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Inhibition of Hepatitis B virus gene expression by single and dual small interfering RNA treatment

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Abstract

RNA interference (RNAi) has been successfully applied in suppression of Hepatitis B virus (HBV) replication. To circumvent the problem that mutation in HBV genome may result in resistance when siRNA is further developed as an anti-viral drug, in this study, we established a dual small interfering RNA (siRNA) expression system, which could simultaneously express two different siRNA molecules that can specifically target two genes. To test the effectiveness of this system, we applied this new approach to express simultaneously two different 21-bp hairpin siRNA duplexes that specifically attack the HBs and HBx genes of HBV, respectively, in Bel-7402 and HepG2.2.15 cells. Results indicated that dual siRNA could simultaneously inhibit the expression of HBs and HBx gene by 83.7% and 87.5%, respectively, based on luciferase assays. In addition, dual siRNA molecules were able to significantly reduce the amount of HBV core associated DNA, which is considered as an intracellular replicative intermediate, and the viral DNA in culture supernatant. Therefore, this dual siRNA system provides a more powerful tool for the study of gene function and implicates a potential application in the treatment of viral infection.

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Keywords: Dual siRNA; HBV; Gene expression

1. Introduction

RNA interference (RNAi) is a natural process of eukaryotic cells by which double-stranded RNA initiates and directs the degradation of homologous mRNA (Hannon, 2002). This RNA silencing mechanism was first described in Caenorhabditis elegans and Drosophila melanogaster (Fire et al., 1998). It has many similarities to the post-transcriptional gene silencing in plants. Specific inhibition of cellular mRNA by RNAi can be triggered in mammalian cells by the introduction of synthetic 21- to 23-nucleotide double-stranded small interfering RNA (siRNA) (Elbashir et al., 2001; Paul et al., 2002) or, alternatively, by the transcrip-
techniques were used more frequently in recent studies. Each vector expresses unique siRNA that can degrade a specific target.

Eight genotypes (A–H) of HBV have been described. The number of HBV carriers worldwide has been estimated to be more than 400 million. These individuals have a 15–25% risk of developing liver diseases such as liver cirrhosis and hepatocellular carcinoma (Kao and Chen, 2002). Although a few drugs were developed against HBV infection, the success rate of these treatments, however, is low and frequently infections reoccur (Carreno et al., 1992; Lai et al., 1997). The fact that RNAi can be applied for blocking the replication of HBV in several reports provided insights into the field of controlling infectious human hepatitis. Nevertheless, mutations in HBV genome may result in viral resistance to siRNA. It has been reported that HIV-1 can escape from RNAi-mediated inhibition due to nucleotide change in the genome (Das et al., 2004). One strategy to circumvent the problem is to choose target in the relatively conserved DNA sequence. The other approach is to produce multiple siRNAs that target different sites or genes on the viral genome. We here established a system that can express two siRNA duplexes simultaneously and target the S and X genes of HBV, respectively. To study the effects of dual siRNA on HBV gene expression in a cell culture model, we used a derivative of the human HepG2 hepatoma cell line, HepG2.2.15, which has been stably transformed with several copies of the HBV genome and used as an in vitro model for HBV replication. The effects of dual siRNA system on HBV gene expression were investigated in this study.

2. Methods

2.1. Cell lines and condition of transfections

Two human hepatoma cell lines, Bel-7402 and HepG2.2.15 were maintained in Dulbecco’s modified Eagle medium (GIBCO/BRL) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated fetal bovine serum at 37 °C under 5% CO₂. Cells were seeded onto 24-well plates at a density of 1.0 × 10⁵ or 4.0 × 10⁵ cells per 24-well plate or 6-well plate and grown to the confluence reaching approximately 60% at the time of transfection. Cells were transfected with 0.1 or 0.4 μg pCMV-HBS together with 0.45 or 1.2 μg pSliencer-2.1-U6-siRNA, using Sofast™ transfection reagent (Xiamen Sunma Biotechnology Co. Ltd., China) according to the protocol provided by the manufacturer. The cells were harvested 48 h after transfection.

2.2. Plasmid construction

Full-length HBV genomic DNA (subtype ayw) was cloned into the HinDIII and SacI sites of pHlusecript (Stratagene) to generate the plasmid pHlue-HBV. HBs gene was cloned into the HinDIII and SacI sites of vector pCMV-tag2A (Stratagene) to yield plasmid pCMV-HBs. HBs gene was cloned into pCMV-Tag2A at EcoRI and XhoI sites to generate pCMV-HBx.

Two pair of primers 5′-CTGGCGAGATCTCTATGGGAGAGC-TCAGATCCAGGATTCT3′ (sense), 5′-GTAGGGCTGACAC-TGTTATACCCAAAGACAAAAAGAA-3′ (antisense) or 5′-GATCCATATGGTAGATCTATGGAGAGC-TCAGATCCAGGATTCT3′ (sense), 5′-GTAGGGCTGACAC-TGTTATACCCAAAGACAAAAAGAA-3′ (antisense) were used to amplify the HBs and HBx gene, respectively. The PCR products were then cloned into SalI and BglII sites of plucF to generate plucF-HBs and plucF-HBx (Fig. 1a), in which the HBs and HBx were fused in frame with the luciferase gene and the expression of the fusion gene was drove by the CMV promoter (Fig. 1a).

2.3. Generation of siRNA expression vectors

Five regions of the HBs gene and three regions of the HBx gene were selected as the targeted sequences of siRNA in this study (Fig. 1b).
To construct single siRNA expression vector, two 64nt primers, each containing a 19nt target sequence in the sense and antisense forms from different regions of the HBs gene or HBx gene as indicated below, were synthesized (Invitrogen): 5′-GCTCCGGCGGTGCTTTGACC-3′ (HBs) siRNA; 5′-GGTGGACTTCCTCAATTT-3′ (HBs2siRNA); 5′-GCCAATCTGGAGTCTTTG-3′ (HBx siRNA); 5′-GTGCTGCTCACAACCTTT-3′ (HBx siRNA); 5′-GCTCAGTTTACTAGTGGCA-3′ (HBx siRNA); 5′-GCACTTGGTCTACCCTGTTG-3′ (HBx siRNA); 5′-GCAATGTCGACCGCCAGC-3′ (HBx siRNA); 5′-GTCTAAAGACTGGGAGGAG-3′ (HBx siRNA).

Sense and antisense primers were then cloned into pSilencer-2.1-U6 plasmid (Ambion) at BamHI and HindIII sites after annealing according to the manufacturer’s instructions.

To generate the dual siRNA expression plasmid, two primers 5′-GCTCATGACGTCAGTGGAAAGACGCG-3′ (sense) or 5′-TCAGCGAATTCACGCCAAGCTTTTCC-3′ (antisense) were designed to amplify a DNA fragment containing U6 promoter and HBX2 siRNA expression cassette from recombinant plasmid pSilencer-2.1-1-U6-HBX2.

The PCR product was then cloned into AaII and EcoRI sites of plasmid pSilencer-2.1-1-U6-HBXX, which carries two independent siRNA expression cassettes (Fig. 1c).

2.4. Luciferase assay

Bel-7402 cells were co-transfected with reporter plasmids and siRNA expression plasmids. Cells were washed with PBS and lysed with luciferase cell culture lysis reagent (Promega). Ten microliters of the cell lysates and 100 μl of luciferase assay substrate (Promega) were mixed and fluorescence intensity was detected by the luminometer (Turner T20/20). Assays were performed in triplicate, and expressed as means ± S.D. relative to vector control as 100%.

2.5. Hepatitis B surface antigen (HBsAg) assay

Bel-7402 cells and HepG2.2.15 cells were transfected with siRNA expression plasmids, the level of HBsAg protein in culture media from transfected cells were then determined by enzyme-linked immunosorbent assay using a HBV diagnostic kit (Shanghai Kehua Biotech Co. Ltd.). Assays were performed in triplicate independent experiments.

2.6. RNA isolation and RT-PCR assay

Bel-7402 cells and HepG2.2.15 cells were transfected with siRNA expression plasmids; total RNA were then extracted from transfected cells by TriZol Reagent (Invitrogen) according to the method described in the manufacturer’s manual. Reverse transcription were performed with total RNA as the template. The cDNAs were synthesized with HBs or HBx gene specific primers, 5′-GCCGGGTTTTTCTGTTGAG-3′ (sense), 5′-CTAGGACCCACTGAGCAAAAT-3′ (antisense) or 5′-CTGGCGGAGAGCCTTTG-3′ (sense), and 5′-CAGTCTTTGAAGTATGCC-3′ (antisense).

2.7. Assays for HBV core associated DNA and HBV DNA by real time-PCR

To assay the effect of siRNAs on HBV replication, intracellular core-associated HBV DNA was extracted by the method described previously (Pugh et al., 1988). Briefly, 1 × 10⁵ transfected HepG2.2.15 cells were lysed and centrifuged at 25 °C. Magnesium chloride was added to the supernatant. DNA not protected by HBV core was treated digested with deoxyribonuclease (DNase I). Then the lysates were treated with proteinase-K and, after phenol/chloroform extraction; core-associated HBV DNA was recovered by ethanol precipitation, and quantified by real time-PCR (RT-PCR) as described by the manufacturer (PG BIOTECH, Shenzhen, China). The HBV DNA in the supernatants was also quantified following the procedure provided by the manufacturer (PG BIOTECH, Shenzhen, China). Primers used in RT-PCR were: P1, 5′-ATCATCCTGCTTGATGCTC-3′ (sense), 5′-CACTTTGGGAAACGGCTTACG-AA-3′ (antisense); P2, 5′-GCTGGCTATCTAGTGGCATTTTT-3′ (antisense). The probe was 5′-TGGCTAGTTTACTAGTGGCATT-3′. PCR reaction was carried out and analyzed by a PE Gene Amp 7700 (Perkin-Elmer, USA).

3. Results

3.1. Effects of HBV-specific siRNA treatment on the expression of HBs-luciferase and HBx-luciferase fusion genes

To efficiently screen siRNA molecules, selected targetting DNA sequences were fused in frame with that of luciferase gene, in which luciferase activity was supposed to represent the level of HBs or HBx mRNA expression. Cells were co-transfected with placF-HBs or placF-HBx and eight single siRNA expression vectors, respectively. Luciferase activities were then determined from those transfected cells. Result showed that HBs siRNA, HBx2 siRNA and HBx2 siRNA strongly inhibited luciferase activities by 81.5%, 80.5%, and 76.5%, respectively, comparing to that of vector control (Fig. 2a and b). These results indicated that the three siRNAs could efficiently degrade the mRNA of HBs-luciferase and HBx-luciferase fusion gene.

To evaluate the effects of dual siRNA expression plasmid on the inhibition of HBs-luciferase or HBx-luciferase fusion gene expression, cells were co-transfected with placF-HBs or placF-HBx and the dual siRNA expression plasmid pHB-SXsiRNA. Result from luciferase activity assays indicated that there was a further reduction in luciferase activity by dual
siRNA duplexes (HBSXsiRNA) comparing to that of single siRNA expression vectors (HBS2siRNA or HBX2siRNA). The reduction rate of luciferase activity caused by HB-SXsiRNA was 83.7% to HBs and 87.5% to HBx, respectively (Fig. 2c and d).

3.2. Effects of HBs specific siRNA treatment on the levels of HBsAg production

To evaluate the influence of RNAi on HBS gene expression, Bel-7402 cells were transfected with pSilence2.1-U6-siRNA, pCMV-HBs or HBSXsiRNA and HepG2.2.15 cells were transfected with pSilence2.1-U6-siRNA or HBSXsiRNA. HBsAg concentrations in the culture media of transfected and control cells were measured 2 days after transfection by ELISA using HBV diagnostic kit. Results showed that HBsAg level was decreased in the Bel-7402 cells after transfection with HBS1siRNA, HBS2siRNA or HBSXsiRNA with reduction rate of 91.5%, 88.5% , and 83.7%, respectively (Fig. 3a and d). In HepG2.2.15 cells, transfection with HBsAg siRNA, HBsXsiRNA or HBXSsiRNA reduced HBsAg level by 75.4%, 85.7%, and 87.6%, respectively (Fig. 3b and c). In addition, transfection with HBsXsiRNA reduced the level of HBsAg production by 65.3% in HepG2.2.15 cells (Fig. 3e).

3.3. Inhibition of HBs and HBx gene expression by siRNA treatment

To determine whether siRNAs specifically degrade HBs or HBx mRNA, we used semi-quantitation RT-PCR analyses to determine the levels of HBs or HBx mRNA in two different cell lines, Bel-7402 (Fig. 4a and b) and HepG2.2.15 (Fig. 4c and d), 2 days after transfection. Results indicate that the levels of HBs mRNA were significantly decreased by the treatment of HBSXsiRNA (Fig. 4a, lane 1), HBS1siRNA (Fig. 4a, lane 2), HBS2siRNA (Fig. 4a, lane 3) in Bel-7402. The levels of HBx mRNA were also decreased by the treatment of HBSXsiRNA (Fig. 4b, lane 1), HBX2siRNA (Fig. 4b, lane 2), but not by that of pSilence-2.1-U6 or untreated cells (Fig. 4b, lanes 3 and 4). Similar results were also obtained in HepG2.2.15 cell lines under the same conditions of siRNA treatments (Fig. 4c and d). Results showed that the levels of HBs mRNA were dramatically reduced in HepG2.2.15 cells after the treatment of HBSXsiRNA (Fig. 4c, lane 6), HBS1siRNA (Fig. 4c, lane 7) and HBSXsiRNA (Fig. 4c, lane 8), respectively. The levels of HBx mRNA in HepG2.2.15 cells were also reduced by the treatment of HBSXsiRNA (Fig. 4d, lane 2), HBXsiRNA (Fig. 4d, lane 3), but not by that of pSilence-2.1-U6 or untreated cells (Fig. 4d, lanes 1 and 4). In addition, our results showed that the inhibition
Fig. 3. Determination of the effectiveness of siRNAs on levels of HBsAg expression. pSilencer-2.1-U6 plasmid was used as vector control in all transfection experiments. HBsAg levels were determined by ELISA using a HBV diagnostic kit. (a) Bel-7402 cells were co-transfected with pCMV-HBs and pSilencer-2.1-U6-siRNA plasmids expressing HBS1siRNA, HBS 2siRNA, HBS 3siRNA, HBS 4siRNA and HBS 5siRNA. (b) HepG2.2.15 cells were transfected with pSilencer 2.1-U6-siRNA plasmids expressing HBS1siRNA, HBS 2siRNA, HBS3siRNA, HBS 5siRNA and HBSXsiRNA. (c) HepG2.2.15 cells were transfected with pSilencer-2.1-U6-siRNA plasmids expressing HBX1siRNA, HBX 2siRNA, HBX 3siRNA. (d) Bel-7402 cells were co-transfected with pCMV-HBs and pSilencer-2.1-U6-siRNA plasmids expressing HBSXsiRNA and HBX5siRNA. (e) HepG2.2.15 cells were transfected with pSilencer2.1-U6-siRNAs plasmids expressing HBX1siRNA, HBX2siRNA, HBX3siRNA.

Effects of dual siRNA, HBXSiRNA on the levels of HBs and HBx mRNA (Fig. 4a (lane 1), b (lane 1), c (lane 6), and d (lane 2)) were more severe than that of single siRNA (Fig. 4a, (lanes 2 and 3), b (lane 2), c (lane 7 and 8), and d (lane 3)).

3.4. Effects of siRNA on the viral DNA replication

3.4.1. Analysis HBV core associated DNA and HBV DNA in the supernatants

To determine the effectiveness of siRNAs on viral DNA replication, HBV core associated DNA (as an intracellular replicative intermediate) and HBV DNA were extracted from HepG2.2.15 cells transfected with HBXSsiRNA, HBX5siRNA, HBX2siRNA, HBX2siRNA, and vector, respectively. The levels of HBV core associated DNA and HBV DNA were determined by real time PCR. Results indicated that the levels of HBV core associated DNA were significantly decreased in the cells transfected by HBXSsiRNA, HBX5siRNA, HBX2siRNA, and HBX2siRNA with reduction rate of 90.2%, 85.7%, 81.3%, and 60.4%, respectively, compared with that of vector control (Fig. 5a). In HepG2.2.15 cells, transfection with HBXSsiRNA, HBX5siRNA, HBX2siRNA and HBX2siRNA reduced the level of viral DNA in supernatants media by 88.7%, 82.6%, 78.4%, and 58.3%, respectively (Fig. 5b).

4. Discussion

It has been attracted considerable attentions in the use of RNAi as therapeutics to treat a variety of diseases, including tumors and viral infections. Hamasaki et al. (2003) demonstrated that RNAi could attenuate the replication of HBV genome in cell culture. Shlomai and Shaul (2003) used
Fig. 4. RNA expression level determined by semi-quantitated RT-PCR analysis. Total RNA was used as template to synthesize cDNA. HBs specific primers were then applied for detection in (a and c), and HBx specific primers in (b and d). (a) Bel-7402 cells were co-transfected with pCMV-HBs and HBXSsiRNA (lane 1), HBXSsiRNA (lane 2), HBXSsiRNA (lane 3), and pSilencer-2.1-U6 (lane 5, as a vector control). As control, Bel-7402 cells were also transfected with pCMV-HBs only (lane 4). (b) Bel-7402 cells were co-transfected with pCMV-HBx and HBXSsiRNA (lane 1), HBXSsiRNA (lane 2) or pSilencer-2.1-U6 (lane 3, as a vector control). As control, Bel-7402 cells were also transfected with pCMV-HBx only (lane 4). Lanes 5–8 were β-actin controls. Lane M indicates DNA markers. (c) HepG2.2.15 cells were transfected with HBXSsiRNA (lane 6), HBXSsiRNA (lane 7), HBXSsiRNA (lane 8) or pSilencer-2.1-U6 (lane 9, as a vector control). Cells without transfection were used as controls (lane 10). Lanes 1–5 were β-actin controls. (d) HepG2.2.15 cells were transfected with HBXSsiRNA (lane 2), HBXSsiRNA (lane 3), or pSilencer-2.1-U6 (lane 1, as a vector control). Cells without transfection were used as controls (lane 4). Lanes 5–8 were β-actin controls.

We can initially select the suitable siRNA duplexes rapidly by simply analyzing the activities of luciferase. By using this approach, we have identified two siRNA molecules (HBXSsiRNA and HBXSsiRNA) having significant impact on the HBs-luciferase fusion gene expression and one siRNA duplex (HBXSsiRNA) having effects on the expression of HBx-luciferase fusion gene. This provides a quick approach to select effective siRNA in the study of gene expression and function analysis.

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To further study the effects of selected RNAi molecules on HBV gene expression and viral replication in a cell culture model, we used a derivative of the human HepG2 hepatoma cell line, HepG2.2.15, which has been stably transformed with several copies of the HBV genome and used as an in vitro model for HBV replication. The effects of dual siRNA system on HBV gene expression and viral replication were studied thoroughly by analyzing the levels of viral protein production through enzyme-linked immunosorbent assay and...
the levels of viral RNA expression by semi-quantitated
RT-PCR analysis. All results indicated that HBS1siRNA,
HBS2siRNA, and HBX2siRNA had significant reduction ef-
facts on viral mRNA expression, and viral protein production.
The fact that mutation in HBV genome may result in re-
sistance if siRNA molecules were further developed as anti-
viral drugs raised our concerns. Our strategies to address
such potential problems are to choose targets in the rela-
tively conserved DNA sequences and to generate multiple
siRNA molecules that can target different sites or genes on
the viral genome. To test our approach, in this study we estab-
lished a system that can simultaneously express two siRNA
duplexes from a single vector that can attack the S and X
genes of HBV, respectively. Results from luciferase activ-
ity assay, enzyme-linked immunosorbent assay and semi-
quantitated RT-PCR analysis were consistently showed that
dual siRNA molecules had synergetic effects or more ef-
ficient on the targeted viral protein production and HBs and
HBx gene expression comparing to that of the single siRNA
molecules. More importantly, dual siRNA could simultane-
ously inhibit the expression of HBs and HBx by 83.7%
and 87.5%, respectively. Therefore, this dual siRNA system
could provide a more powerful tool for the study of gene
function and could be used as a potential application in the
treatment of viral infection.

In the last 20 years, HBV infection affects millions of peo-
ple each year worldwide. Current therapies of HBV infection
including immune modulators such as interferon Alfa, or nu-
cleoside analogs such as lamivudine have provided some de-
gree of cures, but the efficiency of treatment was limited.
As a potential therapy, siRNA seems to be a hopeful alter-
native strategy. We believe that our approach presented in
this study could be broadly used. For example, it could be
used to generate more than two siRNAs duplexes that could
silent more genes in order to study the interactions of genes
and their functions. Such strategies of constructing multiple-
siRNA vectors can confront the evading mechanism of virus
infections. Obviously, this cocktail approach would be benefit
to application of siRNA therapy in viral infections, especially
to those viruses with high mutation rate. In addition, this ap-
proach could also used to deal with two or more viruses,
which are especially useful in the treatment of co-infections
by two or more pathogens, such as HBV–HIV and HCV–HIV.
We are in the process of testing these approaches.

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