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NLRP3 inflammasome activation in liver cirrhotic patients

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ABSTRACT

Aims: NLRP3 inflammasome activation is involved in the mechanism of liver cirrhosis. In this study, we investigated the levels of plasma IL-1β and IL-18 and their relationship to component traits in patients with liver cirrhosis, and NLRP3 inflammasome expression in liver of patients with liver cirrhosis.
Methods: A total of 75 patients with liver cirrhosis and 41 age-matched healthy control subjects were enrolled in this study. NLRP3 inflammasome expression and activation were measured in liver of patients with liver cirrhosis. Plasma IL-1β and IL-18 levels were examined by ELISA in patients with liver cirrhosis.
Results: Plasma IL-1β and IL-18 levels were also significantly higher in patients with liver cirrhosis than in control subjects. Plasma IL-18 levels were significantly positively associated with Child-Pugh classification, IL-1β levels, diastolic blood pressure, aspartate aminotransferase, total bilirubin, direct bilirubin, activated partial thromboplastin timealk and aline phosphatase. Plasma IL-18 levels were significantly negatively associated with albumin. NLRP3 and cleaved caspase-1 expression were increased in the livers of patients with cirrhosis compared with the livers of control subjects.
Conclusions: Plasma IL-1β and IL-18 levels were higher in plasma in patients with liver cirrhosis than in control subjects, and plasma IL-18 levels were significantly positively associated with Child-Pugh classification and IL-1β levels. In addition, NLRP3 and cleaved caspase-1 expression were increased in the livers of patients with cirrhosis.

1. Introduction

Most forms of chronic liver injury and disease progress first to fibrosis and then to cirrhosis, and inflammation is a common element in the pathogenesis of various forms of chronic liver injury and disease. Following liver injury, some hepatic inflammatory cytokines, such as IL-1, are rapidly expressed and directly activate hepatic stellate cells (HSCs), causing the liver to transition from a state of injury to a state of repair or fibrogenesis [1]. Current evidence suggests that NLRP3 inflammasome activation is directly involved in the pathogenesis of liver fibrosis and cirrhosis [2,3]. The NLRP3 inflammasome consists of a protein-nucleotide-binding domain and a leucine-rich repeat pyrin-containing protein-3 (NLRP3), an apoptosis-associated speck-like protein containing CARD (ASC) and the pro-apoptotic protease caspase-1. Innate immune cells express the NLRP3 inflammasome; however, increasing amounts of evidence indicate that the NLRP3 inflammasome also exists and is functionally active in non-immune cells, such as hepatocytes [4,5] and HSCs [6]. NLRP3 inflammasome activation is believed to occur via the following two-step process: an inflammatory signal, or signal 1, upregulates pro-IL-1β and pro-IL-18 expression, and a second inflammatory signal, or signal 2, triggers functional inflammasome (IL-1β and IL-18) activation through an inflammasome-inducing ligand [7,8]. Both IL-1β and IL-18 are classical cytokines driving inflammation after inflammasome activation by binding to their respective receptors. IL-1β through engagement of IL-1R type I and IL-18 through IL-18R, both activate NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) signaling and induce a dedicated proinflammatory gene expression program in the target cell. In vitro studies have shown that IL-1β promotes the proliferation and transdifferentiation of HSCs [9,10] with a substantial increase in levels of their fibrogenic markers, including metalloproteinases [11,12] tissue inhibitor of metalloproteinases-1, collagen-1α1, and transforming growth

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factor-β (TGF-β), and a decrease in the expression of Bambi (a negative regulator of TGF-β signaling) [13].

The aim of this study was to explore the expression of plasma IL-1β and IL-18 in liver cirrhosis patients. Towards that end, we measured the plasma levels of IL-1β and IL-18 and analyzed their association with clinical parameters, and NLRP3 inflammasome expression in liver tissue of liver cirrhosis patients.

2. Methods

2.1. Patients

Patients newly diagnosed with liver cirrhosis (n = 75) were included in this study. Patients were diagnosed with liver cirrhosis according to their histologic or transabdominal ultrasound findings and physical exam findings (signs of portal hypertension, such as ascites, splenomegaly, and fundic or esophageal varices). Liver cirrhosis is caused by chronic hepatitis B, and no patients with viral hepatitis type B (HBV) who were enrolled in our study were treated with anti-HBV drugs. Pregnant women and patients with ketoacidosis, recent infections, severe renal disease, cardiovascular disease or chronic inflammatory or autoimmune diseases were excluded from the study. The sample size was calculated with PASS 19.0 software.

Healthy subjects (n = 41) with no clinical diseases who were undergoing a health checkup in a hospital served as control subjects. This study was reviewed and approved by the Ethics Committee of the Second Affiliated Hospital of Harbin Medical University (reference number KY2016-126) and complied with the Declaration of Helsinki. Informed consent was obtained from all subjects before the study was initiated. Blood samples were collected using tubes containing Na2-EDTA (1 mg/mL) and 500 kIU/mL aprotinin (Sigma, St. Louis, MO, USA) and then centrifuged at 3000 rpm for 10 min at 4 °C to obtain plasma, which was stored at −80 °C until assayed.

The liver tissue specimens were obtained from 8 patients with liver cirrhosis and 8 control subjects (liver graft donors) via biopsy.

2.2. Measurement of other parameters

Blood urea nitrogen, creatinine, aspartate aminotransferase, alanine aminotransferase, albumin (ALB), gamma glutamyl transpeptidase (RGT), alkaline phosphatase (AP), choline esterase (CHE), total bilirubin (TB), direct bilirubin (DB) and indirect bilirubin (IDB) levels; activated partial thromboplastin time (APTT) and prothrombin time (PT); hemoglobin levels; platelet (PLT) counts and white blood cell (WBC) counts were assessed by routine biochemical analyses.

2.3. Measurement of plasma IL-1β and IL-18 concentrations

Plasma IL-1β and IL-18 levels were measured using an ELISA kit (R&D systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

2.4. Western blotting

Frozen liver specimens were homogenized in ice-cold lysis buffer in the presence of protease inhibitor cocktail, after which the homogenates were centrifuged at 12,000 g for 10 min at 4 °C. The protein content of the supernatant was determined using a BCA-200 Protein Assay Kit (Beyotime, China), according to the manufacturer's instructions. Equal amounts of protein (40 μg) were subsequently loaded into a gel and separated by 12% SDS-PAGE before being transferred to a PVDF membrane, which was blocked via incubation with 5% non-fat dry milk for 1 h at room temperature with agitation. The membrane was then incubated with the following primary antibodies overnight at 4 °C: anti-β-actin (1:1000), anti-NLRP3 (1:1000), anti-cleaved caspase-1 (1:1000), anti-IL-1β (1:1000), anti-IL-18 (1:1000), anti-I-collagen (1:1000), and anti-III-collagen (1:1000). The membrane was subsequently washed thrice for 10 min each. The proteins were visualized using a horseradish peroxidase-linked secondary antibody and ECL detection. The levels of the various proteins were normalized to the levels of β-actin.

2.5. Statistical analysis

Clinical data are reported as means (standard deviations) or medians (interquartile range, IQR). Continuous variables were analyzed with Wilcoxon's test, and categorical variables were analyzed with $\chi 2$ tests. The associations between plasma IL-1β and IL-18 levels and other variables were evaluated using Spearman's correlation co-efficient. For comparison between two groups, we used an unpaired Student's t-test, all analyses were performed with GraphPad Prism 5.0 software, $P < 0.05$ was considered statistically significant.

3. Results

3.1. Baseline clinical and demographic characteristics of the study population

We noted no significant differences in age or gender between the control and liver cirrhosis groups. Patients with liver cirrhosis had significantly higher creatinine, aspartate aminotransferase, alanine aminotransferase, TB, DB, IDB and PT levels and significantly lower ALB, CHE, hemoglobin levels and PLT counts than control subjects. Sixty-six patients with cirrhosis had Child-Pugh class A disease, 7 patients had Child-Pugh class B disease, and 2 patients had Child-Pugh class C disease.

Extensive liver fibrosis is a common finding in patients with progressive liver cirrhosis. Further analysis showed that type I and III collagen were increased in patients with liver cirrhosis compared with control subjects by western blotting analysis (Fig. 1).

3.2. Plasma IL-18 and IL-1β levels in patients with cirrhosis

Plasma IL-18 levels were significantly higher in patients with liver cirrhosis than in control subjects, the median plasma IL-18 concentration in patients with liver cirrhosis was 91.3 pg/mL (range, 61.8—116.3 pg/mL), and the median plasma IL-18 concentration in control subjects was 62.5 pg/mL (range, 50.7—75.3 pg/mL)($p = 0.004$)(Fig. 2a). Plasma IL-1β levels were significantly higher in patients with liver cirrhosis than in control subjects($p < 0.001$). Specifically, the median plasma IL-1β concentration in patients with liver cirrhosis was 18.7 pg/mL (range, 14.1—25.3 pg/mL), and the median plasma IL-1β concentration in control subjects was 12.5 pg/mL (range, 11.4—13.6 pg/mL) (Fig. 2b).

Plasma IL-18 levels were significantly positively associated with Child-Pugh classification($rs = 0.386$; $p = 0.001$), IL-1β levels ($rs = 0.243$; $p = 0.036$), diastolic blood pressure($rs = 0.241$; $p = 0.037$), aspartate aminotransferase($rs = 0.336$; $p = 0.003$), total bilirubin($rs = 0.357$; $p = 0.002$), direct bilirubin($rs = 0.465$; $p < 0.000$), activated partial thromboplastin timealk($rs = 0.299$; $p = 0.009$) and aline phosphatase($rs = 0.251$; $p = 0.030$). Plasma IL-18 levels were significantly negatively associated with albumin($rs = -0.290$; $p = 0.011$)(Table 1). However, plasma IL-1β was not significantly associated with clinical parameters in patients with liver cirrhosis.

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