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Short interfering RNA directed inhibition of hepatitis B virus replication

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Abstract RNA interference (RNAi) is the process by which double-stranded RNA directs sequence-specific degradation of mRNA. In mammalian cells, RNAi can be triggered by 21-nucleotide duplexes of short interfering RNA (siRNA). We examined effects of siRNA on hepatitis B virus (HBV) replication. Human hepatoma cells were transfected with HBV DNA and siRNA against HBV-pregenome RNA. Transfection experiments demonstrated that the siRNA reduced the amount of HBV-pregenome RNA and resulted in reduction of the levels of replicative intermediates and viral protein. Our results indicate that siRNA-mediated gene silencing inhibits HBV replication through suppression of viral RNA, which may be useful as a potential therapeutic modality.

Key words: Hepatitis B virus; siRNA; RNAi

1. Introduction

RNA interference (RNAi) is a mechanism of gene regulation in plants, invertebrates and, more recently, in mammalian cells in which target mRNAs are degraded in a sequence-specific manner [1–3]. RNAi is initiated by the double-stranded RNA (dsRNA)-specific endonuclease Dicer, which promotes processive cleavage of long dsRNA into 21–23-mer short interfering RNA (siRNA) [4–8]. siRNAs are incorporated into a protein complex that recognizes and cleaves target mRNA [5–8]. RNAi has been shown to protect against invading genetic elements such as transposons, transgenes and viruses, which potentially share a long dsRNA trigger [9–12].

In mammalian cells, exposure to dsRNAs greater than 30 bp in length induces an antiviral interferon response that generally represses mRNA translation through the activation of dsRNA-dependent protein kinase [13]. However, introduction of shorter siRNA into mammalian cells leads to mRNA degradation with exquisite sequence specificity without activating the interferon system [3]. RNAi-mediated gene silencing offers a potentially powerful tool to inhibit viral replication. Therefore, we examined whether siRNA duplexes specific for hepatitis B virus (HBV) were capable of affecting the degradation of viral RNAs.

2. Materials and methods

2.1. Plasmids and synthesis of siRNA

Full-length HBV DNA was cloned via *Sac*I site into the pGEM-11Zf(+) vector (Promega, Madison, WI, USA) as described previously [14], registered in GenBank (accession number AB050018). According to Harborth's report for selection of siRNA duplexes for the target mRNA sequence [15], we searched for sequences of the type AA (N21) [N, any nucleotide (nt)] from the open reading frame of the 3.5-kb HBV-pregenome RNA, in order to obtain a 21-nt sense and 21-nt antisense strand with 2-nt 3′ overhangs. A selected sequence was also submitted to a BLAST search against the human genome sequence to ensure that the human genome was not targeted. siRNA with the following sense and antisense sequences was used: siHBV, 5′-CAUUGUUCACCUCACCAUATT-3′ (sense), 5′-UAUGGUGAGGUGAACAAUGTT-3′ (antisense), corresponding to HBV sequence in the core region from nt 2137 to nt 2157 (Fig. 1). siRNA against green fluorescent protein was used as control: siGFP, 5′-GGCUACGUCCAGGAGCGCACC-3′ (sense), 5′-UGCGCUCCUGGACGUAGCCTT-3′ (antisense). All siRNAs were purchased from Nihon Bioservice (Saitama, Japan).

2.2. Cells and transfections

The HuH7 and HepG2 cells, human hepatoma cell lines, were maintained in RPMI, supplemented with 10% fetal bovine serum. Prior to transfection, plasmid including HBV genome was cleaved with *Sap*I (New England Biolabs, Beverly, MA, USA) to release the heterologous primer sequences and to create linear HBV monomers with *Sap*I sticky ends, as described previously by Gunther et al. [16]. The digested DNA was purified by a QIAquick PCR purification kit (Qiagen, Hilden, Germany). Transfection of cells using linear monomeric HBV genome with *Sap*I ends can initiate a full replication cycle, including production of viral RNAs, translation of viral proteins and release of virions [14,16]. The HuH7 and HepG2 cells were plated at a density of 2.5×10^5 and 4.0×10^5 cells per 35-mm-diameter dish, respectively. One day later, both cells were transfected with 0.5 μg of HBV DNA and 0.05 μg of pGL3-control vector expressing luciferase (Promega) together with 100 pmol of each siRNA, using oligofectamine (Gibco-Invitrogen, Rockville, MD, USA) according to the protocol provided by the manufacturer. The medium was replaced with a fresh medium 6 h after transfection, and the cells were harvested 2 days after transfection. Experiments were performed in triplicate. Transfection efficiency was standardized by measurement of the luciferase activity in the cell lysate using a luciferase assay system (Promega).

2.3. Quantitative assay of viral marker

The levels of hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) in culture media from transfected cells were quantitatively measured using chemiluminescent immunoassay and radioimmunoassay (Dainabot, Tokyo, Japan), respectively.

2.4. Northern blotting

Total RNA was isolated from the transfected HuH7 cells using the

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Abbreviations: dsRNA, double-stranded RNA; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; RNAi, RNA interference; siRNA, short interfering RNA

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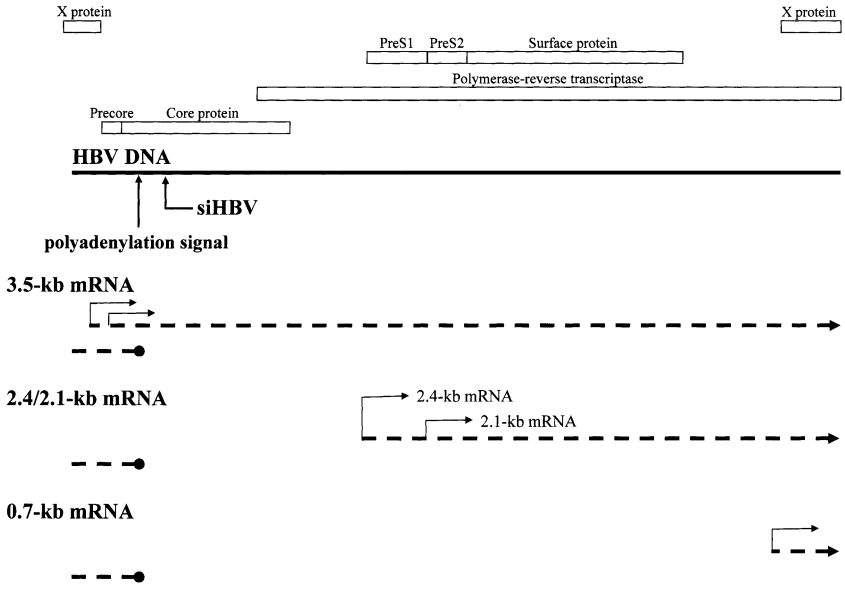

Fig. 1. A schematic diagram depicting the location of the siRNA in association with viral open reading frames and viral mRNAs within the HBV genome.

guanidium isothiocyanate method. The isolated RNA was fractionated on 1.5% formaldehyde agarose gel, transferred onto a nylon membrane (Hybond N^+, Amersham, Little Chalfont, UK), and hybridized with a ^{32}P-labeled full-length HBV fragment. The probe was generated with a random-primed labeling kit (Amersham). Autoradiography was performed and analyzed with a BAS2000 image analyzer (Fuji Photo Film, Tokyo).

2.5. Southern blotting

Purification of HBV DNA from intracellular core particles was performed using the method described by Gunther et al. [16]. Briefly, the cells were washed once with ice-cold phosphate-buffered saline and lysed in 300 μl of lysis buffer [50 mM Tris–HCl (pH 7.4), 1 mM EDTA, 1% Nonidet P-40] per 35-mm-diameter dish. The cell lysates were transferred to 1.5-ml tubes, vortexed, and allowed to stand on ice for 15 min. Nuclei were pelleted by centrifugation for 1 min at 14 000 rpm. The supernatant was adjusted to 10 mM $MgCl_2$ and treated with 100 μg ml^{-1} of DNase I for 30 min at 37°C. The reaction was stopped by addition of EDTA to a final concentration of 25 mM. Proteins were digested with 0.5 mg ml^{-1} of proteinase K and 1% sodium dodecyl sulfate for 2 h at 37°C. Nucleic acids were purified by phenol–chloroform (1:1) extraction and ethanol precipitation with glycogen. DNA isolated from the cytoplasmic core particles was separated on a 1.5% agarose gel, blotted onto a nylon membrane (Hybond N^+), and hybridized with a ^{32}P-labeled full-length HBV fragment.

3. Results

3.1. Quantitative analysis of viral markers in culture media

siRNA duplexes were co-transfected with a full-length HBV DNA into the HuH7 and HepG2 cells. HBsAg and HBeAg secreted into culture media were quantitatively assayed (Fig. 2). The amount of HBsAg in media of cells transfected with siHBV did not differ from that of cells transfected with siGFP (control siRNA). In contrast, medium HBeAg levels in cells transfected with siHBV decreased to 4.6-fold and 4.9-fold in comparison with cells transfected with siGFP in HuH7 and HepG2 cells, respectively.

3.2. Northern and Southern blot analysis

Northern blot analysis was performed 2 days after transfection (Fig. 3). The level of 2.4/2.1-kb mRNA of HBV was not suppressed by siHBV, whereas the level of 3.5-kb mRNA was reduced in cells transfected with siHBV relative to cells transfected with siGFP.

Using Southern blot analysis (Fig. 4), the level of open circular and single-stranded HBV-DNA, which is considered as an intracellular replicative intermediate, was clearly suppressed by siHBV. On the other hand, the level of double-stranded HBV-DNA, which is mostly derived from the transfected DNA, was slightly diminished.

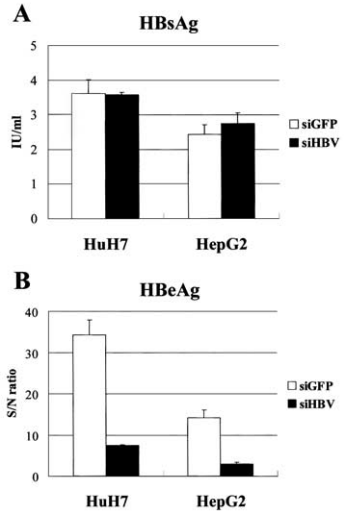

Fig. 2. Quantitative measurements of HBsAg (A) and HBeAg (B) in culture media from HuH7 cells and HepG2 cells co-transfected with HBV DNA and siRNA. Transfection experiments were performed in triplicate as described in Section 2. The levels of medium HBsAg and HBeAg are expressed as mean ± S.D. The S/N ratio denotes the signal-to-noise ratio.

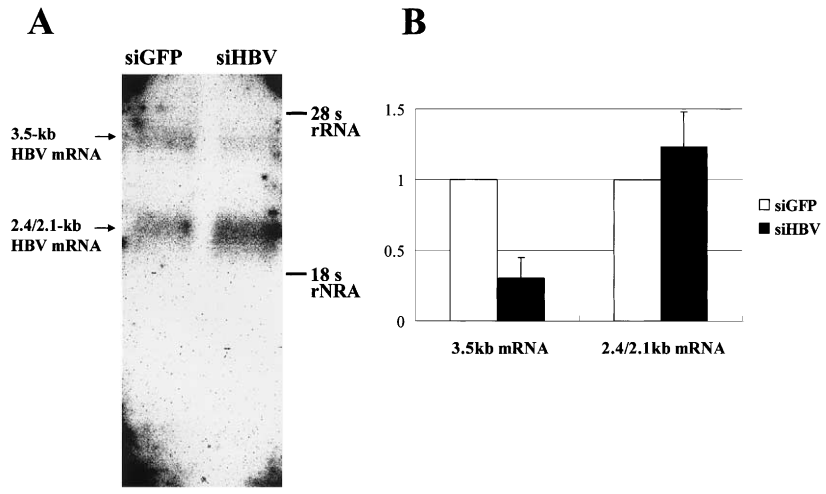

Fig. 3. Northern blot analysis of HBV in cells co-transfected with HBV DNA and siRNA. A: The HuH-7 cells were harvested 2 days after transfection. The amount of 3.5- and 2.4/2.1-kb HBV mRNA was determined by Northern blotting. B: The hybridization signals for the viral transcript were quantitatively evaluated by NIH image analysis software. Data are expressed as mean ± S.D. relative to the value of cells transfected with siGFP in three independent transfection experiments.

4. Discussion

Gene silencing mediated by siRNA is a sequence-specific and highly conserved mechanism in eukaryotes [9–11]. In plants, it serves as an antiviral defense mechanism [12]. Mammalian cells also possess this machinery but its specific function is unclear. More recently, several investigators demonstrated that siRNAs inhibit HIV-1 production by targeting various regions for its genome [17,18]. In this study, we showed that siRNAs directed against HBV genome could effectively block viral replication.

The HBV genome is a partially double-stranded 3.2-kb DNA molecule and is the template transcribed to generate the four viral RNAs [19]. The 3.5-, 2.4/2.1-, and 0.7-kb transcripts encode the core protein/HBeAg and polymerase-reverse transcriptase, HBsAg, and X protein, respectively. All viral transcripts utilize a common polyadenylation signal located within the core protein-coding region. 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. In the present study, the effect of RNAi on HBV replication was examined using a siRNA specific for the 3.5-kb pregenome RNA, which did not bind to other viral transcripts. We demonstrated that the siRNAs reduced the level of 3.5-kb pregenome RNA and resulted in reduction of the levels of secreted HBeAg and replicative intermediates converted from the 3.5-kb pregenome RNA. These findings indicate that mammalian RNAi machinery can be programmed with siRNA corresponding to HBV genome to induce an effective antiviral response. Unsur-

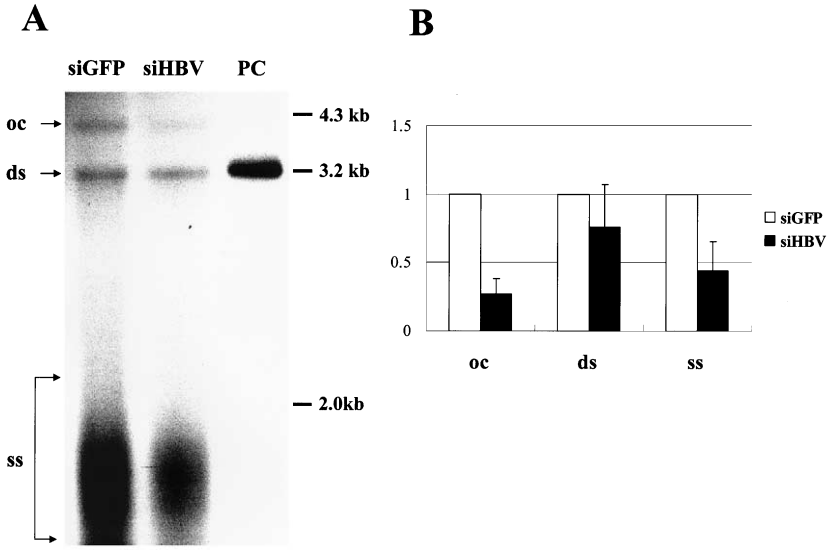

Fig. 4. Southern blot analysis of HBV in cells co-transfected with HBV DNA and siRNA. A: The HuH-7 cells were harvested 2 days after transfection. The level of open circular (oc), double-stranded (ds) and single-stranded (ss) HBV DNA was determined by Southern blotting. The lane denoted PC was loaded with linear full-length HBV. B: The HBV DNA signals obtained from three independent transfection experiments were quantitatively evaluated by NIH image analysis software. Data are expressed as mean ± S.D. relative to the value of cells transfected with siGFP.

prisingly, the levels of 2.4/2.1-kb mRNA and secreted HBsAg was not reduced in cells transfected with siHBV, since the 2.4/2.1-kb mRNA did not include the homologous sequence to siHBV.

Although chronic HBV infection is a major health problem worldwide, there is no completely effective antiviral treatment. siRNA technology may provide a possible therapeutic strategy against chronic HBV infection. Further studies are necessary to determine the antiviral mechanism of siRNA on HBV replication.

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