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Original Article
Recombinant C-terminal heparin-binding domain of fibronectin polypeptide protects against liver damage, reduces serum inflammatory cytokines, and decreases mortality in acute liver failure

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Abstract: This study aimed to evaluate the effect of recombinant C-terminal heparin-binding domain of fibronectin (FNCHBD) polypeptide on live-damage protection, inflammation, and mortality in acute liver failure (ALF) mice. 25 mice were randomly divided into five groups: normal controls, lipopolysaccharide (LPS)/D-galactosamine (GalN), 5 mg/kg FNCHBD, 10 mg/kg FNCHBD and 20 mg/kg FNCHBD groups. Blood samples were obtained at 9 h after treatment for measurement of liver indexes and inflammatory cytokine levels, and livers were acquired for H&E and TUNEL staining assays. 90 mice (18 mice in each group) were randomly divided into five groups for mortality assessment after LPS/GalN administration at 48 h. Compared to LPS/GalN group, levels of blood liver indexes including AST, ALT and TBIL were decreased in FNCHBD polypeptide-treated groups. H&E staining disclosed FNCHBD polypeptide protected cell morphology and histomorphology, and necrosis rates in FNCHBD polypeptide-treated groups were lower compared to LPS/GalN group. TUNEL staining assay revealed cell apoptosis was inhibited in FNCHBD polypeptide-treated groups compared to LPS/GalN group. Serum inflammatory cytokines including TNF-α, IL-1β, and IL-6 were reduced in FNCHBD polypeptide-treated groups compared to LPS/GalN group. As to mortality rate, it was only decreased in 10 mg/kg FNCHBD and 20 mg/kg FNCHBD groups but not in 5 mg/kg FNCHBD compared to LPS/GalN group. In addition, most effects of FNCHBD presented in a dose-dependent manner. FNCHBD polypeptide protects against liver damage, inhibits elevation of serum inflammatory cytokines, and decreases mortality in ALF.

Keywords: C-terminal heparin-binding domain, fibronectin, acute liver failure, liver damage, serum inflammatory cytokines, mortality

Introduction
Acute liver failure (ALF), a life-threatening disease with a sudden loss of liver function occurring in individuals without prior liver disease, is characterized by rapid-evolving hepatic dysfunction and is associated with neurological dysfunction as well as coagulopathy [1-3]. Currently, a variety of managements based on different etiologies are applied in clinical practice to reduce complications and decelerate disease progression of ALF, while the prognosis of these patients is still dismal with the mortality rate up to approximately 50% [1]. In addition, liver transplantation is considered as the only curative treatment, whereas its application is restricted by the lack of donors and immunosuppression-related complications [4, 5]. Hence, exploration of novel therapies for ALF patients is urgently needed.

Fibronectin (FN), a large dimeric glycoprotein that exists as a plasma component and a part of the extracellular matrix of almost every tissue, plays an important role in several biologic and pathologic processes (including wound healing, hemostasis and inflammation) through binding to several biological molecules (such as heparin, collagen, fibrin, and the membrane receptors) [6, 7]. Several studies disclose that plasma FN levels are decreased in ALF patients. Supplementation of plasma FN has therapeutic
effects on ALF, while sufficient FN from plasma is difficult to be obtained due to the lack of blood plasma and the risk of blood-infection disease; also, the synthesis of FN poses obstacles in engineering the whole-molecule FN product [8-12]. Based on these problems, we considered that synthesis of FN fragment might be a potential solution. C-terminal heparin-binding domain of FN (FNCHBD) takes part in various cellular activities, such as increasing cell adhesion and proliferation, promoting the expression of vascular endothelial growth factor and angiogenesis, regulating cell apoptosis, inhibiting the activity of microorganisms, and regulating immune responses [13-18]. In our previous studies, we synthesized human FN polypeptide and N-terminal heparin binding domain of FN polypeptide, which both showed potential to antagonize endotoxin-induced liver failure in mice. We speculated that FNCHBD polypeptide might also have a protective effect on the hepatocytes in ALF mice [11, 19]. Thus, this study aimed to evaluate the effect of recombinant FNCHBD polypeptide on liver-damage protection, inflammation, and mortality in ALF mice.

Methods

Preparation of animals

Nine-week-old BALB/C mice with weight 18-20 g were purchased from Vital River Laboratory Animal Technology Co. Ltd (Beijing, China), which were bred and kept in the Laboratory Animal Center of the Fujian Medical University under standard pathogen-free-material conditions at 24-25°C, and were provided with standard chow and water in a 12-h light-dark cycle. In addition, LPS 100 mg/kg and D-GalN 400 mg/kg were injected intraperitoneally into mice to construct ALF model [11, 19].
Study design and treatments

FNCHBD polypeptide, a 32 KDa protein containing 272 amino acids (Tyr1720-Tyr1991), was synthesized and purified in a yeast expression system by our study group and was applied to treat ALF mice in this present study. This study consisted of two major stages: (1) Stage I, liver damage evaluation and inflammation measurement; (2) Stage II, mortality assessment. In stage I, 25 mice were randomly divided into five groups: normal controls, LPS/GalN, 5 mg/kg FNCHBD, 10 mg/kg FNCHBD, and blood samples were obtained at 9 h after treatment for measurement of liver indexes and inflammatory cytokines levels, then mice were executed and livers were acquired for hematoxylin-eosin (HE) staining and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay (TUNEL). In stage II, 90 mice were randomly divided into five groups: normal controls, LPS/GalN, 5 mg/kg FNCHBD, 10 mg/kg FNCHBD, 20 mg/kg FNCHBD and blood samples were obtained at 9 h after treatment for measurement of liver indexes and inflammatory cytokines levels, then mice were executed and livers were acquired for hematoxylin-eosin (HE) staining and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay (TUNEL). In stage II, 90 mice were randomly divided into five groups: normal controls, LPS/GalN, 5 mg/kg FNCHBD, 10 mg/kg FNCHBD, 20 mg/kg FNCHBD and bred for 48h, and mortality rate at 48 h were calculated.

Measurement of blood liver indexes

Blood liver indexes including aspartate transaminase (AST), alanine transaminase (ALT), and total bilirubin (TBIL) were detected using Automation Biochemist Analyzer (Mairui Medical Company, China).

Measurement of serum inflammatory cytokines

Serum samples were isolated from blood by centrifugation at 4000 rpm for 20 minutes at 4°C. Levels of inflammatory cytokines including TNF-α, IL-1β and IL-6 were determined using commercial enzyme-linked immuno sorbent assay (ELISA) kits (Invitrogen, USA).

H&E staining and TUNEL staining assays

Liver damage was determined by H&E staining using immunohistochemistry (IHC). Cell apoptosis of liver was determined using One Step TUNEL Apoptosis Assay Kit (Promega, China) according to the instructions of the manufacturer, and cell apoptosis rate was calculated using Image J Software (Java, USA).

Statistics

Statistical analysis was performed using SPSS 22.0 software (IBM, USA) and graphs were made using GraphPad Prism 6.01 (GraphPad, USA). Data were mainly presented as mean ±
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standard deviation and count (percentage). Comparison among groups was determined by one-way ANOVA followed by Tukey’s multiple comparisons test. P<0.05 was considered significant.

Results

Effect of FNCHBD polypeptide on reducing blood liver indexes

The levels of blood liver indexes including AST (P<0.001), ALT (P<0.001) and TBIL (P<0.001) were greatly higher in LPS/GalN group compared to normal control group (Figure 1). AST, ALT and TBIL levels were all reduced in 5 mg/kg FNCHBD group, 10 mg/kg FNCHBD group and 20 mg/kg FNCHBD group compared to LPS/GalN group (all P<0.05). In addition, as the dose of FNCHBD polypeptide increased, the decrease of blood liver index level was much greater, indicating that FNCHBD polypeptide was able to reduce the levels of blood liver indexes in a dose-dependent manner.

Effect of FNCHBD polypeptide on alleviating pathologic damage

Various histologic changes that indicate liver damage were observed in LPS/GalN group compared to normal controls group by H&E staining, including extensive hemorrhage, hepatic lobule destruction, disappearance of the hepatocyte cord-like structure, severe denaturation and necrosis of hepatocytes, condensed nuclei, dilatation and congestion of hepatic sinusoids, and infiltration of inflammatory cells in the stroma (Figure 2A). However, FNCHBD polypeptide-treated groups including 5 mg/kg FNCHBD group, 10 mg/kg FNCHBD group and 20 mg/kg FNCHBD group presented reduced degrees of liver damage in cell morphology and histomorphology in a dose-dependent manner compared to LPS/GalN group, and the best hepatic histology was observed in 20 mg/kg FNCHBD group, which showed remarkably alleviated hemorrhage and congestion in liver tissue, more intact hepatic lobule structure and hepatocyte cord-like structure, milder denaturation and less necrosis of hepatocytes (Figure 2A). Liver necrosis rate was further evaluated according to H&E staining, and we observed that it was decreased in the 5 mg/kg FNCHBD group (P<0.01), 10 mg/kg FNCHBD group (P<0.001) and 20 mg/kg FNCHBD group (P<0.001) respectively compared to LPS/GalN group. The effect of FNCHBD on liver necrosis was in a dose-dependent manner (Figure 2B). These data suggested that FNCHBD polypeptide alleviated liver pathologic damage in ALF mice.

Effect of FNCHBD polypeptide on inhibiting liver apoptosis

Increased TUNEL-positive cells were observed in LPS/GalN group compared to normal control group, whereas cell apoptosis was ameliorated.
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were deceased in 5 mg/kg FNCHBD group, 10 mg/kg FNCHBD group and 20 mg/kg FNCHBD group compared to LPS/GaIN group (all P value <0.05), indicating that FNCHBD polypeptide attenuated acute inflammation in ALF mice.

Effect of FNCHBD polypeptide on decreasing mortality

Mortality rate was calculated in 90 mice (18 mice in each group) after LPS/GaIN administration at 48 h. No death was found in normal control group (0%), while the mortality rate in LPS/GaIN group (88.9%, P<0.001) was dramatically higher than that in normal control group (Figure 5). Compared to LPS/GaIN group, mortality rate was undifferentiated in 5 mg/kg FNCHBD group (66.7%), whereas it was decreased in 10 mg/kg FNCHBD group (44.5%, P<0.01) and 20 mg/kg FNCHBD group (33.3%, P<0.001) respectively, suggesting that FNCHBD polypeptide reduced the mortality rate in ALF mice.

**Effect of FNCHBD polypeptide on decreasing serum inflammatory cytokines**

Levels of serum inflammatory cytokines including TNF-α (P<0.001) (Figure 4A), IL-1β (P<0.001) (Figure 4B), and IL-6 (P<0.001) (Figure 4C) were dramatically increased in LPS/GaIN group compared to the normal control group. Conversely, levels of these inflammatory cytokines were deceased in 5 mg/kg FNCHBD group, 10 mg/kg FNCHBD group and 20 mg/kg FNCHBD group compared to LPS/GaIN group (all P value <0.05), indicating that FNCHBD polypeptide attenuated acute inflammation in ALF mice.

*Figure 4.* Assessments of serum inflammatory cytokines. Compared to LPS/GaIN group, TNF-α level was decreased in 5 mg/kg FNCHBD group, 10 mg/kg FNCHBD group and 20 mg/kg FNCHBD group (A); IL-1β level was reduced in 5 mg/kg FNCHBD group, 10 mg/kg FNCHBD group and 20 mg/kg FNCHBD group (B); IL-6 level was lower in 5 mg/kg FNCHBD group, 10 mg/kg FNCHBD group and 20 mg/kg FNCHBD group (C). Comparison between two groups was determined by one-way ANOVA followed by Tukey’s multiple comparisons test. LPS/GaIN, lipopolysaccharide/D-galactosamine; TNF-α, tumor necrosis factor α; FNCHBD, C-terminal heparin-binding domain of fibronectin; IL-1β, Interleukin 1β. P<0.05 was considered significant. **, FNCHBD polypeptide-treated groups vs. LPS/GalN group, P<0.01; ***, FNCHBD polypeptide-treated groups vs. LPS/GaIN group, P<0.001; ###, LPS/GaIN group vs. normal control group, P<0.001.

in the FNCHBD polypeptide-treated groups as indicated by the reduction of TUNEL-positive cells (Figure 3A, 3B). Furthermore, cell apoptosis rate was remarkably lower in 5 mg/kg FNCHBD group (P<0.001), 10 mg/kg FNCHBD group (P<0.001) and 20 mg/kg FNCHBD group (P<0.001) compared to LPS/GaIN group, and the effect of FNCHBD on liver cell apoptosis was in a dose-dependent manner (Figure 3B). These data suggested that FNCHBD polypeptide inhibited liver apoptosis in ALF mice.

*Figure 3.* Assessments of liver cell apoptosis. A: TUNEL-positive cell rate (%) in normal control (N=5), LPS/GaIN (N=5), 5 mg/kg FNCHBD (N=5), 10 mg/kg FNCHBD (N=5) and 20 mg/kg FNCHBD (N=5) groups were 5.6%, 45.0%, 18.9%, 12.7% and 7.6% respectively. **, FNCHBD polypeptide-treated groups vs. LPS/GaIN group, P<0.01; ***, FNCHBD polypeptide-treated groups vs. LPS/GaIN group, P<0.001; ###, LPS/GaIN group vs. normal control group, P<0.001.

Effect of FNCHBD polypeptide on attenuating serum inflammatory cytokines

Levels of serum inflammatory cytokines including TNF-α (P<0.001) (Figure 4A), IL-1β (P<0.001) (Figure 4B), and IL-6 (P<0.001) (Figure 4C) were dramatically increased in LPS/GaIN group compared to the normal control group. Conversely, levels of these inflammatory cytokines were deceased in 5 mg/kg FNCHBD group, 10 mg/kg FNCHBD group and 20 mg/kg FNCHBD group compared to LPS/GaIN group (all P value <0.05), indicating that FNCHBD polypeptide attenuated acute inflammation in ALF mice.

Effect of FNCHBD polypeptide on decreasing mortality

Mortality rate was calculated in 90 mice (18 mice in each group) after LPS/GaIN administration at 48 h. No death was found in normal control group (0%), while the mortality rate in LPS/GaIN group (88.9%, P<0.001) was dramatically higher than that in normal control group (Figure 5). Compared to LPS/GaIN group, mortality rate was undifferentiated in 5 mg/kg FNCHBD group (66.7%), whereas it was decreased in 10 mg/kg FNCHBD group (44.5%, P<0.01) and 20 mg/kg FNCHBD group (33.3%, P<0.001) respectively, suggesting that FNCHBD polypeptide reduced the mortality rate in ALF mice.
Discussion

In this study, we found that: (1) FNCHBD polypeptide attenuated liver damage in ALF mice, including decreasing the levels of blood liver indexes, lowering the pathological damage as well as inhibiting apoptosis; (2) FNCHBD polypeptide decreased levels of serum inflammatory cytokines in ALF mice; (3) FNCHBD polypeptide lowered the mortality rate in ALF mice. These effects of FNCHBD presented in a dose-dependent manner.

FN, a high molecular-weight glycoprotein found in plasma and extracellular matrix, is able to regulate cell proliferation, migration and adhesion. It plays a crucial role in the repair and regeneration of tissue, synthesis of collagen, mediation of reticuloendothelial system, maintenance of capillary integrity, and regulation of the internal environment balance [20-23]. Some previous studies have displayed an effect of FN in ALF [12, 24]. For example, a study reveals that FN suppresses nuclear factor κB (NF-κB) activation, promotes IL-10 level, and enhances B-cell lymphoma-extra large (Bcl-xL) expression, and thereby ameliorates cell apoptosis by reducing TNF-α-triggered apoptosis and blocking intracellular apoptotic signals in ALF mice [12]. Another study discloses that lack of FN induces deficiency of FN-mediated matrix survival signal in ALF mice, which is accompanied by a downregulated level of the antiapoptotic protein Bcl-xL. This causes increased apoptosis [24]. However, the application of FN is limited due to insufficient FN extraction sources and the difficulty of FN synthesis, thus recombinant peptides of FN fragment have been introduced [8, 9]. As genetic technology has achieved many of improvements in the past decades, much attention has been paid to the application of recombinant human FN polypeptide on disease therapy, whereas the evidence about the effect of recombinant FNCHBD polypeptide on disease therapy, whereas the evidence about the effect of recombinant FNCHBD polypeptide on disease therapy, whereas the evidence about the effect of recombinant FNCHBD polypeptide on disease therapy.

Figure 5. Mortality assessment. The mortality rate was unchanged in 5 mg/kg FNCHBD group compared to LPS/GaIN group, whereas it was decreased in 10 mg/kg FNCHBD group and 20 mg/kg FNCHBD group respectively compared to LPS/GaIN group. Comparison between two groups was determined by Chi-square test. FNCHBD, C-terminal heparin-binding domain of fibronectin; LPS/GaIN, lipopolysaccharide/D-galactosamine. P<0.05 was considered significant. **, FNCHBD polypeptide-treated groups vs. LPS/GaIN group, P<0.01; ***, FNCHBD polypeptide-treated groups vs. LPS/GaIN group, P<0.001; ###, LPS/GaIN group vs. normal control group, P<0.001.

Although no studies investigating protective effect of FNCHBD polypeptide on liver damage in ALF have been reported until now, our previous study had shown the value of recombinant polypeptide deriving from FN on protecting the hepatocytes, which reveals that recombinant FN polypeptide greatly inhibits hepatocyte necrosis and apoptosis in endotoxin-induced hepatic failure mice [11]. In this present study, we performed blood liver index measurements, H&E staining assay, and TUNEL staining assay to assess the liver damage in ALF mice, and we found FNCHBD polypeptide decreased levels of blood liver indexes (including AST, ALT and TBIL), attenuated liver pathologic damage (such as more intact structure, lighter denaturation, and less hepatocellular necrosis) and reduced the apoptosis rate in ALF mice. These data suggested that FNCHBD polypeptide was able to protect against liver damage in ALF mice, possibly because: (1) FNCHBD polypeptide promoted the antiapoptotic protein Bcl-xL, thus it reduced cell apoptosis in ALF mice; (2) FNCHBD polypeptide enhanced hepatocyte growth factor, thus it facilitated cell proliferation in ALF mice; (3) FNCHBD polypeptide decreased inflammatory responses and reduced TNF-α-triggered apoptosis, and thereby indirectly protected against liver damage in ALF, so that decreased levels of blood liver indexes, attenuated liver pathologic damage and reduced apoptosis rate were observed [12, 24].
In the pathogenic processes of ALF, the immune system is the first to respond to protect organisms by releasing proinflammatory cytokines, and serum inflammatory cytokines such as TNF-α, IL-1β and IL-6 are considered to be vital factors in ALF [10, 12]. Regarding the LPS/GalN stimulation, it induces Kupffer cells to produce TNF-α, and other inflammatory cytokines such as IL-1, IL-6 and IL-12, which in turn cause liver injury and hepatic infiltration of neutrophils. Furthermore, the TNF-α induced apoptosis of hepatocytes is considered the predominant mechanism for LPS/GalN-induced ALF [27, 28].

Based on these previous studies, we investigated the influence of FNCHBD polypeptide on serum inflammatory cytokines in ALF mice. We observed that FNCHBD polypeptide downregulated the levels of TNF-α, IL-1β and IL-6, suggesting that FNCHBD polypeptide contributed to inhibiting the elevation of inflammatory cytokines in ALF mice. Possible reasons of these results were that: (1) LPS/GalN stimulation induced dysfunction of Kupffer cells, while FNCHBD polypeptide attenuated liver damage and indirectly decreased the degree of Kupffer cell dysfunction in ALF, thus less inflammatory response was triggered and the elevation of inflammatory cytokines was reduced; (2) FNCHBD polypeptide might facilitate anti-inflammatory effects through inhibiting the inhibitor of nuclear factor kappa-B kinase subunit β (IKK-β)/NF-κB dependent pro-inflammatory gene transcription or inducing anti-inflammatory proteins. The elevation of inflammatory cytokines was thereby suppressed in ALF mice [29].

According to some previous studies, higher serum FN levels are correlated with decreased mortality rate. As to recombinant FN polypeptide, our previous study discloses that recombinant FN polypeptide reduces mortality rates of mice after LPS/GalN injection [11]. In this study, we investigated the influence of FNCHBD polypeptide on mortality rate of ALF mice. We found that the mortality rates in FNCHBD polypeptide-treated groups were lower compared to the LPS/GalN group without FNCHBD polypeptide therapy. The possible reasons for the lower mortality rates in FNCHBD polypeptide-treated groups might be that FNCHBD polypeptide downregulated inflammatory cytokine related apoptosis (such as TNF-α) or upregulated some other antiapoptotic proteins (such as antiapoptotic protein Bcl-xL), thereby causing milder disease progression and reduced mortality rates in FNCHBD polypeptide-treated ALF mice [12, 24]. These data indicated that FNCHBD polypeptide might be a promising therapeutic agent to improve the prognosis in ALF.

In conclusion, FNCHBD polypeptide protects against liver damage, inhibits elevation of serum inflammatory cytokines, and decreases mortality in ALF.

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Disclosure of conflict of interest

None.

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