Recombinant fibronectin polypeptide antagonizes hepatic failure induced by endotoxin in mice[1]

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KEY WORDS fibronectins; recombinant fusion proteins; lipopolysaccharides; acetylgalactosamine

ABSTRACT

AIM: To study the preventive effect of recombinant human fibronectin (rhFN) polypeptide on hepatic failure induced by endotoxin in mice. **METHODS:** A cDNA fragment coding for Ile1363-Tyr1725 of human FN was inserted into the *PinPoint Xa-3* plasmid, and the constructed plasmid was transformed into *E coli* BL21 (DE3) cells, and then the expression of rhFN polypeptide in DE3 cells was identified through SDS-PAGE. The target protein from the supernatant of bacteria lysate was purified through biotin-affinity chromatography. The bioactivity of the purified rhFN polypeptide was determined with cell adhesive activity. The survival rate was observed in endotoxemia mice injected with rhFN polypeptide. The tissue damage in hepatocyte was detected using histology, ultrastructure, and DNA fragmentation assay. **RESULTS:** The expression of rhFN polypeptide reached approximately 20 % of the total cellular protein. The adhesion ability of rhFN polypeptide was concentration-dependent. The value of EC_{50} was 0.8 nmol/L. The survival rate of endotoxemia mice sensitized by *D*-galactosamine (*D*-GalN) was 60 % in rhFN polypeptide treated group, while that of endotoxemia mice sensitized by *D*-GalN was 20 % in the control group ($P<0.01$). Histopathology showed that less necrosis occurred on the hepatocyte of endotoxemia mice injected with rhFN polypeptide compared with saline control. Ultrastructure and DNA fragmentation assay showed that no apoptotic hepatocyte was observed in the liver of rhFN-treated endotoxemia mice. **CONCLUSION:** Recombinant fibronectin polypeptide antagonizes hepatic failure induced by endotoxin in mice.

INTRODUCTION

Fibronectin (FN) is known to be a large multifunctional glycoprotein with binding sites for many substances and integrin cell-surface receptors on a variety of cells including fibroblasts, phagocytes and bacteria[1]. Extensive *in vitro* functional analyses indicate that FN modulates cell proliferation, migration and survival[2]. FN interacts with cells in cell-binding domains with RGDS sequence, which is located in the 10th type III repeats of FN, the synergy sequence in the 9th type III repeats, which is a key attachment site for cell-surface receptors, and EDA, a spliced sequence of FN, is connecting segment I (CS-I)[3-5].

Sepsis and endotoxemia are involved in the development of fulminant hepatic failure, the prognosis of which is extremely poor and the mortality is high, with no effective therapy available[6]. Our previous studies indicated that plasma FN (pFN) could prevent hepatic failure induced by endotoxin in mice[7]. However it is almost impossible to obtain sufficient FN from plasma

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due to its high expense, and it is infectious to be treated with FN from plasma due to the high risk of AIDS, hepatitis C, and other infectious diseases. Therefore, genetic technology has been employed to produce FN. In the last decade, much attention has been paid to the effect of recombinant human FN (rhFN) polypeptide on tumor therapy[8,9]. In the present study, we investigated the preventive effects of rhFN polypeptide with RGDS, EDA and the synergy sequence in the 9th type III repeats on hepatic failure induced by endotoxin in mice.

MATERIALS AND METHODS

Vector and host *E coli* strain, BL21 (DE3), and expression vector, *PinPoint Xa-3* were purchased from Promega Company (USA).

Cells Baby hamster kidney (BHK) cells were purchased from ATCC Co, USA. Cells were maintained in MEM, supplemented with 10 % FCS and *L*-glutamine.

Restriction enzymes *Bam*H I and *Eco*R V were purchased from Promega Company (USA), *Taq* Plus I DNA polymerase was from Shanghai Sangon Company (China). PCR purification kit, gel extraction kit, and Miniprep kit for plasmid extraction were obtained from Qiangen Company (USA).

RT-PCR Total RNA was prepared from human normal liver cell line L02 cells by Trizol Reagent kit (Gibco). The primer was designed according to the flanking sequences of cell-binding domains of human FN and synthesized by Shanghai Sangon Company (China). The upstream primer is 5'-TAC AGG ATC CGC CAT CAT CCC GA-3', and the downstream primer is 5'-CTC GAT ATC CAG TGA GCT GAA CAT TGG G-3'. PCR was conducted in a procedure (1) 94 ºC for 5 min; (2) 30 cycles at 94 ºC for 30 s, 58 ºC for 30 s, 72 ºC for 120 s; (3)72 ºC for 10 min.

Sequence analysis Nucleotide sequence of PCR production was sequenced by Shanghai Sangon Company.

Construction of expression vector The obtained product was cut by *Bam*H I and *Eco*R V, and inserted into *PinPoint Xa-3* to obtain pX3-hFN (Fig 1). Digestion and ligation of DNA fragments, cloning, and screening of recombinant DNA were performed according to the methods in literature[10]. Nucleotide sequence of recombinant DNA was confirmed by sequencing.

Induction and expression of rhFN polypeptide The obtained vector was transformed into BL21 (DE3). Cells were grown in LB medium with addition of IPTG. The expression of the recombinant proteins was identified through SD-PAGE and Western blots. The level of expression was determined using SDS-PAGE and then densitometor scanning.

Purification of rhFN polypeptides BL21 (DE3) cells were harvested by centrifugation and ruptured by ultrasound. The desired protein was purified by biotin-affinity chromatography. The purified product was quantified by Bradford reagent, analyzed on a 10 % PAG gel, and dialyzed against PBS buffer at 4 ºC overnight.

Cell adhesion assay According to the method[11], a 96-well plate was coated with rhFN polypeptide and

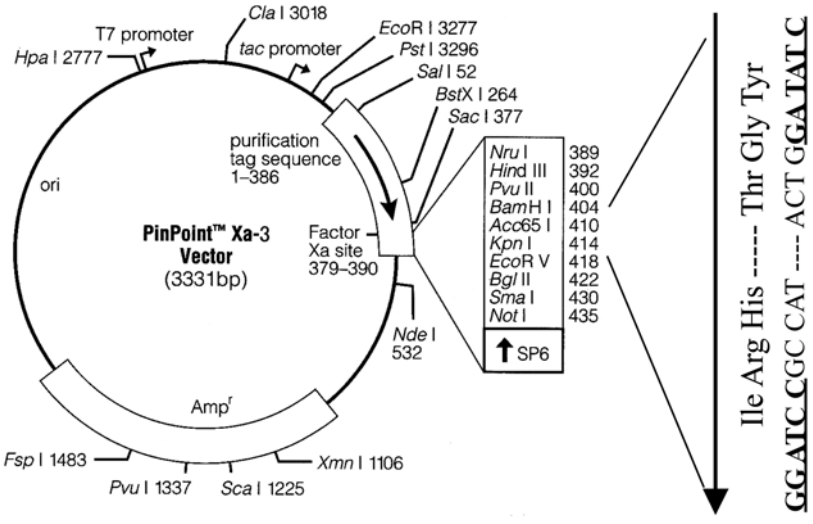

Fig 1. Schematic drawing of the *PinPoint Xa-3* vector with the inserts FN (1086 bp). FN gene sequence was ligated into the *BamH* I and *EcoR* V polylinker sites to create pX3-hFN (4417 bp) recombinant plasmid.

commercial FN polypeptide (GRGDS is a synthetic peptide that is major cell-binding site, and was purchased from Sigma Inc), and washed twice with PBS after it was incubated at 4 ºC overnight. BHK cells in serum free MEM medium (5×10^8/L) was added to the plate, 0.1 mL/well. After being incubated at 37 ºC for 1 h, the unattached cells were removed and the plate was washed twice with PBS. MTT was added to each of the wells to lyse the cells. Absorbance was measured to determine the relevant amount of adherent cells. The concentration that gave 50 % of maximal number of adhering cells (EC_{50}) was estimated from a concentration-response curve.

Effect of rhFN polypeptide on endotoxemia mice sensitized by *D*-galactosamine (*D*-GalN) The model was performed according to the method described previously[12], with minor modifications. Briefly, 9-week-old specific pathogen-free (SPF) male BALB/c mice (22-25 g) were purchased from Experimental Animal Institute, Chinese Academy of Sciences, China. These animals were housed in animal facilities with free access to food and water. In this study, these animals were given an intraperitoneal injection of 500 μL saline containing 300 μg LPS (lipopolysaccharide B, *E coli* 055:B5, Sigma) and 10 mg *D*-GalN (Sigma), and then were randomly divided into 2 groups for experiment. Treatment mice were injected four times with rhFN polypeptide (10 mg/kg per mouse) at 30 min before and 1, 2, and 3 h after the injection of LPS and *D*-GalN. Control mice were injected four times with saline. The mortality of endotoxemia mice sensitized by *D*-GalN was observed for 72 h.

Histopathological analysis The samples of liver tissue were fixed in 10 % formalin, and embedded in paraffin, whose sections were stained with hematoxylin eosin (HE).

Electron-microscopic studies The samples of liver tissue were fixed with 2.5 % glutaraldehyde in 0.1 % mol/L cacodylate buffer (pH 7.4) at 4 ºC for 1 h, post-fixed with 1 % osmium tetroxide at room temperature for 1 h, and dehydrated with increasing concentrations of ethanol before two-stage embedding in LX112 resin. The thin sections (700 nm) stained with uranyl acetate and lead citrate were studied under at HU-12A electron microscope.

DNA fragmentation assay DNA fragmentation was assayed as described previously[13], with minor modifications. The samples of liver tissue were placed in a glass homogenizer in 1 mL lysis buffer containing 200 mmol/L Tris-HCl, pH 8.0,100 mmol/L ethylene diaminotetraacetic acid, 1 % SDS, 50 mg/L proteinase K (Sigma) and homogenized on ice for 5 min. Incubated at 37 ºC for 4 h, the DNA was extracted with an equal volume of phenol-chloroform followed by chloroform-isoamylalcohol (24:1), the aqueous phase was collected, and DNA was precipitated by adding NaCl to 150 mmol/L and 2 volumes ethanol at -20 ºC overnight. The DNA pellet was dried and dissolved in 0.4 mL TNE buffer (10 mmol/L Tris-HCl, 10 mmol/L NaCl, 1 mmol/L ethylene diaminotetraacetic acid). RNAse A (100 mg/L) was added and incubated for 2.5 h followed by proteinase K (120 μg), after 5 h incubation, the DNA was extracted once more and precipitated as above. The DNA pellet was dissolved in 100 μL TNE, and assayed by horizontal electrophoresis for 4 h in 1.2 % agarose gel with TBE (Tris-botalic-ethylene diamine-tetraacetic acid) as running buffer, stained with 0.5 mg/L ethidium bromide and visualized under ultraviolet light.

Statistical analysis χ^2 test was used for comparisons of survival rate between groups. $P<0.05$ was considered statistically significant, and $P<0.01$ as highly significant.

RESULTS

Expression and identification of rhFN polypeptide Based on SDS-PAGE assay, the amount of synthesized rhFN polypeptide reached 20 % of the total cellular protein by densitometry scanning. The expression of a special band was demonstrated by Western blot. rhFN polypeptide was purified from the supernatant of the bacteria lysate by affinity chromatography. The purity of the obtained rhFN was confirmed as high as 95 % by SDS-PAGE, and the molecular weight of the purified product was consistent with the theorial value: 52 kDa. The size of the full fusion protein was 13 kDa larger than the insert portion (39 kDa) due to the presence of the biotin tag portion derived from the plasmid (Fig 2).

Cell adhesive activity of rhFN polypeptide The adhesive ability of rhFN polypeptide and GRGDS was concentration-dependent (Fig 3). EC_{50} of rhFN and GRGDS were 0.8 and 6.4 nmol/L, respectively.

rhFN polypeptides inhibited lethality after LPS/*D*-GalN injection To assess possible beneficial effects of rhFN on the lethality of mice given LPS/*D*-GalN, survival rates were studied. The survival rate of endotoxemia mice sensitized by *D*-GalN was 60 % (12/20)

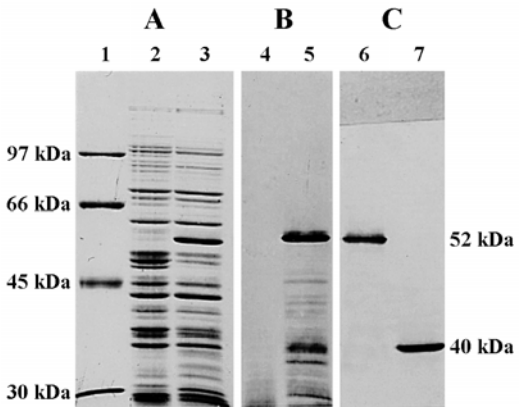

Fig 2. SDS-PAGE and Western blot of total cellular *E coli* protein extract containing the rhFN. A) SDS-PAGE analysis of the bacteria lysate. B) Western blot analysis of the bacteria lysate with FN antibody (CHEMICON). C) SDS-PAGE analysis of the purified rhFN polypeptide. Lane1: low molecular protein markers (Pharmacia Company). Lane 2, 4, 7: BL21 (DE3)/*PinPoint Xa* control vector. Lane 3, 5, 6: BL21 (DE3)/pX3-hFN induced by IPTG. BL21 (DE3)/ *PinPoint Xa* control vector produces a fusion protein of approximately 40 kDa. BL21 (DE3)/pX3-FN produces a fusion protein of approximately 52 kDa.

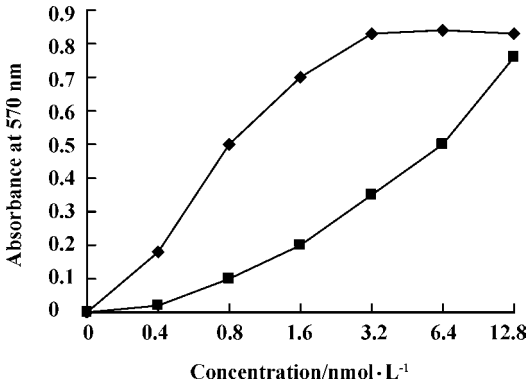

Fig 3. Effect on cell adhesive activity of rhFN(◆) and GRGDS (■).

in the rhFN-treated group, while that of endotoxemia mice sensitized by *D*-GalN was 20 % (4/20) in the control group (χ^2=6.67, P<0.01).

rhFN inhibited massive hepatocyte necrosis after LPS/*D*-GalN injection Histopathological study of the liver was performed to confirm the preventive effects of rhFN against liver injury and to investigate events after LPS/*D*-GalN injection. In control mice, massive necrosis were scattered in the liver. But, less necrosis of hepatocyte was observed in the liver of rhFN treated mice (Fig 4).

rhFN inhibited massive hepatocyte apoptosis after LPS+GalN The chromosomal DNA fragmentation and apoptotic hepatocytes appeared after an LPS/*D*-GalN injection. By contrast, no DNA ladder or apoptotic hepatocyte was observed in the liver of rhFN-treated mice (Fig 5, 6).

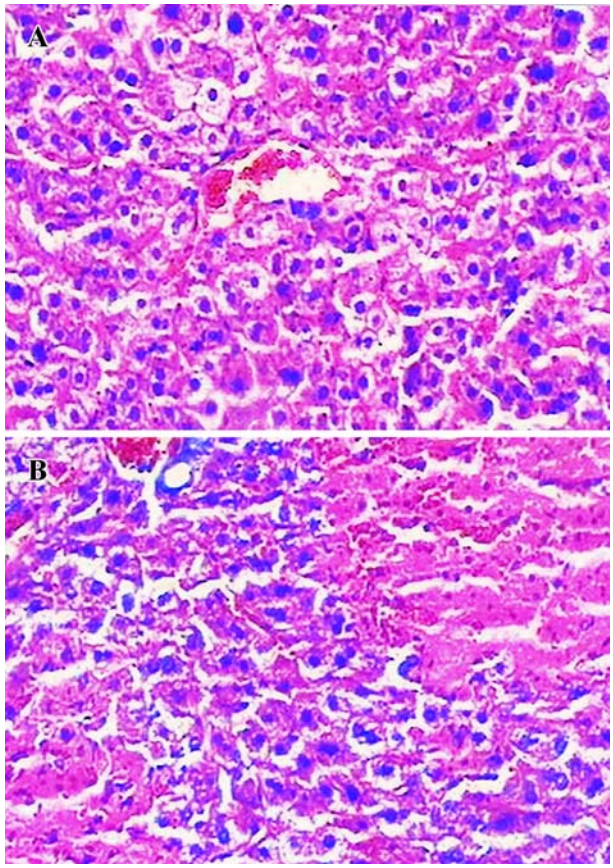

Fig 4. Effect of rhFN polypeptide on endotoxin induced liver injury. Liver histopathology after LPS/*D*-GalN injection with rhFN (A) or saline (B) treatment in mice (HE staining: original magnification×100).

DISCUSSION

The *PinPoint Xa* expression system is designed for the production and purification of fusion proteins that are biotinylated *in vivo*[14]. The advantage of this system is that the transformed *E coli* produces a biotinylated recombinant fusion protein allowing its easy purification based on the interaction between biotin and avidin. Using this system, the expression level of rhFN polypeptide reached approximately 20 % of the total cell protein in the presence of 1 mol/L IPTG. Most of the products of target gene expressed in *E coli* presented as soluble protein, and could be isolated directly by biotin-affinity chromatography. Cell adhesive assay showed that the ability of rhFN polypeptide binding cells was concentration-dependent and was higher than that of commercial FN peptides (GRGDS).

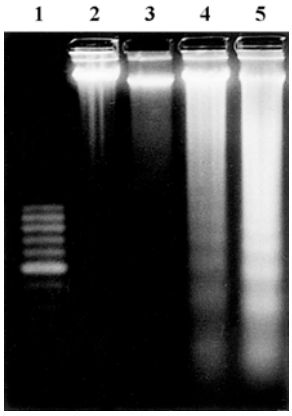

Fig 5. DNA electrophoresis of liver extracts. Lane 1: Marker, 100 bp DNA ladder (MBI). Lane 2, 3: Treated with rhFN after LPS/*D*-GalN injection. Lane 4, 5: Treated with saline after LPS/*D*-GalN injection.

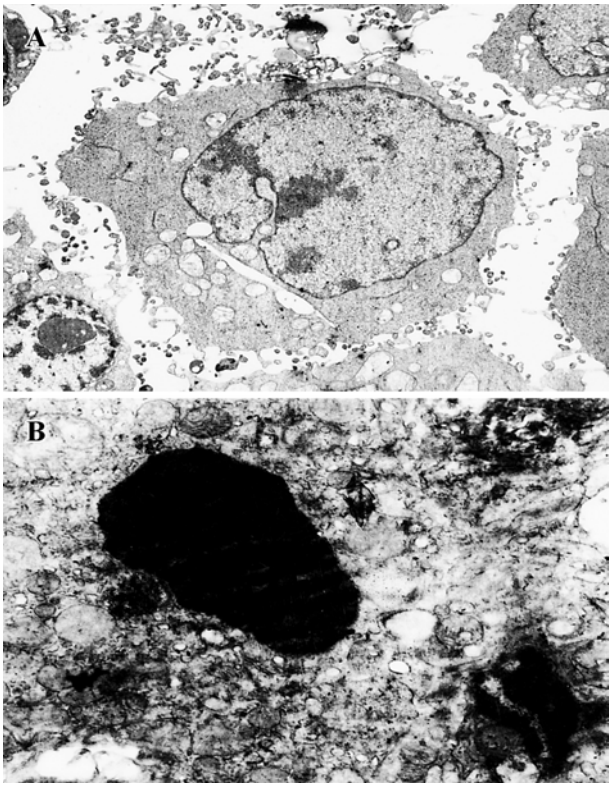

Fig 6. Effect of rhFN polypeptide on endotoxin induced hepatocyte apoptosis. Electron micrographs of liver tissue after LPS/*D*-GalN injection with rhFN (A) or saline (B) treatment in mice (Original magnification×4800).

Up to now, high mortality is still the most important issue of sepsis, particularly in fulminant hepatic failure. Because apoptosis in hepatocyte played a crucial role in the development of hepatic failure, recent approaches to explore therapeutic strategies focused on the prevention of hepatocyte apoptosis. pFN is a multifunctional glycoprotein that is involved in many cellular processes, including cell proliferation, migration and survival. It is expressed at high levels by hepatocyte and secreted in soluble form into plasma[15]. Hepatocyte apoptosis may lead to a lack of pFN. Our previous studies indicated that pFN could prevent hepatic failure induced by endotoxin in mice, and suggested that supplementary pFN might be beneficial to the treatment of hepatic injury[7]. Endotoxin is a gram-negative bacterial LPS, which is involved in the development of fulminant hepatic failure, as well as in multiple organ failure. However, even in the same experimental animal, there was much significant discrepancy after application of LPS. Mice treated with LPS/*D*-GalN selectively developed hepatic failure, in which liver injury was much more severe and more rapid than that in mice induced by a high dose of LPS alone, without sensitiza-tion[12,16,17]. In this study, we used hepatic failure of mice model prepared by injecting *D*-GalN and LPS. The effect of rhFN polypeptide on endotoxin-induced hepatic failure was observed. The results showed that rhFN polypetide treatment could decrease the mortality of endotoxemia mice sensitized by *D*-GalN. It suggested that rhFN could also prevent hepatic failure induced by endotoxin.

However the latent mechanism by which rhFN protected hepatic failure is still unclear. We recently found that pFN abrogated TNF-induced hepatocyte apoptosis[7]. In this study, histopathology showed that treatment with rhFN significantly inhibited massive hepatocyte necrosis in endotoxemia mice sensitized by *D*-GalN. Furthermore, DNA fragmentation assay showed that rhFN treatment significantly inhibited massive hepatocyte apoptosis in mice given LPS/*D*-GalN. The difference was due to the fact that the hepatocyte injury tended to develop from apoptosis at the initial stage to necrosis at later stage. Although “hepatocyte necrosis” was once viewed as “hepatocyte death”, current biochemical and pathological studies suggested that Fas- and/or TNF receptor-mediated hepatocyte apoptosis played an important role in the development of fulminant hepatic failure[12,18]. Massive hepatocyte apoptosis is the predominant model of cell death at the initial stage after *D*-GalN/LPS, while necrosis is the predominant model of cell death at the later stage[12]. By contrast, a lethal dose of LPS in nosensitized mice caused primarily hepatocyte necrosis, accompanied by a low incidence of apoptosis. In various *in vitro* models, the induction of either apoptosis or necrosis has been shown

to depend on the extent and duration of the exposure to LPS. Zhou *et al* showed that a striking correlation between lethal activity and apoptotic DNA fragmentation of liver in response of *D*-GalN-sensitized mice to a non-lethal amount of LPS[19]. In light of our present observations, the potent anti-apoptotic activities of rhFN may be involved in prevention of hepatic failure in such models. However, actual mechanisms of anti-apoptotic activities require further elucidations.

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