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Hepatitis B virus is inhibited by RNA interference in cell culture and in mice

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Abstract

Background and aims: For chronic hepatitis B virus (HBV) infection the effects of current therapies are limited. Recently, RNA interference (RNAi) of virus-specific genes has emerged as a potential antiviral mechanism. Here we studied the effects of HBV-specific 21-bp short hairpin RNAs (shRNAs) targeted to the surface antigen (HBsAg) region and the core antigen (HBcAg) region both in a cell culture system and in a mouse model for HBV replication.

Methods: HBsAg and hepatitis B e antigen (HBeAg) in the media of the cells and in the sera of the mice were analyzed by time-resolved immunofluorometric assay, intracellular HBcAg by immunofluorescence assay, HBsAg and HBcAg in the livers of the mice by immunohistochemical assay, HBV DNA by fluorogenic quantitative polymerase chain reaction (FQ-PCR) and HBV mRNA by semi-quantitative reverse transcriptase PCR (RT-PCR).

Results: Transfection with the shRNAs induced an RNAi response. Secreted HBsAg was reduced by >80% in cell culture and >90% in mouse serum, and HBeAg was also significantly inhibited. Immunofluorescence detection of intracellular HBcAg revealed 76% reduction. In the liver tissues by immunohistochemical detection, there were no HBsAg-positive cells and >70% reduction of HBcAg-positive cells for shRNA-1. And for shRNA-2 the detection of HBsAg and HBcAg also revealed substantial reduction. The shRNAs caused a significant inhibition in the levels of viral mRNA relative to the controls. HBV DNA was reduced by >40% for shRNA-1 and >60% for shRNA-2.

Conclusions: RNAi is capable of inhibiting HBV replication and expression *in vitro* and *in vivo* and thus may constitute a new therapeutic strategy for HBV infection.

Keywords: HBV; RNAi; Hydrodynamics; HepG2.2.15; Animal model

1. Introduction

Hepatitis B is still a major health problem, especially in developing countries such as China. The number of hepatitis B virus (HBV) carriers worldwide is estimated to be 350 million. In China, the infection rate is even as high as 9.8%. It is well known that chronic HBV infection leads to the development of liver cirrhosis and hepatocellular carcinoma in a substantial proportion of patients (Lok, 2000). Currently chronic HBV infection is mainly treated with interferon or nucleoside analogues such as lamivudine, entecavir and adefovir dipivoxil (Dienstag et al., 2003; Lai et al., 2002; Lin and Keeffe, 2001; Marcellin et al., 2003). However, these treatments have some drawbacks, including side effects in the case of interferon, and development of escape mutants after a long period of lamivudine treatment. Therefore, it is urgent to develop novel therapeutic approaches that effectively inhibit HBV replication.

Recent studies have shown that RNA interference (RNAi), which can be induced in mammalian cells by small interfering RNAs (siRNAs), induces posttranscriptional silencing and thus inhibits the replication of HBV (Carmona et al., 2006; Elbashir et al., 2001; Peng et al., 2005; Shlomai and Shaul, 2003). RNAi is a process during which cytoplasmic long double-stranded RNAs (dsRNAs) produced by viral infection, by transposons or by introduced transgenes are targeted for inactivation (Hannon, 2000). The long dsRNAs are processed into 21–23-nucleotide (nt) guide RNA duplexes by an RNase called Dicer and are further incorporated into an RNA-induced silencing complex (RISC) (Bernstein et al., 2001; Hammond et al., 2000). The

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RISC complex uses the guide RNAs to identify homologous RNAs in the cell and proceeds to cleave the RNAs. Synthetic siRNAs and short hairpin RNAs (shRNAs) transcribed *in vivo* from DNA templates trigger specific silencing of genes when transfected into cultured cells (Tuschl, 2002). RNAi is evolutionarily conserved and is found in a wide range of eukaryotic organisms (Hammond et al., 2001; McManus and Sharp, 2002). Therefore, these properties of RNAi open up a possibility in controlling replication processes of infectious viruses such as HBV. In the present study, we selected two different RNAi target sites of HBV and observed the effects of RNAi on the replication of HBV both in cell culture and animal experiments.

2. Results

2.1. Selection of RNAi targets sites

Two RNAi target sequences were chosen on the basis of their conservation among the major HBV genotypes adr, adw, ayr and ayw. Each shRNA targets the pregenomic RNA serving as the template for HBV genomic replication as well as the mRNA for the core antigen and the polymerase. Moreover, shRNA-1 targets the HBV S-antigen mRNAs and shRNA-2 targets the pregenomic RNA in the overlap region encoding the core antigen and the polymerase (Fig. 1).

2.2. Inhibition of HBV antigens in cell culture by shRNAs

To evaluate the effects of shRNAs on HBV gene expression, a dose–response analysis was conducted by HepG2.2.15 cells transfected with different amounts of shRNA-1, shRNA-2 or irrelevant shRNA-3 and the amounts of HBsAg and HBeAg secreted into the medium 72 h after transfection were measured (Fig. 2). The expression levels of HBsAg decreased by 75%, 82% or 89% for shRNA-1, and 74%, 80% or 85% for shRNA-2, respectively, compared with the empty-vector control, when transfected with the different concentrations 1 μg, 2 μg or 4 μg

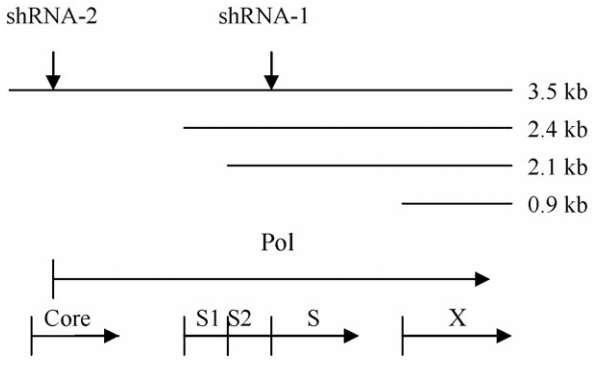

Fig. 1. Location of RNAi target sites. Downward arrows indicate the location of RNAi target sites within the four HBV transcripts. The 3.5-kb transcript is the pregenomic RNA that serves as the template for HBV viral DNA replication. The open reading frames are shown below aligned with the HBV mRNAs. shRNA-1 (9–27) CAUCACAUCAGGAUUCCUAAA, shRNA-2 (193–211) CUAAUGACUCUAGCUACCUAA, shRNA-3 (irrelevant control shRNA) CUUCAUAAGGCGCAUAGCU. Pol, polymerase; Core, HBcAg; S1, large pre-surface antigen; S2, middle pre-surface antigen; S, HBsAg; X, X gene.

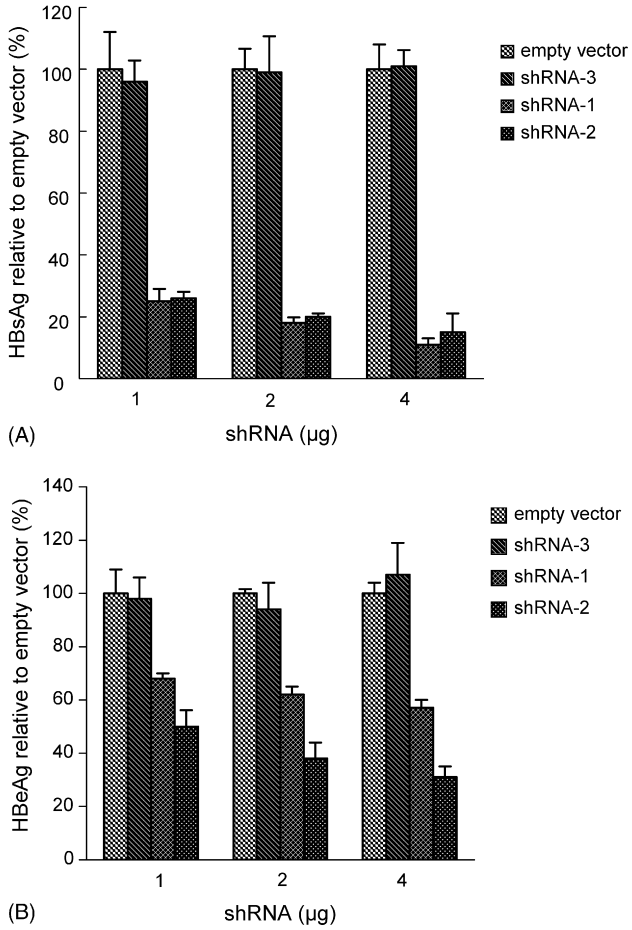

Fig. 2. HBsAg and HBeAg measurements in the medium of shRNA-treated cultured cells. HBsAg (A) and HBeAg (B) levels in the culture medium are significantly reduced by treatment with HBV RNAi. Inhibition of HBsAg and HBeAg is dose-dependent and specific. The data shown are mean ± S.D. based on three independent experiments.

of shRNAs ($P < 0.01$, Fig. 2A). The levels of HBeAg were reduced by 32%, 38% or 43% for shRNA-1, and 49%, 62% or 69% for shRNA-2, respectively, when transfected with the different concentrations of shRNAs ($P < 0.01$, Fig. 2B).

To examine how long shRNAs inhibit HBV gene expression, HBsAg and HBeAg concentrations in the cell culture were measured on days 1, 2, 3, 7, 14 and 21 after transfection with shRNA-1. Treatment of HepG2.2.15 cells with 2 μg shRNA-1 resulted in inhibition of secreted HBsAg by 55%, 81% or 88%, and HBeAg by 30%, 39% or 51%, respectively, on day 1, 2 or 3, compared with the untreated control, but no change of HBsAg and HBeAg was found from day 3 to 7. On day 14, the inhibition rates dropped to only 45% for HBsAg and 32% for HBeAg, respectively ($P < 0.01$, Table 1). There was no inhibitory effect to be found on day 21.

2.3. Effects of shRNA on intracellular HBcAg in HepG2.2.15 cells

The effects of shRNA-2 on intracellular HBcAg were visualized by immunocytochemistry, which was clearly localized both in the cytoplasm and in the nuclei. The staining inten-

Table 1
Kinetics of inhibition of HBsAg and HBeAg expression by shRNA-1

	Days				
	1	2	3	7	14
HBsAg (%)	55 ± 2	81 ± 7	88 ± 10	85 ± 11	45 ± 2
HBeAg (%)	30 ± 1	39 ± 4	51 ± 1	47 ± 1	32 ± 3

The levels of HBsAg and HBeAg secreted into the medium by HepG2.2.15 cells transfected with 2 μg shRNA-1 or with the empty vector were measured on day 1, 2, 3, 7 or 14 after transfection. The amounts of HBsAg and HBeAg are shown as percentages of the amounts secreted by mock-transfected cells. The data shown are mean ± S.D. based on three independent experiments.

sity reflecting the number of intracellular HBcAg was greatly decreased in the cells treated with shRNA-2–metafectene complex (reduced by 76% at 72 h). In contrast, all control cells were strongly stained (Fig. 3).

2.4. Inhibition of HBV RNAs by shRNAs in HepG2.2.15 cells

The HBV shRNA-1 target site is located within the three HBV transcripts, *i.e.* the 3.5-kb, 2.4-kb, and 2.1-kb transcripts. To confirm that the effect of shRNA-1 on HBsAg and HBeAg expression was through the reduction of HBV RNA levels, we performed semi-quantitative RT-PCR analysis with RNA extracted from HepG2.2.15 cells 72 h after transfection with shRNA-1 or with the irrelevant shRNA-3. The observation revealed that treatment with shRNA-1 resulted in a decrease in all transcripts, and the total mRNAs were reduced by 30%, 70% or 90% in shRNA-transfected cells at the concentration of 1 μg, 2 μg or 4 μg shRNA-1, respectively ($P < 0.01$, Fig. 4A), compared with the mock-transfected cells. And the shRNA-2 only reduced the 3.5-kb pregenomic RNA in cells transfected with 1 μg, 2 μg or 4 μg shRNA-2 by 70%, 80% and 90%, respectively ($P < 0.01$, Fig. 4B).

In order to further investigate the specificity of the inhibition of shRNAs on HBV gene expression, the level of HepG2.2.15 c-fos mRNA was measured and no change was found between cells transfected with shRNA-1 or shRNA-2 and the mock-transfected cells (Fig. 4C).

2.5. siRNA-mediated inhibition of HBV DNA in HepG2.2.15 cells

The HBV DNA content in the medium of HepG2.2.15 cells was analyzed by FQ-PCR. Treatment with 1 μg, 2 μg or 4 μg shRNA-1 led to a reduction by 30%, 43% or 49%, respectively, while for shRNA-2 treatment was 36%, 63% or 72%, respectively ($P < 0.01$, Table 2). No reduction was found for HBV

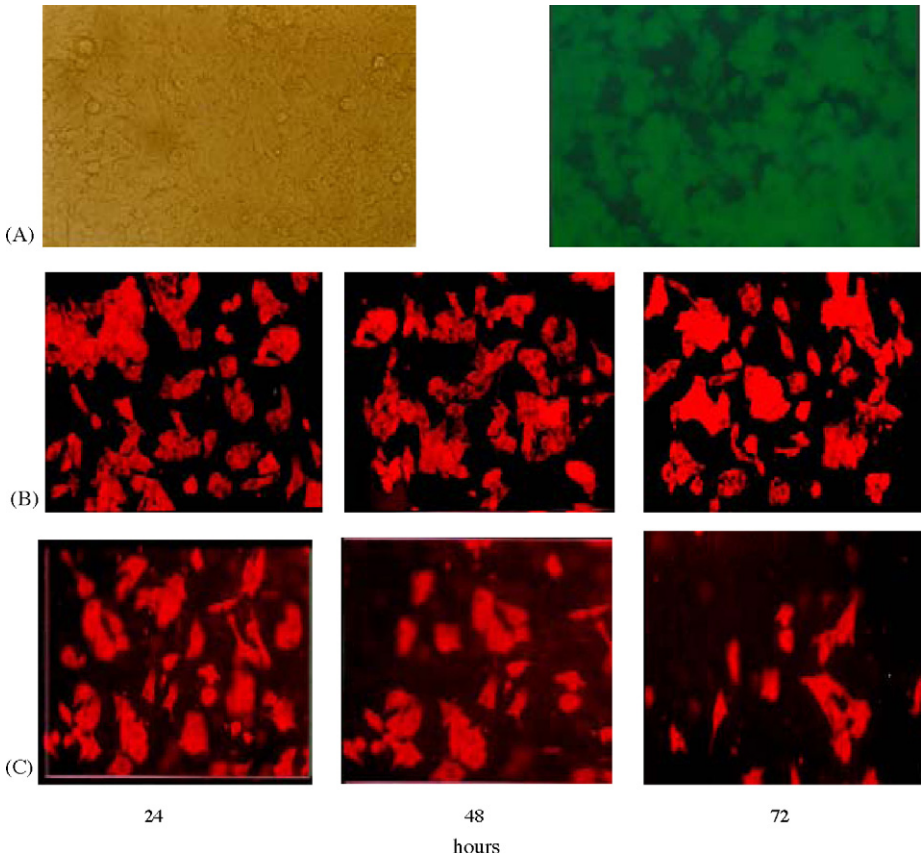

Fig. 3. Intracellular HBcAg in HepG2.2.15 cells by immunocytochemistry. (A) The transfection efficiency. (Left) Before transfection with green fluorescent protein (GFP). (Right) After transfection with GFP and the efficiency is about 76%. Intracellular HBcAg with empty vector (B) and with shRNA-2 (C) is measured 24 h, 48 h and 72 h after transfection (200× original magnification). The number of intracellular HBcAg is much decreased treated with shRNA-2 compared with the control group.

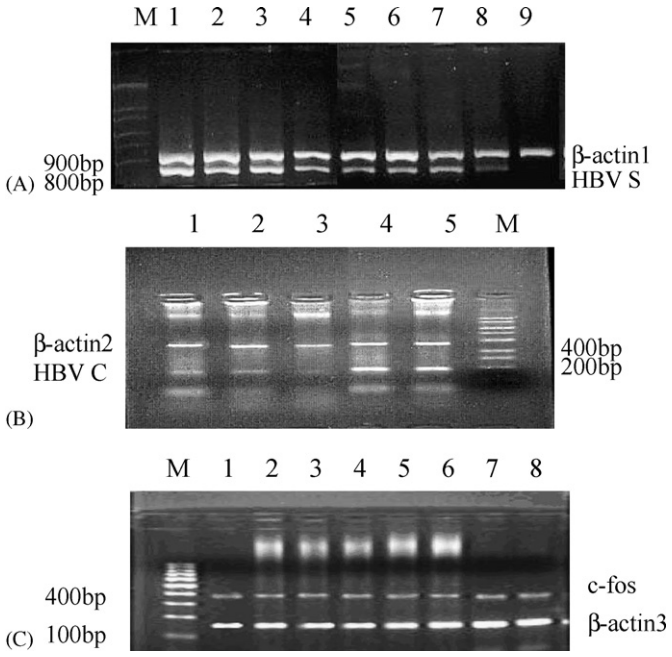

Fig. 4. The inhibitory effects on HBV RNA levels by transfection with HBV shRNAs. HepG 2.2.15 cells were transfected with 1 μg, 2 μg and 4 μg shRNA-1 or shRNA-2. The cells were harvested 72 h after transfection, and RNA was extracted. (A) The inhibitory effects on HBV S-mRNA by transfection with shRNA-1. M, maker; lane 1, empty vector; lanes 2 and 3, 1 μg shRNA-1; lanes 4–7, 2 μg shRNA-1; lanes 8 and 9, 4 μg shRNA-1. (B) The inhibitory effects on HBV C-mRNA by transfection with shRNA-2. Lane 1, 1 μg shRNA-2; lane 2, 2 μg shRNA-2; lane 3, 4 μg shRNA-2; lane 4, irrelevant shRNA-3; lane 5, empty vector; M, maker. Densitometric analysis of these samples shown normalized to β-actin (housekeeping gene) bands. HBV S-mRNA or C-mRNA levels were expressed relative to untreated control taken as 100%. (C) Semi-quantitative RT-PCR for c-fos mRNA by transfection with shRNAs. M, marker; lane 1, control; lanes 2–4, 1 μg, 2 μg and 4 μg shRNA-1; lanes 5–7, 1 μg, 2 μg and 4 μg shRNA-2; lane 8, 2 μg shRNA-1 and 2 μg shRNA-2. Densitometric analysis of these samples shown normalized to β-actin (housekeeping gene) bands. c-fos mRNA levels were expressed as 35% relative to untreated control taken as 100%.

DNA when the cells were treated by the irrelevant shRNA-3. The time-course of the effects of the shRNAs was also examined. Obvious reduction of the viral DNA was observed on day 3 after shRNA transfection.

2.6. Inhibition of HBV antigen in vivo by shRNAs

To determine the effect and the optimal concentration of shRNA for HBV inhibition *in vivo*, the mice were injected via the tail vein with 60 μg pHBV together with 15 μg, 25 μg or 35 μg shRNA-1, and the levels of serum HBsAg were

Table 2
shRNAs inhibit HBV replication in HepG2.2.15 cells

	Dose (μg)		
	1	2	4
shRNA-1 (%)	30 ± 1	43 ± 4	49 ± 2
shRNA-2 (%)	36 ± 4	63 ± 8	72 ± 15

The amounts of HBV DNA are shown as percentages of the amounts secreted by mock-transfected cells for 3 days after transfection. The data shown are mean ± S.D. based on three independent experiments.

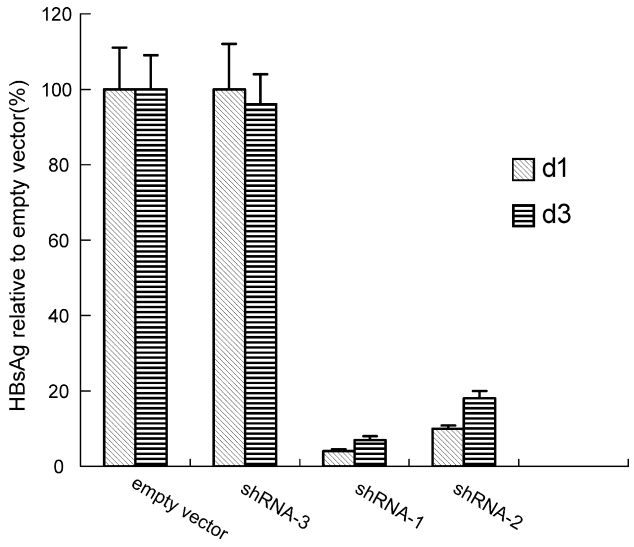

Fig. 5. Serum HBsAg levels by HBV shRNAs in mice. The levels of HBsAg are significantly reduced by treatment with HBV shRNAs (25 μg) in mice. Inhibition of HBsAg is specific and lasts at least 3 days.

28.22 ± 2.24 ng/ml, 4.62 ± 0.18 ng/ml and 16.58 ± 1.39 ng/ml, respectively, after 1 day. Then the most effective inhibitory concentration of shRNA-1 was 25 μg, which caused 96% reduction in the levels of HBsAg, compared with the serum levels (108.88 ± 11.77 ng/ml) in mice injected with the pHBV and an empty vector.

The observation of a kinetic study revealed that the inhibition of shRNA-1 (25 μg) on serum HBsAg levels in the serum was strongest (96%) on day 1 after injection and diminished slightly thereafter, but still reaching 93% inhibition on day 3. The inhibitory effect of sRNA-2 on HBsAg levels was relatively weaker than shRNA-1, with 90% or 82%, respectively, on day 1 or 3 ($P < 0.01$, Fig. 5). The irrelevant shRNA-3 exhibited no effect on HBsAg levels, demonstrating the specificity of the shRNAs effect.

RT-PCR analysis was performed to study further the inhibition of shRNA on viral RNA levels in the livers of the injected mice. shRNA-1-treated mice exhibited an average decrease of 86% or 97% ($P < 0.01$) in total levels of HBV S-mRNA, on day 1 or 3, respectively, compared to the untreated control mice. In addition, shRNA-2-treated mice exhibited an average decrease of 66% or 89% ($P < 0.01$) in HBV C-mRNA total levels, respectively. However, the irrelevant shRNA-3 had no inhibitory effect.

2.7. siRNA-mediated inhibition of HBV DNA in vivo

FQ-PCR analysis was performed with total serum DNA from treated mice. An approximately 3 log reduction in HBV DNA titers on day 1 showed a significant anti-replication effect of shRNA-2. The inhibitory effect diminished on day 3 but over 100-fold inhibition was still observed, while the inhibitory effect of shRNA-1 was 47% or 40%, respectively ($P < 0.01$), and shRNA-3 had no effect on HBV DNA titers. Hence, the results revealed a dramatic inhibitory effect of shRNAs on viral replication for at least 3 days after injection and shRNA-2 was more effective.

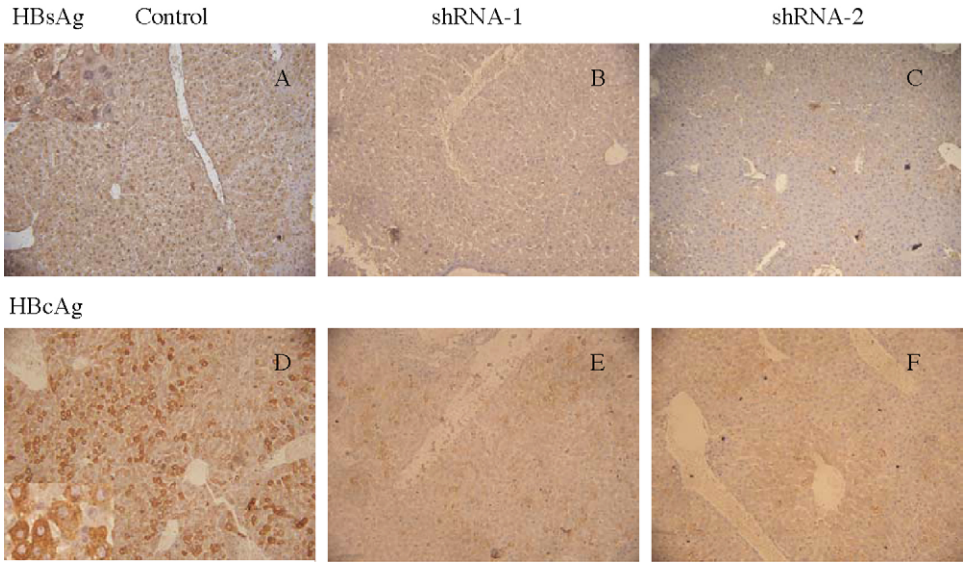

Fig. 6. Immunohistochemical staining for HBsAg and HBcAg in liver sections. (A–F) Treatment with shRNA-1 or shRNA-2 reduces the number of HBsAg or HBcAg-positive cells on day 1 compared to the control. The staining was performed on tissue sections from five animals per group. Representative sections are shown (100× original magnification, insets at 400× original magnification). (A and D) Numerous HBsAg-positive and HBcAg-positive cells are seen in the control group. (B) HBsAg-positive cells are not seen in shRNA-1 group. (C, E and F) Some stained cells are seen in shRNA-1 or shRNA-2 group, but staining is much less intense than that in control group.

2.8. Effects of shRNAs on HBsAg and HBcAg expression in the livers of mice

The effects of shRNAs on HBV antigens were determined histologically by immunohistochemical staining. Compared with the control group, HBsAg-positive cells were absent in the livers of shRNA-1-treated mice on day 1, and the percentage of HBcAg-positive cells was reduced by 79.0%. In shRNA-2-treated mice, the percentage of HBsAg or HBcAg-positive cells was reduced by 55.7% or 92.0%, respectively. Three days after the injection, HBsAg-positive cells were still not seen, and HBcAg-positive cells were scarce in shRNA-1 treated mice, while shRNA-2 treated mice showed similar results to those on day 1 ($P < 0.01$, Fig. 6). In contrast, the mice treated with the irrelevant shRNA-3 exhibited no inhibitory effect on the antigens.

3. Discussion

Although HBV is a DNA virus, its replication requires a key step of reverse transcription for synthesis of viral DNA from pregenomic RNA. Therefore, HBV is assumed to be susceptible to siRNA not only at the level of posttranscription, but also at the level of replication. To study the effect of RNAi on HBV replication in a cell culture model, we used the HepG2.2.15 cell line, which supports viral gene expression and viral replication and serves as an *in vitro* model for HBV replication (Li et al., 2005; Sells et al., 1987). For various reasons, none of the current HBV animal models is ideal. DHBV and WHV are genetically divergent from HBV. Chimpanzee experiments are limited by cost, availability and ethical considerations, whereas transgenic mice are immunologically tolerant to the virus. A mouse model by hydrodynamics (Yang et al., 2002) was reported which alleviates some of these experimental constraints and permits HBV to replicate for a period of time. This kind of convenient animal model has been adopted in some research on RNAi against HBV (Giladi et al., 2003; McCaffrey et al., 2003). *In vivo*, BALB/c mice were injected hydrodynamically via the tail vein (Liu et al., 1999; Yang et al., 2002; Zhang et al., 1999) with the HBV genomic plasmid pcDNA3.1-HBV (Tang et al., 2003), which resulted in HBV DNA production and HBsAg secretion.

HBV S region upstream is an ideal target, as it is not only less sensitive to mutation but also shared by the major viral transcripts. C region plays an important role in viral replication. What is more, the 3.5-kb mRNA not only serves for translation of the core protein/HBeAg and polymerase-reverse transcriptase but also represents the template for reverse transcription. Therefore, in our study, we selected two RNAi target sites, shRNA-1, which targets the region extending from 7 to 29 bases downstream of the ATG translational start site of the small s antigen, and shRNA-2, which targets the region extending from 193 to 211 bases downstream of the ATG translational start site of the C antigen. Both shRNA-1 and shRNA-2 target the 3.5-kb transcript.

In vitro study, the dose–response analysis showed that the inhibition of HBV gene expression in the cell culture by shRNAs is dose-dependent and specific. And the effect sustained for 14 days. Nevertheless, chemically synthesized siRNA is known to be transient and only lasts 3–4 days (McManus and Sharp, 2002). The immunostaining of the cultured cells revealed a strong association between the decline in HBV medium marker levels and the reduced number of HBcAg-positive cells. The RT-PCR analysis demonstrated that the total HBV transcripts were diminished and were dose-dependent and was strongly associated with the reduced levels of medium antigens. And transfection with the shRNAs resulted in reduction of viral DNA synthesis, in parallel.

In vivo study, the shRNAs also caused a significant inhibition in the levels of the viral antigen, viral mRNA and viral DNA relative to the controls. The inhibitory effect of silencing in hepatocytes was higher contrasted with that in the cell lines. The mechanism is not clear and it possibly occurs because hepatocytes are mostly non-dividing, there is no shRNA dilution with cell division (Song et al., 2003).

Our study showed that shRNA-1 exhibited lower level of inhibition both of the amounts of HBeAg and HBV DNA and it is probable that C region plays more important role in viral replication. shRNA-2 also reduced the levels of HBsAg, probably by targeting the 3.5-kb transcript which is the template for reverse transcription. The studies of RNAi against HBV *in vitro* combined with *in vivo* are mainly by Giladi (2003) and McCaffrey (2003). Giladi et al. (2003) had chosen the same HBV S region target site and their results are similar to ours, but in our work, the lasting time of the response is far more longer, possibly because shRNA not siRNA (Yu et al., 2002) is selected in ours. The differences of shRNA and siRNA deserve to be further studied. Compared with the work of McCaffrey et al. (2003), we selected a different C region target site and it also showed a dramatic effect. The information would be useful in future studies of the selection of HBV interfering target sites.

To further confirm the specificity of the inhibitory effect of shRNA on HBV gene expression without affecting the intrinsic genes, we randomly selected the c-fos gene and measured the c-fos mRNA level. c-fos is a well-known rapid-reaction intrinsic gene at early times. We have demonstrated that shRNA-mediated gene silencing has exquisite sequence specificity for target mRNA and does not induce any changes of the intrinsic gene. Chi et al. (2003) also showed the same result.

Hepatitis B still remains a major health problem in China and it is very necessary to find novel approaches to inhibit HBV infection. Current therapies against HBV such as interferon or lamivudine have provided limited cures (Liaw, 2002) with some drawbacks (Lee, 1997). RNAi appears to overcome some disadvantages of these drugs (Uprichard et al., 2005). First, RNAi specifically inhibits the targeted gene without activation of non-specific cellular responses, hence minimizing undesirable side effects. Second, the design for RNAi is directed to the conserved regions, which limits the viral ability to create escape mutants. The potential to introduce a few siRNAs targeting different sequences simultaneously further restricts this ability. Moreover, siRNAs can act even in the absence of active viral replication, making it an optimal candidate as an adjuvant therapy with lamivudine. Although further studies are required, we also show that RNAi is capable of inhibiting HBV replication and expression *in vitro* and *in vivo* and thus may provide a new and potential therapeutic strategy for HBV infection.

4. Materials and methods

4.1. Plasmid and siRNA

The vector pcDNA3.1-HBV (pHBV) contains the HBV genomes of 1013–3182 nt and 1–1986 nt (subtype ayw) (Tang et al., 2003). Synthetic shRNAs were cloned downstream of pSilencer3.1-H1 promoter by Jingsai Corp. (Wuhan, China) and were obtained as annealed duplexes. Target sites of shRNAs were determined by using the on-line tool from Ambion Company (www.ambion.com/techlib/misc/siRNA_finder.html), as shown in Fig. 1 (shRNA-1, shRNA-2 and shRNA-3 which is an irrelevant control shRNA).

4.2. Cell culture and transfection

HepG2.2.15 cells, a derivative of the human HepG2 hepatoma cell line stably transformed with several copies of the HBV genome, which constitutively produce HBV particles (Sells, 1987), were maintained in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 100 ml/l FCS (Hyclone, USA), 400 μg/ml G418, 100 μg/ml streptomycin and 100 IU/ml penicillin at 37 °C in a humidified atmosphere containing 50 ml/l CO_2. The cells were seeded into 6-well plates (60–80% confluence) and were transfected with Liposome metafectene (Biontex Lifetechnology, Germany) according to the manufacturer's instructions in the presence of pSilencer3.1-H1hygro plasmid expressing the shRNA.

4.3. siRNA treatment in vivo

For all the *in vivo* experiments we used 6–7-week-old female BALB/c mice (18–22 g) from the Laboratorial Center of the Academy of Sciences (Shanghai, China). Plasmid HBV and shRNAs were delivered into mice using the hydrodynamic tail vein injection method (Liu et al., 1999; Zhang et al., 1999). Briefly, 60 μg pHBV and 25 μg shRNA dissolved in 1.8 ml Ringer's solutions (147 mM NaCl, 4 mM KCl, 1.13 mM $CaCl_2$) were rapidly injected into the tail vein. In the dose–response experiment, mice were co-injected with 60 μg pHBV and 15 μg, 25 μg or 35 μg shRNA, respectively. The mice were bled, and the sera were separated and examined for HBsAg or HBV DNA content on day 1 or 3 after injection. The livers of the mice were preserved in formalin for histological analysis. All mouse experiments were carried out according to the National Institutes of Health Guidelines for Animal Care.

4.4. HBsAg and HBeAg

The levels of HBsAg and HBeAg in the media of the transfected cells, and in the sera of the treated mice were determined by time-resolved immunofluorometric assay according to the manufacturer's instructions (Sym-Bio Co., Suzhou, China). For immunocytochemistry, the specific antibody against HBcAg (Wuhan Boster Biological Technology, China) was used to detect intracellular HBcAg. The formalin-fixed liver tissues from the mice were embedded in paraffin. Immunohistochemical staining for HBsAg and HBcAg was performed using the PV-6001 kit (PowerVision™ IHC Polymer Detection Reagent, USA) with specific antibodies against HBsAg (rabbit, OBT0990, Oxford Biotechnology Ltd., the United Kingdom) and HBcAg (rabbit, Boster Biological Technology, Wuhan, China). The above

immunological detections followed their own manufacturer's protocol.

4.5. HBV DNA quantitation

Viral particles in the supernatants and in the mice sera were quantified by fluorescent real-time polymerase chain reaction (FQ-PCR) according to the kit's instructions (Shengyou Biotech Company, Shanghai, China). The primers specific for the detection of HBV S region were 5′-CCTCTTCATCCTGCTGCT-3′ and 5′-AACTGAAAGCCAAACAGTG-3′. The fluorescent probe was 6-FAM-TCCCATCCCATCATCCTGGGCTTT-TAMRA. The reaction took place for 40 cycles in the iCycler iQ real-time PCR system (Bio-Rad, USA). The inhibition ratio of HBV DNA was calculated according to the formula, which is [1 − log(treated sample fluorescent intensity)/log(control fluorescent intensity)] × 100%.

4.6. Reverse transcriptase PCR (RT-PCR) assay

Total RNA was extracted from the cells or mice livers using the RNeasy mini kit according to the manufacturer's instructions. Primers were 5′-CTGGGTGGGTGTTAATTTGG-3′ and 5′-TAAGCTGGAGGAGTGCGAAT-3′ for the HBV C region, and 5′-ATGGAGAACATCACATCAGG-3′ and 5′-TTAAA-TGTATACCCAAAGACAAAAG-3′ for the HBV S region. The primers for c-fos were 5′-TCAACGCGCAGGACTTCTGCAC-3′ and 5′-TGGTCTGTCTCCGCTTGGAGTG-3′. The isolated RNA was first subjected to RT-PCR using the access RT-PCR system (Promega, USA). Then, the cDNA was used for PCR. The number of PCR cycles used was the lowest needed to get a product which could be visualized on the gel.

4.7. Statistical methods

Results are presented as mean ± S.D. The data were based on three independent experiments *in vitro* and were on five animals per group *in vivo*. Comparison between groups was performed by an independent-samples *T* test. The level of significance was set to $P < 0.05$.

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