Effect of naked eukaryotic expression plasmid encoding rat augmenter of liver regeneration on acute hepatic injury and hepatic failure in rats

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Abstract

AIM: To study the protective effect of eukaryotic expression plasmid encoding augmenter of liver regeneration (ALR) on acute hepatic injury and hepatic failure in rats.

METHODS: The PCR-amplified ALR gene was recombined with pcDNA3 plasmid, and used to treat rats with acute hepatic injury. The rats with acute hepatic injury induced by intraperitoneal injection of 2 mL/kg 50% carbon tetrachloride (CCl4) were randomly divided into saline control group and recombinant pcDNA3-ALR plasmid treatment groups. Recombinant pcDNA3-ALR plasmid DNA (50 or 200 µg/kg) was injected into the rats with acute hepatic injury intraperitoneally, intraperitoneally, or intravenously and intraperitoneally in combination 4 h after CCl4 administration, respectively. The recombinant plasmid was injected once per 12 h into all treatment groups four times, and the rats were decapitated 12 h after the last injection. Hepatic histopathological alterations were observed after HE staining, the expression of proliferating cell nuclear antigen (PCNA) in liver tissue was detected by immunohistochemical staining, and the level of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) was determined by biochemical method.

RESULTS: The sequence of ALR cDNA of recombinant pcDNA3-ALR plasmid was accordant with the reported sequence of rat ALR cDNA. After the rats with acute hepatic injury were treated with recombinant pcDNA3-ALR plasmid, the degree of liver histopathological injury markedly decreased. The pathologic liver tissues, in which hepatic degeneration and necrosis of a small amount of hepatocytes and a large amount of infiltrating inflammatory cells were observed, and they became basically normal in the most effective group after four times of injection of recombinant pcDNA3-ALR plasmid. The indexes of PCNA significantly increased in the recombinant pcDNA3-ALR plasmid treatment groups compared to model group. The level of serum AST and ALT remarkably reduced in recombinant pcDNA3-ALR plasmid treatment groups compared to model group. The results showed that the effect of 200 µg/kg recombinant pcDNA3-ALR plasmid in the rats with acute liver injury was stronger than that of 50 µg/kg pcDNA3-ALR DNA. The effect of intravenous injection of recombinant pcDNA3-ALR plasmid was better. After the rats with acute hepatic failure were treated with recombinant pcDNA3-ALR plasmid, the survival rate (40%) significantly increased in treatment groups compared to control group (15%, P<0.01).

CONCLUSION: The ALR gene may play an important role in relieving acute hepatic injury and hepatic failure by promoting hepatic cell proliferation and reducing level of AST and ALT in CCl4-intoxicated rats.

Key words: ALR; Acute hepatic injury; Hepatic failure; Gene therapy

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INTRODUCTION

Hepatocytes have strong ability to regenerate, which may be stimulated by a lot of factors. Hepatocyte growth factor (HGF) promotes regeneration of liver cells by stimulating the DNA synthesis of hepatocytes[1]. The augmenter of liver regeneration (ALR) was cloned by Hagiya et al[2], from the weanling rat liver in 1994, is another factor stimulating the hepatocyte regeneration. The length of rat ALR cDNA is 1.2 kb, and contains a 375-bp open reading frame encoding a protein consisting of 125 amino acids. It was found that ALR is different from other stimulating factors of hepatic...
regeneration in structure, but is similar to the hepatic stimulator substance found by LaBrecque and Bachur in physical and chemical characteristics, which could stimulate hepatic generation in vivo. The mouse and human ALR cDNA has been cloned, and the mapping of mouse and human ALR genes on their chromosomes has also been completed. It was found that rat, mouse, and human ALR genes (and protein products) are highly conserved and preferentially expressed in testis and liver. Recombinant human ALR enhances the survival rate, decreases the LDH release rate of hepatocytes injured by CCl4 in vitro, increases the survival rate of liver failure animals, promotes hepatocyte proliferation and decreases the serum level of alanine aminotransferase (ALT) and LDH in vivo. We cloned the rat ALR cDNA in our laboratory and the expression of ALR cDNA in prokaryotes has been studied. In this study, the recombinant eukaryotic expression plasmid of ALR was constructed and the protective effect of the naked recombinant eukaryotic expression plasmid of ALR on rat acute hepatic injury and failure induced by CCl4 was studied.

MATERIALS AND METHODS

Materials

E. coli DH5α was kindly provided by Dr. Yu-Huai Jin (Department of Microbiology of Hebei Medical University) and pcDNA3 vector was provided by Dr. Lin Wei (Department of Immunology of Hebei Medical University). Restriction endonucleases used in the experiment were purchased from Promega Co. (USA) and the primers were synthesized by Sangon Biological Technology Co. (Shanghai, China) according to the reported sequence. Wistar rats were provided by Experimental Animal Center of Hebei Medical University, China. The proliferating cell nuclear antigen (PCNA) detection kit was purchased from Boshide Biological Co. (Wuhan, China).

Construction of eukaryotic expression plasmid of rat ALR

The ALR cDNA was amplified by PCR using pBV220-ALR constructed as template and primer 1, 5'-GCGAAGCTTATGCGGAGCCGAGACG-3' and primer 2, 5'-GCTGAAATTTAGTACAGGACGGCTT-3' (restriction endonucleases of HindIII and EcoRI are boldfaced, respectively), and then digested with HindIII and EcoRI. The PCR product digested by HindIII and EcoRI was ligated with pcDNA3 vector digested by the same restriction endonucleases in the presence of T4 DNA ligase at 16 °C overnight. The recombinant plasmid was transfected into E. coli DH5α and positive clones were screened by color reaction of the recombinant plasmid. The length of insert of recombinant plasmid was determined using dideoxynucleotide chain termination method by Sangon Biological Technology Co. (Shanghai, China), and compared to the reported ALR sequence.

Detection of nucleotide sequence

The nucleotide sequence of insert of the recombinant pcDNA3-ALR plasmid was determined using dideoxynucleotide chain termination method by Sangon Biological Technology Co. (Shanghai, China), and compared to the reported ALR sequence.

Animal experiment of acute hepatic injury

Forty-two male Wistar rats, weighing 180-200 g, were randomly divided into normal control group (6 rats) and acute hepatic injury group (36 rats) induced by intraperitoneal injection of 2 mL/kg 50% CCl4. The rats with acute hepatic injury were randomly divided into six groups: model group and five treatment groups which received intravenous injection of 50 μg/kg recombinant plasmid, intravenous injection of 200 μg/kg recombinant plasmid, intraperitoneal injection of 50 μg/kg recombinant plasmid, intraperitoneal injection of 200 μg/kg recombinant plasmid and intravenous injection of 50 μg/kg recombinant plasmid (100 μg/injection) after 4 h of CCl4 administration, respectively. The recombinant plasmid was injected once per 12 h in all treatment groups four times, and the rats were decapitated 12 h after the last injection.

Histopathological alterations of liver tissue

The activities of serum aspartate aminotransferase (AST) and ALT were determined by the biochemical method using Beckman auto-biochemical instruments after the rats were decapitated. The formalin-fixed tissues were embedded in paraffin and sectioned at a thickness of 4 μm. The sections were deparaffinized and hydrated gradually in alcohol and graded alcohol/water mixture. After being incubated with 3% H2O2 for 10 min at room temperature and unmasked antigens in a microwave oven at 100 °C for 10 min, the sections were blocked with animal serum for 20 min. Specimens were then incubated with mouse anti-rat PCNA monoclonal antibodies (Calbiochem Co., USA) at 37 °C for 2 h and further treated with SABC kits for 20 min at 37 °C. They were visualized by diaminobenzidine color development and counter-stained with hematoxylin. When the cell nuclei were dyied into brown yellow, they were considered to be positive cells. The percentage of positive cells with PCNA staining in six 400× fields was counted as proliferation index under microscope.

Immunohistochemical assay of PCNA

The expression of PCNA in hepatocytes was determined using streptavidin-biotin peroxidase complex (SABC) method which was performed according to the technical manual of the company. The formalin-fixed tissues were embedded in paraffin and sectioned at a thickness of 4 μm. The sections were deparaffinized and hydrated gradually in alcohol and graded alcohol/water mixture. After being incubated with 3% H2O2 for 10 min at room temperature and unmasked antigens in a microwave oven at 100 °C for 10 min, the sections were blocked with animal serum for 20 min. Specimens were then incubated with mouse anti-rat PCNA monoclonal antibodies (Calbiochem Co., USA) at 37 °C for 2 h and further treated with SABC kits for 20 min at 37 °C. They were visualized by diaminobenzidine color development and counter-stained with hematoxylin. When the cell nuclei were dyied into brown yellow, they were considered to be positive cells. The percentage of positive cells with PCNA staining in six 400× fields was counted as proliferation index under microscope.

Detection of liver function

The activities of serum aspartate aminotransferase (AST) and ALT were determined by the biochemical method using Beckman auto-biochemical instruments after the rats were decapitated and serum was isolated.

Animal experiment of acute hepatic failure

Forty male Wistar rats, weighing 180-200 g, were used to induce acute hepatic failure model by intraperitoneal injection of 4 mL/kg 50% CCl4. The rats were randomly divided into model group and treatment group, and then intraperitoneally treated with 500 μL saline and 500 μL recombinant pcDNA3-ALR plasmid (200 μg/kg) 4 h after administration of CCl4, respectively. The number of dead rats was observed every day and the rats living over 96 h were considered as survivals. The survival rate of the two groups was compared.

Statistical analysis

Data were analyzed with SPSS software. Quantitative data...
were presented as mean±SD and compared by one-way ANOVA. Survival data were compared by χ² test. P<0.05 was considered statistically significant.

RESULTS

Clone of rat ALR cDNA

The PCR-amplified rat ALR cDNA from pBV220-ALR plasmid was digested by HindIII and EcoRI from recombinant pcDNA3-ALR plasmid, and then determined by electrophoresis on 1.2% agarose gel. The result showed that one clear band on 1.2% agarose gel. The result showed that one clear band was found as expected (Figure 1). The nucleotide sequence of the insert in recombinant pcDNA3-ALR plasmid was as that reported by Hagiya et al.[2].

Histopathological changes of liver tissue

The liver tissue of normal control group showed normal structure under microscope. Specimens, however, from the liver tissue of model group showed massive necrosis of hepatocytes around the central vein of all hepatic lobules and a few of relatively normal hepatocytes remained around hepatic lobules. After administration of different doses of recombinant pcDNA3-ALR plasmid, the histopathological alterations of hepatic injury were relieved. About one third of the necrotic hepatocytes were found around the central vein of all hepatic lobules, but obvious degeneration and necrosis of hepatocytes could not be found in the tissue intraperitoneally injected with 200 µg/kg recombinant pcDNA3-ALR plasmid. A large amount of infiltrating inflammatory cells were observed around the central vein of hepatic lobules, but obvious degeneration and necrosis of hepatocytes could not be found in the tissue intraperitoneally injected with 200 µg/kg recombinant pcDNA3-ALR plasmid. However, the therapeutic effect of intravenous injection of 200 µg/kg recombinant pcDNA3-ALR plasmid was most remarkable because most pathologic hepatocytes became normal. Degeneration and necrosis were found in a small amount of hepatocytes, while infiltrating inflammatory cells were found in hepatic tissue (Figure 2).

PCNA index of liver tissue

Only few PCNA positive cells were found in the specimens of normal control group. The number of PCNA positive cells increased in the specimens of model group. However, the number of PCNA positive cells spreading all over the liver tissue increased remarkably in the specimens of each recombinant pcDNA3-ALR plasmid treatment group (Figure 3). The PCNA index was higher in model group than in normal group (P<0.01) and much higher in each treatment group compared to model group (P<0.01). However, the indexes of PCNA significantly increased in intravenous injection and combined injection groups compared to intraperitoneal injection group (P<0.01), and enhanced in 200 µg/kg recombinant pcDNA3-ALR plasmid DNA treatment groups compared to 50 µg/kg recombinant plasmid DNA treatment groups (P<0.01, Table 1).

Alterations of serum AST and ALT level

The level of serum AST and ALT markedly increased in the model group of acute hepatic injury, but remarkably decreased after administration of 50 µg/kg recombinant pcDNA3-ALR plasmid by intraperitoneal or intravenous injection. In the groups receiving intraperitoneal or intravenous injection of 200 µg/kg recombinant pcDNA3-ALR plasmid, the reduction of serum AST and ALT level became more remarkable. The results showed that the therapeutic effect of intravenous injection was better than that of the other two injections of recombinant plasmid (Table 1).

| Group                        | n    | AST (U/L)   | ALT (U/L)   | PCNA |
|------------------------------|------|-------------|-------------|------|
| Normal control group         | 6    | 181.8±14.9  | 43.3±5.1    | 0.4±1.3 |
| Model group of hepatic injury| 6    | 3 936.2±685.1| 2 590.0±350.2| 19.7±2.0 |
| IV injection (50 µg/kg)      | 6    | 2 626.2±404.2| 1 579.6±330.1| 54.8±5.3 |
| IV injection (200 µg/kg)     | 6    | 921.6±458.2 | 572.0±254.2 | 66.5±5.3 |
| IP injection (50 µg/kg)      | 6    | 2 717.0±689.8| 1 589.2±581.6| 40.0±4.9 |
| IP injection (200 µg/kg)     | 6    | 1 890.3±610.6| 1 495.3±693.5| 45.8±2.6 |
| Combined injection (200 µg/kg)| 6    | 2 135.5±401.1| 1 457.8±418.2| 61.1±3.8 |

*p<0.01 vs normal control group, *P<0.01 vs model group of hepatic injury.
The survival rate of rats with acute hepatic failure was observed after 200 µg/kg recombinant pcDNA3-ALR plasmid was intraperitoneally injected into the rats with acute hepatic failure induced by injection of 4 mL/kg 50% CCl₄. The results (Table 2) showed that the survival rate of recombinant pcDNA3-ALR plasmid treatment group (40%) was much higher than that of the saline group (15%, \(P<0.01\)).

**DISCUSSION**

Acute hepatic injury (failure) is a kind of serious and life-threatening clinical syndrome of hepatic function damage due to necrosis of a large amount of hepatocytes. Many substances, such as hepatitis virus, drugs, and some chemicals, can give rise to acute hepatic injury or failure due to massive hepatocytic necrosis. The clinical treatment of acute hepatic injury or failure is an urgent problem to be
Table 2 Effect of recombinant pcDNA3-ALR plasmid on survival rate of CCl4-induced acute hepatic failure in rats in 96 h

| Group       | n  | Number of survival | Survival rate (%) |
|-------------|----|--------------------|-------------------|
| Saline      | 20 | 3                  | 15                |
| PcDNA3-ALR  | 20 | 8                  | 40<sup>a</sup>    |

<sup>a</sup>P<0.01 vs saline.

In this paper, the therapeutic effect of naked recombinant pcDNA3-ALR plasmid on acute hepatic injury was demonstrated by histopathological changes of liver tissue, PCNA index and serum level of AST and ALT. After naked recombinant pcDNA3-ALR plasmid was administrated to the rats with acute hepatic injury, the liver pathology remarkably reduced. After four times of injection of recombinant pcDNA3-ALR plasmid, the pathologic liver tissues in which degeneration and necrosis of few hepatocytes and infiltrating inflammatory cells were observed, became normal in the most effective group. PCNA is a nuclear protein and its expression is relative to the duplication of DNA and regeneration of cells. Therefore, PCNA is usually used to assay the DNA synthesis and proliferation of hepatocytes. It was demonstrated that PCNA is induced in acute hepatic injury stage (6 h) and liver regeneration stage (36 h) by intraperitoneal injection of CCl4 into model animals. In this experiment, we used immunohistochemical method of SABC to assay the expression of PCNA in liver tissue. The results showed that a certain degree of hepatocyte proliferation was observed in the liver tissue of model group of acute hepatic injury induced by CCl4, but the expression of PCNA significantly increased in each treatment group compared to model group, suggesting that the products encoded by ALR gene in vivo can remarkably promote the proliferation of hepatocytes. Yang et al<sup>23</sup>, found that the recombinant human ALR expressed by E. coli increases the incorporation of [3H]-TdR into liver DNA of test animals and also has a potent antihistitis effect. In addition, the administration of recombinant pcDNA3-ALR plasmid significantly decreases serum ALT and AST, suggesting that the ALR gene in recombinant pcDNA3-ALR plasmid plays an important role in the recovery of acute hepatic injury by stimulating the hepatic regeneration in vivo and the method of naked recombinant plasmid administration is a new and effective method for the treatment of acute hepatic injury. In this study, we found that the therapeutic effect of intravenous injection of recombinant pcDNA3-ALR plasmid was better than that of the other two injections and the therapeutic effects of the three ways of injection were dose dependent. The reasons why the effect of intravenous injection is superior to that of combination injection are still unknown.

Hwang et al<sup>24</sup>, found that the mice infected with adenovirus carrying cDNA of human HGF (Ad.hHGF) show a dramatic resistance to thioacetamide-induced acute hepatic failure. The survival rate remarkably enhanced in the mice infected with Ad.hHGF. Wang et al<sup>25</sup>, demonstrated that α-melanocyte-stimulating hormone (α-MSH), a potent anti-inflammatory peptide, can prevent fulminant hepatic failure induced by thioacetamide in mice. The mortality in the α-MSH-treated mice was significantly lower compared to the vehicle group 3 d after injury. Liver histology significantly improved and TUNEL-positive hepatocytes decreased in the treated mice. Thus, ALR gene therapy may be potentially useful for the treatment of patients with acute hepatic injury and hepatic failure.

The pcDNA3 plasmid is a mammalian expression vector used widely in experimental studies of gene therapy. The pcDNA3 plasmid carrying purpose gene can nonspecifically transfact tissues of heart, kidney, liver, and prostate by intramuscular or intravenous injection in vivo<sup>26-29</sup>, as well as cell lines such as hepatoma cell line HepG2, human gastric cancer cell line SGC7901 and pancreatic carcinoma cell strain PC-II in vivo<sup>30,31</sup>. Though the pcDNA3-ALR plasmid exhibits therapeutic effect on acute hepatic injury and hepatic failure, but the molecular mechanisms of pcDNA3-ALR mediated hepatic regeneration still need to be established.

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