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Lumbrokinase in a Potential Function to Intervene Chronic Hepatitis B*

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Abstract Fibronectin (FN) is an important molecule that participates in hepatitis B infection and fibrosis. Lumbrokinase (LK) is synthesized by earthworms containing a group of proteolytic isozymes, which were used to hydrolyze fibrin in the treatment of clotting disease. One of the isozymes purified from the LK compound was verified as a hydrolase to degrade FN *in vitro* and was called earthworm fibronectin hydrolase (EFNase). However, whether LK functions to prevent the deterioration of hepatitis B virus (HBV) infection has not been clarified. In this study, we used HepG2.2.15 cells to investigate the effect of LK on the level of hepatitis B surface antigen (HBsAg) or FN, used C57BL/6J-HBV transgenic mice to explore the effect on the level of HBsAg, FN, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum, the pathological changes in livers. The results indicated that LK could inhibit HBsAg production *in vitro* and *in vivo*; decreased FN in serum and liver. Compared with physiological saline, LK improved the state of the livers. Those data provided valuable information to understand the therapeutic role of LK as a potentially effective medicine to treat chronic hepatitis B (CHB).

Key words Lumbrokinase, Fibronectin, HBsAg, Chronic hepatitis B, Organ fibrosis
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1 Introduction

Hepatitis B virus (HBV) infection is not only a serious clinical issue but also a social problem because of its worldwide distribution and possible adverse sequelae, such as hepatic decompensation, cirrhosis and hepatocellular carcinoma[1-2]. The treatment guidelines recommend endpoints that include the suppression of HBV DNA replication to undetectable levels (using a PCR-based assay), normalization of serum aminotransferase levels and HBeAg seroconversion (loss of HBeAg and presence of HBeAb)[3].

There are several therapeutic agents, including IFN-α and oral nucleotide analogues, such as lamivudine, adefovir, entecavir and telbivudine, that are currently approved for the treatment of chronic HBV[4]. In the absence of an ideal therapeutic agent, many chemicals have been used in patients with chronic hepatitis B (CHB), such as chemicals derived from the ascidian Styela plicata[5]. However, all of these drugs have their own limitations. For example, the common side effects of interferon therapy include significant neuropsychiatric complications and flu-like symptoms[6]; lamivudine (LV) is unsuitable for patients with cirrhosis and HBeAg-negative disease who need a long treatment duration[1]; adefovir can result in nephrotoxicity, which may complicate its use in patients with renal insufficiency[7]. Therefore, discovery of new therapeutic drugs to intervene chronic HBV infection is an urgent work to provide more opportunities for clinicals.

Earthworm such as Eisenia fetida and Lumbricus rubellus synthesizes a group of proteases called lumbrokinase (LK) [8] and earthworm fibrinolytic enzyme[9], which has been known for its capacity to

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treat clotting diseases. As described previously, earthworm fibronectin hydrolase (EFNase), which is one effective component of LK, can decrease the levels of hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) in HepG2.2.15 cells[10]. The mechanism of EFNase used to treat HBV infections may be related to its hydrolysis of fibronectin (FN). As a drug, the "Lumbrokinase Capsule", containing the group of earthworm proteolytic isozymes, has been prescribed to treat millions of patients with stroke and myocardial infarction. Since one of the proteolytic isozymes has the role in the hydrolysis of fibronectin, whether LK (compound isozymes) also acts on fibronectin should be clarified.

FN is involved in HBV infection, propagation[11], liver cirrhosis and hepatocellular carcinoma[12]. High concentration of FN in blood improves the transmission frequency of hepatitis viruses[10]. As described by Budkowska and colleagues[13], the FN of human liver sinusoids can bind to HBV *via* the pre-s2 domain, which may play a role in the initial steps of virus entry into target cells. FN also participates in hepatic fibrogenesis and the hepatocellular carcinoma[14] and serves as a biomarker for liver fibrosis staging of CHB patients[15]. FN polymerization is necessary for collagen matrix deposition and is a key contributor to liver fibrosis[16]. Inhibition of FN polymerization ameliorates fibrosis of organs[17-19]. During the early phase of active fibroplasia, FN production increases dramatically, and this augmentation is associated with the fibroblast proliferation responsible for excessive synthesis and deposition of the collagen protein. In other words, the hydrolysis of FN may be helpful for the inhibition of fibrogenesis and prevent liver from fibrosis.

This work focused on the degradation sites on the FN molecule recognized by LK, the effect of LK on the HBsAg in both HepG2.2.15 cells and HBV transgenic mice, and the liver state after the transgenic mouse had been treated with LK, suggesting that there is a potentially therapeutic efficacy for treating CHB.

2 Experimental

2.1 Cell culture

HepG2.2.15 cells were kindly provided by Dr. Ding in the Beijing Institute of Radiation Medicine, who got them from the Mount Sinai Medical Center in New York (USA). The cells secrete not only HBV proteins but also hepatitis B virions[20]. HepG2.2.15 cells were seeded with 1×10^4 cells per well in 96-well cell culture plates or 1×10^5 cells per well in 12-well cell culture plates, and cultured in Dulbecco's modified eagle medium with 10% fetal bovine serum and growth supplement, according to the protocol described in a previous paper[11]. LK (a kind gift from Beijing Baiao Pharmaceuticals Co., Ltd. China) and LV (NDHS. Beijing, China) were added to the media on day 3 after seeding. The cells were grown in the presence of LK or LV for 6 days with changes made to the medium every 3 days. The culture supernatants were collected and subjected to HBsAg analysis. The cells were harvested and subjected to Western blotting analysis of FN protein.

2.2 Animals

The investigation adhered to the principles of care and use of laboratory animals has been approved by the Biological Research Ethics Committee of Institute of Biophysics, Chinese Academy of Sciences.

C57BL/6J-HBV transgenic mice (C57BL/6J-Tg/N(Albl HBV)44Bri) were obtained from the Animals Center of Peking University Health Science Center Beijing (China), which expressed HBsAg in liver cells and secreted HBsAg in serum, as reported previously[21]. They (female, 42−47 days old, with an average body weight approximately 18 g) were housed under standard condition and their body weights and general health were monitored throughout the study. The investigation adhered to the principles of care and use of laboratory animals by the Institutional Animal Care and Use Committee of the Institute of Biophysics, Chinese Academy of Sciences.

According to the HBsAg levels, the transgenic mice were divided into three groups (physiological saline, LK at dose of 50 mg/kg, and LV at dose of 100 mg/kg, 9 per group). Each group was oral gavaged once a day for three weeks. The sera of the mice were collected before administration of drugs, on day 7, 14, 21, and 30 after administration of drugs and stored at −20℃ until determination. After the last sera were drawn, the mice were sacrificed, and their livers were excised. The livers were washed with cool PBS three times then divided into two parts. One part was fixed using 4% buffered formalin for histological analysis, and the other part was preserved in liquid nitrogen for RNA and protein determination.

2.3 FN and HBsAg expression analysis in HepG2.2.15 cells

Cells treated by LK or LV were collected by lysing the cells in a RIPA lysis buffer (Beyotime Biotechnology, China). The total protein amount was determined by the bicinchoninic acid (BCA) method (BCA ™ Protein Assay Kit, Pierce, USA) and then analyzed by Western blotting using FN monoclonal anti-cellular FN clone FN-3E2 (Sigma-Aldrich, Spruce St Louis, USA) according to the protocol described previously[10].

HBsAg in culture media was determined using diagnostic kits for HBsAg (Huameisk, Beijing, China), as described in the manufacturer's manual (Sino-American Biotechnology Co., China). The inhibition rates were calculated according to the following formula: inhibition rate (%) = $(A_{control} - A_{test})/A_{control} \times 100\%$ [11].

2.4 Measurement of FN, HBsAg, AST and ALT in C57BL/6J−HBV transgenic mice

Concentrations of FN in sera and in hepatic tissues were determined by enzyme linked immunosorbent assay (ELISA) as reported[22]. The primary fibronectin antibody (diluted with PBS plus 5% horse serum at a ratio of 1∶10 000) was used for serum FN and the monoclonal anti-cellular FN clone FN-3E2 (1∶2 500) was used as the primary antibody for tissue FN detection.

HBsAg in serum was detected semiquantitatively using the ELISA method, as described in the manufacturer's instructions (Hepanostika® HBsAg Ultra, Biomérieux, Netherlands). The HBsAg levels were expressed in optical density (OD) values. All performance of these measurements strictly followed the manuals from the ELISA kits.

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the serum were detected semiquantitatively with ELISA. The performance of these measurements strictly followed the manuals provided with the kits (Biosino Biotechnology Company Ltd, China).

2.5 Histological analysis

Livers (six per group) from hepatitis B transgenic mice treated with physiological saline, LK or LV were fixed with 4% buffered formalin and embedded in paraffin. Then, the livers were stained with haematoxylin and eosin (HE) and examined under a microscope. The normal and inflamed regions of the specimens were identified using the HE stains. The degree of liver cell degeneration and inflammation were evaluated.

2.6 Enzymolytic sites on fibronectin with LK

Human plasma FN was used to investigate the enzymolysis characteristics of LK on FN. FN (1 g/L, 10 μl) was incubated with LK at different final concentrations at 37℃ for 1 h or 2 h. The cleaved products were electrophoresed on a 10% reduced SDS-PAGE gel and electrotransferred onto a polyvinylidene difluoride membrane (PVDF, Millipore, USA). After staining with Commassie Brilliant Blue R-250, the main digested fragments were cut out for amino acid sequencing to identify the main cleavage sites (State Key Laboratory of Protein & Plant Gene Research, Peking University, Beijing, China).

2.7 Statistical analysis

The experiment results were evaluated using Student's *t*-test or two-factor analysis of variance. Statistical significance was accepted at a level of $P<0.05$.

3 Results

3.1 Effects of LK on FN levels and HBsAg secreted by HepG2.2.15 cells

The level of FN in HepG2.2.15 cells after treatment with LK was determined by Western-blot. It was shown that FN levels decreased depending on the LK concentration (Figure 1a). The level of FN decreased to 59% when the concentration of LK was 5 mg/L (Figure 1b). However, under the same experimental conditions, no obvious decrease in FN levels was observed in the presence of LV (Figure 1c and d). HBsAg levels in the media of HepG2.2.15 cells treated by LK and LV were also assessed with ELISA kit, showing an observable decrease in both groups (Figure S1).

3.2 LK reduces serum FN levels and decreases hepatic FN in transgenic mice

Next, we investigated whether LK suppresses the levels of serum FN and hepatic tissue FN in transgenic mice. The dose of LK (50 mg/kg) was established based on the package inserts of "Lumbrokinase Capsule" used in clinical for stroke and myocardial infarction. As shown in Figure 2a, as transgenic mice, the serum FN increased with time (physiological saline group), might be an outcome of

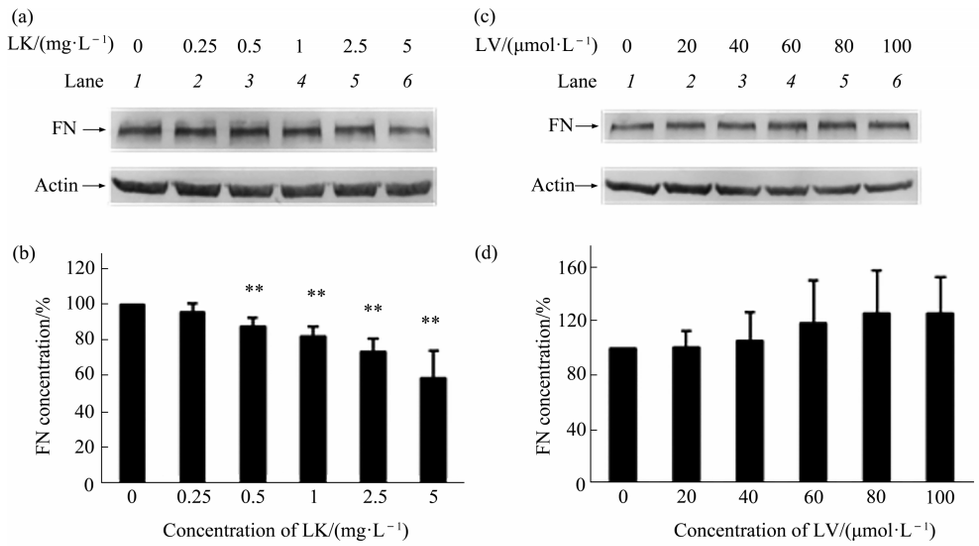

Fig. 1　Effects of LK or LV on FN levels in HepG2.2.15 cells

FN was analyzed with Western blotting (a, LK and c, LV), and the relative concentration of FN was determined with densitometric analysis (b, LK and d, LV). Data represent the $\bar{x}\pm s$ (n = 3), and ** P<0.01 *vs.* control (0 mg/L).

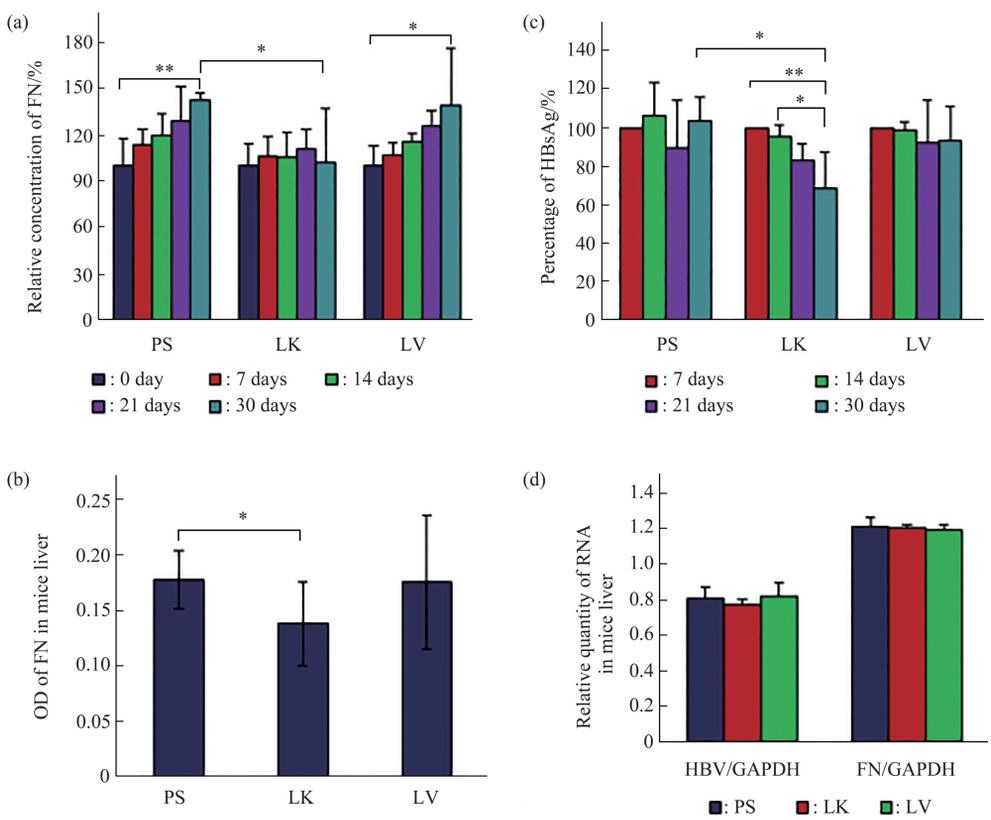

Fig. 2　Relative concentrations of FN and HBsAg in sera and FN concentrations in the hepatic tissues of mice after treatment with physiological saline（PS），LK（50 mg/kg）and LV（100 mg/kg）

(a) FN concentrations in sera. (b) FN concentrations in hepatic tissues. (c) HBsAg in sera ($\bar{x}\pm s$, n=9). (d) Changes in the RNA level of HBV and FN in the livers of mice after treatment with physiological saline, LK and LV ($\bar{x}\pm s$, n=6). * P<0.05 and ** P<0.01 *vs.* their respective controls.

the pathological process. In the LV group, the serum FN also increased with time, showing that LV had no inhibition effect on the FN increase during the pathological process. However, in the presence of LK, the serum FN was maintained at a stable level. Compared to the serum FN level in physiological saline group on day 30, LK-treated group kept its serum FN level at approximately 71.8%, without substantial changes from day 0 to day 30. These data indicate that LK maintains an average level of serum FN that is significantly different from the saline- and LV-treated groups after 30 days of the treatment. Sequentially, we measured the FN level in hepatic tissue after the mice were sacrificed at day 30. As shown in Figure 2b, FN decreased to 77.5% when the mice were treated with LK for 30 days compared to the physiological saline group (P=0.029). The level of FN in the LV-treated group was almost the same as the physiological saline group.

3.3 LK suppresses HBsAg levels in HBV transgenic mice

To investigate whether LK affects HBsAg, we determined the concentration of the antigen in serum. The reduction of HBsAg levels for the LK-treated group was significant (Figure 2c). It was 66.5% (P=0.01) that of physiological saline group after the transgenic mice were treated for 30 days. Both physiological saline group and LV group did not show obvious changes. Furthermore, we determined the FN RNA and HBV RNA with real time PCR, there were no significant difference among these groups (Figure 2d).

3.4 Effects of LK on the liver in transgenic mice

To determine whether LK influences the liver function, we measured the levels of ALT and AST in mouse serum. All the groups showed no significant changes (Figure 3a, b). The histological morphologies of the liver in different treatment groups are depicted in Figure 3c. The histopathological examinations showed that the lobules of liver existed in all the groups. All the hepatic cells showed signs of swelling, hyaline changes, necrosis and inflammation in the liver. Compared to the physiological saline treatment (Figure 3c), both LK and LV improved the liver state. The necrosis for the physiological saline, LK and LV groups were 15.7, 10 and 10.7 (mean number/microscope lowpower field of view), respectively. The areas of necrosis in LK-treated or LV-treated group were smaller than that in the physiological saline group.

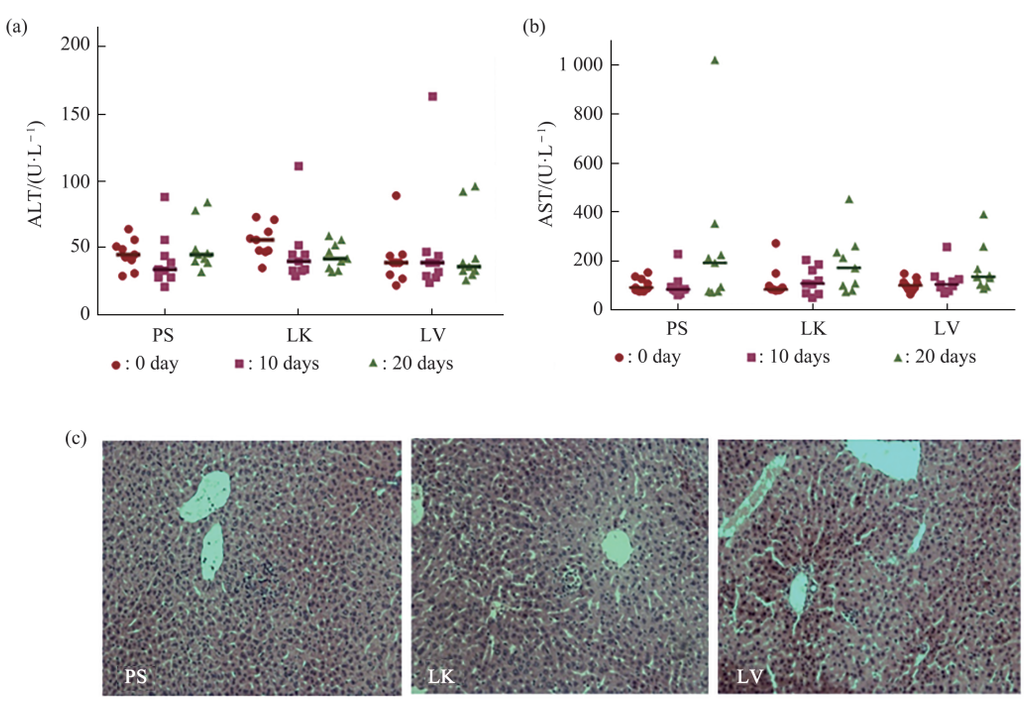

Fig. 3　The ALT and AST in the serum and histological changes in the mouse hepatic tissues after treatment with physiological saline, LK and LV

a, b, and c represent ALT (n=9), AST (n=9) and the histological morphologies of the liver.

3.5 Proteolytic sites on FN with LK

After incubation with LK, FN was digested into fragments (Figure 4a). Human plasma FN existed mainly as a 250 ku monomer on the reduced SDS-PAGE (lane *2*). As the enzyme concentration or the incubation time increased, the ~250 ku monomer was degraded, which resulted in some smaller polypeptides (lanes *3*−*9*). Based on the complete primary structure of human FN (NP002017, GenBank, mature peptide part), four cleavage sites were illustrated (Figure 4b). According to the cleavage sites, LK functions in an alkaline serine-like protease.

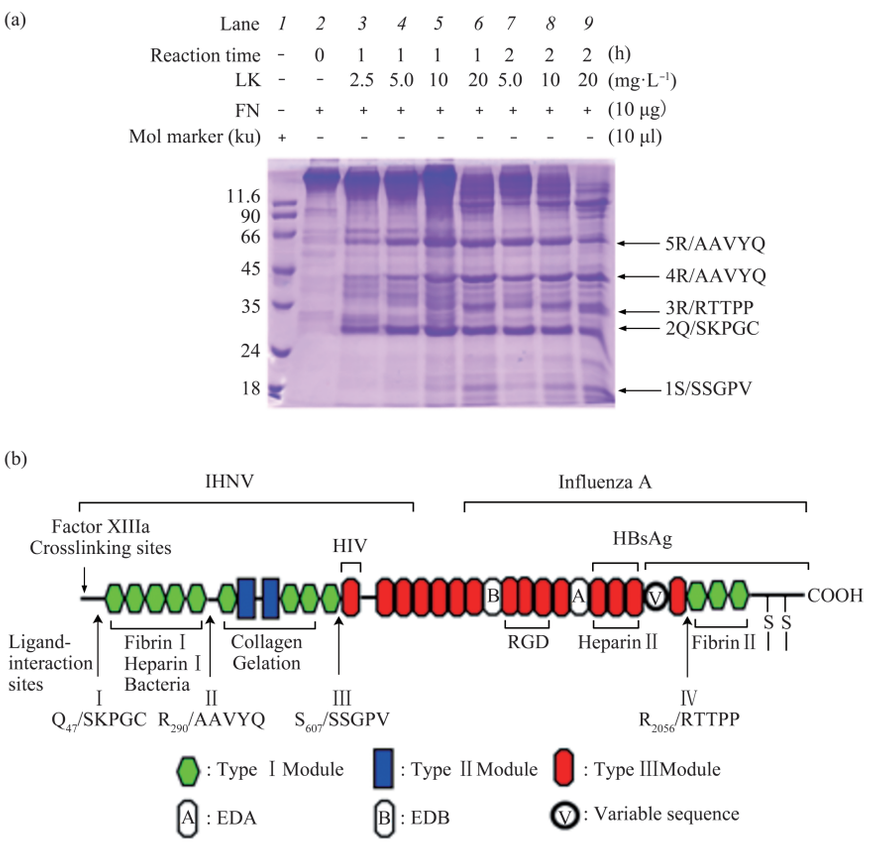

Fig. 4 （a）SDS−PAGE of fibronectin and the fragments digested by Lumbrokinase.（b）The main function domain of fibronectin digested by LK

LK (final concentrations as indicated) was incubated with FN at 37℃, and aliquots were taken at different time intervals for reducing SDS-PAGE during the degradation. The functional domains of FN digested by LK are indicated. Ligand interaction regions and cleavage sites are illustrated. Variably spliced extradomain A(A) and extradomain B(B) modules and the variable sequence(V) are shown in the subunit of FN.

4 Discussion

Fibronectin provides the binding sites for hepatitis B virus and contributes to the virus infection [13,23]. In this work, we employed LK to prevent the infection by HBV through FN. The results showed that FN levels were reduced in the presence of LK both *in vitro* and *in vivo*. Treatment with LK suppressed FN levels in the liver and decreased the levels of HBsAg in serum. LK mainly degraded FN into four fragments and destroyed the HBsAg binding site (R2058/R). Compared to the physiological saline group, administration of LK markedly improved the state of the liver. These data revealed that LK could be used as a potential drug to treat CHB through targeting FN.

The transgenic mouse model of the CHB surface antigen carrier is commonly used in the study on chronic HBV infection. In the mouse genome, the HBsAg S gene, pre S gene and HBxAg gene are

integrated[21]. The symptoms of HBV infection, such as swelling, hyaline changes, necrosis and inflammation, can be observed in liver cells. Hepatocellular cancer can be detected in some transgenic mice after they have been breeding for 48 weeks[24]. In this study, the biochemical results, such as levels of FN and HBsAg, and hepatocellular state showed responses to treatment with LK. All these indexes indicate that this transgenic mouse is suitable for chemotherapeutic experiments and for our work as well.

The aim of this study was to determine that LK had potential therapeutic value for CHB infection. We based this prediction on the following information: first, LK contains 8 earthworm proteases and is the ingredient of the Lumbrokinase capsule, which has been successfully used for treating clotting diseases in clinics. It has been demonstrated that the capsule is efficient and safe for clinical use[25]. Second, human FN is an important protein for HBV infection, and the reduction of FN in the serum and liver should inhibit HBV proliferation[12]. Knockdown of FN expression significantly inhibited HBV DNA replication and protein synthesis by activating endogenous IFN- α production[26]. According to Wang and colleagues[27], downregulation of FN may inhibit the hepatic endocytosis of HBV or affect FN pathways, which ultimately blocks HBV propagation. Third, as discussed above, LK recognized and degraded FN at R2058/R2059, which is located in the HBsAg binding domain. The degradation could destroy the HBV binding site. EFNase, which is one of the isozymes from LK, effectively hydrolyzes FN at R2039/H2040, which is also in the HBsAg binding domain[10]. Fourth, LK decreased FN levels in HepG2.2.15 cells and the transgenic mouse model, especially those in the liver. Finally, administration of LK could ameliorate negative changes to the liver in the C57BL/6J-HBV transgenic mouse.

FN polymerization is not only necessary for forming a collagen matrix, but also a contributor to liver fibrosis[16]. Fibrosis is the scarring response of the liver to injury that occurs in most chronic inflammatory liver diseases, including CHB infections[28]. LK activated metallopeptidase and hydrolyzed matrix molecules[29,10]. Urokinase-type plasminogen activator (uPA) has been widely studied in the treatment of fibrosis. LK is a group of proteases and recognizes and reacts with chromozym U, which is the substrate for uPA[30]. It has been reported that earthworm extracts obviously suppressed liver fibrogenesis in rats induced with CCl_4[31]. Additionally, one of LK iszymes is able to recognize and degrade HBeAg, showing protective effect on hepatic cells[32]. We suggest that LK can be used in the treatment of fibrosis, not only because it hydrolyzes FN but also because it activates the JAK/STAT pathway[33], which is a target for therapeutic intervention of liver fibrosis[34] resulted from CHB and other diseases such as alcoholic and non-alcoholic diseases[35]. In a putative therapy process, proteolytic isozymes are thought to play a primary role in the degradation of the connective tissue generated in fibrosis[36].

In the current study, LK recognizes and degrades FN *in vitro* and *in vivo*. We could not observe a remarkable change in the amount of FN RNA. Thus, decreased FN levels should not be a result from the effect of LK on the transcription of FN RNA. LK quickly decreased FN levels not only in the molecular and cellular experiments but also in the animal tests. A coordinated action of those isozymes that affect FN in animals may be the explanation. However, the mechanism underlying why LK (cock-tail components) show a substantial effect on FN levels in animals needs further investigation.

There were also some drawbacks in the study. We selected C57BL/6J-HBV transgenic mouse as experimental animal. The mouse has the characteristics of HBV infection and is suitable for the study of an HBV infection treatment. However, the model has some limitations. Because the integrated gene is a partial HBV gene with only the HBsAg S gene, pre-S gene and HBxAg gene, no viral replication in the mice. LV used as a positive control in this experiment showed little efficiency in the treatment. In future studies, other animals or other positive controls may be selected. However, the result cannot prevent us from exploring the potential of LK for the treatment of HBV infection, especially for chronic HBV infections.

5 Conclusion

LK has a definite effect on FN in HepG2.2.15 cells and C57BL/6J-HBV transgenic mice that leads to improvements in the liver. Our data provide valuable information for understanding the

therapeutic role and the potential therapeutic mechanism of LK as an effective antiviral medicine to treat CHB.

Supplementary materials 20200151_Figure S1 are available at paper online (http://www. pibb. ac. cn or http://www.cnki.net).

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蚓激酶在慢性乙型肝炎治疗中的潜在作用*

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摘要　纤连蛋白（FN）是参与乙型肝炎病毒感染和肝脏纤维化的重要分子. 蚓激酶（LK）是从赤子爱胜蚓（Eisenia foetide）中提取的一组蛋白水解同工酶，能水解纤维蛋白治疗凝血相关疾病. 从这组同工酶中分离纯化出单一活性成分，在体外可降解纤连蛋白，被命名为蚯蚓纤连蛋白水解酶（EFNase）. 然而，LK 能否预防乙型肝炎病毒感染以及缓解因乙型肝炎导致的肝脏损伤等问题还不清楚. 本研究以人肝癌细胞系 HepG2.2.15 为细胞模型，观察 LK 对乙型肝炎表面抗原（HBsAg）或 FN 水平的影响；以 C57BL / 6J-HBV 转基因小鼠为动物模型，探讨 LK 对小鼠血清中 HBsAg、FN、丙氨酸氨基转移酶（ALT）、天门冬氨酸氨基转移酶（AST）水平及肝脏病理改变的影响. 结果表明，LK 在体内外均能抑制 HBsAg 的生成，降低血清和肝脏 FN. 与生理盐水处理组相比，LK 改善了肝脏的状态. 这些数据为理解 LK 作为治疗乙型肝炎的潜在有效药物的治疗作用提供了有价值的信息.

关键词　蚓激酶，纤连蛋白，乙型肝炎表面抗原，慢性乙型肝炎，组织纤维化

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