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RESEARCH ARTICLE

Downregulation effects of beta-elemene on the levels of plasma endotoxin, serum TNF-alpha, and hepatic CD14 expression in rats with liver fibrosis

Jianguo LIU[1], MB, Zhe ZHANG[2], MM, Jiechang GAO[1], MM, Jiwen XIE (✉)[1], MB, Lin YANG[1], MD, Shenjun HU[1], MM

1 Department of Traditional Chinese Medicine, Union Hospital, Tongji Medical College, Huazhong University of Science & Technology, Wuhan 430022, China
2 Department of Oncology, The Second Staff Hospital of Wuhan Iron and Steel (Group) Corp., Wuhan 430085, China

Abstract It has been demonstrated that β-elemene could protect against carbon tetrachloride (CCl_4)-induced liver fibrosis in our laboratory work, and the aim of this paper is to reveal the protective mechanisms of β-elemene. The hepatic fibrosis experimental model was induced by the hypodermical injection of CCl_4 in Wistar male rats. β-elemene was intraperitoneally administered into rats for 8 weeks (0.1 mL/100 g bodyweight per day), and plasma endotoxin content was assayed by biochemistry. The serum TNF-α level was detected using radioactive immunity. CD14 expression in rat livers was measured by immunohistochemistry and Western blot. The results showed that β-elemene can downregulate the levels of plasma endotoxins, serum TNF-α, and hepatic CD14 expression in rats with liver fibrosis. β-elemene plays an important role in downregulating the lipopolysaccharide signal transduction pathway, a significant pathway in hepatic fibrosis development.

Keywords liver fibrosis; beta-elemene; endotoxin; CD14

1 Introduction

Fibrosis is the essential pathophysiologic consequence of chronic liver injuries. It represents the common underlying mechanism of hepatic insufficiency and most clinical complications of end-stage liver diseases [1]. Historically, hepatic fibrosis treatments, including corticosteroids for autoimmune hepatitis, interferon for hepatitis B and C, and iron depletion for hemochromatosis, have been directed against specific causes of chronic liver injuries. However, there is no effective treatment for most causes of chronic liver diseases [2].

Curcuma zedoaria is a traditional Chinese medicinal herb that is widely used for the clinical treatment of fibrotic diseases. β-elemene, which is a natural sesquiterpene extracted from the rhizome of *C. zedoaria*. Modern pharmacological researches showed that β-elemene is a kind of non-cytotoxic anti-cancer drug. It also is an antioxidant that regulates the immune system, prevents thrombosis and vascular function expansion, and inhibits the growth of tumor cells. Preliminary studies have shown that β-elemene can reverse the pathological progression of carbon tetrachloride (CCl_4)-induced hepatic fibrosis by inhibiting the activation of hepatic stellate cells, down-regulating the expression of α-smooth-muscle actin (α-SMA) and transforming growth factor-β1 (TGF-β1), and decreasing the sediments of extracellular matrices in liver tissues [3,4].

Recently, many studies have demonstrated that lipopolysaccharide (LPS) is capable of exacerbating liver diseases by activating Kupffer cells and enhancing cytokine production [5]. This study aims to investigate the effects of β-elemene on CD14 expression in the LPS signal transduction pathway of rats with CCl_4-induced hepatic fibrosis.

2 Materials and methods

2.1 Animals

Thirty six-week old male Wistar rats weighing 200–220 g (provided by the Experimental Animal Center of Tongji

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E-mail: xiejiwen@126.com

Medical College, Huazhong University of Science and Technology, Wuhan, China) were housed in plastic mesh cages under controlled conditions (23 ± 2)°C and 50% ± 20% relative humidity with light illumination for 12 h/d). After acclimatization for one week, the rats were randomly divided into three groups: CCl_4-treated group (n = 10), β-elemene group (n = 10), and control group (n = 10). The animals were given access to food and tap water *ad libitum* throughout the acclimatization period. Animals in the CCl_4 and β-elemene groups received 3 mL/kg CCl_4 (40% in olive oil) subcutaneously twice a week for eight weeks. Animals in the control group were injected subcutaneously only with isochoric oil. Two weeks after the experiment, rats in the β-elemene group were injected intraperitoneally with 2 mL/kg β-elemene-pyrrolidinone mixture (1∶1) three times a week for six weeks [3,4]. All rats were fed with the basal diet throughout the experiment. At the end of the experiment, all rats were sacrificed using ether anesthesia, according to the recommendations for the poper care and use of laboratory animals [6]. The experiment was approved by the Animal Care and Use Committee of Tongji Medical College, Wuhan, China.

2.2 Endotoxin assay

Blood samples were collected from the portal vein under pyrogen-free conditions and centrifuged at 1200 rpm for 10 min. Plasma was stored at −80°C until measurement. Samples were diluted and heated before assay to minimize the effects of the plasma inhibitors of endotoxins. Endotoxin levels were measured using a *Limulus* amebocyte lysate test kit (Shanghai Institute of Biologic Products).

2.3 Serum tumor necrosis factor-α (TNF-α) measurement

Serum TNF-α was measured by radioactive immunity method (Beijing Northern Institute of Biotechnology).

2.4 Immunohistochemical CD14 examination

In all groups, 4 μm-thick sections of formalin-fixed and paraffin-embedded liver samples were processed routinely for Masson trichrome staining to identify liver fibrosis development. Immunohistochemical staining was performed using a DAB kit (Beijing Zhongshan Golden Bridge Biotechnology, China). Tissue sections were deparaffinized in xylene and rehydrated through a graded alcohol series to water. Endogenous peroxidase activity was quenched with 3% H_2O_2 in methanol for 5 min. Slides were incubated with a 1∶50 dilution of anti-CD14 antibodies (Boshide, China) at 4°C overnight, followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (Boshide, China) for 1 h. Antibody binding sites were visualized by incubation with DAB-H_2O_2 solution. Slides were counterstained with hematoxylin, and tissue sections were mounted. Hepatocytes were viewed under a light microscope (Olympus, Japan) at 400 × magnification. Approximately 400 cells from ten randomly selected fields (0.0506 mm^2 per field) of vision were counted. CD14 labeling indices were expressed as a percentage of positively stained cells relative to the number of counted cells (i.e., CD14 labeling index = CD14 positive hepatocytes/total hepatocytes per high power field × 100).

2.5 Western blot

Liver tissues were lysed in radio-immunoprecipitation assay lysates, and protein concentration was measured using a BCA protein assay kit (Pierce Biotechnology, Inc., USA). The samples were subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Afterwards, the gels were removed and the proteins were transferred to the nitrocellulose membrane using a transfer printing apparatus. The transfer film was incubated with rabbit anti-rat CD14 poly-antibodies and HRP-labeled goat anti-rabbit IgG (diluted at 1∶1000), and then sealed with 5% skimmed milk. Subsequently, the transfer film was visualized using an enhanced-chemiluminescent system kit (Pierce Biotechnology, Inc., USA). The result was evaluated with an image analysis system program (Gel pro, Germany).

2.6 Statistical analysis

Data were expressed as mean ± SD. Statistical analysis was performed by one-way ANOVA using SPSS 12.0. A $P < 0.05$ was considered significant.

3 Results

3.1 Effect of β-elemene on serum endotoxins

Serum endotoxin levels in the control, model, and treatment groups were 0.07 ± 0.01, 0.21 ± 0.02, and 0.12 ± 0.04 U/mL, respectively, indicating that serum endotoxin level in the model group increased significantly compared with that in the control group ($P < 0.01$). Serum endotoxin level in the treatment group decreased significantly compared with that in the model group ($P < 0.01$).

3.2 Effect of β-elemene on serum TNF-α

Serum TNF-α level in the model group (10.49 ± 0.63 μg/L) was significantly higher than that in the control group (4.37 ± 0.66 μg/L, $P < 0.01$). Meanwhile, serum TNF-α level in the treatment group (7.51 ± 0.58 μg/L) was significantly lower than that in the model group ($P < 0.01$).

3.3 β-elemene suppression of hepatic CD14 protein expression

Fig. 1 shows the effect of β-elemene on CD14 expression in the three liver groups. The CD14 labeling index (9.33% ± 1.25%) in the model group was significantly higher than that in the control group (0.28% ± 0.03%) ($P < 0.01$).

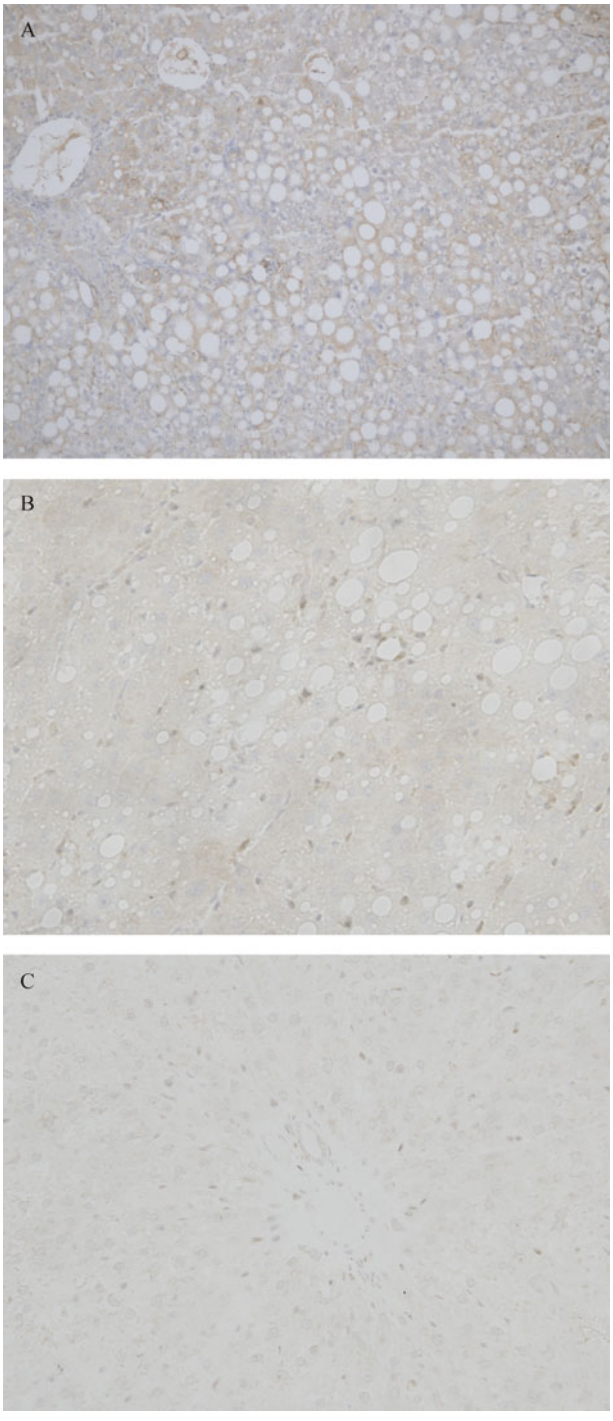

Fig. 1 CD14 immunohistochemical staining of liver sections from rats treated with β-elemene (DAB × 20). **(A)** CCl_4 group; **(B)** β-elemene group; **(C)** control group.

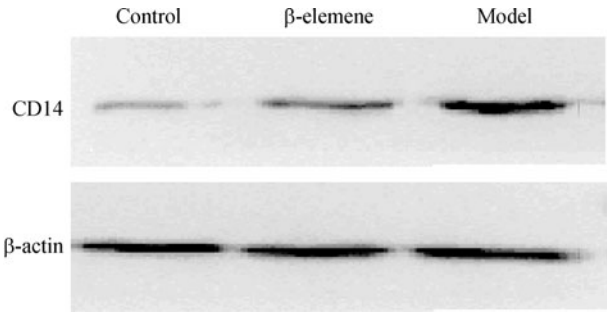

Fig. 2 Western blot of CD14 protein.

Meanwhile, the index in the β-elemene group was only 3.67% ± 0.45% ($P < 0.01$, vs. the control and model groups).

CD14 expression was analyzed by Western blot (Fig. 2). CD14 expressions were measured at 26.72 ± 4.63, 76.46 ± 5.39, 113.42 ± 9.29 in the control, β-elemene, and model groups, respectively. Significant differences were found between any two groups ($P < 0.05$).

4 Discussion

LPS, a glycolipid that is abundantly found on the outer membrane of all gram-negative bacteria, has the ability to incite a vigorous inflammatory response [7]. In experimental studies of healthy animals, LPS is cleared from the blood circulation within a few minutes of intravenous injection, and the majority is traced to the liver [8,9].The primary role of the liver in clearing LPS can be demonstrated in patients with liver failure. Endotoxemia is frequently found in patients with cirrhosis, and the degree of endotoxemia is correlated with the degree of liver failure [10,11]. Multiple lines of evidence point to LPS as a cofactor in liver injuries [12,13]. In animal models of liver injuries, such as those on $CaCl_4$ toxicity, choline deficiency, and alcohol-induced liver diseases, liver injury is mitigated or prevented by adding oral non-absorbable antibiotics, colectomy, or germ-free conditions [14]. In contrast, adding LPS aggravates liver injuries caused by these hepatotoxins [15]. In $CaCl_4$- and galactosamine-induced liver injuries, adding anti-LPS antibody E-5 significantly reduces the hepatotoxicity caused by these agents [16]. These studies demonstrate the role of endogenous LPS in toxin-induced liver injuries.

Endotoxins or LPS itself is not hepatotoxic at low concentrations. However, its ability to stimulate an inflammatory response may account for its pathogenicity in the liver. Although many parameters of the inflammatory response contribute to liver injuries [17], one well-studied pathway is the production of TNF-α. In many injury models, elevated TNF-α levels are present and correlated with injuries [18,19]. The inhibition of TNF-α activity can decrease liver injuries. Adding soluble TNF

receptors that diminish the biologic effects of TNF-α decreases liver enzymes significantly, improves liver histology, and decreases mortality after acute $CaCl_4$ administration [16].

The results of the current study demonstrate that CCl_4 administration upregulates plasma ET and serum TNF-α levels in rats. These findings further confirm that the LPS signal transduction pathway is important in hepatic fibrosis development.

Given the critical importance of LPS and Kupffer cells in many forms of liver injury pathogenesis [20], research has focused on CD14 (CD14 is essential to LPS signaling). CD14 expression on Kupffer cells can be upregulated with multiple stimuli, including LPS. By immunohistochemical staining, low CD14 levels are detected in livers of unstimulated mice. Liver CD14 levels increase rapidly and peak 6 h after intraperitoneal injection with LPS [21,22]. CD14 expression in the liver is also increased in many types of liver diseases, including alcoholic and cholestatic liver injuries in rodents [23,24]. Animals fed with more unsaturated fats have worse liver injuries; the degree of injury is correlated with both the level of endotoxemia and the level of Kupffer cell CD14 expression [25]. *In vivo*, CD14 transgenic mice that overexpress CD14 on monocytes have increased sensitivity to LPS [26]. In contrast, genetically engineered CD14-deficient mice are insensitive to LPS [27,28]. In human diseases, CD14 expression on Kupffer cells is low in the normal human liver, but it increases with different inflammatory liver diseases [29].

This study found that β-elemene can prevent liver fibrosis development. β-elemene can downregulate the levels of plasma ET, serum TNF-α, and hepatic CD14 expression in rats with liver fibrosis.

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