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Vector-mediated expression of interferon gamma inhibits replication of hepatitis B virus *in vitro*

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Summary. – Despite the existence of efficient vaccines against hepatitis B virus (HBV) infections, these still represent a serious threat to human health worldwide. Acute HBV infections often become chronic, marked by liver cirrhosis and hepatocellular carcinoma. Promising results with interferons alpha or gamma (IFN-α, γ) or nucleoside/nucleotide analogs in inhibiting HBV replication *in vitro* have led to therapeutic applications to chronic HBV patients, however, their results so far have not been satisfactory. The treatments were either not effective in all patients or had adverse effects. Certain progress was expected from expression of interferons targeted to liver by adenovirus vectors, however, this approach turned out to be limited by undesired expression of toxic viral genes and high production costs. Therefore, in this study, we attempted to inhibit HBV replication in HepG2.2.15 cells by human IFN-γ expressed through a non-viral vector, an eukaryotic plasmid. The results demonstrated that IFN-γ, targeted to HBV-replicating cells, significantly inhibited the virus growth without inducing apoptosis and indicated that local expression of this kind of cytokine may be a promising strategy of gene therapy.

Keywords: hepatitis B virus; interferon gamma; HepG2.2.15 cells; apoptosis

Introduction

HBV (the species *Hepatitis B virus*, the genus *Orthohepadnavirus*, the family *Hepadnaviridae*) is a small DNA virus with unusual features similar to retroviruses. The infectious HBV virion (Dane particle) has a spherical, double-shelled structure 42 nm in diameter, consisting of a lipid envelope containing hepatitis B surface antigen (HBsAg) that surrounds an inner nucleocapsid composed of hepatitis B core antigen (HBcAg), virally encoded polymerase and the viral DNA genome. The genome of HBV is a partially double-stranded circular DNA of about 3.2 kb pairs. HBV replicates through an RNA intermediate and can integrate into the host genome. The unique features of the HBV replication cycle confer a distinct ability of the virus to persist in infected cells (Liang, 2009).

It has been reported that IFN-γ can inhibit the replication of HBV and is the principal mediator of non-cytolytic inhibition of HBV replication (Guidotti *et al.*, 1999; Schultz and Chisari, 1999; Guidotti and Chisari, 2001). HBV can induce chronic infections characterized by weak and limited T-cell responses against the virus, whereas a strong IFN-γ response by CD4+ T-cells against HBcAg of the virus appears to be important for viral clearance (Chichester *et al.*, 2006; Narayan *et al.*, 2006). Studies in HBV-transgenic mice have shown that IFN-γ mediates most of the antiviral effects of the cytotoxic T-lymphocytes (CTLs), while IFN-α/β is primarily responsible for the early inhibitory effect of HBV replication (McClary *et al.*, 2000).

Clinical application of IFN-α for controlling HBV replication is hindered by troublesome side effects following

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Abbreviations: cccDNA = covalently closed circular DNA; CTL = cytotoxic T-lymphocyte; dsDNA = double-stranded DNA; EGFP = enhanced green fluorescent protein; HBV = hepatitis B virus; HbcAg = hepatitis B core antigen; HbeAg = hepatitis B e antigen; HbsAg = hepatitis B surface antigen; IFN-γ = interferon gamma; rcDNA = relaxed circular DNA; PI = propidium iodide

systemic administration. Because HBV can avoid immune elimination, therapies involving IFN-γ may be potentially useful. Gene delivery of IFN to the liver would focus IFN expression in or near HBV-infected cells, concentrating the therapeutic protein where it is most needed and providing IFN continuously. Previous studies have demonstrated that efficient delivery systems for an intrahepatic and regulated IFN-γ expression to inhibit the replication of hepatitis viruses have been developed successfully (Matskevich *et al.*, 2003; Dumortier *et al.*, 2005; Shin *et al.*, 2005). Despite many successful applications, these delivery systems were established based on a variety of viral vectors. Although novel versions of these viral vectors have been developed for gene transfer, none have fully overcome fundamental limitations of undesired expression of the potentially toxic wild type adenovirus genes and high production costs.

This study represents the first attempt to inhibit HBV replication *in vitro* by IFN-γ expressed through a non-viral eukaryotic expression vector, pcDNA3.1-IFN-γ (IFN-γ vector). Using this vector, we achieved a significant inhibition of HBV replication in HepG2.2.15 cells without inducing apoptosis.

Materials and Methods

Vector constructs. Human IFN-γ gene was generated by PCR from the vector pLY4-γ (Institute of Biochemistry and Cell Biology, Shanghai, China), using the following primers (Takara, Dalian, China):

5′-CTGAATTCATGCAGGACCCGTACGTTAAAGAAGCT-3′ (sense)

5′-AGTGATATCCTATTACTGAGAAGCACGACGACCGCGGAACAGCAT-3′ (antisense).

The PCR product was digested with *Eco*RI and *Eco*RV and inserted into pcDNA3.1(+) digested with the same restriction enzymes, thereby generating pcDNA-IFN-γ (Fig. 1b).

The complete coding region of the enhanced green fluorescence protein (EGFP) gene from pEGFP-N1 (Invitrogen) was generated by *Eco*RI/*Not*I digestion. The EGFP fragment was subcloned into the multiple cloning site of pcDNA3.1(+) to generate the control vector pcDNA3.1-EGFP (Fig. 1a). Transfection efficiency of pcDNA3.1- IFN-γ vector in hepatocytes was tested with the control vector. EGFP expression was monitored by fluorescence microscopy (Nikon Eclipse, Omron, Japan) using a standard FITC-filter set with excitation by blue light (488 nm). All the recombinant vectors were verified by sequence analysis (Takara, Dalian, China).

Cells. The human hepatoma cell line HepG2 was obtained from China Center for Typical Culture Collection (CCTCC) (Wuhan, China). HepG2.2.15, which carries the double full HBV genome and can express stably a series of HBV antigens, is derived from HepG2.

Cultivation and transfection. The cells were cultured at 37°C in a humidified 5% CO_2 atmosphere in Dulbecco′s modified Eagle′s medium supplemented with 10 % fetal bovine serum. For

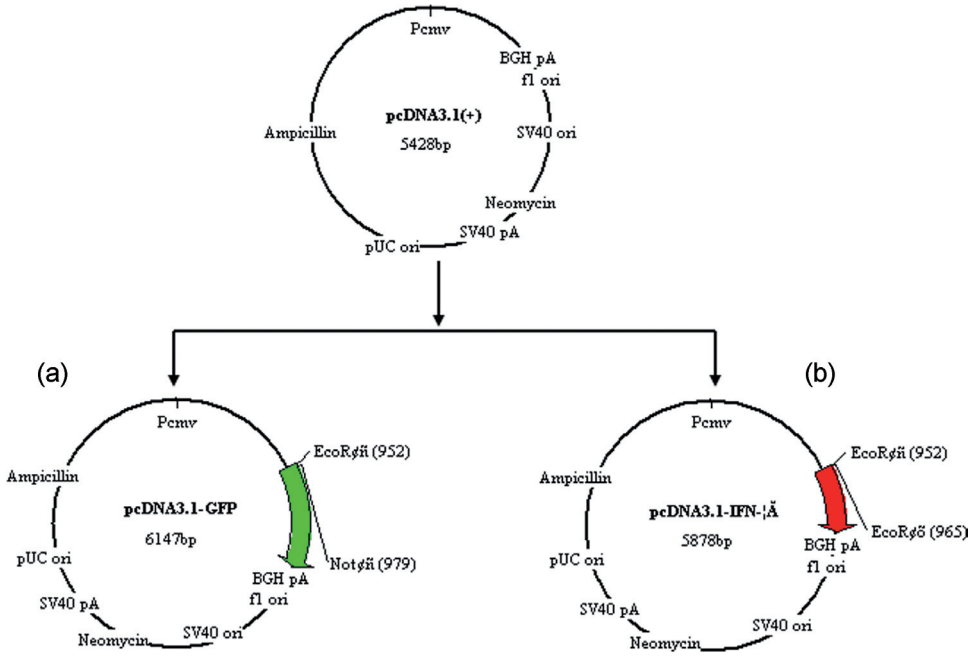

Fig. 1

The scheme of recombinant expression vectors construction

(a) Control vector pcDNA3.1-EGFP. (b) pcDNA3.1- IFN-γ.

HepG2.2.15 cells, G418 (380 μg/ml) was included. Cells were seeded onto six-well culture plates at a density of 6.0×10^5 per well and grown to 70–80% confluence for transfection. Cells were transfected with different doses of plasmid DNA (4 μg, 8 μg, and 16 μg) using Lipofectamine 2000 (10 μl, 20 μl, and 40 μl) (Invitrogen, USA) according to the manufacturer′s protocol.

Antibodies. Anti-hIFN-γ-IgA (InvivoGen, California, USA).

RT-PCR assay of IFN-γ mRNA. To assess the efficiency of the IFN-γ expression by pcDNA3.1-IFN-γ in hepatocytes, the amount of host IFN-γ mRNA was determined by RT-PCR. In brief, after 72 hrs of transfection, total RNA was extracted from the collected HepG2.2.15 cells with the TRIzol reagent (Invitrogen, USA) and was digested with RNase-free DNase (Roche) for 30 min, followed by RT-PCR analysis. PCR products were analyzed by conventional agarose gel electrophoresis and visualized by ethidium bromide. The PCR bands were analyzed by Quantity one software (Bio-Rad, USA). Relative amounts of IFN-γ mRNA were normalized to an endogenous reference (GAPDH). GAPDH gene segment was amplified with primers:

5′-CAACGGATTTGGTCGTATTGGG-3′(sense)

5′-CCTGGAAGATGGTGATGGGATT-3′ (antisense)

The sequences of the IFN-γ primers were:

5′-CTGAATTCATGCAGGACCCGTACGTTAAAGAAGCT-3′ (sense)

5′-AGTGATATCCTATTACTGAGAAGCACGACGACCGCG GAACAGCAT-3′ (antisense).

ELISA of IFN-γ. At 12, 24, 48, 72, and 96 hrs following transfection of pcDNA3.1-IFN-γ into HepG2.2.15 cells, IFN-γ expression in the cell supernatant was measured by a human IFN-γ ELISA kit (R&D, USA) according to the manufacturer′s directions.

ELISA of HBsAg and HBeAg. HBsAg and hepatitis B e antigen (HBeAg) in the cell culture supernatant were detected using a commercially available ELISA Kit (Kehua Bio-engineering Corporation, Shanghai, China) according to the manufacturer′s instructions. All culture supernatants from HepG2.2.15 cells were sampled at 12, 24, 48, 72, and 96 hrs post-transfection for the assay of HBsAg and HBeAg.

Real-time PCR assay of HBV DNA. The quantity of HBV DNA in the culture supernatant of HepG2.2.15 cells was determined by real-time PCR based on the TaqMan technology. Real-time PCR was performed in a LightCycler (Roche, Germany) with the HBV Fluorescent Real-time PCR Detection Kit (PG Biotech, Shenzhen, China) according to the manufacturer′s instructions. All culture supernatants from HepG2.2.15 cells were sampled at 12, 24, 48, 72, and 96 hrs post-transfection for the assay of HBV DNA.

Southern blot analysis of HBV DNA replicative intermediates. Southern blot analysis was performed using a radioactive probe to assess the suppressive effect of the pcDNA3.1-IFN-γ on HBV replicative intermediates in HepG2.2.15 cells. Purification of HBV DNA from transfected HepG2.2.15 cells at 48 hrs post-transfection was performed as described previously (Abdelhamed *et al.*, 2002). Samples were separated by electrophoresis in a 1% agarose gel and substance blotted to the membrane. Membrane-associated HBV DNA was hybridized to [α-^{32}P]-labeled random-primed probes specific for HBV sequences (Roche Diagnostics). The membrane was exposed to an imaging plate and analyzed with an imaging analyzer (Fuji Photo Film, Tokyo, Japan).

Assay of apoptotic cells. Apoptosis was detected using an Annexin V-FITC/propidium iodide (PI) apoptosis detection kit (BD Pharmingen, San Diego, CA) and cells analyzed using a flow cytometer (Beckman Coulter, Inc., Fullerton, USA). At 96 hr after transfection, pcDNA3.1-IFN-γ-transduced, 10 μmol/l Lamivudine-treated, and untreated HepG2.2.15 cells were harvested by trypsinization and washed twice with cold phosphate-buffered saline. Cells were centrifuged at 447 × g for 5 min and resuspended in 100 μl of binding buffer. A total of 5 μl of Annexin V-FITC and 10 μl PI were added and the mixture incubated for 30 min in the dark. Finally, 400 μl of binding buffer was added for analysis with a flow cytometer using the green fluorescence signal detector by filtration through 530 nm and the red fluorescence (PI) signal detector by filtration through 585 nm. For each sample, the apoptotic percentage of 10,000 cells was determined.

Statistical analysis. All statistical analyses were performed using SPSS 10.0 software. Values were compared using Student′s *t*-test and one-way ANOVA. A probability value less than 0.05 was considered significant.

Results

Cells transfected with a recombinant vector express IFN-γ

The transfection efficiency of the pcDNA3.1(+) vector was tested by transfecting pcDNA3.1-EGFP (4 μg, 8 μg, and 16 μg) into the HepG2.2.15 cells. The expression of EGFP after transfection was detected by fluorescence microscopy. Two randomly selected high-power fields were selected from each well of the cell-culture plate at 96 hrs after transfection for photography with ordinary light or green fluorescence. Transfection efficiency was determined using an image analysis system to calculate the average number of fluorescent cells in each HPF. A high transfection efficiency (85–95%) was obtained in the HepG2.2.15 cells at 96 hrs after transfection with 8 μg and 16 μg pcDNA3.1-EGFP (Fig. 2).

HepG2.2.15 cells were transfected with different doses of pcDNA3.1-IFN-γ (4 μg, 8 μg, and 16 μg) to test the production of human IFN-γ mediated by pcDNA3.1-IFN-γ. At 12, 24, 48, 72, and 96 hrs after transfection, secreted IFN-γ in the cell supernatants was measured by ELISA. In the cell supernatants from HepG2.2.15 cells, IFN-γ expression could be measured (259 pg/ml) at the lowest vector dose used (4 μg) at 12 hrs after transfection. The strongest induction of IFN-γ expression was observed at 48 hrs after transfection, and effective expression was observed until 4 days after transient transfection of HepG2.2.15 cells (Fig. 3). No basal IFN-γ expression (8 pg/ml) was detected in the supernatant of HepG2.2.15 cells transfected with any dose of the control vector pcDNA3.1-EGFP, or in untreated cells (data not shown). A clear dose-dependent correlation was

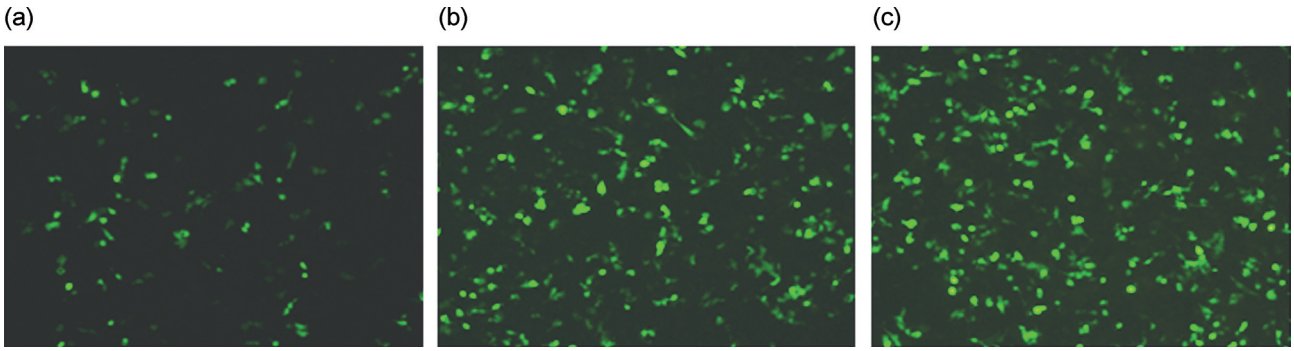

Fig. 2

The transfection efficiency

Cells were transfected with 4, 8, and 16 μg (a–c) of EGFP vector, respectively. Fluorescence microscopy was carried out at 96 hrs post-transfection.

observed between the pcDNA3.1-IFN-γ vector dose and IFN-γ in the supernatant of the HepG2.2.15 cells measured by ELISA (Fig. 3). The dose-dependent IFN-γ expression was confirmed by assay of IFN-γ mRNA in the pcDNA3.1-IFN-γ-transfected HepG2.2.15 cells at serial doses (4 μg, 8 μg, and 16 μg) at 72 hrs after transfection (Fig. 4).

IFN-γ expression inhibits HBV replication (HBsAg, HBeAg, HBV DNA, HBV DNA intermediates)

HepG2.2.15 cells were transfected with different doses of pcDNA3.1-IFN-γ. The amount of HBV DNA, HBsAg, and HBeAg in the culture supernatant was dose-dependently decreased after transfection with pcDNA3.1-IFN-γ. However, there was no significant difference between the amount of HBsAg in the culture supernatant after transfection with pcDNA3.1-IFN-γ at the doses of 8 μg and 16 μg (Fig. 5a). The lowest dose of pcDNA3.1-IFN-γ (4 μg) was sufficient to block HBV replication, with an average inhibition rate of pcDNA3.1-IFN-γ on HBV DNA, HBeAg and HBsAg at 72 hrs post-transfection being 50.2%, 30.9%, and 45.9%, respectively. HepG2.2.15 cells were also transfected with the control vector pcDNA3.1-EGFP. At the highest dose (16 μg), the inhibition rate of the control vector pcDNA3.1-EGFP on HBV DNA, HBsAg, and HBeAg could be ignored (-3.6%–2.9%) during the 96 hrs after transfection (data not shown). No statistical differences were observed in the level of HBV DNA, HBsAg, or HBeAg in the supernatant between HepG2.2.15 cells transfected with the control vector and untreated cells.

The analysis of the time-course of HBV DNA, HBsAg, and HBeAg synthesis inhibition in culture supernatants showed the most dramatic effects on viral expression (HBsAg and HBeAg) and replication (HBV DNA) on day 2, with the decreases of 70.3% in HBsAg, 70.2% in HBeAg, and 67.8% in HBV DNA

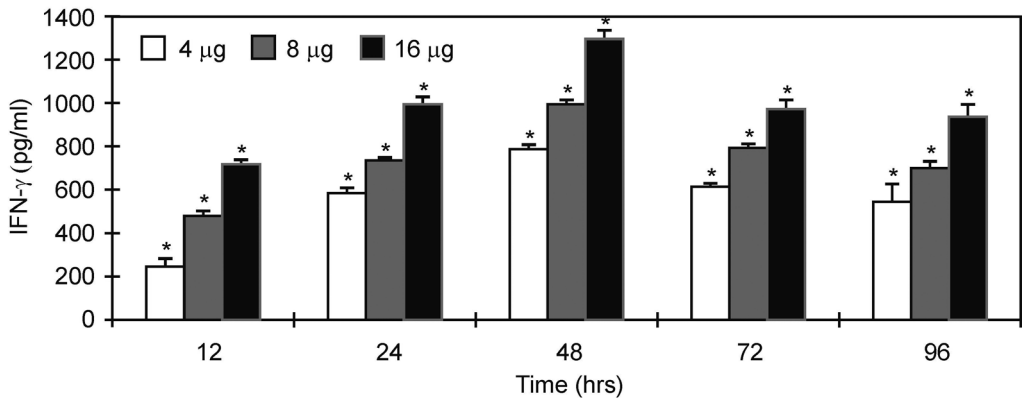

Fig. 3

IFN-γ levels in transfected cell cultures

Cells were transfected with 4–16 μg of IFN-γ vector and IFN-γ in cell culture supernatant was assayed at 12–96 hrs post-transfection. Statistically significant differences (asterisks).

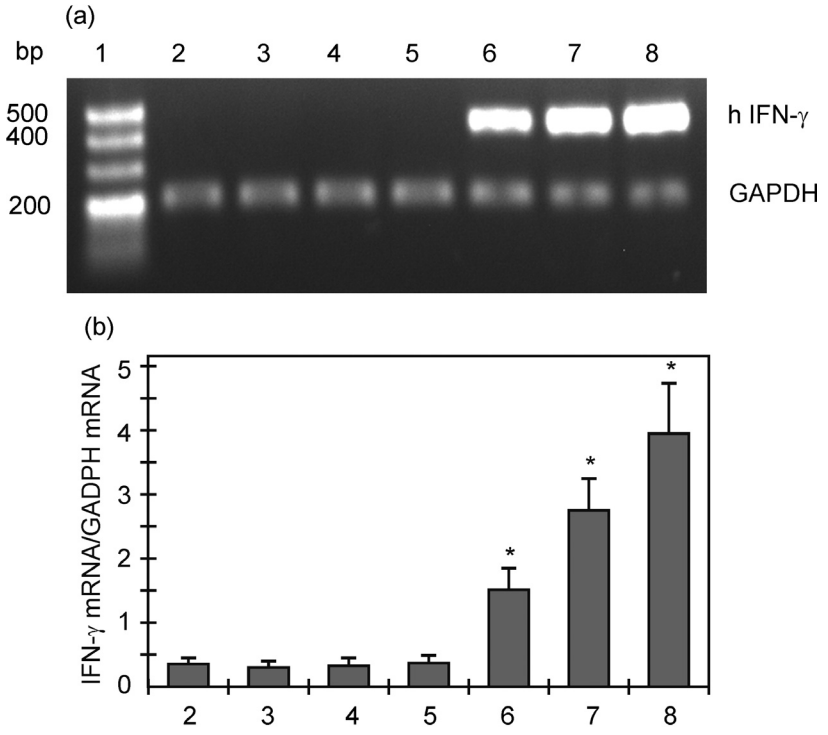

Fig. 4

IFN-γ mRNA levels in transfected cells

(a) Agarose gel electrophoresis of RT-PCR carried out at 72 hrs post-transfection. DNA size markers (lane 1), untransfected cells (lane 2), cells transfected with 4, 8, and 16 μg of EGFP vector (lanes 3–5), cells transfected with 4, 8, and 16 μg of IFN-γ. (b) Quantitation of the results shown above (a). Statistically significant differences (asterisks).

after transfection with 8 μg pcDNA3.1-IFN-γ (Fig. 5b). This correlated with IFN-γ expression, initially indicating that IFN-γ exhibited antiviral activity. To further assess the correlation between diminished viral replication and IFN-γ expression, an experiment was performed where the function of IFN-γ was inhibited with specific neutralizing antibodies. During transient transfection for four days, strong and efficient IFN-γ expression was observed after transfection of HepG2.2.15 cells

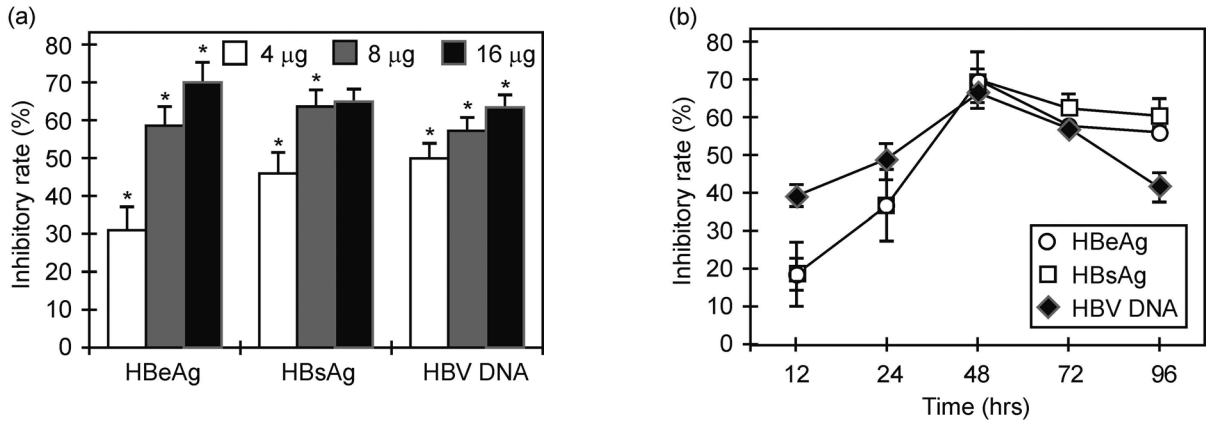

Fig. 5

Inhibition of HBV replication in cells expressing IFN-γ

(a) Cells transfected with 4, 8, and 16 μg, respectively, of IFN-γ vector and assayed for HBV DNA, HBsAg and HBeAg at 72 hrs post-transfection. (b) Time course of inhibition of HBV replication by IFN-γ. Cells transfected with 8 μg of IFN-γ vector and analyzed at 12–96 hrs post-transfection.

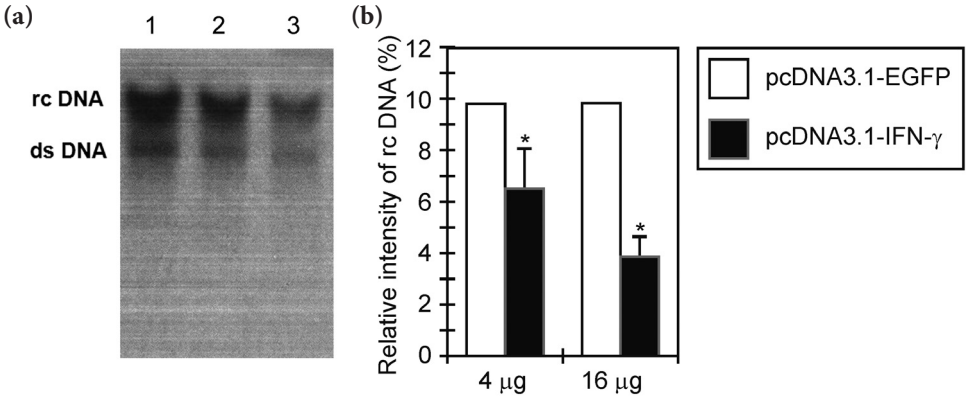

Fig. 6

Inhibition of synthesis of HBV DNA intermediates in cells expressing IFN-γ

(a) Cells transfected with EGFP vector (lane 1), 4 and 16 μg of IFN-γ vector (lanes 2–3) and assayed for rcDNA and dsDNA at 48 hrs post-transfection. (b) Histogram of rcDNA based on Fig. 6a.

with pcDNA3.1-IFN-γ (8 μg). The level of HBV DNA that corresponded to IFN-γ expression in the supernatant of transiently transfected HepG2.2.15 cells decreased significantly compared to untreated control HepG2.2.15 cells (Fig. 7). However, when human anti-IFN-γ neutralizing antibodies (50 μg, AMGEN, CA, USA) were added to the supernatant of HepG2.2.15 cells before transfection, IFN-γ expression decreased sharply, accompanied by a high level of HBV DNA over the 4 days of transfection. No significant difference was found in the level of HBV DNA between transfected cells and control cells (Fig. 8).

The suppressive effect on the HBV replicative intermediates in the HepG2.2.15 cells was assessed by Southern blot analysis. The amount of the intracellular replicative intermediates was also dose-dependently suppressed after transfection with pcDNA3.1-IFN-γ (4 μg and 16 μg). HBV replication was not altered after transfection with pcDNA3.1-EGFP as control vector (Fig. 6).

IFN-γ expression does not induce apoptosis

It has been reported that IFN-γ expression exacerbates inflammation and apoptosis in HepG2.2.15 cells, especially when IFN-γ is applied with tumor necrosis factor alpha (Sasagawa *et al.*, 2000; Reifenberg *et al.*, 2006; Shi and Guan, 2009). Annexin V-FITC/PI staining and flow cytometry were used to quantify the percentage of apoptotic cells in the total

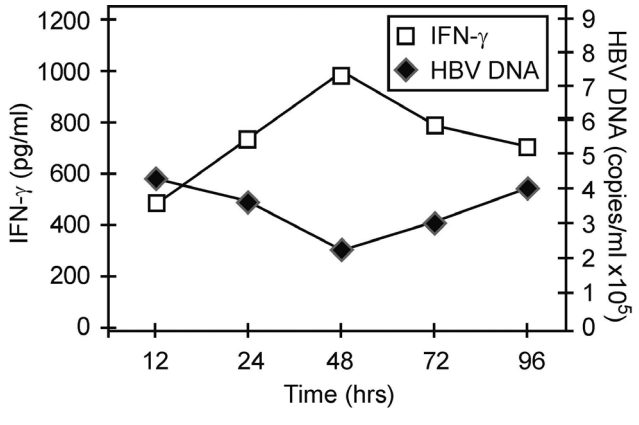

Fig. 7

The relationship between IFN-γ expression and inhibition of HBV DNA synthesis

Cells transfected with 8 μg of IFN-γ vector and assayed for IFN-γ and HBV DNA at 96 hrs post-transfection.

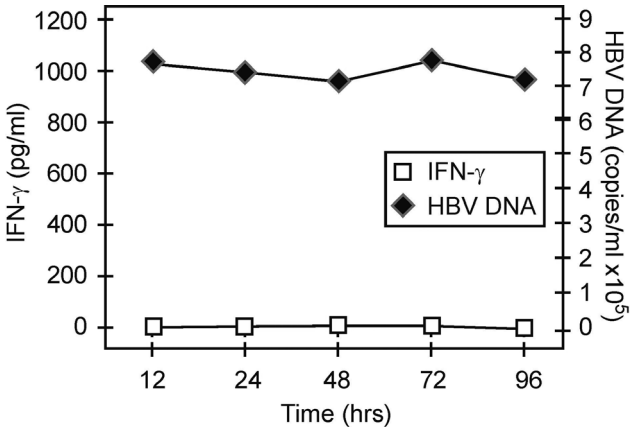

Fig. 8

Effect of IFN-γ antibody on levels of IFN-γ and HBV DNA

IFN-γ-neutralizing antibody (50 μg) was added to the cell culture before the transfection with 8 μg of IFN-γ vector and levels of IFN-γ and HBV DNA were assayed at 12–96 hrs post-transfection.

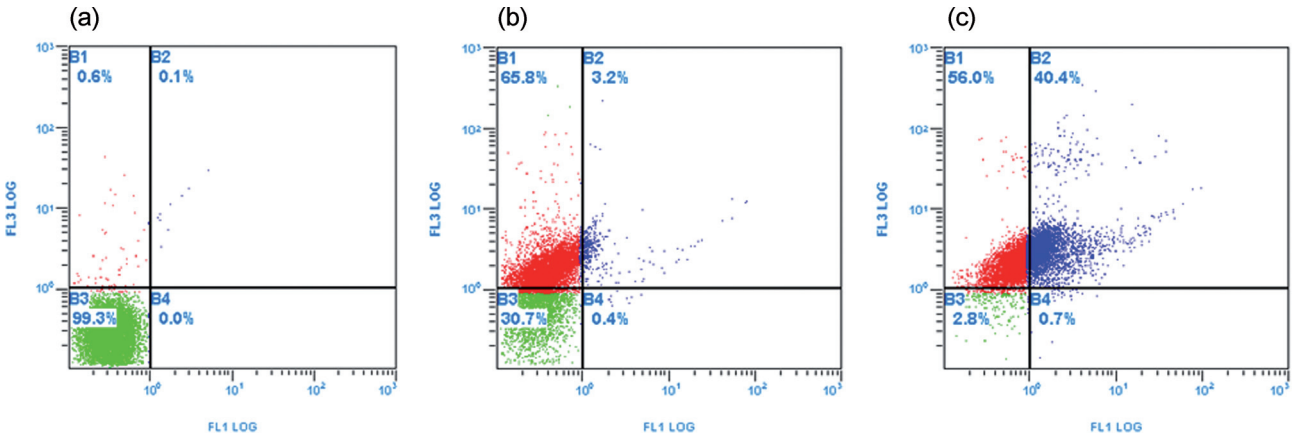

Fig. 9

IFN-γ expression and apoptosis

Untransfected cells (a), cells transfected with 16 μg of IFN-γ vector (b) and treated with 10 μmol/l lamivudine (c) and assayed for apoptosis at 96 hrs post-transfection.

cell population to investigate whether pcDNA3.1-IFN-γ can induce apoptosis in HepG2.2.15 cells. HepG2.2.15 cells exposed to 10 μmol/l Lamivudine for 96 hrs were used as the positive control group. As shown in Fig. 9, 96 hrs after transfection with the highest dose (16 μg) of pcDNA3.1-IFN-γ, a slight increase in the percentage of apoptotic cells (early apoptosis Annexin V+/PI- and late apoptotic/necrotic cells Annexin V+/PI+) was observed between the untreated control group (0.1%) and the pcDNA3.1-IFN-γ-transfected group (3.6%). However, the percentage of apoptotic Lamivudine-treated cells significantly increased to 41.1%. The observation of cell morphology after 96 hrs of treatment showed the HepG2.2.15 cells exposed to Lamivudine gradually shrunk, turned into the round shape and began detach from the culture plate, whereas cells transfected with pcDNA3.1-IFN-γ did not obviously change. The results suggested that pcDNA3.1-IFN-γ was not more effective in inducing apoptosis in HepG2.2.15 cells than Lamivudine.

Discussion

HBV viral infection remains a challenge for modern medicine. IFN-γ, in concert with tumor necrosis factor alpha (TNF-α), leads to a remarkable reduction of intrahepatic replication intermediates and specific mRNAs of HBV by means of a noncytolytic mechanism in the transgenic mouse model (Lu *et al.*, 2002). Another study on primary hepatocytes reveals that IFN-γ treatment does not affect initial covalently closed circular DNA (cccDNA) conversion, but inhibits the synthesis of progeny cccDNA by amplification (Schultz and Chisari, 1999). In the present study, the suppressive effect of IFN-γ on HBV replicative intermediates in HepG2.2.15 cells was demonstrated by Southern blot. Both the relaxed circular DNA (rcDNA) and double-stranded DNA (dsDNA) of the HBV replicative intermediates were inhibited by IFN-γ in a dose-dependent manner. The inhibitory effect on cccDNA of HBV was not observed in this study. However, the results of this study showed that the number of HBV DNA copies in the culture supernatant of HepG2.2.15 cells, which was determined by real-time PCR, was sufficiently suppressed by IFN-γ even at the lowest transfection dose of pcDNA3.1-IFN-γ (4 μg).

This study represents the first application of a non-viral eukaryotic expression vector, pcDNA3.1(+), to express IFN-γ for inhibition of HBV replication. Being safer, more easily accessible and having fewer adverse effects, nonviral eukaryotic expression vectors, such as vector and liposome, are still widely used in the gene transfer. The application of nonviral eukaryotic expression vector is mainly hindered by low transfection efficiency and uncontrollable continual gene expression. In this study, however, the effective transfection capacity of the pcDNA3.1(+) vector was observed. At the lowest dose used (4 μg), effective transfection efficiency (30–45%) was obtained in the HepG2.2.15 cells at 96 hr after transfection with pcDNA3.1-EGFP. Transfection efficiency (85–95%) was very high in HepG2.2.15 cells at 96 hr after transfection with pcDNA3.1-EGFP (8 μg and 16 μg). However, the uncontrollable and continual expression of IFN-γ mediated by pcDNA3.1(+) may lead to excessive activation of macrophages and upregulation of the major histocompatibility complex classes I and II (MHC-I and II) expression, which may eventually result in the inflammation and apoptosis in HBV-infected hepatocytes (Reifenberg *et al.*, 2006). Strategies must be taken to ensure the regulated and local IFN-γ expression based on pcDNA3.1(+) prior to the clinical use.

In this study, an increase in the percentage of early apoptosis (lower right) was observed between the untreated control

group (0.0%) and the pcDNA3.1-IFN-γ-transfected group at the highest dose of 16 μg (0.4%). But since the proportion of the early apoptotic cells (0.4%) was small and no morphological changes were observed, it was concluded that no obvious apoptosis could be induced by pcDNA3.1-IFN-γ. In contrast, significant level of apoptosis was caused in the Lamivudine-treated cells. Most of the studies on the relationship between Lamivudine and the apoptosis of hepatocytes concentrate on whether Lamivudine can increase the apoptosis caused by other apoptotic inducers (Diao *et al.*, 2001; Janssen *et al.*, 2003; Shi and Guan, 2009). The results of these studies show no consistency. This is partly due to the difference in the concentration of Lamivudine and different criteria determining cells apoptosis. In this study, a high concentration of Lamivudine (10 μmol/l) was used to treat the cells, and significant level of apoptosis was observed under these conditions. However, the mechanism, by which Lamivudine induces apoptosis, requires further investigation.

In conclusion, our findings indicated that pcDNA3.1-IFN-γ mediated effective IFN-γ expression in HepG2.2.15 cells, and inhibited HBV replication efficiently without causing significant cytotoxicity. The successful application of the vector to express IFN-γ to inhibit the HBV replication in the HepG2.2.15 cells presented a novel strategy for further study of the effects of local cytokine expression mediated by the nonviral eukaryotic expression vector, pcDNA3.1(+).

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