Laennec Protects Murine from Concanavalin A-Induced Liver Injury through Inhibition of Inflammatory Reactions and Hepatocyte Apoptosis

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The effect of Laennec, a hydrolyte of human placenta, on immune-mediated liver injury was investigated in vivo and in vitro in murine. Vena caudalis administration of concanavalin A (Con A) was employed to establish an in vivo liver-injury model, and in vitro hepatotoxicity was induced by 8 h interaction between Con A pre-treated hepatocytes and Con A-stimulated autologous splenic lymphocytes. Laennec was used for pre-treatment in the two models. Laennec decreased biochemical marker activity (alanine aminotransferase, ALT; lactate dehydrogenase, LDH) in serum and recovered the activity of superoxide dismutase (SOD) and myeloperoxidase (MPO), as well as the content of malondialdehyde (MDA) and nitric oxide (NO) in liver tissue. We also found that the DNA ladder induced by Con A in vivo was attenuated by Laennec. Furthermore, the leakage of aspartate aminotransferase (AST) and LDH in the supernatant of the co-culture system was decreased by addition of Laennec. Potential protective mechanisms were elucidated by DNA fragmentation assay and intercellular adhesion molecule-1 (ICAM-1) induction/inhibition experiments. Results showed that ICAM-1, which is related to the interaction between hepatocytes and lymphocytes, was inhibited by Laennec. These findings indicated that Laennec has potent activity against immune-mediated liver injury.

Key words Laennec; concanavalin A-induced hepatitis; apoptosis; intercellular adhesion molecule-1

In recent years it has become evident that human placenta is the source of a large number of biologically active molecules, such as hepatocyte growth factor (HGF), epidermal growth factor (EGF), transforming growth factor-α (TGF-α) and transforming growth factor-β (TGF-β). Laennec is prepared by purification of human placental extracts involving dialysis, heat treatment and hydrolysis. Although Laennec is a mixture of several anionic acids but does not contain HGF, it markedly stimulates liver regeneration in vivo and in vitro, which suggests that Laennec contains potent mitogens for hepatocytes. Laennec injection has been clinically used to treat chronic hepatic injuries and liver cirrhosis for over 40 years in Japan. However, little is known about its anti-immune effect, therefore, the development of its new pharmacological action is valuable.

Concanavalin A (Con A) induces selective liver failure, which is characterized by polyclonally activated T cells, and follows the systemic release of cytokines. As previously reported, Con A binds strongly to the hepatocyte plasma membrane, which correlates well with the degree of hepatotoxicity induced by Con A. This results from the fact that Con A-binding not only induces a direct toxic effect on primary cultured hepatocytes independent of the presence of T cells, but also enhances the susceptibility of hepatocytes to activated autologous lymphocytes. Both activation of lymphocytes and Con A-binding to hepatocytes are essential for hepatic cytotoxicity. Hepatocytes are first sensitized by Con A or even killed by Con A at a high concentration, and then interact with polyclonally activated T cells, which results in cell apoptosis or even necrosis. The interaction between hepatocytes and lymphocytes is mainly mediated by intercellular adhesion molecule-1 (ICAM-1)/lymphocyte function associated antigen (LFA-1) interaction. To a certain extent, the pathogenesis of this hepatitis model is similar to human immune-mediated hepatitis, such as autoimmune and viral hepatitis.

The present study was conducted to assess the protective effect of Laennec on Con A-induced hepatitis. The results in vivo and in vitro demonstrated that prophylactic administration of Laennec significantly ameliorated liver injury by reducing inflammatory reaction and inhibiting hepatocyte apoptosis.

MATERIALS AND METHODS

Animals and Reagents Female BALB/c mice and female Wistar rats (from the Experimental Animal Center of Dalian Medical University, Dalian, China) were handled and treated in accordance with the strict guiding principles of the National Institution of Health for experimental care and use of animals. Con A and collagenase were obtained from Wako (Japan); Laennec was provided by Japan Bioproducts Industry Co., Ltd. (Tokyo, Japan). Anti-rat ICAM-1 antibody was purchased from Boster Biological Technology Co., Ltd. (Wuhan, China). Other reagents will be further specified when mentioned.

Experimental Protocol. In Vivo Mouse liver damage was induced by injection of Con A (10 mg/kg), dissolved in saline, through the tail vein. Laennec (3.6 ml/kg) was intramuscularly injected to animals 30 min before Con A was used. Hepatocyte damage was assessed 8 h after Con A administration. Control mice were given the same volume of saline.

In Vitro Hepatocytes were isolated from female Wistar rats weighing 130—160 g by a two-step in situ collagenase perfusion method. The spleen was removed from the same rat, and the splenic lymphocytes were obtained by using Lymphocyte Separation solution (Lymphocyte-Rat; Tianjin

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Haoyang Bio Co., Ltd., China). Mixed lymphocytes were incubated for 3 h of cell attachment, and then the attached cells were discarded. Hepatocytes were pre-treated with Con A (20 μg/ml). Twenty-four hours later, the autologous lymphocytes (1.25×10^6 cells/ml) activated with Con A (20 μg/ml) for 48 h were added to the hepatocytes, and co-incubated at a lymphocyte/hepatocyte ratio of 10:1 for 8 h. In the ICAM-1 induction experiment, the pooled supernatant from Con A (20 μg/ml)-treated lymphocytes was added to the hepatocytes and incubated for 8 h (medium-stimulated group). Laennec (1 μl/ml) was used to pre-treat hepatocytes 1 h before addition of Con A.

**Determination of Biochemical Marker Leakage**

Mouse serum samples were collected 8 h after Con A administration and the supernatants were collected at the end of the incubation periods. The activities of aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) in the serum and supernatant were measured with assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The assays were performed exactly as described in the manufacturer’s instructions.

**Liver Tissue Superoxide Dismutase (SOD), Malondialdehyde (MDA), Myeloperoxidase (MPO) and Nitric Oxide (NO) Determination**

Liver samples were obtained, weighed, and homogenized on ice with saline 8 h after Con A administration. The assays for SOD, MDA, MPO, and NO were carried out using assay kits (Nanjing Jiancheng Bioengineering Institute) and performed exactly according to the manufacturer’s instructions. Proteins were determined using the Lowry method.

The inhibition rate (I) was calculated using the following formula (Eq. 1), where \( V \) represents the measured values of AST, LDH, SOD, MDA, MPO and NO in the control group Con A alone/model group, or Laennec treated group, respectively.

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I(\%) = \frac{V_{\text{Con A/model group}} - V_{\text{Laennec}}}{V_{\text{Con A/model group}}} \times 100
\]

**DNA Gel Electrophoresis Assay**

Mouse liver was removed and homogenized with lysis buffer (50 μg/ml proteinase K in 1 mM EDTA, 100 mM Tris–HCl, pH 8.0, and 1% SDS). After incubation for 2 h at 50 °C (or 37 °C overnight), the tissue lysate was collected, and the supernatant was extracted with a mixture of hydroxybenzene/chloroform/isooamyl alcohol (25:24:1). DNA was deposited with dehydrated alcohol and sodium acetate, and dissolved in TE buffer (1 mM EDTA, 10 mM Tris–HCl, pH 8.0). Electrophoresis was performed in 1.5% agarose gel containing 0.5 μg/ml ethidium bromide for 1 h, and the bands were visualized and photographed under transmitted ultraviolet light.

**Two-Step Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**

Total RNA of rat hepatocytes was extracted using the TRIZOL reagent (Invitrogen, Shanghai, China). The sequences of the primers for rat were as follows: ICAM-1 (forward): 5’CAGGACGACACTGTGCTTTGA3’; (reverse): 5’GTGACGCTTCAGGACCCTAGT3’; and β-actin (forward): 5’ATTTAGAACGCGATTGTAC3’; (reverse): 5’CATGGGAGACGATCTC3’. The sequences of the primers for mouse were as follows: (reverse) 5’GGCCGGCTGGGGATGACTTCT3’ and (forward) 5’CAGCAGCCCGCCCCAGGATG3’ and (forward) 5’GAGCATCTGACACACCAGTTGCAC3’ for bcl-2, and (reverse) 5’GAGCAGCCGCCCCAGGATG3’ and (forward) 5’GGTGACGGCGGGTGAGGAC3’ for bax, (reverse) 5’GGGCACA-GTGTGGGGTGCAC3’ and (forward) 5’CGGGCACCCACCCCA-CTTCTAC3’ for β-actin. Two-step RT-PCR was performed according to the protocol of the kit (Takara, Dalian, China), and amplified in a GeneAmp PCR system (Techne TC512, U.K.). RNA samples were first reverse transcribed and then immediately amplified by PCR. Quantity One (version 4.40) software (Bio-Rad, U.S.A.) was used to analyze the OD values of the electrophoresis bands. To exclude variations due to RNA quantity and quality, the data for caspase-3 and ICAM-1 were adjusted to β-actin expression (OD of caspase-3 or ICAM-1 mRNA versus OD of β-actin mRNA).

**Immunocytochemistry**

Immunocytochemistry staining for ICAM-1 was performed in situ on the 24-well collagen-coated plates using the streptavidin–biotin–peroxidase complex. After the medium was removed at different time points, the cells were fixed with 70% alcohol and pre-treated with 0.3% H$_2$O$_2$–methanol solution and 3% normal goat serum, respectively. The cells were then incubated at 4 °C overnight with rabbit anti-rat caspase-3 or ICAM-1 antibody (1:500 dilution), followed by incubation in biotinylated goat anti-rabbit IgG and then streptavidin–biotin–peroxidase complex both for 20 min at 37 °C. 3,3-Diaminobenzidine–H$_2$O$_2$ was used in color development, and the cells were counterstained with hematoxylin and visualized by inverted digital image light microscopy (Nikon Imaging Sales Co., Ltd., China). The cells stained brown were considered as positive, and the green levels were scanned using SimplePCI 6.2 software (Key Bond Int. Ltd., Hong Kong, China).

**Statistical Analysis**

Data were expressed as means±S.E.M. (in vivo) and means±S.D. (in vitro), and analyzed by analysis of variance (ANOVA) and Student’s t test using the statistical software SPSS 11.5. (SPSS Inc. Chicago, U.S.A.) Differences were considered significant at \( p<0.05 \).

**RESULTS**

**Effect of Laennec on the Activity of Cytosolic Enzymes in Con A-Intoxicated Mice in Vivo**

After intramuscular administration of Laennec to Con A-intoxicated mice, the activity of liver cytosolic enzymes alanine aminotransferase (ALT) and LDH in serum was reduced by 48.96 and 65.73%, respectively (Fig. 1).

**Effect of Laennec on the Activity of Biochemical Marker Leakage in Vitro**

To examine whether Laennec...
Con A-Intoxicated Mice

To determine whether Laennec tent was decreased by 50% (Fig. 4).

Treating mice. After administration of Laennec, the MPO content of liver tissue was measured after intramuscular injection of Laennec in Con A-treated mice. After administration of Laennec, the MPO content was decreased by 50% (Fig. 4).

Effect of Laennec on MPO Activity in Liver Tissue of Con A-Intoxicated Mice To examine the effect of Laennec on the levels of NO in liver tissue in Con A-treated mice after intramuscular injection of Laennec in Con A-treated mice. After administration of Laennec, the NO content was reduced by 56.02% (Fig. 5).

Inhibition of Hepatocyte Apoptosis by Laennec Pretreatment in Vivo To examine whether the protective effects of Laennec correlated with apoptosis inhibition, the inhibitory effect on DNA fragmentation was investigated. A typical DNA ladder was found in the Con A alone group; however, the DNA ladder was significantly attenuated in the Laennec-treated group (Fig. 6). We also determined the effect of Laennec on the expression of apoptosis-associated genes bcl-2 and bax. The RT-PCR results showed that Con A significantly increased the ratio of bcl-2/bax and Laennec depressed the ratio markedly (Fig. 7).

Reduction of ICAM-1 Expression by Laennec Pretreatment Evidence of Laennec showing a protective effect on cytotoxicity induced by interaction between hepatocytes and lymphocytes prompted us to determine whether it regulated ICAM-1 expression. Expression of ICAM-1 was induced by addition of the pooled supernatant from Con A-activated lymphocytes and then levels of AST and LDH (Fig. 2B) induced by the co-culture, compared to the untreated group or to the Con A (20 μg/ml) alone group. Laennec decreased the level of AST and LDH by 44.93 and 44.53%, respectively.

Effect of Laennec on SOD Activity and MDA Content in Liver Tissue in Con A-Intoxicated Mice To examine the antioxidative effect of Laennec on Con A-induced hepatitis, we analyzed SOD activity and MDA production in liver tissue. Compared with the control group, the level of liver SOD in the Con A alone group was increased by 41.94% (Fig. 3A) and the MDA content was decreased by 47.86% (Fig. 3B).

Effects of Laennec on SOD Activity and MDA Content in Liver Tissue of Con A-Intoxicated Mice To examine the antioxidative effect of Laennec on Con A-induced hepatitis, we analyzed SOD activity and MDA production in liver tissue. Compared with the control group, the level of liver SOD in the Con A alone group was increased by 41.94% (Fig. 3A) and the MDA content was decreased by 47.86% (Fig. 3B).

Effects of Laennec on MPO Activity in Liver Tissue of Con A-Intoxicated Mice To examine the effect of Laennec on Con A-induced liver injury in response to hepatic neutrophil recruitment, the MPO content of liver tissue was measured after intramuscular injection of Laennec in Con A-treated mice. After administration of Laennec, the MPO content was decreased by 50% (Fig. 4).

Effect of Laennec on NO Content in Liver Tissue of Con A-Intoxicated Mice To determine whether Laennec can affect inflammatory mediator expression, we examined...
(20 μg/ml)-treated lymphocytes (PSL) to hepatocytes, then we determined the effects of Laennec on this. As predicted, after incubation with Laennec for 24 h, the expression of ICAM-1 mRNA was reduced by 53.13% (Fig. 8). Similarly, albeit to a lesser extent, the expression of ICAM-1 protein by immunocytochemical staining in hepatocytes was also reduced by Laennec (Fig. 9).

**DISCUSSION**

We examined the effect of Laennec on hepatic cytotoxicity induced by Con A and the possible molecular mechanisms in vivo and in vitro. Our results indicated that Laennec significantly inhibited the activity of cytoplasmic enzymes (ALT, LDH, MPO and NO) and recovered the cellular oxy-associated index (SOD and MDA). At the same time, Laennec inhibited the apoptosis of hepatocytes induced by Con A. Furthermore, our results also indicated that Laennec inhibited ICAM-1 expression in hepatocytes, which suggests that Laennec is involved in regulating immune-associated molecules.

In vivo results showed that Laennec pretreatment markedly alleviated Con A-induced acute liver injury, as demonstrated by a reduction of ALT and LDH (Fig. 1). In line with our findings in vivo, Laennec can also suppress the cytotoxicity induced by the interaction between Con A-treated hepatocytes and Con A-stimulated splenic lymphocytes (Fig. 2). ICAM-1/LFA-1 is thought to be critical in inducing further apoptosis via the Fas/perforin-mediated pathway in T-cell-mediated cytotoxicity. The presence of anti-ICAM-1/LFA-1 antibodies almost completely inhibits liver injury in vivo, while it partially inhibits hepatic cytotoxicity (ca. 50%) in vitro, which indicates that there are still other molecules involved in the interaction. Based on early reports, Con A binds to murine major histocompatibility complex (MHC) molecules to mimic their antigenic properties, which explains the fact that activated T cells only recognize Con A-stimulated hepatocytes. Therefore, both Con A binding to hepatocytes and ICAM-1/LFA-1 interaction are essential for hepatic-cytotoxicity-activated lymphocytes, which is the primary theoretical basis of our in vitro model—cytotoxicity induced by the interaction between Con A-treated hepatocytes and Con A-activated lymphocytes. Cytotoxicity induced by interaction between hepatocytes and lymphocytes was markedly mitigated by Laennec pre-treatment. Further experiments are planned to elucidate whether Laennec regulates ICAM-1 expression in hepatocytes. Inflammatory cytokines up-regulate ICAM-1 expression in cultured hepatocytes and T-lymphocyte adhesion. It is inevitable that hepatocytes will be affected by cytokines derived from activated lymphocytes and Con A-stimulated splenic lymphocytes (Fig. 2).

![Fig. 7. Effect of Laennec on the Expression of bcl-2 and Bax mRNA in Hepatocytes](image)

Lane 1: Con A (10 mg/kg) alone. Lane 2: Con A + Laennec (3.6 ml/kg). Lane 3: control. *p<0.05 vs. control group; #p<0.05 vs. Con A alone group. Each value represents the mean of three replicates; error bars represent S.E.M.

![Fig. 8. Effect of Laennec on the Expression of ICAM-1 mRNA in Hepatocytes](image)

Fresh isolated hepatocytes (2.5 × 10^5) were seeded in 24-well plates. 24 h later, the culture medium was carefully replaced with pooled supernatant from Con A (20 μg/ml)-treated lymphocytes. Hepatocytes were pre-treated with Laennec (1 μl/ml) 1 h before the medium of lymphocytes was added. Lane 1: control. Lane 2: medium-stimulated group. Lane 3: Laennec (1 μl/ml) pre-treated group. ***p<0.001 vs. control group; *p<0.05 vs. medium-stimulated group. Each value represents the mean of three replicates; error bars represent S.D.

![Fig. 9. Immunocytochemical Staining of ICAM-1 in Hepatocytes](image)

The cell stained brown were considered as positive cells. Grey level reflects the extent of ICAM-1 expression and exhibits an inverse proportional relationship between them. Photos A—C ×200 magnification, bar=50 μm. ***p<0.01 vs. control group; *p<0.05 vs. medium-stimulated group. Each value represents the mean of three replicates; error bars represent S.D.
cytes when co-cultured with Con A-pre-treated lymphocytes, based on which, we used medium of Con A (20 μg/ml)-treated lymphocytes to induce ICAM-1 expression in hepatocytes. Results in vitro showed that Laennec down-regulated ICAM-1 expression either at the gene (Fig. 7) or at the protein (Fig. 8) level. This finding may explain the increased hepatocyte survival in co-cultures of hepatocytes and lymphocytes (Fig. 2).

Laennec suppressed lipid peroxidation and increased antioxidant level. During inflammation, neutrophils as well as Kupffer cells produce several reactive oxygen species to cause lipid peroxidation.\(^{20}\) Although the severity of lipid peroxidation in vivo is not sufficient to cause direct cell damage, lipid peroxidation is a potent chemotactic factor for neutrophils and is involved in neutrophil recruitment and aggravation of the injury.\(^{21,22}\) As our in vivo results showed, Laennec increased the activity of SOD and decreased MDA levels in liver tissue in Con A-intoxicated mice (Fig. 3). This finding, as well as the fact that Laennec down-regulated expression of ICAM-1, helps explain the inhibition of apoptosis in vivo (Figs. 6, 7). Bcl-2 protein is an anti-apoptotic factor, and bax inactivates Bcl-2 by binding to it to form a heterodimer. The ratio of bcl-2 to bax mRNA is a pivotal factor in determining whether or not apoptosis occurs in cells.\(^{23,24}\) NO mediates tissue injury through pathways including inhibition of mitochondrial respiration, inactivation of proteinase inhibitors, and formation of free radicals,\(^{25}\) that is to say, NO is a substance extremely toxic to cells. Laennec pretreatment significantly decreased liver NO content, which resulted in an evident reduction in liver damage when compared with the control group. Moreover, Laennec markedly reduced the MPO level induced by Con A in vivo (Fig. 4), which indicated that Laennec reduced infiltration of leukocytes into inflammatory sites. These above findings indicated that Laennec might exert a hepatoprotective effect by improving activity of the endogenous antioxidant enzyme.

In conclusion, Laennec exerted a protective effect on liver injury induced by Con A in vivo and in vitro, possibly through suppression of the inflammatory reactions and apoptosis. Besides, Laennec also down-regulate the expression of ICAM-1. Our results provide the novel insights into the mechanisms of Laennec as anti-hepatitis candidate in the immune-mediated hepatitis and further investigation will provide more information to help establish its safety and clinical potentials.

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**REFERENCES**