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Intraperitoneal Administration of Fetuin-A Attenuates d-Galactosamine/Lipopolysaccharide-Induced Liver Failure in Mouse

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Abstract

Background Fulminant hepatic failure (FHF) is a devastating syndrome, which sometimes results in death or liver transplantation, in which inflammation would aggravate the development of fetuin-A which would act as an anti-inflammatory factor and may be an available approach to attenuate FHF.

Aims The purpose of this study was to investigate the effects of fetuin-A on d-galactosamine/lipopolysaccharide (d-GalN/LPS)-induced liver failure in mice.

Methods A mouse model of FHF induced by d-GalN/LPS was established and fetuin-A was injected intraperitoneally prior to d-GalN/LPS treatment. At different time points after d-GalN/LPS intervention, serum TNF-α and IL-6 levels were measured by ELISA. Fetuin-A mRNA and protein expression in liver tissues was assessed by RT-PCR, Western blotting and immunohistochemical staining. Besides, an observation of liver tissue injury, the apoptosis of hepatocytes, was analyzed by TUNEL assay.

Results Expression of fetuin-A mRNA and protein in liver tissue were significantly and gradually decreased after d-GalN/LPS administration. A pre-intervention of exogenous fetuin-A significantly improved the liver function, decreased TNF-α and IL-6 expression in peripheral blood, and liver tissue inhibited hepatocyte apoptosis responded to d-GalN/LPS induction so as to decrease the mortality rates of FHF mouse. Meanwhile, fetuin-A was negatively correlated with the hepatic pathological score and TNF-α protein staining in FHF mouse.

Conclusions An intraperitoneal injection of fetuin-A attenuates d-GalN/LPS-induced FHF in mice. Fetuin-A might be a protective agent of liver damage partly through inhibiting liver inflammatory response and hepatocyte apoptosis.

Keywords Liver failure · Fetuin-A · TNF-α · Lipopolysaccharide · Endotoximia

Introduction

Fulminant hepatic failure (FHF) is a condition with rapid deterioration of hepatocyte function resulting in hepatic encephalopathy and/or coagulopathy in the patients [1]. This is a devastating syndrome, which results in death or the need for liver transplantation in over 50% of the cases [2]. Urgent liver transplantation has become the standard care for most FHF patients in some countries where FHF survival rates have shown progressive and substantial improvement with 1-year survival exceeding 80% [2, 3]. However, in some other countries, access to liver transplantation and other extracorporeal liver-assist devices is severely limited. On the other hand, FHF also has a positive reversible potential, where survivors recover
completely without any sequelae. Accordingly, if the individual can be supported properly throughout the acute event, recovery will follow the rapid regeneration of hepatocytes. Thus, it is of important clinical significance to find a more effective approach to treat patients with FHF when a liver transplantation is not available.

The mechanisms of FHF are quite complex and still not fully defined. It is generally acknowledged that immunologic injury, hypoxic ischemic lesion and endotoxemia are the three main causes. Normally, the transition from stable cirrhosis to FHF is caused by systemic inflammatory response, characterized by a predominantly proinflammatory cytokine profile [4]. Therefore, removal of proinflammatory cytokines such as TNF-α in plasma might be beneficial to FHF patients.

Fetuin-A (formerly named as α2-Heremans-Schmid glycoprotein, AHSG) is an abundant serum protein that is exclusively produced by the liver, tongue and placenta [5, 6]. As a glycoprotein, fetuin-A carries two N-linked and three O-linked oligosaccharide chains that terminate with sialic acid residues and can bind biogenic cationic ions (e.g., Ca2+) and other anti-inflammatory molecules (e.g., spermine) [7, 8]. Accordingly, fetuin-A has been proposed as an endogenous inhibitor of pathological mineralization or calcification [9–12] and an opsonin of cationic molecules (such as spermine) [7]. During FHF, it was found that liver would orchestrate a defensive response to inflammation by altering the synthesis and release of “acute phase proteins” (APPs), such as fetuin-A [13]. Data showed that circulating fetuin-A level decreased significantly (about 30–50 %) during the acute phase of inflammation and injury [7], while some classic proinflammatory cytokines such as TNF-α, IL-1, and IL-6 increased [14]. Moreover, higher concentrations of fetuin-A would act as an anti-inflammatory factor, inhibiting bacterial endotoxin-induced production of proinflammatory mediators (such as TNF, IL-1, and nitric oxide) in macrophage cultures effectively implying that fetuin-A is associated with inflammation activity [15]. In an animal model of arrageenan-induced paw edema, intraperitoneal administration of fetuin-A attenuated local TNF production and inflammation [16]. In early cerebral ischemic injury, fetuin-A played a protective role partly by attenuating the brain inflammatory response [17]. Therefore, fetuin-A may be an available approach to control serious inflammation response in FHF. To verify our hypothesis, we established a mouse model of FHF to explore roles of fetuin-A in the pathogenesis of FHF.

D-GalN is a commonly used hepatotoxic drug in the FHF model that induces dose-dependent liver injury while LPS play an important role in liver injury, and the mechanism is as follows: (1) Through inflammatory factors, LPS can damage the completeness of the vascular endothelial cell and results in microvascular damage, formation of microthrombus and leads to intrahepatic hemorrhage and hepatocyte necrosis. (2) LPS promotes secretions of multiple pro-inflammatory cytokines by Hepatic stellat cells, e.g. TNF-α, IL-1β and IL-6 etc. Combination of D-GalN and LPS created a better simulation of FHF and has been widely used recently. In our previous work, we established a stable and effective mouse model of fulminant hepatic failure (FHF) induced by D-GalN (600 mg/kg), and LPS (0.5 mg/kg) through factorial experiment [18–20].

Methods

Animal Studies

Eight-week-old BALB/c mice (SPF, female, 28–30 g) were obtained from the Hunan Agro-Techniques University Laboratories (Changsha, China). To induce acute liver failure, the mice (except for the controls) were injected intraperitoneally (i.p.) with D-GalN (600 mg/kg, Department of Biomedical Engineering, Chongqing Medical College, China) and LPS (0.5 mg/kg; Escherichia coli, Sigma, St. Louis, MO) dissolved in phosphate-buffered saline (PBS). Fetuin-A (100 mg/kg body weight, ICN, Sigma) was injected intraperitoneally 1 h prior to D-GalN/LPS treatment.

The mice were then randomly assigned into five groups (n = 6 for each): Blank control: no intervention; FHF group: 600 mg/kg D-GalN with 0.5 mg/kg LPS, i.p.; Sham group: physiologic saline with 600 mg/kg D-GalN and 0.5 mg/kg LPS, i.p.; Fetuin-A protection group: 100 mg/kg fetuin-A with 600 mg/kg D-GalN and 0.5 mg/kg LPS, i.p.; Anti-fetuin-A group: 100 mg/kg rabbit-anti-mouse fetuin-A with 600 mg/kg D-GalN and 0.5 mg/kg LPS, i.p.

For continuous stimulation in vivo, BALB/c mice were maintained under 12-h light/dark cycles with a regular diet of LPS (0.5 mg/g body weight), D-GalN or bovine serum albumin (100 mg/g body weight), intraperitoneal bolus of bovine fetuin-A (100 mg/kg body weight), rabbit anti-mouse fetuin-A and comparable amount of sterile saline respectively (except for blank control groups).

At selected time points of the 3rd, 6th and 9th hour after D-GalN/LPS treatment, the mice were anesthetized and blood samples were collected and the animals were sacrificed. The liver tissues were harvested by resections and either stored in liquid nitrogen for RT-PCR and Western blot or fixed in 10 % formaldehyde for immunohistochemistry and TUNEL analysis. The mortality rate of the mice was observed 24 h after the experiment began in another parallel experiment group. Furthermore, to observe the potential toxicity and side effects of fetuin-A in cardiac, renal and hepatic tissue, fetuin-A (100 mg/Kg) was injected intraperitoneally in normal mouse (n = 6), the animals were sacrificed and the tissues of liver, heart and kidney were harvested by resections after 24 h. The pathological

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changes were observed in a general way. The Animal Use Committee of Xiangya Hospital approved all protocols for treating animals and all mice were treated humanely during the whole study period.

RT-PCR

For quantification of fetuin-A mRNA expression in mouse liver tissue, total RNA was isolated and the first strand cDNA was synthesized by the Advantage RT-for-PCR kit (BD Biosciences, Palo Alto, CA). The primers for mouse fetuin-A (GenBank Accession No.: BC012678) as: 5′-TCGGAGTGTATGAGATGGAAG-3′ (sense) and 5′-GGCAGTGTTGACGGTGTCGGAC-3′ (antisense) were synthesized by Boya Company (Shanghai, China). The regular PCR producing a 281 bp fragment was carried out and glyceraldehydes 3-phosphate dehydrogenate (GAPDH, GenBank Accession No.: BC029618) was used as an internal control.

Western Blotting

Mouse fetuin-A-specific antibodies were obtained from Dr. Haichao Wang (USA). Goat anti-rabbit aquaporin-2 antibodies were obtained from Santa Cruz (Santa Cruz, CA). After SDS-PAGE, the proteins were transferred onto PVDF membrane and hybridized with specific primary antibodies, and subsequently were incubated with HRP-conjugated sheep anti-mouse IgG. Bands were visualized using the ECL kit (Amersham, Piscataway, NJ) per the manufacturer’s instruction [21, 22].

Histological Assessment and Immunohistochemical (IHC) Staining

Four μm-thick formalin-fixed liver tissue sections were incubated with the primary antibody, subsequently incubated with the secondary antibody conjugated with streptavidin–biotin–peroxidase complex (Zhongshan Goldenbridge Biotechnology, Beijing, China) and a color reaction was developed using 3,3-diaminobenzidine tetrahydrochloride (Sigma). The negative control slides were probed with normal rabbit serum under the same experimental conditions.

The histological characteristics of liver tissues were scored by Ishak Classification [23]. In each case, the immunoreactivity was determined in five random fields and the IHC scoring was made independently by two experienced pathologists. The images were analyzed using Image J, a digital image processing system.

TUNEL Assays

Apoptosis of hepatocytes was detected using terminal deoxynucleotidyl transferasemediated dUTP nick-end labeling (TUNEL, red fluorescence) kit (Doctor Biotechnology, Wuhan, China). Images were observed on a Nikon Eclipse E800 fluorescent microscope. The average number of apoptotic cells per high-power field (200× magnification) was calculated in ten random visual fields for each sample and six samples for each time point, and the apoptotic index (AI) of each specimen was scored.

Liver Function Tests and Cytokine Quantification

The serum concentrations of TNF-α and IL-6 in mice were measured by commercial enzyme-linked immunosorbent assays (ELISA, Bio Vendor Laboratory Medicine, Brno, Czech Republic). The level of alanine aminotransferase (ALT) in mice serum was determined using an automatic biochemical analyzer (HITACHI-7170A, Hitachinaka City, Japan).

Statistical Analysis

The continuous data were presented as mean ± SD. Using SPSS 15.0 software (SPSS, Chicago, IL), one-way ANOVA tests were employed for comparison among all groups, and Student–Newman–Keuls test was subsequently applied to compare the statistical difference between two groups. Meanwhile, multivariate linear regression analysis was performed using log-transformed data and Pearson’s correlation coefficient to test the correlation between the relevant covariates (such as pathological score, grey scales of TNF-α and fetuin-A, and apoptotic index). All tests were two-sided and p value <0.05 was considered to be statistically significant.

Results

Liver Injury, Inflammation, and Hepatocyte Apoptosis in FHF Mouse

At the third hour after t-GalN/LPS administration, the mice in the FHF group exhibited slightly disordered liver structure, active blood sinusoid response, ballooning degeneration of hepatocytes, spot necrosis around the central veins of the hepatic lobules, scattered inflammatory cell infiltration, and eosinophilic bodies. After 6 h, the liver tissues showed aggravated structural disorder, active blood sinusoid response, lamellar necrosis, eosinophilic hepatocytes, moderate to severe inflammatory cell infiltration and numerous apoptotic bodies. After 9 h, the tissue showed widespread necrosis, abundant erythrocyte obturation, piles of inflammatory cell infiltration, numerous apoptotic bodies and eosinophilic bodies (Fig. 1a). The Ishak score
showed that the injury in liver tissue aggravated over time (Table 1). The IHC staining of TNF-α staining in liver tissue reached a maximum at the ninth hour after d-GalN/LPS injection in the FHF group (Fig. 1b). The peak of apoptotic index of hepatocytes was observed in the sixth hour in the FHF mouse model (Figs. 1c, 3e).

Table 1 The Ishake scores of liver tissue at different time points after injection of d-GalN/LPS in the FHF group

| Time point | Mean value | Standard error (SE) |
|------------|------------|---------------------|
| 0 h        | 0.000      | 0.000               |
| 3rd hour   | 5.833      | 0.654               |
| 6th hour   | 9.833      | 0.654               |
| 9th hour   | 17.333     | 0.333               |

TNF-α in hepatic tissues at the 0, third, sixth, and ninth hours after d-GalN/LPS induction in the FHF mouse (×400). Highly concentrated staining of TNF-α in brown color was obvious both in the hepatocyte cytoplasm and on the cell membrane during the ninth hour. c Apoptosis of hepatocytes in FHF mouse (×400); the stained apoptotic cells were indicated with arrowheads.

Fetuin-A Expression in Liver Tissue was Reduced by d-GalN/LPS Induction in FHF Mouse

In FHF mouse model, both mRNA and protein expression (Fig. 2a, b) of fetuin-A in liver tissue were decreased significantly and gradually from the sixth to the ninth hour after d-GalN/LPS administration. Meanwhile, the significantly decreased fetuin-A protein expression, which was induced by d-GalN/LPS, was further identified by IHC staining in the FHF group (Fig. 2c).
Exogenous Fetuin-A Decreased the Mortality of FHF Mouse Caused by d-GalN/LPS Injection

At the 24th hour of d-GalN/LPS treatment, 83% (5/6) of the mice in the FHF group and 100% (6/6) of the mice in the groups of sham and anti-fetuin-A were dead. In contrast, only 16.7% (1/6) of the mice in the fetuin-A protection group died. The administration of fetuin-A has significantly decreased the mortality rates of the mouse with FHF induced by d-GalN/LPS (Fig. 3a).

Fetuin-A Improved Liver Function in FHF Mouse

Following d-GalN/LPS injection, the serum ALT significantly increased in a time-dependent manner in FHF mice (data not shown). At the ninth hour after d-GalN/LPS induction, serum ALT levels in groups of FHF (291.370.76 ± 29.241.68 nkat/L), sham (265.975.30 ± 26.652.21 nkat/L) and anti-fetuin-A (301.256.25 ± 6.258.38 nkat/L) were significantly higher than that in the groups of blank (1,055.21 ± 84.68 nkat/L) (p < 0.0001, p < 0.0001, p < 0.0001, respectively) and fetuin-A protection (31,585.15 ± 4,086.35 nkat/L) (p < 0.0001, p < 0.0001, p < 0.0001, respectively) (Fig. 3b).

Fetuin-A Attenuated Hepatic Inflammatory Response to d-GalN/LPS Intervention in FHF Mouse

The peak of serum proinflammatory factors was observed 3 h after d-GalN/LPS injection. As shown in Fig. 3c, serum TNF-\(\alpha\) in fetuin-A protection group (233.87 ± 48.03 pg/mL) was significantly lower than that in both groups of FHF (515.48 ± 46.31 pg/mL, p = 0.034), sham (545.39 ± 62.14 pg/mL, p = 0.001), and anti-fetuin-A (692.39 ± 310.59 pg/mL, p < 0.001) groups. Compared with blank control (9.36 ± 6.28 pg/mL), serum TNF-\(\alpha\) level in the anti-fetuin-A group with an antagonism against endogenous fetuin-A in mouse liver was significantly higher (p < 0.0001, respectively).
Meanwhile, the serum level of IL-6 in the fetuin-A protection group (546.73 ± 56.42 pg/mL) was significantly lower than that in both the FHF (1,093.93 ± 153.15 pg/mL, \( p < 0.0001 \)), sham (1,189.26 ± 134.72 pg/mL, \( p < 0.0001 \)) and anti-fetuin-A groups (1,034.61 ± 266.12 pg/mL, \( p < 0.0001 \)), indicating that an intervention of fetuin-A significantly decreased the serum level of IL-6 in the mouse with FHF. Similar to serum TNF-\( \alpha \) comparison, an antagonism against endogenous fetuin-A of mouse liver in the anti-fetuin-A group significantly increased serum IL-6 levels compared with blank control (56.87 ± 34.98 pg/mL, \( p < 0.0001 \)) (Fig. 3c). Thus, an administration of fetuin-A significantly decreased the serum concentrations of proinflammation factors, such as IL-6 and TNF-\( \alpha \) in the mouse with FHF.

Fetuin-A and TNF-\( \alpha \) Protein Expression in the Liver Tissues of FHF Mouse

Since d-GalN/LPS intervention inhibited fetuin-A expression in a time-dependent manner and reached the lowest level at the ninth hour in the liver tissue of FHF mouse (Fig. 2), the ninth hour was subsequently chosen as the check point to compare fetuin-A and TNF-\( \alpha \) expression levels among different experimental groups (\( n = 6 \)). As shown in Fig. 3d, the relative IHC scores of fetuin-A in the
groups of the blank control (114.68 ± 0.67,) and fetuin-A protection (113.29 ± 1.98) were significantly higher than that in the groups of FHF (110.89 ± 0.86, p < 0.0001, respectively), sham (110.45 ± 0.67, p < 0.0001, respectively) and anti-fetuin-A (109.77 ± 1.01, p < 0.0001, respectively) groups.

After d-GalN/LPS injection in mouse, IHC staining of TNF-α in the liver tissue of FHF mouse was gradually increased and reached the highest level at the sixth hour. The relative IHC staining intensities of TNF-α in the groups of the blank control (112.14 ± 3.07) and fetuin-A protection (111.95 ± 2.59) were significantly lower than that in the groups of both FHF (117.01 ± 3.42, p = 0.002, p < 0.0001 respectively), sham (116.45 ± 2.60, p < 0.0001, respectively) and anti-fetuin-A (115.55 ± 0.85, p = 0.016, p = 0.0004, respectively) (Fig. 3e). An intraperitoneal injection prior to d-GalN/LPS treatment effectively attenuates TNF-α expression in the liver tissues of the FHF mouse. Moreover, TNF-α expression in the anti-fetuin-A group was significantly higher than that in the fetuin-A protection group, indicating that an antagonism against endogenous fetuin-A promoted liver inflammation in FHF mouse.

Fetuin-A Inhibited the Apoptosis of Hepatocytes in FHF Mouse

After d-GalN/LPS injection, an obvious hepatocyte apoptosis was observed in the liver tissue of FHF mouse (Fig. 2f). Apoptosis was found occasionally in the blank control (0.10 ± 0.32) group. However, the hepatocyte apoptosis index (AI) in the groups of FHF (23.10 ± 7.39, p < 0.0001), anti-fetuin-A (13.50 ± 3.47, p < 0.0001) and fetuin-A protection (2.20 ± 2.97) was statistically significant (Fig. 3f). Thus, the interference of fetuin-A significantly inhibited hepatocyte apoptosis induced by d-GalN/LPS treatment in FHF mouse.

Toxicity and Side Effects of Fetuin-A on Normal Mouse Liver, Kidney and Heart

As a potential therapeutic drug for FHF, fetuin-A at the experimental dosage was further evaluated in terms of potential toxicity and side effects in cardiac, renal and hepatic tissues. As shown in Fig. 4, all the histological appearances of cardiac, renal and hepatic tissues were normal after fetuin-A injection at 100 mg/kg for 24 h (Fig. 4).

Correlation Among Hepatic Pathological Scores, Expression of Fetuin-A, TNF-α, and Hepatocyte Apoptosis

For a multiple linear regression analysis, the hepatic pathological scores of FHF mouse were adopted as the dependent variable (Y), while the relative IHC staining scores of fetuin-A and TNF-α and hepatic AI were respectively used as the independent variables X1, X2, X3; a regression equation was constructed as: Y = -4.388 - 2.682 X1 + 0.073 X2 + 0.032 X3 (R² = 0.603; F test: F = 7.075, p = 0.004).
Moreover, a correlation test was carried out among these four aforementioned indices. The Pearson’s contiguous coefficients showed that the relative IHC score of fetuin-A was negatively correlated with the pathological score, while the relative IHC score of TNF-α was positively correlated with the pathological score and the relative IHC scores of fetuin-A and TNF-α were negatively correlated with each other (Table 2).

### Discussion

In response to infection or injury, the liver re-prioritizes the synthesis and systemic release of many APPs, one of which is fetuin-A [24, 25]. In the present study, we found that fetuin-A expression in liver tissue was time-dependently decreased during FHF. Meanwhile, fetuin-A expression was negatively correlated with TNF-α in the liver of FHF mouse. During FHF, the severe necrosis and apoptosis directly reduced the number of hepatocytes and also affected the functions of living cells so that fetuin-A produced by hepatocytes was decreased and the hepatic functions were severely injured. An exogenous injection of fetuin-A prior to d-GalN/LPS intervention could lower the circulating levels of TNF-α and IL-6, decrease hepatic apoptosis, improve liver function and eventually lower the fatality rate of FHF mice. With regard to the safety of intraperitoneal injection of fetuin-A, our data did not find obvious pathological damage to mouse liver, kidney and heart tissues at the dose of 100 mg/kg. As a result, an intraperitoneal administration of fetuin-A attenuated d-GalN/LPS-induced liver failure in mice.

Although the mortality and serum ALT levels of FHF mouse were elevated by a certain dose of anti-fetuin-A (100 mg/kg) to interfere the endogenous fetuin-A, no statistical significance was found between the groups of FHF and anti-fetuin-A (100 mg/kg). The possible reasons may be the abundant endogenous fetuin-A in mice (about 500 mg/kg), so that 100 mg/kg of anti-fetuin-A was not enough for antagonism. Secondly, an extended sample size might be better for exhibiting the statistical differences among different experimental groups.

The hepatic apoptosis was obvious in our FHF mouse model. When apoptosis occurred in a large amount of hepatocytes, apoptotic cells were not endocytized promptly by phagocytes. The cellular contents were delivered after these cells were distended and split, which stimulated neutrophilic granulocytes to release and recruit inflammatory factors. Then inflammatory response was generated, aggravated and prolong. Previous studies have shown that fetuin-A played an important role in recognition and clearance of apoptotic cells [26], inhibited the apoptosis of vascular smooth muscle cells and reduced the cleavage of caspases 3, 8, and 9 into their active subunits [27]. So fetuin-A could be a protective agent for tissue injury through apoptosis inhibition. Moreover, we found that fetuin-A could also decrease hepatic apoptosis indirectly by depressing TNF-α, which could induce hepatic apoptosis.

On the other hand, fetuin-A was documented to be critical in phagocytosis [28] and inhibit the inflammatory response of macrophages after phagocytosis [29]. In our study, multiple linear regression analysis demonstrated that fetuin-A, which was correlated with TNF-α closely but oppositely, was probably a positive regulator of pathological damage of hepatic tissue in FHF mouse. The decreased fetuin-A in liver tissue ensures a rigorous innate immune response manifested by excessive accumulation of proinflammatory mediators. Supplementation with exogenous fetuin-A could tilt the balance towards inhibiting the release of activated TNF-α and IL-6.

Fetuin-A is an abundant serum protein that is exclusively produced by the liver, tongue and placenta. It has biological homology, high affinity and no significant side effects. Furthermore, administration of fetuin-A confers protection against fulminant hepatic failure in the FHF mouse model, and thus calls for need to further explore its therapeutic potential for the clinical management of acute liver failure.

In summary, the present study demonstrates fetuin-A as a negative APP in FHF. Fetuin-A exerts an important protective role against FHF by counter-regulating systemic accumulation of proinflammatory mediators.

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Conflict of interest None.

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