

Midorigaoka higashi 2-1-1-1, Asahikawa 078-8510, Japan

Tel: +81-166-68-2454   Fax: +81-166-68-2459

E-mail: yokohama@asahikawa.hosp.go.jp (S.Yokohama)

Short title: The role of MIP-1α in Con A-induced liver injury

Abstract

***Background/Aims*:** The chemokines play roles in the development of immune mediated liver diseases. In this study, we investigate the involvement of macrophage inflammatory protein-1α (MIP-1α), one of the CC chemokines in concanavalin A (Con A)-induced liver injury in mice. ***Methods*:** Liver injury was induced by intravenous injection of Con A. Anti-mouse MIP-1α antibody, recombinant murine-MIP-1α, and gadolinium chloride (GdCl₃) were administrated prior to Con A injection. Plasma alanine aminotransferase (ALT), MIP-1α, tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) levels were determined and histological assessment of the liver was performed. ***Results*:** Plasma MIP-1α level was elevated after Con A injection. The elevated plasma ALT level, mortality rate and histological change after Con A injection were inhibited by anti-MIP-1α antibody pretreatment. The elevated plasma ALT level after Con A injection was further enhanced by recombinant murine-MIP-1α. The elevated plasma TNF-α and IFN-γ levels after Con A injection were inhibited by anti-MIP-1α antibody, and enhanced by recombinant murine-MIP-1α. The elevated plasma MIP-1α and ALT levels were inhibited by GdCl₃ pretreatment. ***Conclusions*:** These findings suggest that MIP-1α is produced from Kupffer cells after Con A injection, and this CC chemokine plays a crucial role in Con A-induced liver injury through induction of proinflammatory cytokines.

Keywords: CC chemokine, tumor necrosis factor-α, interferon-γ, T-cell, macrophage, Kupffer cell

1. Introduction

Activated T cells play a pivotal role in many liver diseases, including viral hepatitis, autoimmune hepatitis, drug-induced hepatitis or allograft rejection. Recently, an animal model of T cells-mediated liver injury was developed [1]. In this model, concanavalin A (Con A), a plant lectin known to mitogenically activate T cells, leads to polyclonal T cells activation and induces liver-specific necrotic injury [1-3]. Among various proinflammatory cytokines released from Con A-activated T cells, macrophages and natural killer T cells [4-9], tumor necrosis factor α (TNF-α) and interferon γ (IFN-γ) have been shown to play an important role in the progression of massive hepatocellular apoptotic and necrotic injury [7,9-14].

Chemokines are 8-12 kDa peptides that belong to low-molecular cytokine family and have leukocytes chemoattractic and stimulatory activities. Chemokines are focally produced at inflammatory and immune responsive sites, and play a pivotal role in many inflammation, and allergic and immunological disorders [15-17]. These chemoattractic cytokines are subdivided into four subfamilies including CXC, CC, CX3C and C, according to the positions of cysteines in their N termini. In these subfamilies, CXC chemokines (especially ELR motif positive CXC chemokines) have demonstrated to recruit neutrophils, and CC chemokines to recruit lymphocytes and monocytes-macrophages mainly. We have recently shown that the plasma levels of several chemokines are elevated after Con A administration, and macrophage inflammatory protein-2 (MIP-2), one of mouse CXC chemokines, is induced by TNF-α after Con A injection and contributes the development of Con A-induced liver injury mediated through attracting and activating neutrophils [18]. On the other hand, the function of CC chemokines, another main subfamily of chemokines,   in Con A-induced liver injury model is still poorly understood.

To investigate the further role of the chemokines in Con A-induced liver injury model, we focused on macrophage inflammatory protein-1α (MIP-1α), one of CC chemokines, and studied whether this chemokine was released in plasma after Con A administration. We further investigated the effect of anti-MIP-1α antibody, recombinant murine-MIP-1α, and gadolinium chloride hexahydrate

(GdCl₃) on Con A-induced liver injury. Our results suggest that MIP-1α is released from Kupffer cells, hepatic residual macrophages, after Con A administration and enhances the liver damage through induction of proinflammatory cytokines. These findings indicate that MIP-1α, one of CC chemokines may play a critical role in Con A-induced liver injury.

2. Materials and methods

2.1. Animals

Female specific pathogen-free BALB/c mice (7-8 weeks old) were purchased from Japan SLC Co. (Shizuoka, Japan). Mice were housed under conditions of controlled temperature (22-24 ºC) and illumination (12-h light cycle starting at 06:00 h) for at least 7 days before experiments. Protocols describing the use of mice were approved by the Animal Care Committee of Asahikawa Medical College, and were in accordance with the Minister of Education, Culture, Sports, Science and Technology of Japan *'Guide for the Care and Use of Laboratory Animals'*.

2.2. Chemicals

Con A type IV (Jack Bean) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The monoclonal mouse MIP-1α antibody was purchased   from Genzyme/Techne Co. (Cambridge, MA, USA) and the recombinant murine-MIP-1α was purchased from Peprotech EC Co. (London, UK). Purified rat myeloma IgG2b was purchased from ZYMED Laboratories, Inc. (San Francisco, CA). GdCl3 was purchased from Wako Co. (Osaka, Japan).

2.3. Experimental protocols

2.3.1. Plasma transaminase and MIP-1α levels after Con A injection; Con A, 20 mg/kg in a volume of 0.3 ml, dissolved in pyrogen-free saline was injected to mice via the tail vein. The plasma alanine aminotransferase (ALT) level was determined enzymatically using a commercially available kit (Wako Co.) 8 and 24 h after Con A injection. Plasma MIP-1α level was determined 0, 1, 2, 4, 6, 8, 12, and 24 h after Con A treatment by enzyme linked immunosorbent assay (ELISA) using commercially available kit (Genzyme Co.). The sensitivity of detection in the ELISA was 1 pg/ml.

2.3.2. Effects of anti-MIP-1α antibody on Con A-induced liver injury and plasma cytokine levels after Con A injection; To investigate whether MIP-1α released after Con A treatment plays a role in Con A-induced liver injury, we administered anti-MIP-1α antibody prior to Con A injection. Anti-mouse MIP-1α antibody (50 or 200 µg) and vehicle (200 µg purified rat IgG2b) were dissolved in

pyrogen-free saline in a volume of 0.2 ml and intravenously administered 30 min before Con A injection. The plasma ALT level was determined enzymatically 8 and 24 h after Con A treatment. The mortality rates were also observed 24 h after Con A injection. The plasma TNF-α and IFN-γ levels were determined 2 and 8 h after Con A treatment by ELISA (Genzyme Co.). In the ELISAs, the sensitivities of detection were 15 pg/ml for TNF-α and 5 pg/ml for IFN-γ, respectively.

2.3.3. Effect of recombinant murine-MIP-1α on Con A-induced liver injury and plasma cytokine levels after Con A injection; To confirm the role of MIP-1α on Con A-induced liver injury, we administered recombinant murine-MIP-1α prior to Con A (15 mg/kg) injection. We chose the dose for Con A by pilot experiment, because 15 mg/kg of Con A injection induces a submaximum liver injury assessed by serum ALT level. Recombinant murine-MIP-1α (0.5 or 2 µg) was dissolved in pyrogen-free saline in a volume of 0.5 ml and intraperitonealy administered 1 h prior to Con A injection, and vehicle (pyrogen-free saline) was administered similarly. The plasma ALT level was determined 8 h after Con A treatment, and the plasma TNF-α and IFN-γ levels was determined at 2 and 8 h after Con A treatment.

2.3.4. Effect of gadolinium chloride pretreatment on Con A-induced liver injury and plasma MIP-1α level after Con A injection; To investigate the role of Kupffer cells, hepatic residual macrophages, on Con A-induced liver injury and MIP-1α release after Con A injection, we administered GdCl$_3$ prior to Con A (20 mg/kg) injection. GdCl$_3$ (40 mg/kg) was dissolved in pyrogen-free saline in a volume of 0.5 ml and intraperitonealy administered 24 h prior to Con A injection, and vehicle (pyrogen-free saline) were administered otherwise under identical condition. The plasma MIP-1α level was determined 2 h after, and the plasma ALT level was determined 8 h after Con A injection.

2.3.5. Blood sampling and histology

Under ether anesthesia, the abdomen was opened and the peripheral blood was obtained from the vena cave inferior with heparinized syringe. The plasma was obtained after a 10-min centrifuging at

3000 rpm, and was kept at -70 ᵒC until assay. The liver sample was removed 8 h after Con A injection by total bleeding due to cutting of the abdominal aorta and fixed in 10 % (v/v) neutral buffered formalin. The specimens were stained with hematoxylin and eosin for assessment of liver injury.

2.4. Statistical analysis

All results are expressed as mean ± SEM. Comparison between two independent groups was performed by Student's t-test, and multiple group comparisons were done by analysis of variance followed by Fisher's protected least significant difference test. $P<0.05$ was considered statistically significant.

3. Results

3.1. Time course of plasma MIP-1α level after Con A injection

MIP-1α was not detected in the plasma before Con A treatment. Plasma MIP-1α level had already started to elevate 1 h (87.2 ± 9.6 pg/ml) postinjection, and reached peak levels at 2 h (213.5 ± 39.7 pg/ml) and gradually declined thereafter to baseline 18 h after Con A injection (Fig. 1).

3.2. Effects of anti-MIP-1α antibody on Con A-induced liver injury, mortality rate and plasma cytokine levels after Con A injection

Anti-MIP-1α antibody pretreatment dose-dependently suppressed the Con A-induced elevation of plasma ALT levels 8 h after Con A administration, and 200 µg of anti-MIP-1α antibody further decreased plasma ALT level even at 24 h after Con A injection (Fig. 2). The liver specimens were obtained 8 h after Con A treatment from vehicle- and anti-MIP-1α antibody (200 µg)-pretreated mice, and examined under light microscope. Midzonal hepatocellular necrosis was markedly reduced in the liver of anti-MIP-1α antibody-pretreated mice compared with those of vehicle-pretreated mice (Fig. 3). The mortality rate 24 h after Con A treatment was completely inhibited in the anti-MIP-1α antibody-pretreated mice compare with the vehicle-treated mice (control, 33.3% [4/12]; Anti-MIP-1α, 0% [0/8]). Anti-MIP-1α antibody pretreatment significantly decreased the plasma TNF-α level 2 and 24 h, and plasma IFN-γ level 8 h after Con A injection (Fig. 4).

3.3. Effect of recombinant murine-MIP-1α on Con A-induced liver injury and plasma cytokine levels after Con A injection

Recombinant murine-MIP-1α pretreatment dose-dependently increased the Con A-induced plasma ALT levels (Fig. 5). Recombinant murine-MIP-1α (2 µg/mouse) pretreatment also significantly enhanced the plasma TNF-α level 2 h after Con A injection and plasma IFN-γ level 8 h after Con A injection, respectively (Fig. 6).

3.4. Effect of GdCl₃ pretreatment on Con A-induced liver injury and plasma MIP-1α level after Con A injection

GdCl₃ (40 mg/kg) pretreatment significantly reduced the plasma MIP-1α level by 54 %, 2 h after Con A injection (Fig. 7A) and also significantly inhibited the elevated plasma ALT level by 26 %, 8 h after Con A injection (Fig. 7B)

4. Discussion

The Con A-induced liver injury model in mice was established by Tieges et al, and is known as an animal model for autoimmune and fulminant hepatitis [1]. In this model, the reproducible liver injury is easily induced by a one-shot intravenous injection of Con A without any further sensitization. CD4+ T cells accumulate in the liver immediately after Con A administration and interact with monocytes-macrophages. These cells activate each other and progress the liver injury mediated through induction of proinflammatory cytokines [1,19,20]. Hepatic natural killer T cells activated by Con A produce interleukin 4 and play a critical role in this model [21]. These cells are recruited by specific chemokines and by themselves produce various chemokines [15-17,22]. We have recently shown that MIP-2, one of mouse CXC chemokines, is induced by TNF-α after Con A injection, and this chemokine contributes the development of Con A-induced liver injury mediated through attracting and activating neutrophils [18]. However, the function of CC chemokines, which attracts T cells and monocytes-macrophages that play main role in this model, are poorly understood.

MIP-1α, one of CC chemokines, has chemoattractic activity for T helper 1 cells, monocytes-macrophages, natural killer cells, and dendritic cells [23-28]. This CC chemokine plays a pivotal role in diverse forms of organ injury models and clinical diseases, such as lung fibrosis, rheumatoid arthritis, inflammatory bowel disease and glomerulonephritis [17,29-32]. In regard to liver diseases, the involvement of this chemokine has recently been demonstrated. MIP-1α is expressed in human liver allografts after transplantation [33], and induces migration of CCR5-expressing CD8+ T cells into the portal area in graft-versus-host disease [34]. In experimental granulomatous liver disease, MIP-1α attracts circulating dendritic precursors to the liver [25]. Thus, we focused on the role of MIP-1α in Con A-induced liver injury, and found that plasma MIP-1α level was elevated and reached a peak level 2 h after Con A administration. Pretreatment of anti-MIP-1α antibody reduced Con A-induced elevation of plasma ALT levels in a dose-dependent manner. Anti-MIP-1α antibody also diminished necrotic change in the liver and mortality rate. On the other hand, pretreatment of

recombinant murine-MIP-1α dose-dependently enhanced the Con A-induced elevation of plasma ALT levels. These results suggest that MIP-1α plays an important role in Con A-induced liver injury. Although several studies reported that administration of anti-MIP-1α antibody reduced the organ injury in experimental animal models [34-36], the effect of exogenous MIP-1α has not been investigated. In the present study, we at the first time demonstrate recombinant MIP-1α exacerbates Con A-induced liver injury, suggesting a regulatory role of MIP-1α for liver pathophysiological processes in this model. Proinflammatory cytokines, such as TNF-α and IFN-γ, are demonstrated to play a critical role in progression of Con A-induced liver injury [5-7,37]. We investigated the interaction between MIP-1α and these proinflammatory cytokines, and demonstrated that pretreatment of anti-MIP-1α antibody reduced plasma TNF-α level 2 h after and IFN-γ level 8 h after Con A injection, respectively. On the other hand, pretreatment of recombinant murine-MIP-1α enhanced plasma TNF-α level 2 h after and IFN-γ level 8 h after Con A injection, respectively. It remains unclear why plasma TNF-α and IFN-γ levels are significantly elevated by recombinant MIP-1α pretreatment only 2 h after and 8 h after Con A injection, respectively. Our previous study also showed that plasma TNF-α and IFN-γ reached their peak levels 2 h and 8 h after Con A injection, respectively [18]. Con A-induced MIP-1α may attract and activate producing cells of these proinlammatory cytokines. It is of interest to investigate which cells are recruited or activated by MIP-1α in this model.

Pretreatment of anti-MIP-1α antibody could not   completely block Con A-induced liver injury and the elevation of plasma TNF-α   and IFN-γ. These results suggest the existence of alternative pathways that induce the liver injury in this model. It is known that there are complex networks among many cytokines and chemokines in viral hepatitis, autoimmune liver diseases, fulminant liver failure and alcoholic liver injury [38-41]. Recent study reported that monocyte chemotactic protein-1 (MCP-1), one of CC chemokines, diminished Con A-induced liver injury by inhibiting interleukin-4 produced from natural killer T cells [42]. It is very interesting that different CC chemokines has conflicting effect in this liver injury model. Many chemokines and cytokines induced by Con A treatment

[5,7,18,37,42,43] may make a network and cascade, and regulate progression of liver injury in this model.

Activated monocytes and macrophages are known as major producing cells of MIP-1α [44]. To investigate whether Kupffer cells, the residual macrophages in the liver, produce MIP-1α in Con A-induced liver injury, we pretreated mice with GdCl3, which is known to selectively inactivate Kupffer cells [45], prior to Con A administration. Pretreatment of GdCl3 significantly decreased plasma ALT and MIP-1α levels after Con A injection. These results suggest that MIP-1α is at least partially produced by Kupffer cells after Con A injection. Interestingly, MIP-1α is known to be a strong chemoattractant for monocytes and macrophages [20,44]. In macrophage cell line, MIP-1α   acts as an autocrine activator of macrophages [46]. Furthermore, MIP-1α produced by alveolar macrophage of itself induces late production of MIP-1α mediating through the induction of TNF-α and interleukin-1β in bleomycin-induced lung fibrosis models [35]. Shanley TP et al. further showed that MIP-1α acts as an autocrine activator of pulmonary macrophages in acute lung injury induced by immune complexes or lipopolysaccharide [47]. From these data, it is suggested that MIP-1α produced from Kupffer cells enhances the production of itself through autocrine or paracrine mechanisms. Recently, Ajuebor MN et al. reported that MIP-1α produced in the liver, recruited CCR1-expressing CD4+ T cell, and IFN-γ released by the cells progressed Con A-induced liver injury [48]. Although our data supports their results, we think Kupffer cell and TNF-α also play an important role in MIP-1α mediated liver cell injury in this model.

In conclusion, our results suggest that MIP-1α produced from Kupffer cells, plays an important role in progression of Con A-induced liver injury mediated through the production of TNF-α and IFN-γ. We demonstrate the crucial role of MIP-1α, one of CC chemokines, in progression of liver injury in this model. Development of targeting therapy for chemokines may establish potential and safe therapeutic intervention in patients with autoimmune hepatitis and fulminant hepatitis.

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Figure legends

Figure 1   Time course of plasma MIP-1α level after Con A injection. Mice were intravenously injected with 20 mg/kg of Con A. Plasma MIP-1α levels were determined at various time points by ELISA.

Figure 2   Effect of anti-MIP-1α antibody on plasma ALT level after Con A injection. Anti-MIP-1α antibody (50 or 200 µg) or vehicle (200 µg purified rat IgG2b) were intravenously administered 30 min prior to Con A (20 mg/kg) injection. Plasma ALT levels were determined enzymatically 8 and 24 h after Con A treatment.

Figure 3   Effect of anti-MIP-1α antibody on histological changes in livers by Con A. Anti-MIP-1α antibody (200 µg) or vehicle (200 µg purified rat IgG2b) were intravenously administered 30 min prior to Con A (20 mg/kg) injection. The liver samples were removed 8 h after Con A injection. The specimens were fixed by 10 % (v/v) formalin, stained with H&E, and assessed under the light microscope (100).

Figure 4   Effect of anti-MIP-1α antibody on plasma TNF-α and IFN-γ levels after Con A injection. Anti-MIP-1α antibody (200 µg) or vehicle (200 µg purified rat IgG2b) were intravenously administered 30 min prior to Con A (20 mg/kg) injection. Plasma TNF-α and IFN-γ levels were determined 2, 8, 24 h after Con A injection by ELISA.

Figure 5   Effect of recombinant murine MIP-1α on plasma ALT level after Con A injection. Recombinant murine MIP-1α (0.5 or 2.0 µg) or vehicle (pyrogen-free saline) were intraperitonealy administered 1 h prior to Con A (15 mg/kg) injection. Plasma ALT levels

were determined enzymatically 8 h after Con A treatment.

Figure 6   Effect of recombinant murine MIP-1α on plasma TNF-α and IFN-γ levels after Con A injection. Recombinant murine MIP-1α (2 µg) or vehicle (pyrogen-free saline) were intraperitoneally administered 1 h prior to Con A (15 mg/kg) injection. Plasma TNF-α and IFN-γ levels were determined 2 and 8 h after Con A injection by ELISA.

Figure 7   Effect of GdCl3 pretreatment on Con A-induced liver injury and plasma MIP-1α level after Con A injection. GdCl3 (40 mg/kg) or vehicle (pyrogen-free saline) were intraperitonealy administered 24 h prior to Con A (20 mg/kg) injection. The plasma MIP-1α level (A) was determined 2 h after Con A treatment by ELISA, and the plasma ALT level (B) was enzymatically determined 8 h after Con A treatment.