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TARGETED INHIBITION OF HEPATITIS B VIRUS GENE EXPRESSION: A GENE THERAPY APPROACH

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1. ABSTRACT

In this study, we employ antisense RNA technology to block Hepatitis B Virus (HBV) gene expression in cell culture by gene transfer as an approach to block immune recognition and pathogenic sequelae. Retroviral vectors encoding antisense and sense copies of the HBV surface antigen gene (HBsAg) were constructed, respectively. To assay the inhibition of HBV gene expression by antisense RNA, the antisense retroviral construct was co-transfected with HBV expression vector (pTHBV) in hepatoma cell line, HepG2 cells. Expression of surface antigen was assessed by a standard HBsAg assay. The results indicated that HBsAg expression was reduced (40-50%) in antisense co-transfected cells as compared to the control vector co-transfected cells. Furthermore, HepG2 was transduced with antisense retroviral vector and tranfected with pTHBV. HBsAg expression was reduced 75% in the antisense retrovirus transduced HepG2 cells as compared to control vector transduced cells.

The retroviral vectors developed in this study can be used to identify the target antigen of cytotoxic T lymphocytes, which contribute to the immune mediated damage in chronic HBV patients. The retroviral mediated antisense gene transfer combined with liver (or hepatocyte) transplant could also provide a molecular targeting approach for treating chronic hepatitis patients.

2. INTRODUCTION

HBV is an enveloped, hepatotropic DNA virus that causes acute and chronic liver cell injury, inflammation and hepatocellular carcinoma (HCC). Primary HBV infection is usually self-limited, with clearance of viral antigens and infection from liver and blood and the development of lasting immunity to reinfection (1). However, 5-10% of individuals do not recover from primary infection, but develop a persistent, usually lifelong, hepatic infection (2). As in primary infections, such individuals may be asymptomatic or experience varying grades of chronic liver injury (3). Even though it is a minority outcome, persistent infection occupies a central role in the pathogenesis of HBV. In severe chronic hepatitis, liver transplant is the only curative remedy for patients suffering from liver failure. Unfortunately, reinfection of donor liver is almost unavoidable.

More than 250 million people throughout the world are chronically infected by this virus, serve as its reservoir, and have a 200-fold greater risk of developing HCC than their non-infected counterparts (4). HBV replicates episomally within infected cells by a process involving reverse transcription of an RNA pregenome (5). During prolonged infection, viral DNA sequences integrate into the host cell genome where they and the flanking cellular sequences are commonly rearranged in association with the development of HCC (6,7). Moreover, most of the mortality from HBV infection results in chronic rather than acute disease (8,9). Severe chronic hepatitis B frequently leads to premature death from liver failure (10). Viral replication itself does not appear to be cytotoxic; variation in the severity of liver damage between individuals has been attributed instead to differences in host immune responses to virally infected cells (11-14). However, the implicated target antigens and host effect mechanisms remain elusive. The only established therapy for chronic hepatitis B is interferon-alpha, with an efficacy of only 30-40% in highly selected patients (15).

Analysis of the complete nucleotide sequence of HBV genome indicated four major open reading frames (ORFs) which are encoded by minus-strand DNA. The coding regions for HBsAg revealed unanticipated complexity. The coding region for HBsAg (ORF S) proved to be the 3' portion of a large coding region: upstream of ORF S is an in-phase reading frame (Pre S) with two conserved in-phase ATG codons that can direct the synthesis of additional HBsAg-related proteins, termed pre-S1 and pre-S2. Similarly, the coding region for HBcAg (ORF C) is also preceeded by a short upstream in-phase ORF (termed pre-C). The ORF X encodes a transactivator protein which can trans-activate transcription from the HBV core promoter (16) as well as other viral promoters (17,18). Overlapping these coding regions is a large open reading frame, ORF P, which is believed to encode the viral polymerase.

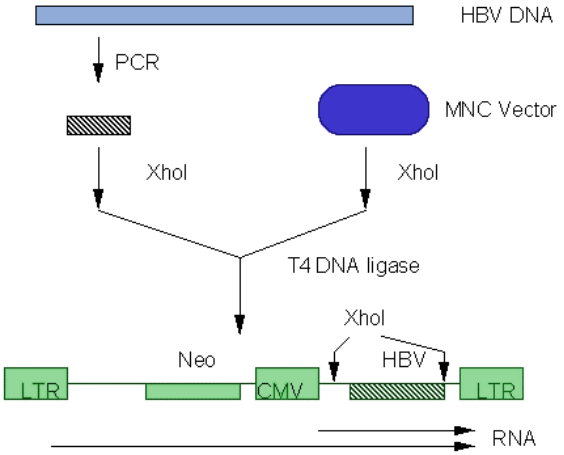

Figure. 1. Construction of recombinant retroviral vector. Two RNA transcripts (3.8 and 1.8 kilobases) encoding HBsAg were driven by Long Terminal Repeat (LTR) and cytomegalovirus (CMV) early promoters, respectively (also see Fig.3). The Neo resistant gene was driven by the LTR promoter.

One rational strategy for inhibiting virus replication would be to use nucleic acids of defined HBV gene sequences that would "hybridize" to virus genetic material, either regulatory signals or coding information, inside the infected cells and thereby block viral expression or replication in a very specific manner (19-21). Synthetic antisense oligonucleotides to HBV surface antigen have been used to inhibit HBV replication in vitro and in animal models, however, the antiviral effect is short-term and transient (22-24). Antisense RNA expressed by a retroviral vector is not immunogenic and will not elicit immune responses. One very attractive feature of using antisense nucleic acids for inhibition of HBV is the possibility that virus replication can be completely stopped without killing the host cells. Such an approach is especially attractive for cells that have a regenerative ability, such as liver cells. The progeny derived from antiviral gene protected liver cells may replace the damaged cells caused by viral gene expression or host immune responses.

Retroviral vectors have been widely used as a highly efficient method for gene transfer into eukaryotic cells and for gene therapy strategies(25,26). Retroviral vectors offer a number of advantages compared to other gene transfer vectors, including stable integration of the transferred genes and a lack of retrovirus protein coding sequences in the transfer vector. Thus, we describe here the construction of a novel retroviral vector to express HBV antisense RNA and its application to block HBV antigen expression in culture hepatic cells.

3. MATERIALS AND METHODS

3.1. Construction of retroviral vectors and producer lines

We chose HBsAg gene as target for antisense RNA mediated inhibition because the HBsAg has been identified as target antigen for cytotoxic T lymphocytes (27,28) and the quantitative ELISA kit for HBsAg expression is commercially available. The Moloney murine leukemia virus-based expression vector pMNC (figure 1) was used to construct antisense and sense constructs. A PCR product comprising the HBsAg gene (820 bp) was prepared using a plasmid containing single copies of the complete genome of HBV (pTWL1) as the template DNA (29). Synthetic oligonucleotides (FT7:5' GGATCCTCGA GTCAGGGGTCTGTA and FT8:5'GTTTCTCGAGG GTT T AAATGTATACCCA) corresponding to 5' and 3' ends of HBsAg gene were used for PCR. An additional XhoI restriction site was added to primers to facilitate subcloning. The amplified fragment was digested with XhoI, then ligated into XhoI-linearized murine retroviral vector pMNC (30). The recombinant clones containing the HBsAg sequence were identified with ^{32}P-labeled HBsAg probe. The orientation of the insert was confirmed by restriction enzyme analysis. pYT7$^-$ contains a copy of HBsAg in the antisense orientation and pYT7$^+$ contains a copy of HBsAg in the sense orientation. Plasmid DNA was purified by CsCl ultracentrifugation. Virus producer lines were generated as described (31). In brief, 5 µg DNA was transfected into packaging cell line GP+envAm12 by electroporation. Individual stable transfected cells were selected in 500 µg/ml G418 (75% active, Life Technologies, USA). The viral titers of cell-free viruses from 30 clonal cell lines were determined on NIH3T3 cells as described (31). A high-titer producer line (5x10^6 colony forming unit/ml) was selected for further transduction experiments.

3.2. Transient assay of antisense-mediated inhibition of HBsAg

The hepatoma cell line, HepG2, was co-transfected with HBV molecular clone (pTHBV,32) and pYT7$^-$ or vector control (pMNC) at different molar ratios by calcium phosphate precipitation method. Co-transfected cells were incubated for approximately 10.5 hrs, after which they were rinsed with PBS and the cells further incubated in growth medium for 68 hrs. Samples were then collected for assay of HBsAg secreted into the medium. The medium taken from the dish was centrifuged briefly to remove any dead cells and debris, and stored at -20 °C until ready to be used. At the time of assay, 1 ml samples were subjected to evaporation by Speed Vac to 0.1 ml (10-fold concentration) and added into anti-HBsAg antibody-coated wells using a commercially available ELISA kit (United Biochemical Inc., Mountain View, CA, USA). The antibody-antigen-antibody conjugate sandwich assay system was performed according to the manufacturer's protocol.

3.3. Northern blot analysis of antisense and sense RNA expressed in transduced HepG2 cells

Total RNA was extracted from transduced HepG2 cells with an RNA Extraction Kit (Stratagene, La Jolla, CA, USA). RNA was fractionated on a 1.2 % formaldehyde gel and capillary blotted onto charged nylon paper (MicroSeparation Inc. Westboro, MA, USA). The blot was probed with a ^{32}P-labeled HBsAg DNA fragment prepared by PCR.

4. RESULTS

4.1 Construction of retroviral vectors and producer lines encoding an antisense and sense copy of surface antigen

Recombinant clones encoding HBsAg gene were identified by colony hybridization with a ^{32}P-labeled probe. Plasmid DNA was isolated from each positive clone and subjected to restriction enzyme analysis. The clones which released the 0.8 Kb fragment after XhoI digestion were further digested with SspI. The clones (named pYT7$^+$) containing the HBsAg gene in the sense orientation released 6.2 and 3.3 Kb bands. The clones containing antisense HBsAg (named pYT7$^-$) released 5.5 and 4.1 Kb bands.

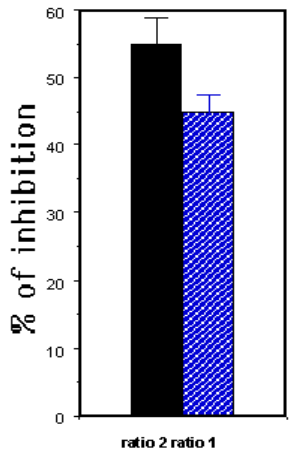

Figure 2. Inhibition of HBsAg expression pYT7⁻ was co-transfected with pHBV in the molar ratio of 2 and 1. The level of HBsAg was compared to the cells co-transfected with pTHBV and pMNC.

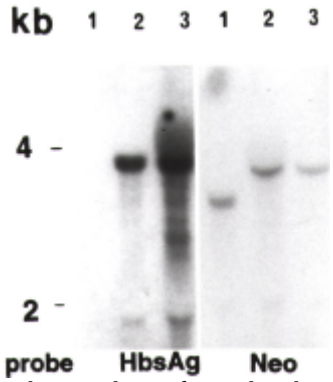

Figure 3. Northern analysis of transduced HepG2 cells. Total cellular RNA was extracted from transduced cells and probed with ^{32}p labeled HBsAg or Neo gene. Lane 1: retroviral vector MNC, lane2: sense copy of HBsAg, lane3: antisense copy of HBsAg retroviral transduced cells.

4.2 Inhibition of HBsAg production by co-transfection assay

HepG2 cells were co-tranfected with pYT7⁻ and pTHBV at molar ratios of 1:1 and 1:2 by calcium phosphate method. When a higher molar ratio was used, lower transfection efficiency was observed. Because HBsAg was detectable in pYT7+ transfected HepG2 cells, only vector (pMNC) was used as control DNA for co-transfection. The inhibition of HBsAg expression was measured by ELISA as described in Materials and Methods.

The results indicated that the expression of HBsAg was inhibited by 45-55 % in antisense construct (pYT7⁻) co-transfected cells as compared to vector (pMNC) co-transfected cells (figure 2). The data shown represents the average of three independent (duplicate) experiments.

4.3 Inhibition of HBsAg production in the antisense-transduced HepG2 cells

It is possible that multiple copies of antisense constructs and HBV plasmids could enter single co-transfected cells in previous transient co-transfection assays. To test whether the inhibition of surface antigen expression will still take place in hepatocytes transduced with single copies of antisense gene, the human hepatoma cell line HepG2 was transduced with cell-free virus harvested from the producer lines of MNC and YT7⁻ having the highest titers. Transduced cells were selected in the presence of G418 (750 µg/ml) for 14-16 days. The resulting G418 resistant colonies were pooled and expanded to a cell line. The presence of HBsAg gene in the transduced cells was comfirmed by PCR (data not shown). The transduced HepG2 cells were analyzed for antisense RNA expression by Northern analysis. The results indicated that two antisense RNA transcripts (3.8 and 1.8 kilobases) were driven by LTR and CMV promoters, respectively (figure 3). Both RNA transcripts, including approximately 1 kilobase RNA, were derived from the vector and poly A signal. The YT7⁻ and MNC transduced cells were challenged with HBV by transfecting 5 µg of pTHBV and subsequently measuring the levels of ELISA-detectable HBsAg products secreted into the growth medium as previously described. The results showed that HepG2 cells transduced with the antisense HBsAg construct resulted in an approximate 75% inhibition in HBsAg expression as compared to the control cells, MNC transduced cells 3 days after transacting with pTHBV (figure 4).

5. DISCUSSION

In this report, we describe a potential gene therapy approach for persistent HBV infection. Antisense RNAs of the HBV surface antigen gene expressed from a retroviral vector can effectively inhibit HBsAg expression by 50-75%. The advantage of using long antisense RNA (0.8 kilobases in this case) to inhibit the target sequence is that variant viruses should also be inhibited by antisense RNA due to complementation to the target sequence. Many HBV variants have been identified and shown to contribute to differences in severity of disease (33). Recently, Yu et al, (34) reported a ribozyme-mediated inhibition of HBsAg by co-transfection assay which is similar to this paper. In that report, high molar ratio (at least >5) of ribozyme construct to HBV expression construct was used to achieve 80% inhibition by measuring endogenous polymerase activity. Due to a different HBV expression construct being used, the efficiency of inhibition can not be compared directly. However, it would be difficult to deliver high copy number of ribozyme to liver cells in practice.

The unique features of retroviral vectors for gene transfer are that they can provide long-term gene expression and are non-immunogenic to the host immune system. To achieve long lasting anti-HBV effect, the antisense transduced cells have to stably express antisesnse RNA in chronic HBV patients. Using current *ex vivo* gene transfer protocol, only a small percentage of hepatocytes can be transduced with a retroviral vector. However, this small percentage of antisense transduced liver cells may have a selective advantage over unprotected HBV infected cells as the result of immune-mediated damage. With the regenerating ability of normal hepatocytes, the antisense RNA protected cells may dominate the cell population in the liver.

In our assay system, the multiple copies of HBV genome still can enter the antisense transduced cells by co-transfection. Therefore, the anti-viral effect may be more significant in natural HBV infection. In that case, fewer copies of replicative RNA intermediates and/or viral

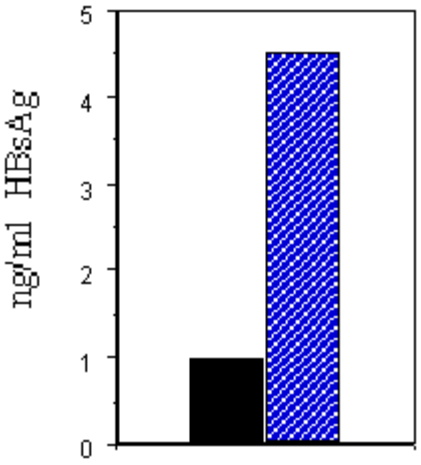

Figure 4. Inhibition of HBsAg expression in the transduced HepG2 cells. Transduced HepG2 cells were transfected with 5 µg of pTHBV. The data shown is an average of 6 assays.

mRNA are present in the infected cells as the targets of antisense RNA.

6. ACKNOWLEDGMENT

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