Antithrombin III prevents concanavalin A-induced liver injury through inhibition of macrophage inflammatory protein-2 release and production of prostacyclin in mice.

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ANTITHROMBIN III PREVENTS CONCANAVALIN A-INDUCED LIVER INJURY THROUGH INHIBITION OF MACROPHAGE INFLAMMATORY PROTEIN-2 RELEASE AND PRODUCTION OF PROSTACYCLIN IN MICE.

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Running title: The protective effect of AT-III on Con A-induced liver injury

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Background/Aims: Recently, we have reported that macrophage inflammatory protein-2 (MIP-2) plays a pivotal role in concanavalin A (Con A)-induced liver injury. In this study, we investigated the effect of antithrombin III (AT-III) on liver damage, and production of MIP-2 and prostacyclin in this model. ***Methods:*** Liver injury was induced by intravenous injection of Con A (15 mg/kg) and AT-III was administered (50, 250 and 500 units/kg, iv) 30 min before Con A injection. Plasma levels of alanine aminotransferase (ALT), MIP-2 and 6-keto-prostaglandin F1α (6k-PG-F1α), stable metabolite of prostaglandin I2 (prostacyclin), were determined. ***Results:*** The elevated plasma ALT levels 8, 16, 24 h after Con A injection were inhibited by AT-III pretreatment. The elevated plasma MIP-2 levels were significantly inhibited by AT-III pretreatment compared with vehicle treatment. The inhibitory effect of AT-III on plasma ALT and MIP-2 in Con A-induced liver injury was attenuated by indomethacin (5 mg/kg, ip). Plasma concentration of 6k-PG-F1α at 2 h after AT-III injection was significantly elevated compared with baseline and vehicle pretreatment. ***Conclusion:*** These findings suggest that AT-III prevents Con A-induced liver injury through an inhibition of MIP-2 release and a production of prostacyclin.

Introduction

In many liver diseases including viral hepatitis, autoimmune hepatitis and allograft rejection, activated T lymphocytes appear to play responsible roles. Tiges et al, reported that an intravenous injection of concanavalin A (Con A) induced T-cell activation-mediated liver injury in mice (1). Among various cytokines released during Con A-induced T cell activation, tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) are considered to play critical role in the development of massive hepatocellular apoptosis and necrosis, whereas interleukin 10 (IL-10) is demonstrated to prevent liver injury through inhibition of TNF-α and IFN-γ (2-12). Moreover, a role of intrasinusoidal hemostasis, caused by the sinusoidal endothelial cell damage mediated through TNF-α and IFN-γ, was noted for one of the important mechanisms in this model (13,14).

Antithrombin III (AT-III), an inhibitor of the serine proteases that are generated within the coagulation cascade, is reported to attenuate lung and liver injury induced by sepsis, endotoxemia and ischemia-reperfusion (15-18). Uchiba et al, reported that the intravenous administration of AT-III prevented the pulmonary accumulation of neutrophils and the pulmonary vascular injury induced by endotoxin (15). In the ischemia-reperfusion injury of rat liver, it is proposed that AT-III prevent liver injury by inhibiting the hepatic accumulation of neutrophils through an increase in the hepatic prostacyclin (prostaglandin I_2 , PGI_2) levels and inhibition of cytokine-induced neutrophil chemoattractant (rat CXC chemokine) production (16,17). Furthermore, in baboons administered with a lethal dose of Escherichia coli, AT-III has been shown to improve survival through inhibition of interleukin-8 (IL-8) production as well as anticoagulant action (18). We have recently found that macrophage inflammatory protein-2 (MIP-2), one of mouse CXC chemokines, plays a pivotal role in Con A-induced liver injury (19). MIP-2 is considered as functionally analogous to human IL-8 and rat cytokine-induced neutrophil chemoattractant (20,21). MIP-2 has been reported as an important mediator involved in liver injury induced by endotoxemia or ischemia-reperfusion through inhibition of neutrophil accumulation and activation in mice (22, 23). These studies prompted us the hypothesis that AT-III may be an beneficial agent in Con A-induced liver injury by interacting with local MIP-2 release and production of PGI_2.

In this study, we investigated the effect of AT-III on liver damage, and the

production of MIP-2 and 6-keto-prostaglandin F1α (6k-PG-F1α), stable metabolite of PGI$_2$, in Con A-induced liver injury model. Furthermore, we studied whether the action of AT-III was mediated through PGI$_2$ by pretreating indomethacin (cyclooxygenase inhibitor). Our data demonstrated that AT-III attenuated liver injury, MIP-2 production and hepatic neutrophil accumulation induced by Con A treatment and produced PGI$_2$, and these beneficial effects of AT-III were abolished by pretreatment of indomethacin.

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). AT-III was kindly provided by Hoechst Marion Roussel Co. (Tokyo, Japan). Recombinant mouse MIP-2 and TNF-α were purchased from IBL, Co. (Fujioka, Japan). Indomethacin and all other chemicals used in this study were of regent grade and were purchased from Wako Co. (Osaka, Japan).

Experimental protocols

Effect of AT-III on plasma transaminase and cytokine production in Con A-induced liver injury; 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. AT-III was dissolved in distilled water (50, 250 and 500 units/kg in a volume of 0.2 ml) and administered intravenously 30 min before Con A injection. <u>Plasma alanine aminotransferase (ALT) level at 8, 16 and 24 h after Con A injection was determined enzymatically using commercially available kit (Wako Co.).</u> To evaluate the effect of AT-III on the production of cytokines, plasma MIP-2, TNF-α, IFN-γ and interleukin 10 (IL-10) levels were determined 0, 2, 4 and 8 h after Con A treatment by enzyme-linked immunosorbent assay (ELISA) using commercially available ELISA kits (MIP-2; IBL

Co., TNF-α, IFN-γ and IL-10; Genzyme Co., Cambridge, MA, USA, respectively). The sensitivities of detection in the ELISAs were 1 pg/ml for MIP-2, 15 pg/ml for TNF-α, 5 pg/ml for IFN-γ and 4 pg/ml for IL-10, respectively.

Effect of recombinant MIP-2 and indomethacin on AT-III-induced cytoprotection against Con A-induced liver injury; To investigate an involvement of MIP-2 and prostacyclin in the protective effect of AT-III on Con A-induced liver injury, recombinant mouse MIP-2 (4 μg/mouse dissolved in 0.5 ml of saline), indomethacin (5 mg/kg dissolved in 0.5 ml of NaHCO3 solution) or respective vehicle solution was intraperitoneally co-injected with AT-III (500 units/kg,iv) 30 min before Con A administration. Plasma ALT level was determined 8 h after Con A.

Effect of AT-III on the plasma prostacyclin production; To investigate whether AT-III produces prostacyclin (PGI2), the concentration of plasma 6k-PG-F1α, stable metabolite of PGI2 was determined. AT-III (500 units/kg) or vehicle (distilled water) was intravenously administered 30 min before Con A injection. Plasma concentration of 6k-PG-F1α was determined before and 2, 4, 8 h after Con A treatment by using commercially available ELISA kit (Assay Designs, Inc., MI, USA). The sensitivity of detection in the ELISA for 6k-PG-F1α was 1.4 pg/ml.

Effect of indomethacin on the modulation of plasma prostacyclin and MIP-2 induced by AT-III in Con A-induced liver injury; To investigate a role of prostacyclin in the modulation of plasma MIP-2 level by AT-III in Con A-induced liver injury, indomethacin (5 mg/kg dissolved in 0.5 ml of 1% NaHCO3 solution) or vehicle (1% NaHCO3 solution) was intraperitoneally co-injected with AT-III (500 units/kg, iv) 30 min before Con A, and plasma MIP-2 and 6k-PG-F1α levels were determined 8 h later.

Effect of AT-III and indomethacin on the plasma MIP-2 production induced by recombinant TNF-α; We have reported that the intravenous injection of recombinant mouse TNF-α induces MIP-2 in plasma (19). To investigate whether AT-III affects MIP-2 production induced by TNF-α, indomethacin (5 mg/kg dissolved in 0.5 ml of 1% NaHCO3 solution) or vehicle (1% NaHCO3 solution) was intraperitoneally co-injected with AT-III (500 units/kg, iv) 30 min before recombinant mouse TNF-α (2 μg/mouse dissolved in 0.2 ml pyrogen-free saline) or vehicle (pyrogen-free saline) injection. Plasma MIP-2 level was determined 2 h after TNF-α treatment by ELISA.

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. The plasma was obtained after 10-min centrifuging at 3000 rpm, and was kept at -70 o C 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 an assessment of hepatic damage. Hepatic neutrophils were stained by the naphthol AS-D chloroacetate esterase technique using commercially available kit (Sigma) and randomly chosen 25 high-power fields (HPF; magnification of 600) of each sample were counted by blind fashion (19).

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

Effect of AT-III on plasma transaminase and cytokine production

Plasma ALT levels 8 h after Con A injection were dose-dependently inhibited by AT-III pretreatment (Mean ± SE, KU/L: vehicle 1397 ± 209; AT-III 50 units/kg 673 ± 230; 250 units/kg 165 ± 66; 500 units/kg 53 ± 10, respectively) (Fig. 1a). Furthermore, AT-III (500 units/kg) pretreatment significantly decreased plasma ALT levels at each time point of 8, 16, 24 h after Con A injection (Fig. 1b). Although MIP-2, TNF-α, IFN-γ and IL-10 were not detectable in plasma before Con A treatment, plasma MIP-2 and TNF-α elevated and reached the peak level at 2 h after Con A injection and gradually declined thereafter. However, plasma IFN-γ and IL-10 were kept elevating 8 h over a observation period (Table 1). The elevated plasma MIP-2 level after Con A treatment was significantly inhibited by AT-III (500 units/kg) pretreatment at all time points compared with vehicle treatment, whereas plasma TNF-α, IFN-γ and IL-10 levels were not significantly changed by AT-III at all time points (Table 1).

Effect of recombinant MIP-2 and indomethacin on AT-III-induced cytoprotection

The inhibitory effect of AT-III (500 units/kg) on Con A-induced liver injury

was partially reversed by simultaneous administration of recombinant mouse MIP-2 protein (4 μg/mouse, ip) (Fig. 2), and that almost completely attenuated by indomethacin (5 mg/kg, ip) assessed by plasma ALT level 8 h after Con A treatment (Fig. 3). Hemostasis in the hepatic sinusoid and degenerative changes in hepatocytes at midzonal zone were observed 8 h after Con A injection (Fig. 4a) and these histological changes decreased in the liver of AT-III pretreated mice compared with vehicle pretreated mice (Fig. 4b).These beneficial effects of AT-III on histological findings were also attenuated by indomethacin (Fig. 4c).

Effect of AT-III on the plasma prostacyclin production

Plasma concentration of 6k-PG-F1α 2 h after AT-III (500 units/kg) injection was significantly elevated compared with baseline or vehicle treatment. Furthermore, plasma concentration of 6k-PG-F1α 4 h after Con A injection was significantly increased by AT-III pretreatment compared with baseline. Plasma concentration of 6k-PG-F1α 2 and 4 h after Con A injection was increased by AT-III pretreatment compared with vehicle treatment, but the difference was not statistically significant (Fig. 5).

Effect of indomethacin on the modulation of plasma prostacyclin and MIP-2 production by AT-III

Plasma concentration of 6k-PG-F1α 8 h after Con A injection was slightly increased by AT-III pretreatment (500 units/kg) , whereas indomethacin completely abolished the inducible effect of AT-III on plasma 6k-PG-F1α (Fig. 6a). Furthermore, elevated plasma MIP-2 level 8 h after Con A injection was significantly decreased by AT-III, and indomethacin also attenuated this inhibitory effect of AT-III on plasma MIP-2 induction (Fig. 6b).

Effect of AT-III and indomethacin on the plasma MIP-2 production induced by recombinant TNF-α

MIP-2 was not induced in plasma 2 h after distilled water vehicle plus 1% NaHCO3 solution or indomethacin pretreatment (Fig. 7). Plasma MIP-2 level 2 h after recombinant TNF-α injection was increased to 140.1 ± 21.2 pg/ml, and this increase in plasma MIP-2 was partially reversed by AT-III (500 units/kg) pretreatment (77.8 ± 15.7 pg/ml). However, indomethacin pretreatment attenuated this inhibitory effect on plasma MIP-2 by AT-III pretreatment (Fig. 7).

Effect of AT-III and indomethacin on the infiltration of hepatic

neutrophils

Neutrophils in the liver before Con A injection were 1.8 ± 0.1 counts/HPF. Although hepatic neutrophils were accumulated and increased at 8 h by Con A treatment, these cells were significantly reduced by AT-III (500 units/kg) pretreatment. However, indomethacin pretreatment also abolished this inhibitory effect of AT-III on the infiltration of hepatic neutrophils (Fig. 8).

Discussion

AT-III, an important physiological inhibitor of the coagulation cascade, is reported to attenuated lung and liver injury induced by sepsis, endotoxemia and ischemia-reperfusion (15-18,24,25), suggesting that AT-III has anti-inflammatory effects and a potency of prostacyclin induction. Therefore, we investigated the effect of AT-III on liver damage, and production of MIP-2 and prostacyclin in Con A-induced liver injury model. In the present study, we showed that pretreatment of AT-III dose-dependently inhibited the elevated plasma ALT levels and AT-III (500 units/kg) pretreatment decreased plasma ALT level each time point (8, 16, 24 h) after Con A injection (Fig. 1). Furthermore, AT-III pretreatment decreased hemostasis in the hepatic sinusoid and degenerative changes in hepatocytes at midzonal zone induced by Con A treatment in mice. Several studies showed that CD4+ T-cells were stimulated and various cytokines were produced by Con A injection. Among these cytokines TNF-α and IFN-γ are considered to play critical role in the development of massive hepatocellular apoptosis and necrosis, whereas IL-10 prevents liver injury through inhibition of TNF-α and IFN-γ production (2-12). We have recently found that MIP-2, one of mouse CXC chemokines, plays a pivotal role in Con A-induced liver injury (19). In the present study, the elevation of plasma MIP-2 levels were significantly reduced by AT-III pretreatment compared with vehicle pretreatment, while plasma TNF-α, IFN-γ and IL-10 levels were not significantly changed by AT-III pretreatment at all time points (Table 1). We further demonstrated that AT-III pretreatment decreased plasma ALT level 8 h after Con A injection, whereas this inhibitory effect was partially reversed by simultaneous administration of recombinant mouse MIP-2 protein (Fig. 2). These findings suggest that the beneficial effect of AT-III on liver injury is at least partially mediated through an inhibition of MIP-2 release. However, TNF-α , IFN-γ and IL-10 are unrelated to the effect of AT-III.

In ischemia-reperfusion and endotoxin-induced liver injury models, AT-III attenuated the liver injury not only by anticoagulant action, but also by inhibiting accumulation and activation of neutrophils. These studies proposed several mechanisms of AT-III: 1) AT-III promotes the release of prostacyclin (PGI_2) from the endothelial cell (15,24); 2) AT-III inhibits the production of CXC chemokine and prevents leukocyte activation (16,17); 3) AT-III reduces the expression of selectin and integrin families on endothelial cell and prevents leukocyte recruitment (18,25); and 4) AT-III inhibits T cell proliferation (26). In the present study, we also investigated whether the beneficial effects of AT-III participate in the induction of PGI_2. Consequently, plasma concentration of 6k-PG-F1α, stable metabolite of PGI_2, was increased by AT-III and decreased by indomethacin pretreatment (Fig. 5 and 6a). Furthermore, indomethacin pretreatment attenuated the protective effect of AT-III on liver injury and the inhibitory effect of AT-III on MIP-2 production (Fig. 3 and 6b). Therefore, we suggested that these beneficial effects of AT-III on Con A-induced liver injury were at least partially mediated through the production of prostacyclin. Since it is reported that prostacyclin is directly produced from endothelial cells by AT-III (27) and suppresses ischemia-reperfusion liver injury and stress-induced gastric mucosal damage (28-30), it is of interest to study the effect of prostacyclin on Con A-induced liver injury and MIP-2 production.

In the previous study, we reported that Con A induced a TNF-α release, and this TNF-α may stimulate MIP-2 induction, contributing to the liver injury mediated through the recruitment of neutrophils (19). In the present study, we demonstrated that plasma MIP-2 induced by recombinant TNF-α and hepatic neutrophils accumulated by Con A treatment were significantly reduced by AT-III pretreatment (Fig. 7 and 8). Furthermore, indomethacin attenuated this inhibitory effect on plasma MIP-2 and hepatic neutrophils by AT-III pretreatment. Moreover, AT-III decreased plasma MIP-2 level but not TNF-α level stimulated by Con A. Thus, our present findings suggest that AT-III inhibits MIP-2 release induced by TNF-α and this inhibition of MIP-2 further decreases the accumulation of hepatic neutrophils, and these beneficial effects of AT-III may also be mediated partially through prostacyclin synthesis.

Recently, the role of intrasinusoidal hemostasis in the development of Con A-induced liver injury and a protective effect of heparin (thrombin inhibitor) on this

injury through improvement of microcirculation have been reported (13,14,31). In our study, the effect of AT-Ⅲ on the prostacyclin induction was not potent, however AT-Ⅲ completely prevented the liver injury induced by Con A, indicating that AT-Ⅲ may have another mechanism such as an improvement of intrahepatic microcirculation through an anticoagulant effect. It is generally recognized that activation of coagulation is closely linked to immune and inflammatory responses. Thrombin plays a central role in the blood coagulation system, and is considered to be involved in the regulation of various diseases including thrombosis, atherosclerosis and inflammation (32,33). Several studies have demonstrated that thrombin induces the expression of IL-8 and leukocyte adhesion molecules such as P-, E-selectin and intercellular adhesion molecule-1 in endothelial cell culture at the protein and mRNA levels (34-36). Therefore, it may be suggested that the thrombin produced by intrasinusoidal hemostasis after Con A injection is also involved in MIP-2 production and neutrophil accumulation in the liver. Furthermore, AT-III may inhibit MIP-2 production and neutrophil accumulation by reducing the production of the thrombin in hepatic sinusoids.

In conclusion, present study suggests that AT-III attenuates liver injury and MIP-2 production induced by Con A treatment, and these beneficial effects of AT-III, at least in part, mediating through prostacyclin synthesis.

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Figure legends

Fig. 1. Effect of AT III on plasma ALT levels after Con A treatment. AT-III was dissolved in distilled water (50, 250, 500 units/kg in a volume of 0.2 ml), and administered intravenously 30 min before Con A injection. Plasma ALT levels were determined 8 h after Con A treatment (a). AT-III (500 units/kg) or vehicle (distilled water) was administered intravenously 30 min before Con A injection and plasma ALT levels were determined 8, 16, and 24 h after Con A treatment (b). *p<0.05, **p<0.01, ***p<0.001 compared with distilled water vehicle treatment.

Fig. 2. Effect of recombinant MIP-2 on AT-III-induced cytoprotection against Con A-induced liver injury. Recombinant mouse MIP-2 (4 μg/mouse) or vehicle (pyrogen-free saline) was intraperitoneally co-injected with AT-III (500 units/kg) 30 min before Con A injection. Plasma ALT levels were determined 8 h after Con A treatment. *p<0.05, **p<0.01 compared with pyrogen-free saline plus distilled water vehicle treatment. #p<0.05 compared with pyrogen-free saline plus AT-III treatment.

Fig. 3. Effect of indomethacin on AT-III-induced cytoprotection against Con A-induced liver injury. Indomethacin (Indo, 5 mg/kg) or vehicle (1% NaHCO3 solution) was intraperitoneally co-injected with AT-III (500 units/kg) 30 min before Con A injection. Plasma ALT levels were determined 8 h after Con A treatment. **p<0.01 compared with 1% NaHCO3 solution plus distilled water vehicle treatment. #p<0.05 compared with 1% NaHCO3 solution plus AT-III treatment.

Fig. 4. Effect of AT-III on light micrographic changes of the liver after Con A treatment. The liver samples were obtained 8 h after Con A treatment from vehicle pretreated (a), AT-III (500 units/kg, iv) pretreated (b), AT-III (500 units/kg, iv) plus indomethacin (5 mg/kg, ip) pretreated (c). Hematoxylin-eosin staining; original magnification x100.

Fig. 5. Effect of AT-III on the plasma prostacyclin production. AT-III (500 units/kg) or vehicle (distilled water) was administered intravenously without Con A treatment (Con A(-)) and plasma concentration of 6k-PG-F1α was determined before and 2 h after AT-III treatment by ELISA. Furthermore, AT-III (500 units/kg) or vehicle (distilled water) was administered intravenously 30 min before Con A injection and plasma

concentration of 6k-PG-F1α was determined 2, 4, 8 h after Con A treatment. *p<0.05 compared with plasma concentration at 0 h, #p<0.05 compared with distilled water vehicle treatment.

Fig. 6. Effect of indomethacin on the modulation of plasma prostacyclin and MIP-2 production by AT-III. Indomethacin (Indo, 5 mg/kg) or vehicle (1% NaHCO3 solution) was intraperitoneally co-injected with AT-III (500 units/kg) 30 min before Con A injection. Plasma 6k-PG-F1α (a) and MIP-2 (b) levels were determined 8 h after Con A treatment. **p<0.01 compared with 1% NaHCO3 solution plus distilled water vehicle treatment. #p<0.05 compared with 1% NaHCO3 solution plus AT-III treatment.

Fig. 7. Effect of AT-III and indomethacin on the plasma MIP-2 production induced by recombinant mouse TNF-α. Indomethacin (Indo, 5 mg/kg) or vehicle (1% NaHCO3 solution) was intraperitoneally co-injected with AT-III (500 units/kg) or vehicle (distilled water) 30 min before recombinant mouse TNF-α (2 μg/mouse) or vehicle (pyrogen-free saline) injection. Plasma MIP-2 level was determined 2 h after TNF-α treatment by ELISA. *p<0.05 compared with TNF-α plus distilled water vehicle treatment.

Fig. 8. Effect of AT-III and indomethacin on the infiltration of hepatic neutrophils. Indomethacin (Indo, 5 mg/kg) or vehicle (1% NaHCO3 solution) was intraperitoneally co-injected with AT-III (500 units/kg) 30 min before Con A injection. 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<0.05 compared with 1% NaHCO3 solution and distilled water vehicle treatment.