PIBB 生物化学与生物物理进展
Progress in Biochemistry and Biophysics
2022,49(1):202~218
www.pibb.ac.cn

Compound Lumbrokinase Isozymes Decrease Hepatitis B Antigens and Protect Hepatic Function*

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Abstract **Objective** Lumbrokinase isozymes (LKIs), which were isolated from earthworm called Dilong in traditional Chinese medicine (TCM), have been used as the active ingredient of the enteric-coated capsule to treat clotting disease approximately 30 years. Recently, the study of LKIs on other critical diseases received much attention. **Methods** To demonstrate the efficacy of LKIs on hepatitis B proteins, we incubated surface antigen (HBsAg), core antigen (HBcAg) and e antigen (HBeAg) with LKIs at different concentrations for different time intervals, and then estimated their cleavage sites. HepG2.2.15 cells were incubated with LKIs and their HBsAg, HbeAg were determined by ELISA and Western blotting. HBV-transgenic mice (Balb/c) were gavaged with LKIs for 30 days. HBsAg and HBeAg in serum were detected by ELISA and Western blotting, and HBcAg in hepatic tissues were immunohistochemically stained. Hematoxylin-eosin (HE) staining was used to exhibit liver endolysis of LKI-treated HBV-transgenic mice. Serum glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) were semi-quantitatively detected with ELISA. After intraperitoneal injection of LKIs into Sprague Dawley rats, LKIs in serum and liver tissue were assayed. Longyan sheldrakes (LYS) were gavaged with LKIs for 30 days, and their serum HBV DNA were assayed by PCR. **Results** We observed that the capsule ingredient is a compound drug containing 6 LKIs. By incubating with the HBV proteins, LKIs were probably estimated to degrade HBsAg at K141/P142 and R160/F161, HBcAg at R142/E143, and HBeAg at R122/E123. LKIs significantly inhibited HBsAg and HBeAg secretion from HepG2.2.15 cells. Levels of HBsAg and HBeAg in serum and those of HBcAg in hepatic tissues decreased in HBV-transgenic mice gavaged with LKIs, suggesting a suppression of viral assembly. Levels of GOT and GPT and the number of endolysis in liver exhibited by HE staining were decreased in the LKI-treated HBV-transgenic mice, demonstrating LKIs' protecting mice hepatic cells. The activity of LKIs could be detected in serum and hepatic tissues of Sprague Dawley rats after being intraperitoneally injected with LKIs. After gavaged with LKIs, the ducks showed a decrease in their serum HBV DNA levels. **Conclusion** The current work indicates that LKIs degrade HBs, HBc and HBe proteins and may interfere with the virion assembly and release, leading to decrease in the virus transmission between hepatocytes, and to hepatic protection.

Key words HBeAg, HBcAg, HBsAg, lumbrokinase, hepatitis B virus, isozyme
DOI: 10.16476/j.pibb.2022.0003

Hepatitis B virus (HBV) infection remains a major public health problem worldwide. Acute and chronic hepatitis B (CHB) are associated with significant morbidity and mortality worldwide[1]. While efficient vaccines are now available against HBV infection, it is still estimated that 2 billion people have been infected with HBV[2], and 257 million people or 3.5% of the population, live with chronic HBV infection worldwide (Global hepatitis

* This work was supported by a grant from The National Natural Science Foundation of China (31470036).
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Received: October 21, 2021 Accepted: January 5, 2022

report, WHO 2017), with 240 million people being chronic HBV surface antigen (HBsAg) carriers[3]. Chronic HBV infection can lead to hepatic cirrhosis and hepatocellular carcinoma[4], and around 650 000 people die each year from the complications of CHB[5], which remains a major health problem worldwide[6].

HBV genome codes several distinct proteins such as HBsAg and HBcAg. HBsAg is composed of 3 HBV envelope proteins, called small, middle, and large proteins[7-8]. The small and large proteins are necessary for virion production[9]. In infectious virions, the relaxed circular DNA (rcDNA) is enclosed within a protein capsid composed of HBcAg[10], which is further surrounded by an outer coat consisting of a host-derived lipid bilayer studded with viral envelope or HBsAg. As described previously, high rates of sub-viral particles (SVPs) bearing HBsAg is essential for the HBV assembly after DNA duplication[11]. Therefore, it still needs a novel anti-HBV regimen, in which the medicine targets the HBsAg, HBcAg, and HBeAg proteins to interfere with the viral assembly, and even inhibits the HBV infection.

Dilong (earthworm) has long been used as a material to develop antipyretic and diuretic drugs[12]. Earthworm extract is also employed to treat abdominal jaundice, as recorded in “Compendium of Materia Medica” a primary indication in traditional Chinese medicine (TCM)[13]. These 6 lumbrokinase isozymes (LKIs) are isolated from earthworms (*Eisenia fetida*, *E. fetida*) fibrinolytic activities[14-15]. Fan and Wu have used LKIs as the ingredient for the enteric-coated capsule called lumbrokinase capsule (LKC) to treat patients with stroke[16-18] and cardiovascular disorders[19-20]. The capsule contains 6 LKIs based on their fibrinolytic function[14]. Wang *et al*. [21] found a fibronectinase one of LKIs from *E. fetida* rapidly degrading fibronectin that is involved in HBV infection. LKIs also decrease fibronectin in the hepatic cells, indicating that LKIs potentially function in ameliorating liver fibrosis[22]. Recently, we observed that *Ef*P-III-1 degrades HBeAg *in vitro*[23]. It is imperative to clarify whether LKIs affect and decrease HBsAg, HBcAg, and HBeAg proteins, and protect hepatic cells.

This study focuses on the pharmaceutical ingredient of LKIs, as a compound drug isolated from *E. fetida*. LKIs administration disturbs viral assembly, protects hepatic cells and interferes with HBV infection, through targeting and degrading HBsAg, HBeAg, and HBcAg *in vivo* and *in vitro*. The multi-functional LKIs may be used as a potential drug to treat CHB on clinical in the future.

1 Materials and methods

1.1 Lumbrokinase isozymes and Dilong

LKIs were a kind gift from Beijing Baiao pharmaceuticals Co., Ltd (Beijing, China). They isolated LKIs from Dilong (*E. fetida*) in Beijing. The enteric-coated capsule has been approved by the China Food and Drug Administration (CFDA, H11021129) for the treatment of clotting disease for instance stroke.

The animal experiments followed the principles of care and use of laboratory animals and were approved by the Animal Welfare and Research Ethics Committee of the Institute of Biophysics, Chinese Academy of Sciences (SYXK2019-01).

1.2 Assay of LKIs activities

Different LKI concentrations were analyzed on 12% fibrin-SDS-PAGE (F-SDS-PAGE) as described previously[24]. The F-SDS-PAGE was formed by adding 0.12% fibrinogen (Sigma Aldrich, St Louis, MO, USA) and mixing 50 μU thrombin (Sigma Aldrich, St Louis, MO, USA). After electrophoresis, the zymogram was obtained when the gel was renatured with 2.5% TritonX-100 for 30 min, incubated with PBS buffer (pH 7.2) at 37° C for 30 min, stained with Coomassie Brilliant Blue R-250, and de-colored with 7% acetic acid. The images were captured using ChampGel 5000 imager (Sage Creation Science Co., Ltd, Beijing, China). The densities were measured to estimate the relative activities using Photoshop-2019 (Adobe Photoshop Inc; CA, USA) as described previously[24].

1.3 Hydrolysis of HBeAg，HBcAg，and HBsAg with LKIs

A recombinant HBeAg (Beijing Huamei Bioscience Technology Ltd, Beijing, China) was incubated with 0, 0.25, 0.5, 1, or 2 mg/L LKIs at 37℃ for 60 min. The reaction was terminated by adding 6× sample loading buffer and boiling for 10 min. Samples (12 μl each) were electrophoresed on 15% SDS-PAGE and the protein bands in the gel were visualized using Coomassie Brilliant Blue R-250. Then, under the same conditions, HBeAg degradation

in the presence of LKIs (final concentration 1 mg/L) was observed at different time intervals. HBeAg in the absence of LKIs and presence of trypsin (final concentration 2 mg/L) was used as the negative and positive controls, respectively.

Under the same experimental conditions, HBsAg (adr, recombinant N-truncated; a.a. 226, Abnova, Taipei, Taiwan), and truncated HBcAg (recombinant; a. a. 1−183, RayBiotech, Inc., GA, USA) were also incubated with LKIs and then assayed by SDS-PAGE. For convenience, we have stated small HBsAg as HBsAg in this work unless stated otherwise.

To estimate the proteolytic site on HBeAg in the presence of LKIs, HBeAg (0.2 g/L, 3 μl) was incubated with the isozyme (final concentration 2 mg/L) at 37℃ for 1 h. The cleaved products were electrophoresed on 15% SDS-PAGE and their apparent molecular masses were analyzed as described[25]. The proteins were transferred on polyvinylidene fluoride (PVDF) membrane (Millipore, MA, USA). After staining with Coomassie Brilliant Blue R-250 for 10 s, the bands were cut out for amino acid sequencing to identify the main cleavage sites on the Applied Biosystem Automated Protein Sequencer (Applied Biosystem Inc.; CA, USA). In addition, the reaction mixture was subjected to mass spectrometry (REFLEX III, Bruker; Bremen, Germany). The proteolytic sites were estimated based on the intact sequences of HBeAg (GenBank CAA01610.1, https://www. ncbi. nlm. nih. gov/protein/CAA01610.1), HBcAg (GenBank AUR80754.1, https://www. ncbi. nlm. nih. gov/protein/AUR80754.1), and HBsAg (GenBank ACX36042.1, https://www. ncbi. nlm.nih.gov/protein/ACX36042).

1.4 Cell culture and treatments

HepG2.2.15 cell line was provided by the Animal Center of Academy of Military Science of the Chinese People's Liberation Army (Beijing, China). HepG2.2.15 cells were clonal cells derived from HepG2 cells, transfected with a plasmid containing HBV DNA. The HepG2.2.15 cells were cultured on Dulbecco's modified eagle medium (high glucose, Gibco, Invitrogen Corporation; Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (PAA; Pasching, Austria), antibiotic G-418 sulfate (final concentration 380 mg/L, Amresco; Solon, OH, USA) and penicillin-streptomycin solution (final concentrations 100 units/ml penicillin and 100 mg/L streptomycin; HyClone; Logan, UT, USA).

HepG2.2.15 cells were seeded at 1.5×10^5 cells per tissue culture dish (Corning; NY, USA)[26]. LKIs were added to the culture medium three days after seeding and incubated in the presence of LKIs for 6 days with changes of media every 3 days. After treatment, the culture supernatants were collected and subjected to HBeAg and HBsAg analysis by Western blotting and ELISA as described previously[21, 27]. The inhibition ratio was calculated according to the following formula: inhibition rate (%) = [(Ac − As)/Ac]×100%, where Ac and As represent the absorbance of control, and that of sample, respectively[21]. As described[28], cell viability was also examined using cell-counting kit-8 (DOJINDO; Kumamoto, Japan).

1.5 Treatment of HBV−transgenic mice and Longyan sheldrakes

HBV-transgenic mice came from Transgenic Engineering Lab, Infectious Disease Center, Guangzhou, China[29]. Inbred Balb/c mice were selected as the transgenic receptor mice. After HBV genome plasmid amplification, the plasmid DNA was extracted, digested by enzyme, purified, and microinjected into male prokaryotes of fertilized eggs in Balb/c mice. Lamivudine (LV, GlaxoSmithKline; Philadelphia, PA, Britain) was selected as positive control because it is a common drug in treating chronic HBV infections on clinic[30]. Thirty HBV-transgenic mice were divided into three groups randomly and received 50 mg/kg LKI-, 100 mg/kg LV-, or 0.9% NaCl (saline)-gavage daily. Then, 6 mice of each group were euthanized. The treatment time (d) for transgenic mice were as indicated in the figure legends. The remaining four mice were raised without drugs for 7 days. During the medication, mice were weighed from the beginning of medication (D_0) to their euthanasia (P_7).

Longyan sheldrakes (LYS) came from School of Basic Medical Sciences, Guangzhou University of Chinese Medicine (China), which are naturally infected with duck HBV (DHBV)[31-32]. They were randomly divided into 5 groups and received gavage administration each day for 28 days (D_{28}) with 100, 50, and 25 mg/kg LKIs, 100 mg/kg LV, or saline (n= 14). Then 8 ducks from each group were euthanized, and 6 were left without administration for 5 days (P_5). The ducks were weighed at D_0, D_7, D_{14}, D_{21}, D_{28}, and P_5. In this experiment, we provided the LKC and Beijing Kangyuyao Biological Company (Beijing,

China) managed and provided the duck experimental data.

1.6 Sample collection

Blood of HBV-transgenic mice was collected as described previously[33] at different time intervals as indicated in Table S1. Sera were prepared and stored at −70°C until determination. After the animals were euthanized, the liver, spleen, and kidney were quickly dissected and fixed in 4% paraformaldehyde. The liver tissues were also immediately homogenized in lysis buffer (Beyotime; Shanghai, China) and centrifuged to collect supernatants for Western blotting. Blood of LYS were collected at different time intervals (D_0, D_7, D_{14}, D_{21}, and D_{28}), they were rested and observed at P_7.

1.7 Assay of HBeAg，HBsAg，and HBcAg by Western blotting and ELISA

Western blotting was performed to detect HBeAg (recombinant HBeAg, Beijing Huamei Company, China), HBsAg, and HBcAg levels. The samples (reaction mixture of HBeAg and LKIs, cell culture media, or serum) were first analyzed using 15% SDS-PAGE. The proteins in the gel were then transferred on PVDF membrane (Millipore; MA, USA). The membrane was blocked in Tris-HCl buffered saline (TBS) containing 5% nonfat milk and then incubated with primary antibody (anti-HBeAg and anti-HBsAg, Santa Cruz; Biotechnology, CA, USA; and anti-HBcAg, Millipore; MA, USA) at 4℃ overnight. After rinsing with TBS plus 0.2% Tween-20 TBS (TBST), the membrane was subsequently exposed to peroxidase-conjugated IgG (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd, Beijing, China) in the same solution at 37℃ for 2 h. The blots were washed and then developed using a super enhanced chemiluminescence detection kit (Applygen Technologies Inc., Beijing, China). The protein bands were visualized after exposing the membranes to Kodak X-ray film. ELISA was immediately performed with diagnostic kits for HBeAg and HBsAg (Beijing Wantai Biological Pharmacy Enterprise Co., Ltd, Beijing, China) as described in the manual.

1.8 HBV RNA and DNA measurements

Total cellular RNA was extracted using TRIzols Regent (Invitrogen; CA, USA), treated with RNase-free DNase I (Promega; Madison, WI, USA), and used for cDNA synthesis by Super Script™ cDNA Synthesis Kit (Invitrogen) according to the manufacturer's protocols. The RNA expression was measured using SYBR green-based real-time PCR performed on Corbett 6200 Real-time PCR system (Corbett Research Pvt Ltd, Sydney, Australia). The optimized amplification protocol consisted of an initial denaturation step of 94℃ for 5 min, followed by 35 amplification cycles at 94℃ for 15 s, annealing at 54℃ for 15 s, and extension at 72℃ for 10 s. To ensure full extension of the products, every cycle was followed by an extra incubation at 72℃ for 10 min.

HBV DNA from the culture media and HepG2.2.15 cells was extracted using TIANamp Virus DNA Kit (TIANGEN biotech Co., Ltd, Beijing, China) and amplified under the same conditions as cDNA samples except annealing at 57°C for 15 s and extension at 72℃ for 30 s.

Serum samples from HBV-transgenic mice were boiled for 10 min to remove the proteins and the supernatant was used as the PCR template. HBV DNA in the serum was detected under the same conditions as that in HepG2.2.15 cells.

LYS blood samples were collected from the posterior tibial veins and centrifuged (2 000 *g*, 20 min, 4℃). The serum was subjected to PCR to screen DHBV positive ducks. DHBV was amplified through 40 cycles, each cycle consisting of 94℃ for 30 s, annealing at 55℃ for 60 s, and extension at 72℃ for 60 s [34].

Viremia was assessed throughout the treatment and follow-up period by semi-quantitative DHBV DNA detection in duck serum using dot-blotting hybridization. Serum (50 μl) was spotted directly on nitrocellulose filters (Pall Corporation, Ann Arbor, MI, USA). After denaturation and neutralization, the filters were hybridized with a full-length DHBV genomic DNA probe labeled with ^{32}P using a nick translation system kit (Invitrogen; CA, USA) [35]. The DNA blots were placed side up in the film cassette and a sheet of autoradiography film was placed on top of the blots. The blots on the film were scanned at 490 nm to analyze the concentration of DHBV DNA in serum[36]. Primers used in this study were synthesized by Sangon Biotech Co., Ltd, Shanghai, China (Table S2).

1.9 Immunohistochemistry and hematoxylin–eosin（HE）staining

As described previously, the mice livers were

immersed in 4% paraformaldehyde for 48 h immediately after dissection[37]. After fixation, the livers were embedded in paraffin blocks and 5−8 μm thick sections were processed for immunohistochemistry. De-paraffined and hydrated sections were incubated in target retrieval solution (Dako, Glostrup, Denmark) at 95℃ for 30 min for enhancing immunoreactivity. After permeabilization with 0.3% H_2O_2 in absolute methanol for 10 min to block endogenous peroxidase, the sections were incubated in 10% normal goat serum (Burlingame, CA, USA) in PBS at room temperature (~20℃) for 30 min. The specimens were then incubated with anti-HBcAg monoclonal antibody solution (Millipore; Bedford, MA, USA) diluted in PBS at 4℃ overnight. After washing with PBS, sections were subsequently incubated with biotin-labeled secondary antibodies (Invitrogen, Carlsbad, CA, USA) (37℃, 1 h). The immunoreaction was detected using horseradish peroxidase-labeled antibodies (GE; Piscataway, NJ, USA) (37℃, 1 h) and red staining was visualized using an AEC system (Nikon Optical; Tokyo, Japan).

Semi-quantitative immunohistochemical detection was used to determine the HBcAg levels as described previously[38]. The immunoreactivity for HBcAg was scored by evaluating the sum of positive stained zones and staining intensity over the total number of the zones. Intensity was scored as "+" (weakly stained), "++" (moderately stained), and "+++" (strongly stained). HE staining was carried out in the immunodeficiency animal laboratory of Institute of Biophysics, Chinese Academy of Sciences, Beijing, China.

1.10 Glutamic−oxaloacetic transaminase (GOT), glutamic−pyruvic transaminase (GPT), and creatinine level analysis

GOT and GPT levels in the serum were semi-quantitatively detected with ELISA. Creatinine level was determined using mouse creatinine assay kit (picric acid). The performance of these measurements strictly followed the manuals provided with the kits (Sangon Biotech Co., Ltd, Shanghai, China).

1.11 Data analysis

All values reported are means ± standard errors (SE) unless otherwise indicated. Data analysis was performed by one way analysis of variance (ANOVA) using Origin 7.0 (Origin Lab, Northampton, MA, USA) statistical software. Differences with 95% probability level (P<0.05) were considered significant.

2 Results

2.1 LKIs cleaved HBsAg, HBcAg and HBeAg

First, we prepared the fibrin polyacrylamide gel electrophoresis (F-PAGE) to assay the activities of LKIs. F-PAGE exhibited 6 fibrinolytic bands with different apparent molecular masses (40.79, 36.49, 34.03, 30.44, 28.40, and 26.49 ku; Figure 1a, b). Density-based scanning showed 6 main peaks of proteolytic activity in percentage (Figure 1c, Table 1). As described previously, *L. rubellus*[39] or *E. fetida*[40] contains 6 LKIs with fibrinolytic activity. These data indicate that the ingredient of the enteric-coated capsule contains 6 LKIs used in this work.

As we know, infectious HBV is spherical consisting of HBsAg that envelops the viral nucleocapsid. To identify whether LKIs interfere with HBV assembly, we incubated HBsAg with LKIs at 37°C and collected aliquots at different time intervals. As exhibited in 15% SDS-PAGE, HBsAg was hydrolyzed and two protein bands with the apparent molecular masses of 18.45 and 12.35 ku for HBsAg (23.90 ku) were released in the presence of LKIs (averagely 32.77 ku) (Figure 1d). The cleavage sites were estimated at K141/P142 and R160/F161 (Figure S1), based on the amino acid sequence of HBsAg (adr, Abnova, GenBank APY18030.1).

HBcAg constructs the nucleocapsid involving viral assembly[10]. To demonstrate that LKIs disturb the HBV assembly, we incubated HBcAg (~18.31 ku; Figure 1e) with the proteases for different time intervals. Different from HBsAg, hydrolysis of HBcAg by LKIs was slow, and generally produced a peptide (~12.72 ku) in 4 days under current experimental conditions. According to our previous work[23], the N-terminal sequence of the released peptide was identical to that of the intact HBcAg peptidyl chain (n—MDIDPYKEFG—). Thus, the cleaved site was estimably at 142R/143E (Figure S2). HBcAg degradation suggests that LKIs may impede viral assembly, leading to the suppression of HBV infecting ability.

Serum HBeAg has been widely used to monitor viral infection and treatment response as it is usually associated with viral replication[10]. To identify whether LKIs decrease HBeAg levels, we incubated a recombinant HBeAg with LKIs (2 h at 37° C). As

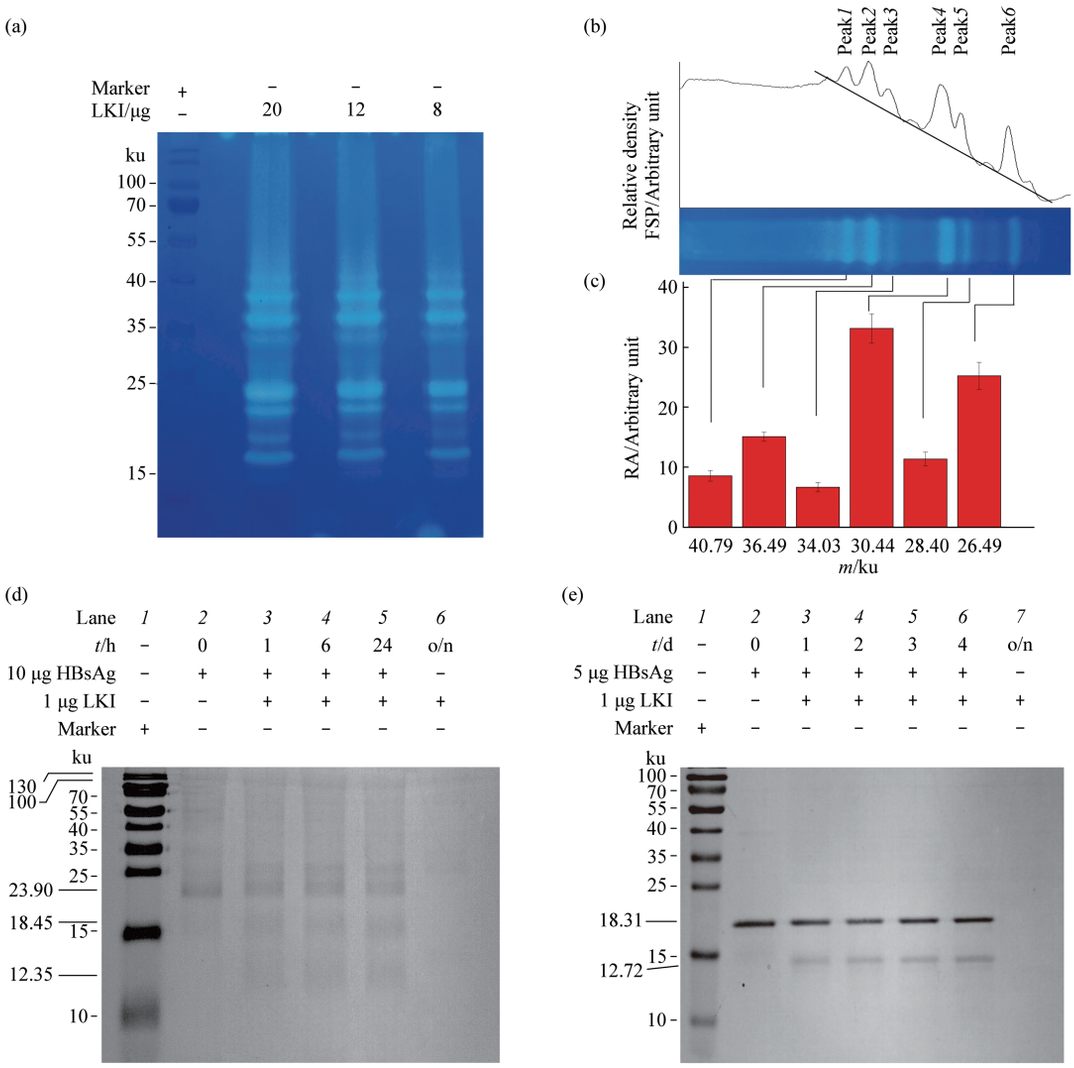

Fig. 1　Assays of lumbrokinases （LKIs），and hepatitis B surface antigen（HBsAg）and core protein（HBcAg）hydrolysis

The LKIs were isolated on 12% SDS-PAGE mixed with 0.15% fibrinogen and 50 μU thrombin at 37°C for 30 min. Different amounts of LKIs (20, 12, and 8 μg) were added to lanes at 37°C, respectively (a). After fibrinolytic reaction, the gel was stained with Coomassie Brilliant Blue R-250, followed by density-based scanning to evaluate the relative activity (in percentage) of each isozyme (b, c). Conditions were as indicated, HBsAg (d) and HBcAg (e) were incubated with LKIs at 37°C and aliquots were collected at different time intervals for assays on 15% SDS-PAGE.

Table 1　Relative activity and apparent molecular masses of LKIs[1)]

Peak	*m*/ku	Density based scanning		Assay of activity	
		Area	Ratio/%	Area	Ratio/%
1	40.79	12±1.8	15.29	9±0.6	9.13
2	36.49	9±2.2	11.6	15±1.1	15.64
3	34.03	18±5.7	23.2	9±1.4	9.86
4	30.44	12±4.1	14.89	27±3.1	27.74
5	28.40	9±3.0	11.65	13±3.3	14.16
6	26.49	18±4.4	23.37	14±1.8	14.65
aMW	32.77	-	-	-	-

[1)]Data from Figure 1, and measurements of samples (n=3) shown in means ± *SD*.

shown, HBeAg (10 μg, ~14.50 ku) was degraded and released a major cleaved protein band about 12.48 ku in the presence of 1.0, 2.0, 4.0, and 10 μg LKIs (Figure S3a). The cleaved peptide N-terminal sequence was identical to that of HBeAg (n—Thr—Met—Ile—Thr—Asn—), and the cleaved site was estimated at R122/E123 (Figure S2). The density of HBeAg protein band markedly decreased in 120 min in the presence of 1 mg/L LKIs (Figure S3b). Hydrolysis of HBeAg underwent in an LKI-concentration- and time-independent manner detected by Western blotting (Figure S3c, d) and ELISA (Figure S3e, f).

2.2　LKIs decrease HBsAg and HBeAg secreted by HepG2.2.15 cells

HepG2.2.15 cells with HBV genomic DNA release HBsAg and HBeAg into the cultured media[41]. Before studying the effect of LKIs on secreted HBsAg and HBeAg, we assessed the potential toxicity of those isozymes on HepG2.2.15 cells. As shown in Figure S4, 0.25−5.0 mg/L LKIs, or LV (positive control), did not affect cell viability significantly as examined by cell-counting kit-8 under the experimental conditions.

To investigate whether LKIs affects the secreted antigen levels, we added different concentrations of LKIs to the HepG2.2.15 cell culture media and collected aliquots for observing the changes in HBV proteins. Both Western blot and ELISA showed that secreted HBsAg levels decrease with the increase of LKIs concentration in the culture media (Figure 2a, a′). The decreased levels of secreted HBeAg in the presence of LKIs were also observed using WB and ELISA (Figure 2b, b′).

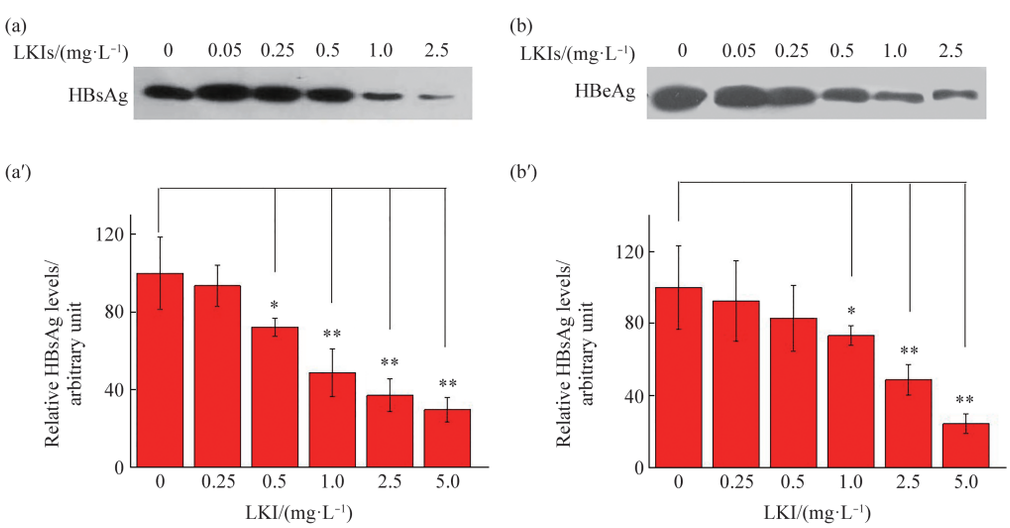

Fig. 2　Decreased HBsAg and HBeAg levels in HepG2.2.15 cell culture media

HepG2.2.15 cells were cultured with different lumbrokinase isozyme (LKI) concentrations for 6 days, and the media were collected for determining the HBsAg and HBeAg levels by Western blotting (a, b) and ELISA (a′, b′) as indicated. The inhibition ratio was calculated as described in Materials and Methods. The data represent means±*SD* (n=3). *, P<0.05; **, P<0.01.

2.3　LKIs decrease levels of HBsAg, HBeAg, and HBcAg to protect the hepatic cells in HBV–transgenic mice

HBV-transgenic Balb/c mice were divided into three groups: LKI-, LV-, and saline-treated (n=10 each group). During the drug administration course, there were no statistically significant differences in body weights among the three groups (Figure S5). No obvious side effects were observed in the animals on antiviral therapy or control animals during the 35-day treatment.

Serum HBsAg levels decreased to ~34% (using Western blotting, Figure 3a) and ~64% (using ELISA, Figure 3c) in the LKI-treated transgenic mice (LKIs, 50 mg/kg) at D_{23} compared with that at D_0 (P=0.039 1). Interestingly, the serum HBsAg levels significantly bounced back (P<0.05) at P_7 compared with that at D_{23}. However, serum HBsAg levels in the LV-treated (Figure 3d) or saline-treated (Figure 3e) transgenic mice had little changes, even after withdrawing the administration.

HBeAg was significantly decreased (P<0.05) in

the LKI-treated transgenic mice (Figure 3b). At D_{15} and D_{23}, the HBeAg levels decreased to ~55% (P=0.016 5) and ~28% (P=0.003 4) (Figure 3f), compared with that at D_0. However, at P_7, HBeAg levels in the blood returned to 69% (P<0.05) compared with that at D_{30}. Neither the serum HBeAg levels in LV (100 mg/kg/d)-treated HBV-transgenic mice, nor the control group showed significant decreases (Figure 3g, h).

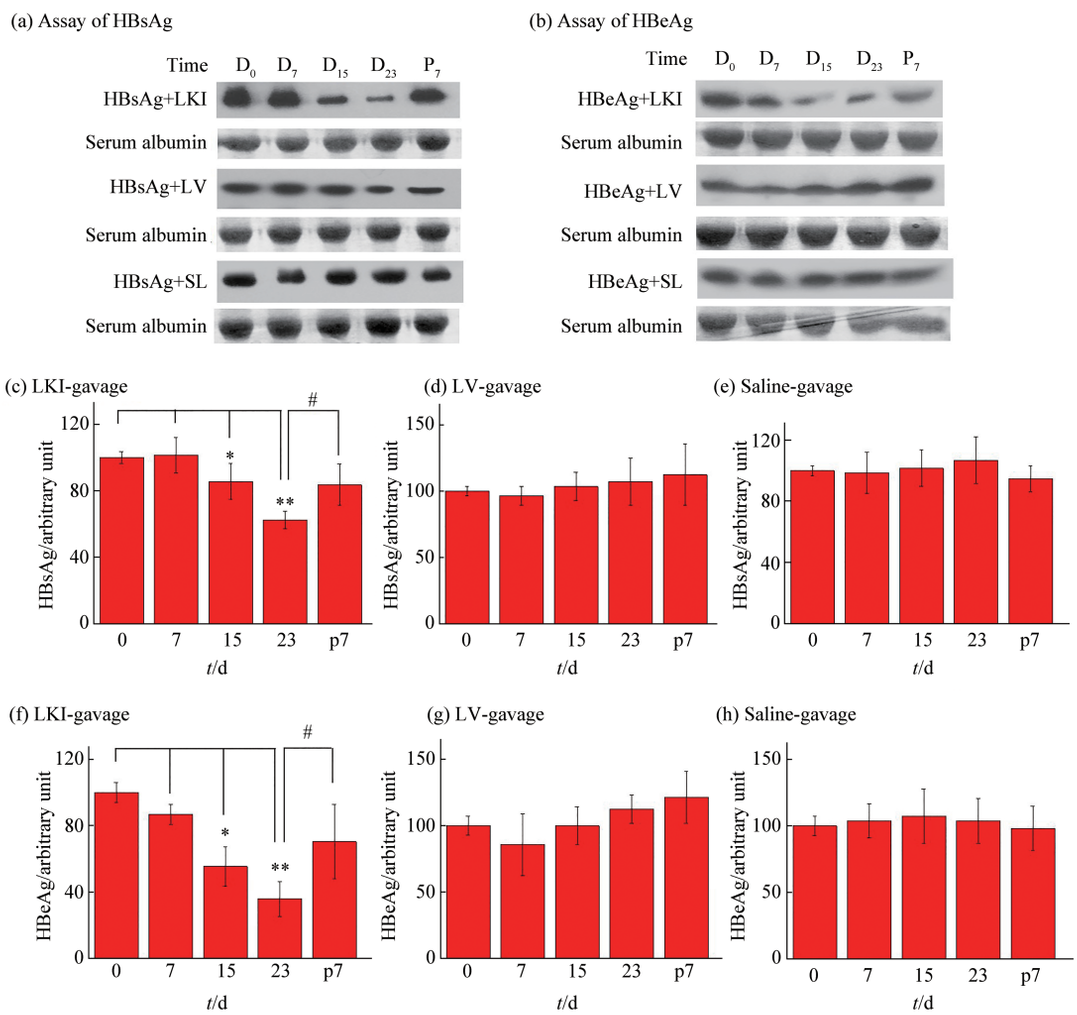

Fig. 3　Effects of LKIs on serum HBsAg and HBeAg in HBV−transgenic mice

HBV-transgenic Balb/c mice (n=6, each group) were gavaged with LKIs, lamivudine (LV), or saline (SL) once daily for 30 days, and their sera were collected at different time intervals (0, 7, 15, and 23 d; then gavage withdrawal for further 7 days) for examining the HBsAg level using Western blotting (a) and ELISA (c, LKI-gavage; d, LV-gavage; e, saline-gavage group). Under the same conditions, the HBeAg level was determined using Western blotting (b) and ELISA (f, LKI-gavage; g, LV-gavage; h, saline-gavage group). Serum albumin was used as loading control. P, withdrawal of LKIs, LV, or saline for 7 days, respectively. n=6, *, #, P<0.05; **, P<0.01.

2.4　HBcAg changes on LKI treatment

HBcAg within the hepatocytes is associated with the inflammatory response, liver cell damage, and HBeAg expression[42]. To clarify whether LKIs target HBcAg *in vivo*, we detected HBcAg levels in the hepatic tissues of LKI-treated transgenic mice for 30 consecutive days (n=6 in each group) and with gavage withdrawal for 7 days after the continuous gavage for 30 days (n=4 in each group) (Figure 4a). HBcAg levels in LKI-treated mice reduced to 80.0% (P=0.048 5) compared with that in control mice at D_{30} (Figure 4b). However, HBcAg levels rebounded to ~87.6% at P_7. LV observably suppressed HBcAg levels (decreased to ~88%), which rebounded after gavage withdrawal as well. HBcAg levels in saline-gavage transgenic mice (control) did not change

significantly before and after gavage withdrawal. These data indicate that LKIs can decrease HBcAg levels in the hepatic tissues of the transgenic mice.

To further demonstrate that LKIs decrease HBcAg levels in hepatic tissues, immunohistochemical examination was performed. The hepatic tissues collected from LKI-gavage mice had small and light cell clusters stained positive for HBcAg (Figure 4c, c′), while those from saline-gavage mice (control) were strongly stained (Figure 4d, d′). The staining of hepatic tissues from LV-gavage mice was stronger than that from LKI-gavage mice (Figure 4e and 4e′). Normal wildtype mice did not show HBcAg signals (Figure 4f, f′). Semi-quantitative immunohistochemical statistics from 6 sections of each group is summarized in Figure 4g and Table S3. These data indicate that LKI administration decreases HBcAg levels in the hepatic tissues of HBV-transgenic mice. In other words, LKIs may disturb HBV assembly in the hepatic cells.

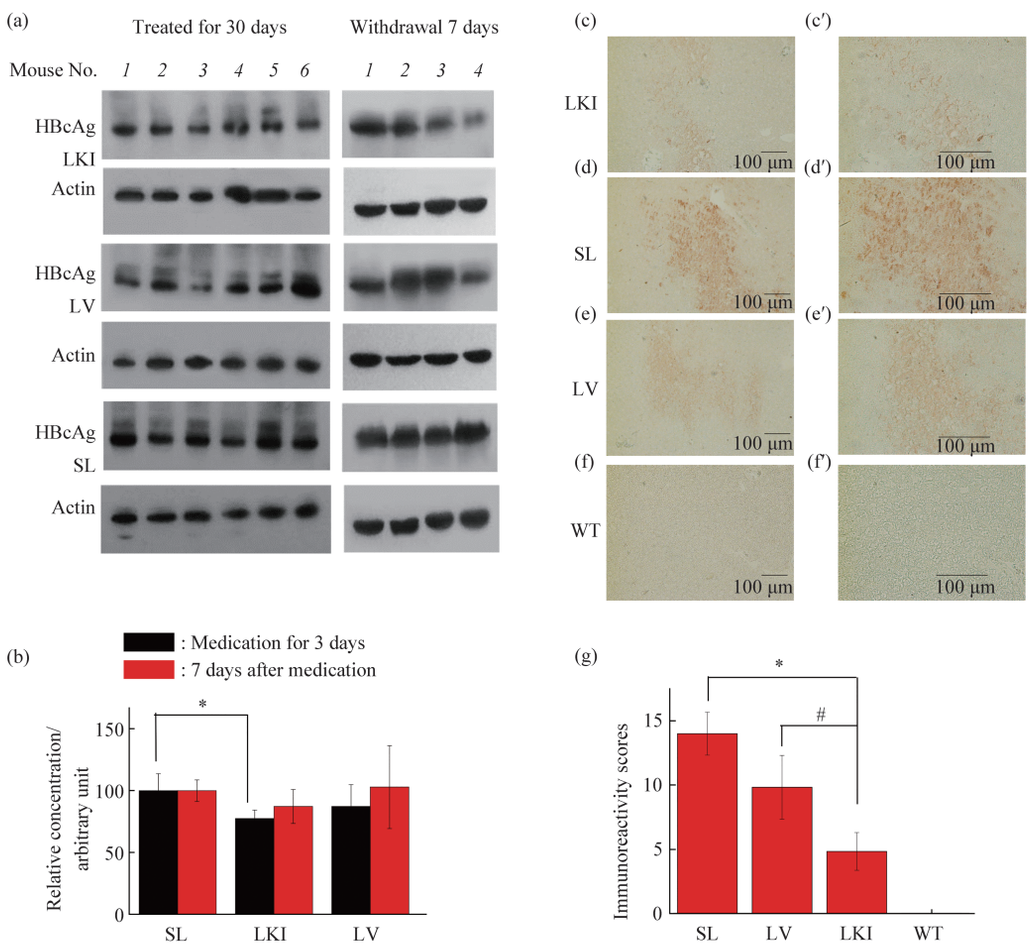

Fig. 4　The effect of LKIs on HBcAg in the hepatic tissues of the transgenic mice

After gavage with LKIs, lamivudine (LV), or saline for 30 days, 6 HBV-transgenic mice Balb/c in each group were euthanized and their sera were collected for detecting HBcAg by Western blotting (a). Four mice in each group were sacrificed for detecting HBcAg by Western blotting after the withdrawal of LKIs, LV, and saline for 7 days. Actin was used as a control. The densitometric analyses of HBcAg are shown (b). The hepatic tissues were sectioned and HBcAg was detected by immunohistochemistry with the monoclonal antibody in LKIs-treated (c, c′). Saline-treated (d, d′), and LV-treated (e, e′) transgenic mice. Untreated wildtype C57/bl6 mice (f, f′) were used as controls. n=6. The immunoreactivity for HBcAg was scored by evaluating the sum of positive stained zones and the staining intensity over the total number of the zones as described[38] (g).

2.5　LKIs protect the structure and function of hepatic cells in HBV−transgenic mice

To investigate whether LKIs protect the structure of hepatic cells, we sectioned the liver samples of transgenic mice and stained them with HE (Figure 5) in the treatment on day 28. In the normal liver (wildtype), the hepatic lobule structure was integrated, the central vein and its radially arranged liver cells

were clearly visible, the boundaries of liver cells were clear, and the cytoplasm was abundant (Figure 5a). However, in saline-treated transgenic mice liver, hydropic degeneration was prominently observed in the lobular center, accompanied by a significant cytoplasmolysis of hepatic cells (Figure 5b). Mild and diffuse hepatocyte degeneration and partial necrosis of liver cells were observed in LV-treated transgenic mice (Figure 5c). Marked hepatocyte hydropic degeneration, except lymphocytic infiltration, was not observed in partial liver parenchyma and perivascular space in LKIs-treated transgenic mice (Figure 5d). Kidney and spleen of the HBV-transgenic mice were also sectioned and stained, which did not exhibit significant morphological changes (Figure S6), similar to the creatinine levels (Figure 5g). Thus, LKIs may prevent the hepatic cells from the hydropic degeneration.

Serum GOT and GPT levels were also measured to detect the function of the hepatic cells of transgenic mice after the treatment of LKIs[43]. GOT levels (Figure 5e) were significantly decreased (P<0.05) in the HBV-transgenic mice serum during LKI gavage from D_{25} to D_{30}, compared with that at D_0. LV as a positive control which also suppressed GOT levels. Serum GPT levels on D_{25} were significantly decreased (P<0.05) in LKI-gavage mice, but those of LV-gavage mice were not (P>0.05) (Figure 5f). Thus, LKIs suppress both GOT and GTP levels, further suggesting their protective role on hepatic cells.

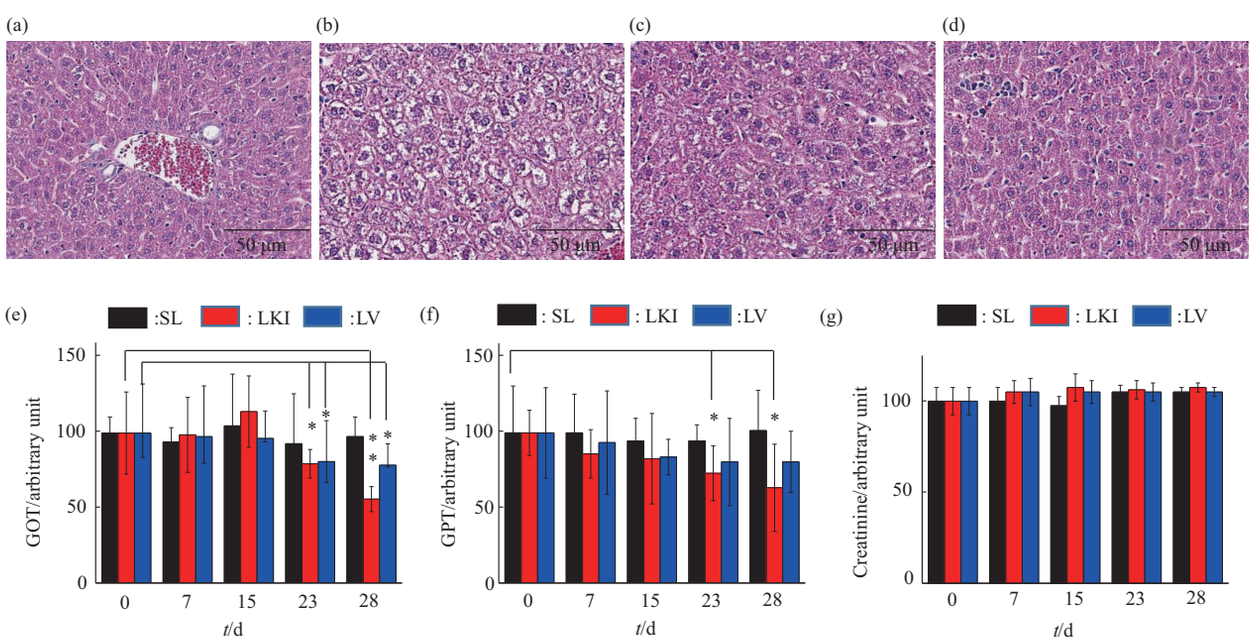

Fig. 5 Hematoxylin−eosin（HE）staining of the liver of HBV−transgenic mice and changes in serum GOT，GPT，and creatinine levels after medication

Conditions were similar to that reported in Figure 3. The liver sections collected from wildtype mice (a) and saline-treated (b), lamivudine (LV)-treated (c), and lumbrokinase isozyme (LKI)-treated (d) transgenic mice were stained with HE. Scales were as indicated. Moreover, serum aliquots were collected from saline-, LV-, and LKI-treated transgenic mice at different time intervals for measuring the GOT (e) and GPT (f) levels by ELISA and creatinine level (g) by picric acid-kit. *, P<0.05; **, P<0.01.

2.6 LKIs decreases DHBV DNA levels in LYS serum

We then checked whether LKIs affect the viral DNA in an animal naturally infected with HBV. We employed LYS because it is naturally infected with DHBV[44]. LYS was gavaged with LKC and sera DHBV DNA were examined at different time intervals. During intragastric medication, the LYS gained weights, but not significantly different in the weights among the three groups (Figure 6a). Sera DHBV DNA decreased markedly after intragastric LV administration for 7 days (P<0.05) and kept at low levels during medication (P<0.01), but it increased rapidly back to the start level at P_5 (Table S4). Interestingly, DHBV DNA was significantly inhibited at D_{21} in 100 mg/kg LKI-treated LYS (P<0.05)

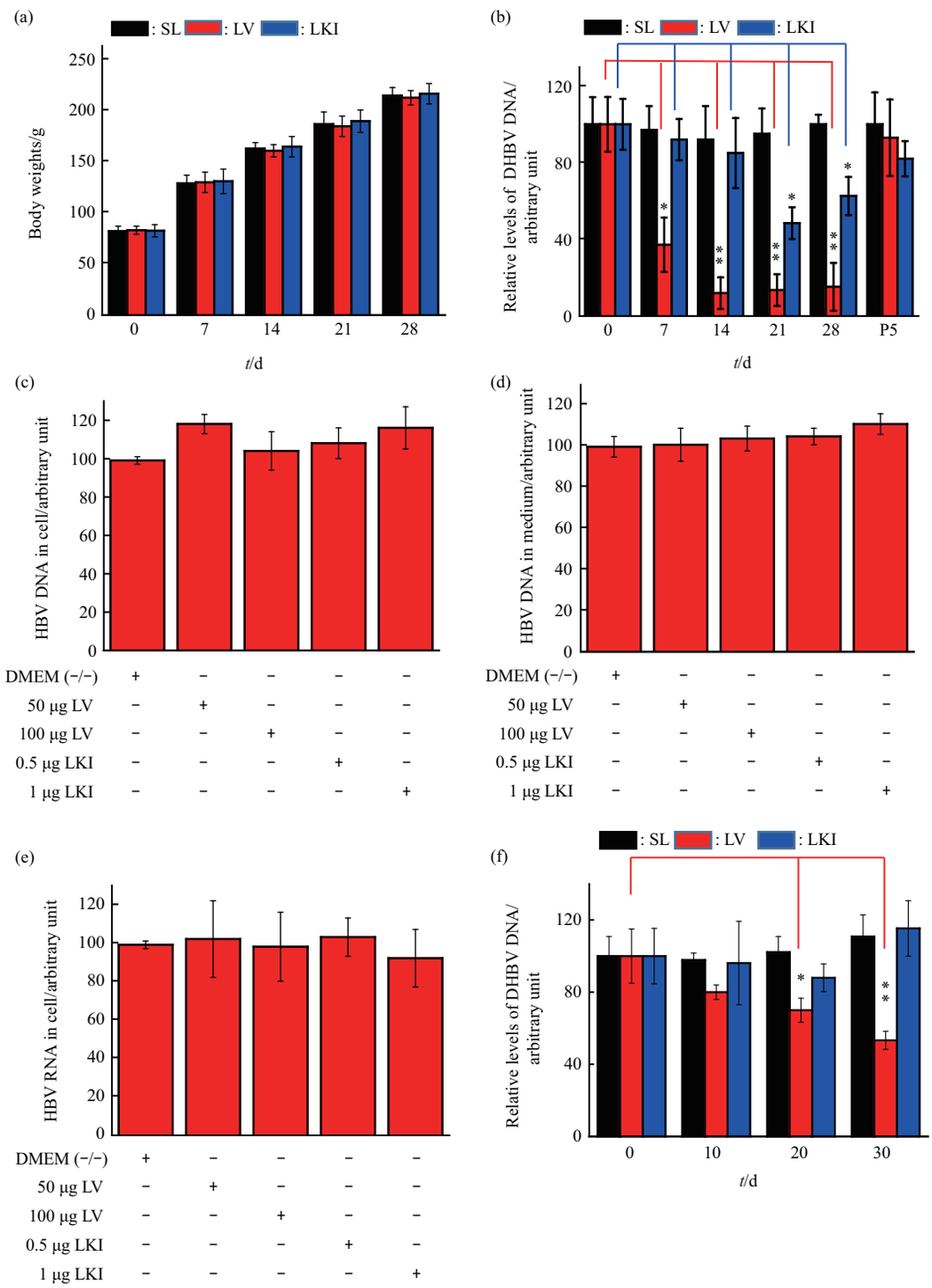

Fig. 6　Changes in HBV DNA copies in LKIs−treated LYS，mice，and HepG2.2.15 cells

DHBV-infected LYS were randomly divided into three groups (n=14) and gavaged with 100 mg/kg LKI in enteric capsule, 100 mg/kg lamivudine (LV), or 0.9% saline (once daily) for 28 days. Then 8 ducks from each group were euthanized and 6 were left for stopping medication for 5 days. Their bodies were weighed (a) and sera HBV DNA were determined with TIANamp virus DNA kit (b) at different time intervals. Cell culture conditions were similar to that reported in Figure 2, however the HBV DNA in HepG2.2.15 cells (c) and culture media (d) and cellular HBV RNA (e) were determined by fluorescent qPCR. Serum from saline-, LV-, and LKI-treated transgenic mice were employed for determining HBV DNA copies (f). n=6; *, P<0.05; **, P<0.01.

compared with that at D_0 or that at D_{21} in saline-treated LYS (Figure 6b). DHBV DNA levels also rebounded significantly at P_5. These data manifest that oral LKI medication suppresses DHBV DNA in LYS serum.

2.7 LKIs did not decrease HBV RNA and DNA in HepG2.2.15 cells and transgenic mice

We determined the HBV DNA and RNA levels in HepG2.2.15 cells using fluorescent qPCR. The HBV DNA levels in HepG2.2.15 cells (Figure 6c) or their cultured media (Figure 6d) under LKI-treatment did not significantly decrease, compared to those in LV-treated cells or medium only. The RNA levels in the three groups were also similar (Figure 6e). Moreover, the treatment of cells with LKIs combined with LV at different concentrations did not change the HBV DNA levels significantly compared to that in the controls (data not shown).

We also investigated the impact of LKIs on HBV DNA levels in the serum of transgenic mice. As shown in Figure 6f, the serum viral DNA level was slightly increased, but was stable in saline-treated mice during the gavage. No significant reduction in viremia was found in LKI-treated mice at day D_{10}, D_{20} and D_{30}, compared with the baseline at D_0. However, serum HBV DNA levels were markedly decreased with LV medication (positive control), with the significant decrease at D_{20} (P<0.05) and D_{30} (P<0.01). It is likely that LKIs cannot remove the HBV DNA which is integrated into chromosome genomes of HepG2.2.15 cells and transgenic mice.

Together, these results suggest that the decreased DHBV DNA may be due to hydrolysis of HBsAg and HBcAg by LKIs, disturbing the assembly of virion and life cycle, which leads to indirectly reducing of the serum DHBV DNA.

3 Discussion

As described in the previous and current work, LKIs decreases not only fibrin and fibronectin functioning in anti-hepatic cirrhosis[21], but also HBsAg, HBeAg and HBcAg, in their disturbing viral assembly. Gavage of LKIs significantly reduced GOT and GPT levels and relieved the hepatic pathological changes in the transgenic mice. Administration of LKIs also decreased DHBV DNA level in LYS. These *in vivo* and *in vitro* results indicate that LKC may be employed as a candidate drug to treat CHB. This viewpoint is based on the following observations. First, HBsAg is cleavaged and decreased to impede HBV envelope assembly. Second, HBcAg degradation interferes with HBV capsid formation. Third, *Ef*P-III-1 purified LKIs significantly decrease HBeAg levels in molecular and cellular studies *in vitro*[21, 23, 45]. HBeAg hydrolysis affects HBV immunoregulation[46], particularly immune tolerance of T-lymph reactions[47]. Fourth, LKIs protect the liver tissues through alleviating hepatic cell endolysis and decreasing the levels of GPT and GOT. Fifth, DHBV DNA level decline in naturally HBV-infected LYS serum. Sixth, LKIs have high activity on fibronectin to affect hepatic cirrhosis[21-22]. Seventh, LKIs have fibrinogenase activity for not only anti-thrombus in stroke and myocardial infarction[13, 48], but also disturbing liver cirrhosis with a high fibrinogen level[49]. And eighth, oral LKC administration for treating stroke had mild side effects in a clinical trial for 69 patients, though their limb movements and language abilities were markedly improved[50].

One of the important goals of HBV therapy is HBV DNA clearance[51]. As mentioned above, LKIs gavage significantly decreased DHBV DNA levels in LYS serum, and withdrawal led to a rebound, demonstrating that LKIs can suppress DHBV in serum. However, LKIs cannot suppress HBV DNA and RNA levels in HepG2.2.15 cells and HBV DNA levels in the transgenic Balb/c mice (Figure 6c−f). Since HBV DNA has been integrated in the chromosomes of HepG2.2.15 cell[52] and transgenic mice[53], all hepatic cells are infected, containing the HBV genome which cannot be cleared[41, 52]. Although one of the LKIs was observed to function as a deoxyribonuclease (DNase), the optimal pH for the isozyme is acidic[54]. The DNase activity of the LKI was too low to be detected after absorbed into serum under weak alkaline (pH>7.0) conditions.

The spread of HBV infection between hepatocytes in the liver is mainly due to cell-free virus[55], which is the virion released from the infected cells after assembly[56]. We hypothesized that LKIs prevent HBV cell-to-cell transmission between hepatic cells by interfering with capsid and envelope assembly (Figure 7). HBcAg, which self-assembles to form the viral capsid, is degraded in the presence of LKIs. HBcAg damage should disturb capsid assembly, thus affecting the HBV life cycle[57]. Degradation and decrease of HBs and HBc protein

and may disturb the cell-to-cell transmission of the cell-free virus between hepatocytes[57]. Furthermore, HBsAg degradation also affects HBV life cycle because HBV requires HBsAg for enveloping and release [58]. LKIs decrease not only HBsAg but also HBeAg levels *in vitro* and *in vivo*. LKIs probably decrease the serum DHBV DNA by degrading the proteins essential for viral assembly and interfering with virons spread between hepatocytes (Figure 6b).

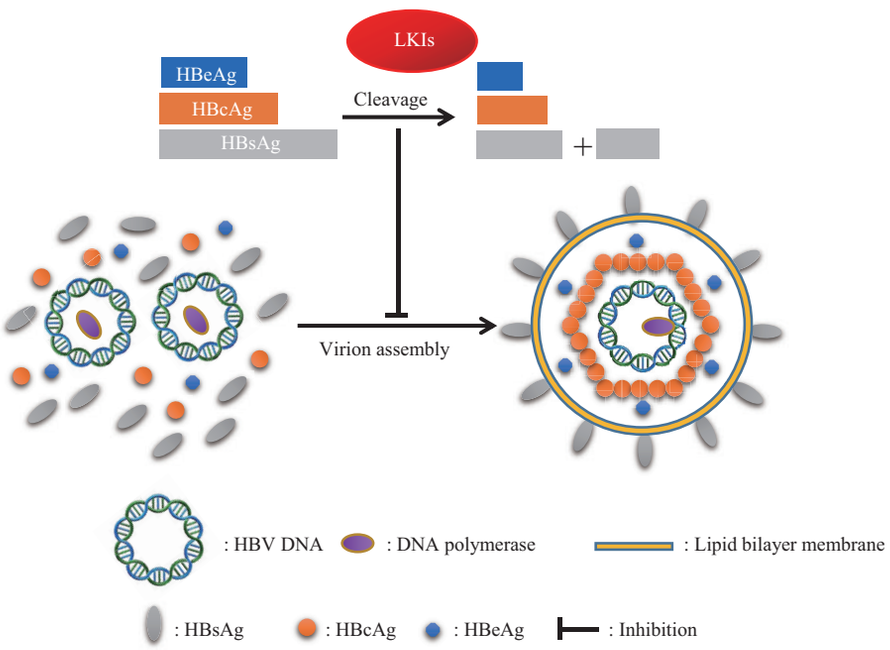

Fig. 7　Putative mechanisms of LKIs in anti−infection of HBV and protection of hepatic tissues

Infectious HBV is spherical and consists of hepatitis B surface antigen (HBsAg) that envelops the viral nucleocapsid, which is formed by the core protein (HBcAg). LKIs attack and degrade HBsAg, HBcAg, and HBeAg, markedly decreasing their levels to impede new virion assembly. Thereby, LKIs resist hepatitis B infection and protect hepatic cells.

From the HBcAg sequence (GenBank AUR80754.1), we estimated the cleavage site of LKIs in HBcAg to be around R142, since a ~12.72 ku fragment was released by the degradation. HBcAg is a multiple-function protein required for HBV replication and pathogenesis[59]. The arginine-rich domain (ARD, C-terminal) of HBcAg has distinct nuclear localization (NLS) and cytoplasmic retention signals (CRS) [60]. Cleavage by LKIs results in ARD loss, which affects several HBcAg functions, such as regulating host gene transcription, interacting with host proteins, and acting in allosteric modulation[61].

Two degradation sites in HBsAg were estimated to be located in the first cytosolic loop (CL1) and major hydrophobic region (MHR). This is because LKIs belong to carboxy-peptidase and recognize lysine and arginine residues[20]. The full-length HBsAg releases 18.45 and 12.35 ku peptides after treatment, which suggests that the cleavage sites should be K141 and R160 based on the amino acid sequence provided by Abnova (http://www. abnova. com/products/products_detail. asp? catalog_id=P4874). We have repeatedly tried to determine LKIs' cleavage sites on HBsAg using mass spectrometry, but we could not successfully detect the hydrolytic sites on HBsAg in the presence of LKIs. Approximately, we have to estimate the cleaved sites from HBsAg molecular mass according to the amino acid sequence.

For the *in vivo* experiments, an easily generated immunocompetent animal model is instrumental for the systematic investigation of HBV therapy[62]. We adopted a gavage administration route of LKIs as earthworm proteases have been formulated into the oral enteric-soluble capsule to protect LKIs from degradation under the acidic conditions in stomach. This formulation has been successfully used in clinics with certified efficiency[16, 18]. Clinically, LKIs have mild side effects and require a 21-day treatment

course[63]. In this study, HE-stained spleen and kidney sections showed no pathological change after LKI administration for 30 days and the serum creatinine levels did not change significantly during the medication. These results indicate the relative safety of LKIs. However, whether the 21-day course can be continued in the patients with hepatitis needs further clinical trials.

LKIs may transport through the intestinal wall into blood because LKIs (at least *Ef*P-III-1 and *Ef*P-III-2) adhered to and crossed the intestinal wall[64] using duodenum segment protocol[65], which can be detected in rat serum after intraperitoneal injection[64]. Further, LKIs were reported to maintain their biological activity in serum after oral administration, which was the most at about 1.5 h, while several LKIs were distributed in stomach and duodenum[66]. Figure S7a, a′ showed the section of duodenum wall markedly adhered with LKIs using duodenum segment protocol and immunohistochemical staining. Moreover, intraperitoneal injection resulted in LKI activity in rat serum and liver (Figure S7b−d). It should be noted that LKI dose (25 mg/kg body weight of rat) used was reported previously[67-68]. Thus, LKIs can transport across the intestinal wall to at least partially reach the liver via the blood[21, 64, 69-70].

Human HBV and duck HBV, which are members of the same family of Hepadnaviridae, share several common features[71]. Unavailability of primary animal models and inefficient and unreliable *in vitro* infection process[72] are major limitations in HBV research and restrain the study of human pathogen. However, establishing the duck model has helped greatly to overcome the shortcomings in HBV research[71, 73]. Unlike the HBV-transgenic mice, the ducks are naturally infected with DHBV, which simulates a process similar to that in humans infected with HBV, suggesting that it is an ideal model for investigating anti-HBV therapy. As mentioned above, DHBV DNA significantly decreased in LV-treated ducks at D_7 through D_{28} and 100 mg/kg LKI-treated ducks starting at D_{21}. This is to say, LKC can inhibit DHBV DNA *in vivo*, but requires longer time for its effect than LV. Further studies should focus on not only DHBV DNA but also other indicators, such as pathological change of liver.

4 Conclusion

LKIs degrade HBsAg, HBcAg, and HBeAg *in vitro*, and decrease their levels in cell and HBV-transgenic mice, protecting hepatic tissues. LKIs also decrease serum DHBV DNA levels in LYS. These effects may be due to LKIs' suppressing virion assembly and release, which is involved in the viral spreading between hepatocytes. LKC may be used as a potential drug to target the HBV proteins for long-term CHB treatment although there have been different developing and potential therapies such as siRNA[74], vesicles[75] , nanoproducts[76], and other natural products[77].

Acknowledgments We deeply thank Beijing Kangyuyao Biological Company (Beijing, China) for the collaboration of studying LKIs in the treatment of hepatitis B and their providing the data of duck DNA experiment.

Supplementary PIBB_20220003_Doc_S1. pdf is available online (http://www.pibb.ac.cn or http://www.cnki.net).

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蚓激酶同工酶降解乙型肝炎抗原并保护肝功能*

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摘要　目的　蚓激酶同工酶（LKIs）作为肠溶胶囊的有效成分，用于治疗血栓性疾病已有30多年历史。近年来，LKIs在其他危重疾病中的研究时有报道。本文关注LKIs在乙型肝炎方面的作用。**方法**　乙型肝炎表面抗原（HBsAg）、核心抗原（HBcAg）和e抗原（HBeAg）分别与不同浓度LKIs孵育，观察这些蛋白质的降解和估计肽链的切割位点。HepG2.2.15细胞与LKIs孵育，采用酶联免疫吸附测定（ELISA）和蛋白质印迹（Western blotting）检测细胞分泌的HBsAg和HbeAg。LKIs灌胃Balb/c小鼠30天，采用ELISA和Western blotting检测其血清HBsAg和HBeAg，免疫组化染色检测肝组织中的HBcAg。采用苏木精-伊红染色分析乙肝病毒转基因小鼠肝组织的损害，并通过ELISA定量分析血清谷草转氨酶（GOT）和谷丙转氨酶（GPT）。腹腔注射后，取大鼠血清和肝组织，测定其中的LKIs含量，从而观察LKIs的吸收。采用LKIs给龙岩麻鸭灌胃30天，通过PCR检测其血清HBV DNA。**结果**　蚓激酶肠溶胶囊的有效成分是含有6种LKIs的复方蛋白酶药物，可以降解HBV编码的蛋白质。LKIs降解HBsAg的位点为K141/P142及R160/F161；HBcAg为R142/E143；HBeAg为R122/E123。LKIs可显著抑制HepG2.2.15细胞分泌HBsAg和HBeAg。LKIs灌胃，HBV转基因小鼠血清HBsAg和HBeAg水平及肝组织的HBcAg水平均降低，提示病毒的组装和释放可能受到了抑制。在LKIs处理的转基因小鼠中，血清GPT和GOT水平降低，肝组织溶解数量减少，表明LKIs对小鼠肝细胞具有保护作用。LKIs灌胃龙岩麻鸭，血清中DHBV DNA水平明显下降，停药后出现反弹。**结论**　LKIs通过降解HBs、HBc和HBe蛋白，可能干扰HBV的装配和释放，减少病毒在肝细胞之间的传递，从而对肝细胞起到保护作用。

关键词　HBeAg，HBcAg，HBsAg，蚓激酶同工酶，乙肝病毒，同工酶
中图分类号　Q55，R511　　**DOI**：10.16476/j.pibb.2022.0003

* 国家自然科学(31470036)基金资助项目。
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收稿日期：2021-10-21，接受日期：2022-01-05