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Macrophage inflammatory protein-2 induced by TNF-alpha plays a pivotal role in concanavalin A-induced liver injury in mice.

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MACROPHAGE INFLAMMATORY PROTEIN-2 INDUCED BY TNF-α PLAYS A PIVOTAL ROLE IN CONCANAVALIN A-INDUCED LIVER INJURY IN MICE.

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Short title: The role of MIP-2 in Con A-induced liver injury

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Background/Aims: Macrophage inflammatory protein-2 (MIP-2), one of CXC chemokines is involved in the recruitment of neutrophils in several tissue injury. In this study, we investigated a role of MIP-2 in concanavalin A (Con A)-induced liver injury in mice. ***Methods:***Liver injury was induced by intravenous injection of Con A (15 mg/kg) and plasma alanine aminotransferase (ALT), MIP-2 levels were determined and histological assessment of the liver was performed. Anti-mouse MIP-2 antibody was intravenously administered 30 min before Con A injection. ***Results:*** Plasma ALT level significantly elevated and reached a maximum at 8 h after Con A injection. Plasma MIP-2 level was also elevated and reached peak value at 2 h after Con A injection. The elevated ALT level by Con A injection was significantly inhibited by MIP-2 antibody**.** The elevated plasma MIP-2 level after Con A injection was significantly reduced by TNF-α antibody, and MIP-2 was induced in plasma after recombinant TNF-α injection. Hepatic necrosis and infiltration of neutrophils were observed after Con A injection, and these histological changes were attenuated by MIP-2 antibody. ***Conclusion:*** These findings suggest that Con A induces TNF-α release, and this TNF-α stimulates MIP-2 induction, at least partially contributing to the liver injury mediated through the recruitment of neutrophils.

Key words: Concanavalin A , Macrophage inflammatory protein-2, Cytokine, Chemokine, Liver injury, Neutrophil.

Abbreviations:

Con A, Concanavalin A; MIP-2, Macrophage inflammatory protein-2; ALT, Alanine aminotransferase; TNF-α, Tumor necrosis factor alpha; IFN-γ, Interferon gamma; ELISA, Enzyme-linked immunosorbent assay.

Introduction

In many liver diseases including viral hepatitis, autoimmune hepatitis and allograft rejection, activated T lymphocytes appear to play responsible roles. Recently, an animal model of T-cell activation-mediated liver injury was developed (1). Injection of mice with concanavalin A (Con A), a plant lectin known to mitogenically activate T lymphocytes, leads to a polyclonal T-cell activation and to a liver-selective necrotic injury (1-3). Among various cytokines released by Con A-activated T cell (4-9), tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) are demonstrated to play a critical role in the development of massive hepatocellular apoptosis and necrosis (7,9-14). The proinflammatory cytokines, TNF-α and IFN-γ have been shown to exert pleiotrophic effects in a variety of pathophysiologic states including sepsis, ischemia-reperfusion injury, viral infection and other inflammatory states such as acute immune complex-induced lung injury and glomerulonephritis (15-17). On the other hand, the importance of neutrophil in development of the liver injury has been shown in several experimental models such as endotoxin shock (18-20), hepatic ischemia-reperfusion injury (21-23) and alcoholic hepatitis (24). Cytokines specifically chemotactic for inflammatory cells, called chemokines are considerd to play important roles in leukocytes accumulation toward the inflammatory site. Chemokines are subdivided into two major families, the CXC and CC, and the CXC chemokines are typically chemotactic for polymorphonuclear leukocyte (neutrophil), whereas the CC chemokines are more important in mononuclear leukocyte (monocyte and lymphocyte) chemotaxis (25,26).

Murine macrophage inflammatory protein-2 (MIP-2) is one of CXC chemokines which is considered functionally analogous to human interleukin-8 and rat neutrophil-chemoattractant (26), and mostly induced by TNF-α (27,28). Recent studies on hepatic ischemia-reperfusion injury in mice have been shown that the enhanced production of TNF-α plays an important role in the initiation of a cascade of events that causes significant liver injury mediated by neutrophil (22,27-30). Furthermore, one of the main functions of TNF-α is revealed to be the up-regulation of neutrophil-attracting CXC chemokines (MIP-2, KC and ENA-78; epithelial neutrophil activation protein-78). Among these CXC chemokines, MIP-2 is reported to be a most important mediator involved in hepatic injury induced by ischemia-reperfusion in mice (22). These findings prompted us the hypothesis that MIP-2 may play a pivotal role in Con A-induced liver

injury.

In this study, we investigated whether MIP-2 was released in plasma after Con A administration and an antibody to MIP-2 prevented Con A-induced liver injury by reducing the accumulation of neutrophil into the liver. Our results demonstrated that Con A induced TNF-α release, and this TNF-α stimulated MIP-2 induction and anti-MIP-2 antibody pretreatment inhibited hepatic neutrophil accumulation and Con A-induced liver injury. These findings indicate that MIP-2, one of CXC chemokines, may play a pivotal role in Con A-induced liver injury.

Materials and Methods

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 6:00 AM) 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 National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Chemicals

Con A type IV (Jack Bean) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The monoclonal mouse MIP-2 antibody (rat IgG2b class) was purchased from R&D Systems Inc. (Minneapolis, MN, USA) and purified rat myeloma IgG2b was from ZYMED laboratories Inc. (San Francisco, CA, USA). The polyclonal rabbit antibodies to mouse TNF-α and IFN-γ, and recombinant mouse TNF-α and IFN-γ were purchased from IBL, Co. (Fujioka, Japan). The monoclonal anti-mouse neutrophils antibody (lyophilized ascitic fluid) was purchased from CEDARLANE, Co. (Ontario, Canada).

Experimental protocols

Time course of plasma transaminase and cytokines levels after Con A injection; Con A, 15 mg/kg in a volume of 0.3 ml, dissolved in pyrogen-free saline was injected to mice via the tail vein. Plasma ALT level was determined enzymatically using commercially available kit (Wako Co., Tokyo, Japan) at various time points after Con A injection (0, 1, 2, 4, 6, 8, 12, 18 and 24 h). Plasma MIP-2, TNF-α and IFN-γ levels were

determined at 0, 1, 2, 4, 8 and 24 h after Con A treatment by enzyme-linked immunosorbent assay (ELISA) using commercially available kits (MIP-2; IBL Co., TNF-α and IFN-γ; Genzyme Co., Cambridge, MA, USA, respectively). The sensitivities of detection in the ELISAs were 1 pg/ml in MIP-2, 15 pg/ml in TNF-α and 5 pg/ml in IFN-γ, respectively.

Protective effect of anti-MIP-2, TNF-α and IFN-γ antibodies on Con A-induced liver injury; To investigate whether MIP-2 released after Con A treatment plays a role in Con A-induced liver injury, we compared the protective effect of anti-MIP-2 antibody with that of anti-TNF-α and anti-IFN-γ antibodies which were already shown to protect against Con A-induced liver injury (4-7). Anti-mouse MIP-2 antibody (200 μg or 400 μg/mouse), anti-mouse TNF-α antibody (100 μg/mouse) and anti-mouse IFN-γ antibody (100 μg/mouse), and vehicle (400 μg purified rat IgG2b/mouse) were dissolved in pyrogen-free saline in a volume of 0.2 ml and intravenously administered 30 min prior to Con A injection. We chose the doses and administration methods for the antibodies based on previous studies (14,22). Plasma ALT level was determined enzymatically 8 h after Con A treatment.

Effect of anti-TNF-α and IFN-γ antibodies on plasma MIP-2 release; To determine whether TNF-α and IFN-γ are involved in the induction of plasma MIP-2, anti-mouse-TNF-α (100 μg/mouse), anti-mouse-IFN-γ antibody (100 μg/mouse) dissolved in pyrogen-free saline in a volume of 0.2 ml or vehicle (pyrogen-free saline) was intravenously administered 30 min prior to Con A injection. Plasma MIP-2 levels were determined 2 and 8 h after Con A treatment.

Induction of plasma MIP-2 by TNF-α and IFN-γ; We also investigated whether MIP-2 can be induced in plasma by TNF-α or IFN-γ. Mice were intravenously injected with 2 or 10 μg of recombinant mouse TNF-α, 10 μg of recombinant mouse INF-γ dissolved in pyrogen-free saline in a volume of 0.2 ml or vehicle (pyrogen-free saline). We chose the doses of TNF-α and IFN-γ based on previous study (27). Plasma MIP-2 level was determined 2 h after these cytokines or vehicle treatment.

Effect of anti-neutrophils antibody on the protective effect of anti-MIP-2 antibody against Con A-induced liver injury; To clarify an involvement of neutrophils in the protective effect of anti-MIP-2 antibody on Con A-induced liver injury, effect of anti-MIP-2 antibody was investigated in mice pretreated with anti-neutrophils antibody. Anti-mouse neutrophils antibody (200 mg/mouse) dissolved in pyrogen-free saline in a

volume of 0.4 ml or vehicle (pyrogen-free saline) was intraperitonealy administered 18 h before, and following anti-mouse MIP-2 antibody (100 μg/mouse) or vehicle (100 μg purified rat IgG2b/mouse) was intravenously injected 30 min before Con A injection. Plasma ALT level was determined enzymatically 8 h after Con A treatment.

Blood Sampling and Histology

Under ether anesthesia, the abdomen was opened and the peripheral blood was obtained from the vena cava inferior with heparinized syringe 0, 1, 2, 4, 6, 8, 12, 18 and 24 h after Con A injection. The plasma was obtained after 10-min centrifuging at 3000 rpm, and was kept at -70 o C until assay. The liver sample was obtained 8 h after Con A injection by total bleeding due to cutting of abdominal aorta and fixed in 10% (v/v) neutral-buffered formalin. The specimens were stained with hematoxylin and eosin for an assessment of hepatic necrosis. Hepatic neutrophils were stained by the naphthol AS-D chloroacetate esterase technique (18,21) using commercially available kit (Sigma) and randomly chosen 25 high-power fields (HPF; magnification of 600) of each sample were counted by blind fashion.

Statistical analysis

All results are expressed as Mean ± SEM. Comparison between two independent groups was made by Student's t-test. Multiple group comparisons were performed by ANOVA followed by Fisher's protected least significant difference test. $P<0.05$ was considerd statistically significant.

Results

Time course of plasma ALT and cytokines levels after Con A injection

Plasma ALT level before Con A injection were 33 ± 8 KU/l, and started to elevate 4 h after 15 mg/kg Con A administration, and reached a maximum at 8 h (1634 ± 425 KU/l) and decreased thereafter (Fig. 1). MIP-2, TNF-α and IFN-γ were not detected in plasma before Con A treatment. Although plasma TNF-α and MIP-2 levels were elevated and reached peak levels at 2 h after Con A injection, plasma TNF-α level already started to elevate at 1 h while MIP-2 level was still at baseline at a same time point. Both plasma MIP-2 and TNF-α levels gradually declined thereafter to base line at 24 h after Con A injection. On the other hand, plasma IFN-γ level was kept elevating until 8 h and still detected at 24 h after Con A injection (Fig. 2).

Protective effect of anti-MIP-2, TNF-α and IFN-γ antibodies

Anti-TNF-α and anti-IFN-γ antibodies pretreatment significantly suppressed the elevated plasma ALT level induced by Con A injection. Furthermore, anti-MIP-2 antibody pretreatment also significantly suppressed the Con A-induced elevation of plasma ALT level and these effects were dose dependent (Fig. 3).

Effect of antibodies and recombinant proteins of TNF-α and IFN-γ on plasma MIP-2 induction

The elevated plasma MIP-2 level 2 and 8 h after Con A injection was significantly suppressed by anti-TNF-α antibody pretreatment compared with vehicle treatment at both 2 and 8 h after Con A injection. However, anti-IFN-γ antibody pretreatment did not inhibit the elevation of plasma MIP-2 level at both time point. (Fig. 4). Although MIP-2 was not detected in plasma 2 h after pyrogen-free saline and 10 μg/mouse recombinant IFN-γ injection, recombinant TNF-α dose-dependently increased plasma MIP-2 level (Mean ± SE, pg/ml: 140.1 ± 21.2 in 2 μg and 231.8 ± 36.8 in 10 μg/mouse recombinant TNF-α pretreatment, respectively) (Fig. 5).

Accumulation of hepatic neutrophil after Con A injection

Neutrophils in the liver before Con A injection were 1.8 ± 0.1 counts/HPF. Hepatic neutrophils significantly increased at 2 h after Con A injection compared with vehicle treatment. This increase in hepatic neutrophils reached the maximal level at 8 h, and maintained until 24 h after Con A injection (Fig. 6).

Effect of anti-MIP-2 antibody on liver histology and hepatic neutrophil

The liver specimens were obtained 8 h after Con A treatment from vehicle or anti-MIP-2 antibody (400 μg/mouse) pretreated mice and examined under light microscopy. Midzonal hemostasis and hepatocellular necrosis were markedly reduced in the liver of anti-MIP-2 antibody-treated mice compared with those of vehicle-treated mice. In addition, the infiltration of hepatic neutrophils was attenuated by anti-MIP-2 antibody pretreatment (Fig. 7 and 8). However, 400 μg/mouse of anti-MIP-2 antibody did not induce further inhibition of hepatic neutrophil infiltration compared with 200 μg/mouse of anti-MIP-2 antibody (Fig. 8).

Effect of anti-neutrophils antibody on the protective effect of anti-MIP-2 antibody against Con A-induced liver injury

Pretreatment of anti-mouse neutrophils antibody decreased peripheral blood neutrophils by 70%, and infiltrated neutrophils from 15.2 ± 0.7 counts/HPF to 9.2 ± 0.8

counts/HPF at 8 h after Con A treatment. With pretreatment of anti-mouse neutrophils antibody, the elevated plasma ALT level 8 h after Con A injection was partially suppressed and anti-MIP-2 antibody further suppressed elevated ALT level after Con A injection in mice pretreated with anti-mouse neutrophils antibody. (Fig. 9).

Discussion

Con A-induced liver injury model in mice was established by Tiges et al (1). In this model, the reproducible liver injury is easily induced by one shot intravenous injection of Con A without any further sensitization. Several studies showed that CD4+ T-cells were stimulated and various cytokines were also produced by Con A injection (1-3). Among various cytokines released by Con A activated T-cells (4-9), TNF-α and IFN-γ are demonstrated to play a critical role in the development of massive hepatocellular apoptosis and necrosis by several studies using neutralizing antibodies and knockout mice against these cytokines (7,9-14).

In this study, we first demonstrated a role of MIP-2, one of CXC chemokines, in Con A-induced liver injury. After Con A injection, MIP-2 was increased in plasma as well as TNF-α and IFN-γ by using specific ELISAs. Thus, we investigated whether MIP-2 released after Con A injection is involved in Con A-induced liver injury. We compared the protective effect of anti-MIP-2 antibody with that of anti-TNF-α and anti-IFN-γ antibodies. Pretreatment of anti-TNF-α antibody and anti-IFN-γ antibody significantly inhibited the elevation of plasma ALT level after Con A injection, and anti-MIP-2 antibody pretreatment also dose-dependently suppressed the elevation of plasma ALT level and reduced hepatic necrosis (Fig. 3 and 7). These findings suggest that MIP-2 also plays a crucial role in this model. However, since plasma ALT elevation was not completely inhibited by MIP-2 antibody pretreatment, other mediators may also be involved. Recently, a role of intrasinusoidal hemostasis, caused by the sinusoidal endothelial cell damage mediated by TNF-α and IFN-γ, was indicated for one of the mechanisms in Con A-induced liver injury model and a protective effect of heparin (thrombin inhibitor) on Con A-induced liver injury through improvement of microcirculation have been reported (14,31-33).

CXC chemokines are 8- to 10- kDa peptides which have significant neutrophil chemotactic and stimulatory activity (25,26). Various animal models of inflammation have demonstrated the direct correlation of neutrophil accumulation into the

inflammatory tissue with the presence of MIP-2 (15,17,22,24,27,28). In experimental immune complex-induced lung injury and glomerulonephritis, anti-MIP-2 antibody pretreatment reduces the influx of neutrophils to inflammatory sites and prevents the neutrophil-mediated injury of lung alveoli and glomeruli (15-17). Furthermore, recent studies on hepatic ischemia-reperfusion injury in mice have been shown that MIP-2 is involved in the initial recruitment of neutrophil to the ischemic lobe and the infiltrated hepatic neutrophil is thought to promote the progressive hepatocellular damage caused by the sinusoidal hemostasis (22,23,29), and releases of neutrophil-generated oxygen free radicals and protease (30,34,35). Thus, our present findings that MIP-2 plays a pivotal role in Con A-induced liver injury is consistent with hepatic ischemia-reperfusion injury.

MIP-2 is regarded as functionally analogous to human interleukin-8 and rat neutrophil-chemoattractant (26), and several studies have reported that mouse MIP-2 is mostly induced by TNF-α (26-28). MIP-2 is reported to be released from several hepatic cells, including Kupffer cells, sinusoidal endothelial cells and hepatocytes, under various pathologic conditions (22,24,36,37). In the present study, the elevation of plasma MIP-2 level was preceded by that of plasma TNF-α level (Fig. 2). Furthermore, the elevated plasma MIP-2 level after Con A injection was significantly reduced by anti-TNF-α antibody pretreatment but not by anti-IFN-γ antibody (Fig. 4), and MIP-2 was induced in plasma after recombinant TNF-α administration (Fig. 5). These findings suggest that Con A induces TNF-α and this TNF-α stimulates MIP-2 synthesis in Con A-induced liver injury. Although our present results and this hypothesis are very consistent with and supported by previous study (27), mechanisms through which TNF-α induces MIP-2 is unclear.

In this study, hepatic neutrophils were significantly accumulated following MIP-2 induction and the accumulation was significantly inhibited by anti-MIP-2 antibody pretreatment. However, dose-dependent effects of anti-MIP-2 antibody on neutrophils recruitment were not shown, whereas the antibody dose-dependently protected against Con A-induced liver injury. This discrepancy suggests that besides MIP-2, other mediators such as selectin families, integrin families, platelet-activating factor and another CXC chemokines (KC and ENA-78, etc) may also be involved in neutrophil recruitment (17-24,27,28), because anti-MIP-2 antibody further suppressed the elevation of plasma ALT level induced by Con A injection in mice pretreated with

anti-neutrophils antibody (Fig. 9). These results may suggest that anti-MIP-2 antibody suppresses not only the neutrophil accumulation into the liver but also the activation of hepatic neutrophils. Activated neutrophil releases a variety of inflammatory mediators, including proteolytic enzymes, arachidonic acid metabolites, reactive oxygen species and several cytokines (30,34,35). It is of interest to investigate the mechanism through which hepatic neutrophil recruited by MIP-2 produces the liver injury in this model.

In conclusion, present studies suggest that Con A induces TNF-α release, and this TNF-α stimulates MIP-2 induction, at least partially contributing to the liver injury mediated through the recruitment of neutrophil. These findings indicate that MIP-2, one of CXC chemokines, may play a pivotal role in Con A-induced liver injury.

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Figure legends

Fig. 1. Time course of plasma ALT level after Con A injection. Mice were injected with 15 mg/kg of Con A or pyrogen-free saline vehicle. Plasma ALT level was determined at various time points. **p<0.01 compared with vehicle (saline) treatment.

Fig. 2. Time course of plasma cytokines levels after Con A injection. Plasma MIP-2, TNF-α and IFN-γ levels were determined at various time points by ELISA. **p<0.01 compared with before Con A treatment (0 h).

Fig. 3. Protective effect of MIP-2, TNF-α and IFN-γ antibodies on Con A-induced liver injury. Anti-mouse-MIP-2 antibody (200 or 400 μg/mouse), anti-mouse-TNF-α antibody (100 μg/mouse), anti-mouse-IFN-γ antibody (100 μg/mouse) and vehicle (400 μg purified rat IgG2b/mouse) were dissolved in pyrogen-free saline in a volume of 0.2 ml and administered intravenously 30 min prior to Con A injection. Plasma ALT level was determined 8 h after Con A treatment. *p<0.05, **p<0.01 compared with vehicle (rat IgG2b) treatment.

Fig. 4. Effect of anti-mouse TNF-α and IFN-γ antibodies on plasma MIP-2 release after Con A injection. Anti-mouse-TNF-α antibody (100 μg/mouse), anti-mouse-IFN-γ

antibody (100 μg/mouse) dissolved in pyrogen-free saline in a volume of 0.2 ml or vehicle (pyrogen-free saline) was administered intravenously 30 min prior to Con A injection. Plasma MIP-2 level was determined 2 and 8 h after Con A treatment by ELISA. **$p<0.01$ compared with vehicle (saline) or anti-INF-γ treatment.

Fig. 5. Induction of plasma MIP-2 after recombinant TNF-α and INF-γ injection. Two or 10 μg of recombinant mouse TNF-α, 10 μg of recombinant mouse INF-γ dissolved in pyrogen-free saline in a volume of 0.2 ml or vehicle (pyrogen-free saline) was intravenously administered. Plasma MIP-2 level was determined 2 h after cytokines or vehicle treatment by ELISA.

Fig. 6. Time course of the infiltrated hepatic neutrophil after Con A injection. Mice were injected with 15 mg/kg of Con A or pyrogen-free saline vehicle. Hepatic neutrophils were stained using the naphthol AS-D chloroacetate esterase technique and randomly chosen 25 high-power fields (HPF; magnification of 600) of each sample were counted by blind fashion. **$p<0.01$ compared with vehicle (saline) treatment.

Fig. 7. Effect of anti-MIP-2 antibody on light micrographic changes in livers. The liver samples were obtained 8 h after Con A injection from control mice (a and b) or anti-mouse-MIP-2 antibody (400 μg/mouse)-treated mice (c and d). The liver specimens were stained with hematoxylin and eosin (a and c, x100) or naphthol AS-D chloroacetate (b and d, x600).

Fig. 8. Effect of anti-MIP-2 antibodies on the infiltrated hepatic neutrophil after Con A injection. Anti-mouse-MIP-2 antibodies (200 or 400 μg/mouse) dissolved in pyrogen-free saline in a volume of 0.2 ml or vehicle (400 μg purified rat IgG2b/mouse) was administered intravenously 30 min prior to Con A injection. Hepatic neutrophils were stained and counted 8 h after Con A treatment. **$p<0.01$ compared with vehicle (rat IgG2b) treatment.

Fig. 9. Effect of anti-neutrophils antibody on the protective effect of anti-MIP-2 antibody against Con A-induced liver injury. Anti-mouse neutrophils antibody (200 mg/mouse) dissolved in pyrogen-free saline in a volume of 0.4 ml or vehicle

(pyrogen-free saline) was intraperitonealy administered 18 h before, and following anti-mouse MIP-2 antibody (100 μg/mouse) or vehicle (100 μg purified rat IgG2b/mouse) was intravenously injected 30 min before Con A injection. Plasma ALT level was determined enzymatically 8 h after Con A treatment. *$p<0.05$ compared with vehicle treatment.