Interleukin-22 Protects against Acute Alcohol-Induced Hepatotoxicity in Mice

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The protective effects of interleukin-22 (IL-22) on acute alcohol-induced liver injury were investigated. Mice were gavaged with 7 doses of alcohol (56% wt/vol, 15.2 mL/kg of body weight for each dose) over the 24 h, and IL-22 (0.5 mg/kg BW) was given to the mice by injection into the tail vein 1 h after alcohol administration. The results indicated that acute alcohol administration caused prominent hepatic microvesicular steatosis and an elevation of serum transaminase activities, induced a significant decrease in hepatic glutathione in conjunction with enhanced lipid peroxidation, and increased hepatocyte apoptosis as well as hepatic TNF-alpha production. IL-22 treatment attenuated these adverse changes induced by acute alcohol administration. The protective effects of IL-22 on alcohol-induced hepatotoxicity were due mainly to its anti-inflammatory, anti-oxidant, and anti-apoptotic features.

Key words: alcoholic liver disease; interleukin-22; oxidative stress; apoptosis; TNF-alpha

Alcoholic liver disease (ALD) is a common medical consequence of chronic alcohol abuse, which ranks among the major causes of morbidity and mortality around the world.1) Although much progress has been made in understanding the pathogenesis of alcoholic liver disease, there remains no effective therapy for it. Accumulated evidence indicates that oxidative stress, abnormal cytokines, especially TNF-alpha, and hepatocyte apoptosis play important etiological roles in the development of alcoholic liver disease.2–4) Alcohol administration causes accumulation of reactive oxygen species (ROS), including superoxide, hydrogen peroxide, and hydrogen peroxide.5) Reactive oxygen species in turn cause lipid peroxidation of cellular membranes and protein and DNA oxidation, which results in hepatocyte injury.6–8) The significant role of tumor necrosis factor alpha (TNF-alpha) has been confirmed for both clinical and experimental alcoholic liver disease.9,10) Previous investigations indicated that rats chronically fed alcohol had much higher TNF-alpha than control rats, and that liver injury can be attenuated by agents such as prostaglandin analog, which downregulate TNF-alpha production.11) Hepatocyte apoptosis has been observed in both clinical and experimental alcoholic liver disease, and the apoptotic index directly correlates with disease severity.12–14) It has been found that inhibition of apoptosis can alleviate ethanol-induced liver injury in experimental models.15) Agents that have anti-oxidant, anti-inflammatory, and anti-apoptotic properties represent promising therapeutic interventions for ALD.

IL-22 is a member of the IL-10 family of cytokines and represents an important effector molecule of activated T cells and NK cells. Unlike interleukin-10, IL-22 does not affect cells of hematopoietic lineage. Instead, its target cells are certain tissue cells of the skin, liver and kidney. The main biological roles of IL-22 include boosting of innate immunity, protection from damage, and enhancement of regeneration. It can play a protective or a pathogenic role in chronic inflammatory diseases depending on the nature of the affected tissue and the local cytokine milieu.16) In the liver, it appears to be a survival factor for hepatocytes. Previous reports have suggested that it might have protective effects through anti-oxidant and anti-apoptotic functions in the case of liver injuries induced by FasL, ConA, CCl4, and chronic alcohol administration,17–20) but there have been few reports on its effects in an acute alcohol exposure model.

This study was undertaken to determine whether interleukin-22 can ameliorate acute alcohol-induced hepatotoxicity and to explore possible mechanisms.

Materials and Methods

Materials. Alcohol (56% wt/vol) was purchased from a commercial brewery of HongXing (Beijing, China).21) hIL-22 was prepared in our laboratory.22) MDA kits were from the Jiancheng Bioengineering Institute (Nanjing, China). An enzyme-linked immunosorbent assay (ELISA) kit for TNF-alpha was from eBioscience (San Diego, CA, USA). Trizol was from Invitrogen (Carlsbad, CA, USA). Reverse transcriptional polymerase chain reaction (RT-PCR) kits were from Takara Biotechnology (Shiga, Japan). All other chemicals were of analytical grade.

Animals and experimental design. Male ICR mice (25 ± 30 g, aged 9 weeks) were purchased from Beijing Vital River Experimental Animal (Beijing, China). The mice were maintained at 22 °C with a 12-h light/dark cycle. All experimental procedures were in compliance with the guidelines of China for animal care, which conform to internationally accepted principles for the care and use of experimental animals. Since we did not detect any adverse effects under IL-22 treatment alone in a preliminary experiment, the mice were randomly divided into three groups, control, alcohol treatment, and IL-22/alcohol treatment, eight mice per group. The mice daily received alcohol (15.2 mL/kg BW) by gavage for a total of 7 d. The control mice received an isocaloric maltose solution. In the IL-22/alcohol group, IL-22 was dissolved in PBS and administered at 0.5 mg/kg BW by tail vein injection 1 h after alcohol administration. The mice in the other two groups received an isocaloric maltose solution.
groups were given the same volume PBS by injection. During the entire process, the mice had free access to a standard laboratory chow and tap water. At 12 h after final alcohol administration, the mice were anesthetized and blood samples were taken for measurement of serum transaminase activities. Liver samples were taken at the same time for biochemical assays and histopathological examination.

Serum enzyme assay. Serum ALT, AST, and GGT activities were measured colorimetrically with diagnostic kits (Beijing BHKT Clinical Reagent, Beijing, China) following the instructions provided.

Histopathological examination. For histopathological analysis, fresh liver tissues were cut into 3 mm slices and fixed with 10% neutral formalin. The tissue slices were embedded in paraffin. Tissue sections of 5 μm were stained with hematoxylin and eosin, and were observed under light microscopy at a magnification of 200×. Pathological assessment involved examining four random fields on each slide, and was scored by a pathologist, who was blinded with respect to the treatment group, by a method described previously.23) The numbers of cells containing fat were estimated as follows: steatosis <25%, 1; 25–50%, 2; 50–75%, 3; >75%, 4.

Measurements of hepatic lipid peroxidation and glutathione content. Lipid peroxidation was evaluated by the thiobarbituric acid reactive substances method (TBARS), and was expressed as MDA concentration, as assayed with a commercial kit following the manufacturer’s instructions. Briefly, this method was used to obtain a spectrophotometric measurement of the color produced during the reaction of TBA with MDA at 535 nm. The MDA level was expressed as nmol/g of tissue. The hepatic glutathione content was determined in the liver homogenates after precipitation with 1% picric acid using yeast glutathione reductase, 5,5'-dithio-bis-(2-nitrobenzoic acid), and NADPH at 412 nm. Hepatic glutathione values were expressed as nmol/mg of protein.

Determination of hepatic TNF-alpha level. Liver samples were disintegrated in 5 volumes of iced-cold RIPA buffer. After incubation on ice for 30 min, the samples were centrifuged at 20,000 × g for 15 min at 4 °C. The resulting supernatants were used in the quantification of intracellular TNF-alpha levels with murine ELISA kits. The results were expressed as pg per mg of liver.

Isolation of total liver RNA and detection of TNF-alpha mRNA expression. Total RNA from liver tissue was isolated using Trizol reagent according to the manufacturer’s instructions. Total RNA (500 ng) was used for cDNA synthesis, and 10 μL of each reverse transcription product was added to 40 μL of reaction mixture containing 10 μL of 5 × PCR buffer, 0.25 μL of 5 μM Ex Taq® DNA polymerase, 1 μL of 100 μM of the corresponding primers, and 27.75 μL of ddH₂O for PCR amplification. The following primers were synthesized by Beijing Biomed (Beijing, China): GAPDH, 5'-ATT CAA CGG CAC ACT CAA-3' and 5'-CTT CTG GGT GGC AGT GAT-3'; and TNF-alpha, 5'-GGG TCC GGG CAG GTC TA-3' and 5'-GGG GGC TGG CTC TG TGA-3'. The sizes of the amplified PCR products were 394 bp for GAPDH and 458 bp for TNF-alpha. PCR was initiated at 94 °C for 3 min, followed by 30 cycles at 94 °C for 30 s, 50–59 °C for 30 s, and 72 °C for 1 min. Annealing temperatures for GAPDH and TNF-alpha were 50 °C and 59 °C respectively, and a final extension of 72 °C for 7 min was included. The amplified PCR products were subjected to electrophoresis at 100 V through 1.5% agarose gels for 30 min. A 100-bp DNA ladder was used as molecular marker. The bands were visualized with ethidium bromide and analyzed using Glyko BandScan software (Glyko, Novato, CA, USA).

Detection of apoptotic cells and caspase-3 activity assay. Apoptotic cells were detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) method using an in situ apoptosis detection kit (Roche, Mannheim, Germany). TUNEL-positive cells were counted under a ×40 objective, and data were expressed as the number of TUNEL-positive cells per 100 hepatocytes. To determine caspase-3 activity in the mouse liver, freshly harvested organs were subjected to a cell lysis buffer 12 h after final alcohol administration. To prepare whole-cell extracts, the tissue was homogenized on ice and subsequently centrifuged at 12,000 × g for 15 min at 4 °C. Ten μg of protein from the supernatant was used to measure caspase-3 activity using Caspase-3 Colorimetric Assay kit (KeyGen Biotech, Nanjing, China).

Statistical analysis. All data were expressed as means ± SD (n = 8). Statistical analysis was performed by Student’s t-test and ANOVA where appropriate. Differences between groups were considered to be statistically significant at p < 0.05.

Results

Effects of IL-22 on serum enzyme levels and histopathological changes

Since the effect of cytokine is dependent on its concentration, four dose groups of IL-22 (0.125 mg/kg, 0.25 mg/kg, 0.5 mg/kg, and 1 mg/kg) were chosen to evaluate their effects in acute alcohol-induced liver injury. We found that doses of 0.5 mg/kg and 1 mg/kg had more significant protective effects than the other two groups (data not shown). Hence IL-22 at a dose of 0.5 mg/kg was used in further study. The present study indicates that acute alcohol administration causes elevation of plasma ALT, AST, GGT activities, but IL-22 treatment attenuated alcohol-induced increases in serum transaminase activities (Table 1). Liver sections from mice treated with alcohol showed prominent microvesicular steatosis (Fig. 1B, arrows), which is charac-

Table 1. Effects of IL-22 on Plasma ALT, AST, and GGT Levels in Acute Alcohol-Intoxicated Mice

<table>
<thead>
<tr>
<th>Treatment (n = 8)</th>
<th>ALT (U/L)</th>
<th>AST (U/L)</th>
<th>GGT (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>29.5 ± 3.3</td>
<td>108.5 ± 11.1</td>
<td>12.8 ± 5.3</td>
</tr>
<tr>
<td>Alcohol</td>
<td>69.8 ± 13.9b</td>
<td>164.6 ± 33.6b</td>
<td>20.3 ± 8.8b</td>
</tr>
<tr>
<td>IL-22/Alcohol</td>
<td>44.5 ± 10.0b</td>
<td>132.6 ± 11.9b</td>
<td>14.5 ± 8.3b</td>
</tr>
</tbody>
</table>

The mice were treated as described in "Material and Methods." Blood was collected 12 h after alcohol administration. Values are means ± SD (n = 8). a, Significant difference from control group (p < 0.05); b, Significant difference from alcohol group (p < 0.05).

Fig. 1. Histological Appearance of Livers in All Groups. A, Normal histological appearance of livers of control mice. B, Histological appearance of livers of mice given alcohol. Appearance of prominent microvesicular steatosis (arrows). C, Livers from the IL-22-treated group showed less extensive microvesicular steatosis. D, Pathology was scored as described in "Materials and Methods." Data are presented as means ± SD. a, Significant difference from the control group (p < 0.05); b, Significant difference from the alcohol group (p < 0.05). H&E staining, magnification ×200.
characterized by the accumulation of small lipid droplets in the hepatocytes. In the IL-22 treatment group, less extensive microvesicular steatosis was observed (Fig. 1C).

Effects of IL-22 on hepatic lipid peroxidation and GSH concentration

Liver lipid peroxidation was assessed by measuring the malondialdehyde content in the liver. In comparison with control, alcohol administration caused a significant increase in the MDA content of the liver. Under IL-22 treatment, the alcohol-induced elevation of MDA was significantly attenuated (Fig. 2). In addition, the alcohol-treated mice showed a significantly decreased concentration of hepatic GSH in comparison with the control mice, but IL-22 treatment effectively attenuated the hepatic GSH decrease (Fig. 3).

Effects of IL-22 on the expression of hepatic TNF-alpha in alcohol-intoxicated mice

The overexpression of liver TNF-alpha induced by alcohol was evaluated at both the protein and the gene level by ELISA and RT-PCR respectively. The results showed that the level of hepatic TNF-alpha in the alcohol group was significantly higher than that in the control group 12 h after alcohol administration, and this increase was attenuated by IL-22 treatment (Fig. 4). A similar observation was made regarding TNF-alpha mRNA levels in the hepatic tissues (Fig. 5).

Discussion

IL-22, a dual-nature cytokine, has either inflammatory or protective properties depending on the inflammatory context, including the duration and amount of IL-22 present, the overall cytokine milieu, and the tissue involved. IL-22 plays a pro-inflammatory role in dermal inflammation. In contrast, it plays a protective role in hepatitis, experimental autoimmune myocarditis (EAM), and ulcerative colitis. Although hepatoprotective...
Abnormal cytokine metabolism, especially TNF-alpha, is another major feature of ALD. It has been found that alcohol exposure leads to increases of endotoxin (lipopolysaccharide, LPS) in the plasma, which binds to the LPS CD14/toll-like receptor 4 complex on Kupffer cells, causing NF-κB activation and TNF-alpha expression.9,29,30) In patients with alcoholic hepatitis, high plasma TNF-alpha levels correlate with disease severity and mortality.31) Several groups have found that anti-TNF-alpha antibody prevents liver injury in alcohol-fed rats, and that mice lacking the TNF-type I receptor did not develop alcoholic liver injury.32,33) Our results showed that acute alcohol administration enhanced hepatic TNF-alpha production, and that in vivo IL-22 treatment attenuated this increased TNF-alpha production. Thus, IL-22 might ameliorate acute alcohol-induced liver injury partly through inhibition of the TNF-alpha signaling pathway.

Hepatocyte apoptosis has been well documented in alcoholic liver disease in both clinical and experimental studies.12-14) It has been found that inhibition of apoptosis alleviated ethanol-induced liver injury in experimental models.15) Mechanistic studies have demonstrated that oxidative stress and death receptors (TNF-R1/TNF-alpha and Fas/FasL) are the major mediators in ethanol-induced hepatocyte apoptosis.34) Previous studies showed that IL-22 had an anti-apoptotic function through induction of Bcl-2 and Bcl-XL in vivo and in vitro.17,19) Our study show that IL-22 attenuates the increase in hepatocyte apoptosis and the elevation of hepatic caspase-3 activity induced by acute alcohol administration. This indicates that the hepatoprotective functions of IL-22 are related to its anti-apoptotic action.

In conclusion, the cytokine concentration is critical to its effects, and the present study indicates that IL-22 at a dose of 0.5 mg/kg offers significant protection against acute alcohol-induced liver injury.

Acknowledgments

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References