Lethiferous effects of a recombinant vector carrying thymidine kinase suicide gene on 2.2.15 cells via a self-modulating mechanism

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INTRODUCTION

Chronic hepatitis B virus (HBV) infection remains a major public health problem worldwide[1-7]. The World Health Organization estimates that there are still 350 million chronic carriers of the virus who are at risk of developing chronic hepatitis, liver cirrhosis and hepatocellular carcinoma[8, 9]. This issue is also an important problem in China. The carrier rates of HBsAg are estimated to be as high as more than 10 % of Chinese population and a substantial proportion of liver cancer incidence is associated with chronic HBV infection[10-12]. Currently interferon-alpha (IFN-α), an immunomodulator, and two synthetic nucleoside analogues, lamivudine and adefovir dipivoxil, are the only licensed antiviral agents for the treatment of chronic HBV infection. However, this therapeutic modality is still problematic. In addition to the standard treatment endpoints being achievable only in approximately 20-30 % of those treated with either type of agents, interferon usually confers serious side effects, and lamivudine is associated with the occurrence of viral mutations and drug-resistant strains[13-17]. Thus, there is an urgent need for the development of more effective modalities to deal with HBV infection. As yet, several therapeutic protocols with molecular biological techniques have been tried in experimental studies of this disease in the last few years[18-25], although significant progress remains to be made in such kinds of explorations. In the present study, we tried to construct a recombinant vector carrying TK suicide gene and harboring a self-modulating property for gene therapy in an attempt to vanquish HBV infection in a fire-new strategy.

RESULTS

The structural accuracy of pcDNA3-SCI TK was confirmed by restriction endonuclease digestion, PCR with specific primers and DNA sequencing. The HBsAg levels in the supernatant of transfected 2.2.15 cell culture were significantly decreased on the 6th day post-transfection as compared with that of the mock control (P<0.05). The lethiferous effect of pcDNA3-SCI TK expression on 2.2.15 cells was initially noted on the 3rd day after transfection and aggravated on the 6th day post transfusion, in which the majority of transfected 2.2.15 cells were observed shrunken, round in shape and even dead. With assessment by the trypan blue exclusion test, the survival cell ratio on the 6th day post transfection was 95 % in the negative control and only 11 % in the experimental group.

MATERIALS AND METHODS

Materials

Plasmid pcDNA3-TK containing TK gene and pcDNA3-S carrying HBV-S gene were maintained in our laboratory. Eukaryotic expression vector pcDNA3 was from Invitrogen Company, Netherlands. Purification kit for PCR product was obtained from Qiagen Company (Germany). Reverse transcriptase and PCR amplification system were purchased from Shenzhen Jingmei Company (China) and Expand PCR kit form Stratgene Company (USA). Taq and Pfu enzymes were purchased from Bio-Star Company (Canada). Restriction endonucleases such as EcoR I, BamHI and HinD III, T7 DNA ligase and ELISA kit for detecting HBsAg were from Huamei Bioengineering Company (Luoyang, China) for the 10th Five-Year Plan Period (No. 2001BA705B05)

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CONCLUSION: The results indicate that suicide gene expression of pcDNA3-SCI TK can only respond to HBV-S gene transcription, which may be potentially useful in the treatment of HBV infection and its related liver malignancies.

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Preparation of gene fragments

The fragments of HCV-IRES element and core gene were obtained by RT-PCR. Total HCV RNA was extracted and purified from 100 µl samples of HCV-positive sera using a guanidium isothiocyanate-acid phenol extraction procedure (Jingmei Company, Shenzhen, China). Precipitated RNA was dissolved in 20 µl of diethyl pyrocarbonate-treated water and quantified by UV spectrum analysis. Specific cDNA for HCV IRES element and core gene were amplified respectively with RT-PCR kit by adding their corresponding primers according to the protocols of Reverse Transcription System (Jingmei Company, Shenzhen, China). The primers for the HCV IRES were sequenced as sense: 5’-GCGCGGATCCGGGCGACAC TCCACCATAG-3’ (nt 17 to 36) and antisense: 5’-GCGAATTCGTTTTTCTTTGAGGTTTAGGATTC-3’ (nt 347 to 371). The primers for the HCV core gene were 5’-TAGGAATCCTGATGAGCACGAATCCTAAACCTC-3’ (sense) and 5’-GCGCGGATCCTTAAGCGGAAGCTGGGATG-3’ (antisense). PCR was performed on the Real-time PCR apparatus (Roche, USA) with a program for 30 cycles at 42 °C for 45 min and at 94 °C for 5 min followed by at 92 °C for 30 s, at 55 °C for 30 s and at 72 °C for 30 s. After prepared by routine procedures, the PCR products were analyzed by agarose gel electrophoresis and stored at -20 °C until use.

The antisense fragment of HBV-S gene lack of initiating codon (ATG) was amplified with the PCR protocol using the oligonucleotide sequence 5’-GCGCGTGCAAGCTTATAAAACGCCGACACATC-3’ (sense) and 5’-ATTCGTGCTCATCAGGATTCCTAGGACCCC-3’ (antisense). PCR was performed on the Real-time PCR apparatus (Roche, USA) with a program for 45 cycles at 95 °C for 15 s, at 70 °C for 5 s and at 72 °C for 15 s each cycle. The PCR products were identified by electrophoresis on an 1% of agarose gel stained with ethidium bromide and stored at -20 °C until use.

TK gene segment was prepared by digestion of plasmid pcDNA3-TK with EcoR I and purified with the routine procedures.

Construction of self-modulated expression vector

The construction scheme for the self-modulated expression vector that can respond to HBV infection and eliminate the infected hepatic cells is shown in Figure 1. Briefly, ligation of antisense fragment of HBV-S gene with HCV-C gene segment was performed by SOEing PCR[26-28], in which the sense sequence of HBV-C gene primer 5’-TAGGAATCCTGATGAGCACGAATCCTAAACCTC-3’ and a truncated antisense sequence of HBV-S gene primer 5’-ATTCTGCTCATCAGGATTCCTAGGACCCC-3’ were used as primers. The major SOEing PCR round comprised of 30 cycles at 92 °C for 30 s, at 50 °C for 45 s and at 72 °C for 60 s each cycle, and a final extension at 72 °C for 10 min. The ligated PCR products were purified and analyzed by agarose gel electrophoresis and used to link with the HCV-IRES fragment by T4 ligase after both segments were digested by BamH I. The resultant HBV antisense S-HCV C-HCV IRES (SCI) fragment was further amplified by PCR with the major round to be at 92 °C for 30 s, at 53 °C for 45 s and at 72 °C for 60 s for 30 cycles, and the final extension at 72 °C for 10 min, in which HBV-S gene sense primer, 5’-GCGCGTGCAAGCTTATAAAACGCCGACACATC-3, and HCV-IRES antisense primer, 5’-GCGAATTCGTTTTTCTTTGAGGTTTAGGATTC-3’, were employed as primers. After digested by corresponding restriction endonucleases, the amplified HBV antisense S-HCV C-HCV IRES fragment was cloned into Hind III and EcoR I sites of pcDNA3 to generate eukaryotic expression plasmid pcDNA3-SCI (Figure 1). In the same way, the expression plasmid pcDNA3-SCITK was yielded by cloning TK fragment into EcoR I site of pcDNA3-SCI. Both of the newly constructed plasmid were confirmed by restriction endonuclease digestion, PCR with specific primers and finally by DNA sequencing (Baosheng Company, Dalian, China).

Self-restricted expression of recombinant plasmid

HepG2 and its derived cell line 2.2.15 that carries the full HBV genome and can stably express series of HBV antigen were maintained in our laboratory. They were cultured in a modified Eagle’s essential medium (Sigima) supplemented with 100 U·ml^-1 penicillin, 100 U·ml^-1 streptomycin, and L-glutamine, nonessential amino acids and sodium bicarbonate.
in doses recommended by the China Type Culture Collection (Wuhan, China) at 37 °C in a humidified atmosphere containing 5% of CO₂. One day prior to transfection, cells were transferred onto a 8-well plate and incubated in complete Dulbecco’s modified Eagle’s medium (DMEM) containing 10% (v/v) fetal calf serum (FCS) and 10 µg/ml of ganciclovir. When the cells grew to 50-80% confluence, they were transfected with pcDNA3-SCITK or pcDNA3-SCI plasmid in a ratio of per 10⁶ cells to 1 µg of plasmid DNA by LipofectAMINE transfection reagent following the manufacturer’s instructions. (Invitrogen Co. USA). 2.2.15 cells transfected with pcDNA3-SCITK or pcDNA3-SCI plasmids were designated as the experimental group or the mock group. The HepG2 cells transfected with pcDNA3-SCI plasmids were functioned as the negative control. All of the transfected cells were maintained in DMEM medium with 10% (v/v) fetal calf serum (FCS) and 10 µg/ml of ganciclovir for at least 7 days. The HBsAg levels in the supernatant of cell culture were detected by ELISA on the 1st, 3rd and 6th day after transfection. Meanwhile, the morphology of transfected cells were recorded by the photograph and the survival cell ratio was assessed by the trypan blue exclusion test on the 6th day of posttransfection.

**Statistical analysis**
Experimental data were processed by analysis of variance and t-test for comparison between groups. Results were expressed as mean ± SE. *P*<0.05 was selected as the level of statistical significance.

**RESULTS**

**Structural identification of constructed plasmids**

The segment analysis of recombinant plasmid pcDNA3-SCITK by restriction endonuclease digestion and PCR with specific primers demonstrated that the inserted gene sequences in the plasmid were completely consistent with that of the theoretical

![Figure 2](image)

**Figure 2** A: RT-PCR products of HCV IRES element. M: marker (2 kb), Lanes 1-3: HCV IRES element (330 bp). B. Component segment analysis of pcDNA3-SCITK by agarose gel electrophoresis. M: marker (2 kb), Lanes 1-2: SCI-TK fragment demonstrated by PCR with HBV-S gene sense primer and TK antisense primer (5'-ACTTCCGTGGCTTCTTGCTG-3' (nt 150-170). Lane 3: SCI fragment verified by PCR with HBV-S gene sense primer and HCV IRES antisense primer. Lane 4: SI segment from the ligation of HBV-S and HCV-Core gene by SOEing PCR. Lane 5: RT-PCR product of HCV-Core gene. Lane 6: Partial antisense segment of HBV-S gene.

![Figure 3](image)

**Figure 3** Effects of pcDNA3-SCITK expression on morphological alterations of 2.2.15 and HepG2 cells. Photographs a, b and c in each group exhibited respectively the cell changes on the 1st, 3rd and 6th day post transfection. Lethiferous changes of 2.2.15 cells transfected with pcDNA3-SCITK were noted initially on the 3rd day of posttransfection and aggravated on the 6th day after transfection, in which the majority of cells were observed shrunken, rounded in shape and even dead. However, the negative control HepG2 cells grew well.
calculations as shown in Figure 2, which were further confirmed by the DNA sequencing (data not shown).

Effects of pcDNA3-SCITK expression on HBV-infected cells
The expressive efficiency of TK protein by 2.2.15 cells transfected with pcDNA3-SCITK was confirmed in our previous investigations (data to be published). In the present study, the effects of pcDNA3-SCITK transfection on the expression of HBsAg by 2.2.15 cells was observed. The results showed that HBsAg in the supernatant of transfected 2.2.15 cell culture was significantly decreased on the 6th day post-transfection as compared with that of the mock group (P<0.05, Table 1).

Table 1 HBsAg expression in supernatants of cell culture by experimental cells

| Groups         | OD Value | 1st day | 3rd day | 6th day |
|----------------|----------|---------|---------|---------|
| Mock group     | 0.32±0.13| 0.61±0.23| 0.61±0.25|
| Experimental group | 0.31±0.11| 0.51±0.22| 0.41±0.16*|

*P<0.05 vs Blank control.

The lethal effect of pcDNA3-SCITK expression on 2.2.15 cells was first noted on the 3rd day after transfection and aggravated on the 6th day posttransfection, in which the majority of transfected 2.2.15 cells were observed shrunk, round in shape and even dead (Figure 3). With assessment by the trypan blue exclusion test, the survival cell ratio on the 6th day posttransfection was 95% in the negative control and only 11% in the experimental group.

DISCUSSION
Chronic hepatitis B infection remains a major public health problem worldwide. Hepatitis B virus belongs to the family of hepatoviruses that replicate their DNA genome via a reverse transcription pathway. The chronicity of infection in infected hepatocytes is maintained by the persistence of the viral covalently closed circular DNA. Traditionally, the strategy to combat chronic HBV infection depends mainly on the stimulation of specific antiviral immune response or on the inhibition of viral replication, or on both. However, it has been found that prolonged administration of therapeutic agents is often associated with the production of resistant mutants and serious side effects, as well as a control of viral replication rather than eradication. To further search for effective therapeutic modalities to deal with the chronic infection of HBV is therefore urgently needed to design a brand-new strategy to clear the causative virus.

Hepatitis C virus, a member of the Flaviviridae family and an enveloped virus with a single-stranded 9.6 kb RNA genome of positive polarity,[29] utilizes a cap-independent mechanism to initiate translation on its genomic RNAs. This process could involve an internal ribosome entry sites (IRES) located in the 5'-untranslated RNA (5'-NTR), and both canonical and noncanonical translation initiation factors.[30-32] The former could be constructed in some of the bicistronic systems with a higher efficiency in the initiation of protein translation and has been used to construct recombinant vectors[33-36]. It has been recently found that HCV-C protein, another functional modulator localized both in cytoplasm and in perinuclear regions, could interact with IRES and down-regulate IRES-directed translation,[37-42], which has been considered to be involved in the establishment or maintenance of the virus persistence.[40-43]. Based on these observations, we hypothesized that a self-modulating mechanism by the interaction of HCV-C protein with IRES element was possible to be employed in gene therapy for the eradication of chronic HBV infection.

In the present study, we constructed a recombinant plasmid pcDNA3-SCITK by cloning HCV-C gene and IRES element, partial antisense sequence of HBV-S gene, and TK suicide gene into the eukaryotic expression vector pcDNA3, in which the expression of TK gene was driven in turn by human cytomegalovirus (CMV) promoter, HBV-antisense and HCV core- IRES sequences. The prominent feature of this plasmid was the temperate expression of effector gene in transfected cells by a self-modulating mechanism. When HBV in the infected cells began to replicate, the transfected mRNA of HBV-S gene could be combined with its counterpart produced by HBV-antisense- S gene in the recombinant plasmid to form a DNA primer prior to the initiation codon AUG of HCV-C gene, which brought about a decreased expression of HCV-C protein. The consequently reduced HCV core protein-IRES combination could enhance the IRES element to promote the expression of TK suicide gene and further to cause a lethal effect on HBV infected cells. In contrast, the expression of effector gene TK was inhibited in non-infected cells because of the normal level of HCV-C protein expression and its normal combination with HCV- IRES element, which would not cause any damage to the healthy cells.

In order to identify the anti-HBV efficiency of pcDNA3-SCITK, 2.2.15 cells that carry the full HBV genome and can stably express series of HBV antigen were transfected with the plasmid. The results showed that HBsAg level in the supernatant of transfected 2.2.15 cell culture was significantly decreased on the 6th day post transfection as compared with that of the mock cells (Table 1). The lethal effect of pcDNA3-SCITK expression on 2.2.15 cells was noted initially on the 3rd day after transfection and aggravated on the 6th day posttransfection, in which the majority of transfected 2.2.15 cells were observed shrunk, round in shape and even dead. However, the negative control HepG2 cells grew well (Figure 3). With assessment by the trypan blue exclusion test, the survival cell ratio on the 6th day post transfection was 95% in the negative control and only 11% in the experimental group. All these observations confirmed that plasmid pcDNA3-SCITK could exert a lethal effect on HBV infected cells via a self-modulating mechanism, which might also be served as a potential therapeutic strategy for the liver cancers derived from chronic HBV infection.

The limitation of the present study is the neglect of anti-HBV infection by preventing the pathogen from invading target cells or by interrupting the virus replication at the beginning of the disease. How to apply our present therapeutic strategy in combination with other treatment protocols to the eradication of chronic HBV infection is to be further investigated.

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