**In vitro resistance to interferon of hepatitis B virus with precore mutation**

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**INTRODUCTION**

Chronic hepatitis B virus (HBV) infection is predominantly treated with interferon alpha (IFN-α), which results in an efficient reduction of the viral load only in 20-40% of treated patients. Mutations at HBV precore prevail in different clinical status of HBV infection. The roles of precore mutation in the progression of chronic hepatitis and interferon sensitivity are still unknown. The aim of this study was to explore if there was any relationship between HBV precore mutation and sensitivity to interferon in vitro.

**METHODS:** HBV replication-competent recombinant constructs with different patterns of precore mutations were developed. Then the recombinants were transiently transfected into hepatoma cell line (Huh7) by calcium phosphate transfection method. With or without IFN, viral products in culture medium were collected and quantified 3 d after transfection.

**RESULTS:** We obtained 4 recombinant constructs by orientation-cloning 1.2-fold-overlength HBV genome into pUC18 vector via the EcoRI and HindIII and PCR mediated site-directed mutagenesis method. All the recombinants contained mutations within precore region. Huh7 cells transfected with recombinants secreted HBSAg and HBV particles into the cell culture medium, indicating that all the recombinants were replication-competent. By comparing the amount of HBV DNA in the medium, we found that HBV DNA in medium reflecting HBV replication efficiency was different in different recombinants. Recombinants containing precore mutation had fewer HBV DNAs than wild type. This result showed that recombinants containing precore mutation had lower replication efficiency than wild type. HBV DNA was decreased in pUC18-HBV1.2-WT recombinants after IFN was added while others with precore mutations were not, indicating that HBV harboring precore mutation was less sensitive to IFN in cell culture system.

**CONCLUSION:** These data indicate that HBV harboring precore mutation may be resistant to IFN in vitro.

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**Key words:** Hepatitis B virus; Mutation; Interferon; Viral resistance
length of HBV. Therefore we introduced recombinant constructs harboring more than one copy of HBV genome into hepatoma cell line by transient transfection. Since the recombinants contained different mutations at the precore region, we could study the relationship of these mutations and interferon sensitivity in vitro.

MATERIALS AND METHODS

Construction of plasmids

Cloning of plasmid constructs used in transfection experiments was performed by standard techniques[13]. Mutagenesis reactions were performed through polymerase chain reaction (PCR)-mediated site-directed mutagenesis with the designed mutagenetic primers (Table 1). For the calculation of the nucleotide (ntd.) positions, the first thymine of the putative EcoRI site of HBV was defined as position 1 and then anti-clockwise was counted (GenBank accession number is AY040627, genotype C). Different kinds of mutations were introduced by amplification with Pwo-Taq DNA polymerase mix (expand high fidelity PCR system, Roche Molecular Biochemical, Germany).

First, pUC18-HBV1.2-TA was constructed. Plasmid pUC18-HBV1.2-TA was derived from pALTER-HBV1.2 containing a 1.2×HBV full length (T1762/A1764 mutant type). The 1.2×HBV full length from pALTER-HBV1.2 and linearized pUC18 vector were obtained by restrictive endonuclease EcoRI and Hind III digestion. Both of them were purified by QIAquick gel extraction kit (Qiagen, Germany). Then the 1.2×HBV full length was orientedly subcloned into the linearized vector pUC18 to acquire pUC18-HBV1.2-TA (T1762/A1764).

Second, we constructed wild type recombinants. Plasmid pUC18-HBV1.2-WT was produced by the large primer PCR method. In brief, fragments which were prepared by expand high fidelity PCR system (Roche) were used as large primers. In the PCR system, pUC18-HBV1.2-TA was used as a template with repair primer (wild type) A1896For and primer PS4Rev. The PCR products were purified by QIAGEN gel extraction kit (Qiagen, Germany). Then the 1.2×HBV full length was orientedly subcloned as described above. Plasmids containing the mutated site A1896 were obtained and named as pUC18-HBV1.2-A1896. Accordingly, plasmids pUC18-HBV1.2-A1899 and pUC18-HBV1.2-AA were obtained later. The detailed mutation patterns of all the produced plasmids are shown in Table 2 and Figure 1. Correctly introduced nucleotides were verified by sequencing.

Table 2 Mutation patterns of the 4 recombinant constructs

| Number | Recombinant constructs | Mutation pattern |
|--------|------------------------|------------------|
| 1      | pUC18-HBV1.2-WT        | -                |
| 2      | pUC18-HBV1.2-A1896     | +                |
| 3      | pUC18-HBV1.2-A1899     | -                |
| 4      | pUC18-HBV1.2-AA        | +                |

Note: “+” indicates the construct containing the corresponding mutation. “-” indicates the constructs containing no corresponding mutation instead of wild type.

Determination of the most optimum concentration of interferon-α in vitro

Hub7 cells were seeded at a density of 6×10^5/well in six-well plates (10 cm² per well, Falcon) in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and cultured at 37 °C in 5% CO₂ for 24 h. To determine the most optimal concentration of interferon-α, different concentrations of interferon-α were added 4 h before transfection. Five ug plasmid was transfected into nearly confluent Huh7 cells. Sixteen hours after transfection, Huh7 cells were shocked with 15% glycerol solution, and then incubated with a fresh culture medium for 24 h.

Transfection of HBV DNA by calcium phosphate precipitation

All plasmid DNAs were prepared and purified by QIAGEN.
EndoFree maxi kit (EndoFree™ Plasmid Mega Kit, Qiagen, Germany). The DNA was then quantified spectrophotometrically (a ratio of the optical density at 260 nm to that at 280 nm of 1.7 to 1.8).

Transfection was performed by the calcium phosphate precipitation method (Promega ProFection® mammalian transfection system-calcium phosphate) according to the supplied protocol. Human hepatoma cell line Huh7 was grown as a monolayer in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum at 37 °C in 5% CO2. Huh7 cells were plated at a density of 3.0×10^6 cells per Petri dish (Falcon) 1 d before transfection to reach 80% confluence at transfection. The medium was changed 4 h before transfection. Twenty ug corresponding HBV replication-competent plasmid was mixed with CaCl2 solution, then added dropwise to 2× HEPES-buffered saline (pH 7.05), and transfected into confluent Huh7 cells. Sixteen hours after transfection Huh7 cells were shocked with 15% glycerol solution, and then incubated with a fresh culture medium for 24 h. Medium with or without 1000 IU/mL IFN-α (interferon alfa-2b, intron A, Schering-Plough Research Institute) was then added and the culture medium was harvested 3 d after transfection.

Transfection efficiency was measured by cotransfection of 1.0 μg of reporter plasmid pSEAP2-Control-SV40 (Clontech) expressing secretable alkaline phosphatase under the control of the SV40 early promoter and the SV40 enhancer expressing secretable alkaline phosphatase under the control to the recommendation after transfection by performing a colorimetric assay according to the manufacturer. The SEAP activity of the culture media was measured 24 h after transfection. The SEAP coding sequence was followed by the SV40 late polyadenylation signal to ensure proper and efficient processing of the SEAP transcript in eukaryotic cells. The secreted SEAP enzyme was assayed directly from the culture medium. The reporter plasmid served as an internal control to monitor transfection efficiency and potential IFN-α-mediated cytotoxic effects or nonspecific inhibitory effects. The transfection efficiency in each cell culture plate was carefully controlled by determination of the SEAP enzymatic activity in the cell culture medium before addition of IFN-α, and was found to vary by less than 10% (data not shown).

The SEAP activity of the culture media was measured 24 h after transfection by performing a colorimetric assay according to the recommendation\(^{[13]}\). Briefly, twenty microliters of heat-treated (at 65 °C for 5 min) medium was adjusted to 1× SEAP assay buffer (1.0 mol/L diethanolamine pH 9.8, 0.5 mmol/L MgCl2, 10 mmol/L L-homoarginine) in a final volume of 200 μL and prewarmed to 37 °C for 10 min in a 96-well flat-bottom culture dish. Twenty microliters of prewarmed 120 mmol/L p-nitrophenylphosphate (pNPP) dissolved in 1× SEAP assay buffer was then added and mixed. A405 of the reaction mixture was read in a microplate reader at 5-min intervals. The change in absorbance was plotted and the maximum linear reaction rate was determined. The SEAP activity was expressed in A405 (data not shown). The amount of both HBsAg and HBV DNA was corrected by the SEAP activities.

**Detection for HBsAg secreted to cell culture medium**

The amount of HBV surface antigen (HBsAg) secreted into the cell culture medium was determined by enzyme-linked immunosorbent assay (ELISA, Hepanostika HBsAg Uni-Form II, Biomérieux bv, Netherlands) after removal of cell debris and stored at -20 °C until analysis. Total amount of HBsAg was calculated by comparison with a standard HBsAg-positive human serum and negative control sera provided by the manufacturer. If the A450 for HBsAg was more than 3.0, a 1:10 dilution of culture medium was made for further assay.

**Real-time quantitative PCR to detect HBV DNA secreted to culture medium**

Cell culture medium was collected 3 d after transfection and centrifuged at 14 000 r/min 10 min at 4 °C to remove cell debris. The supernatant was adjusted to 10 mmol/L MgCl2 and treated with 100 μg/mL DNase I for 60 min at 37 °C to remove the remaining recombinants. The reaction was stopped by adding EDTA to a final concentration of 25 mmol/L. HBV DNA in the cell medium was detected by fluorescent real-time quantitative PCR (commercially available assay kit, Roche Light cycler).

**RESULTS**

**Replication-competent constructs containing HBV genome with mutations at precore region**

By PCR-mediated site-directed mutagenesis method and molecular cloning, we obtained different HBV fragments containing varied mutations with different mutagenetic primers. The agarose gel electrophoresis of different HBV fragments is shown in Figure 1. Finally we acquired four kinds of replication-competent constructs containing HBV genome with mutations at the precore region. All the mutated sites were ascertained by sequencing. The sequences were analyzed by Chromas and Bioedit software. Therefore the desired mutations instead of the unrelated mutations were introduced. Part of the sequencing results at the mutation site are shown in Figure 2.

![Figure 1](image) **Electrophoresis of mutagenesis PCR product.** Lanes A-D: Different HBV fragments using different mutated primers, Lanes E-G: The second round PCR products.

![Figure 2](image) **Local sequences of mutagenesis sites of 4 recombinants.** Arrowhead indicates the mutated nucleosides. A: pUC18-HBV1.2-WT; B: pUC18-HBV1.2-A1896; C: pUC18-HBV1.2-A1899; D: pUC18-HBV1.2-AA.
Dose-dependent sensitivity to interferon in vitro

As described in Materials and Methods, the plasmid containing wild type HBV was transfected to Huh7 in six-well plates. Different concentrations of interferon-α (62.5 IU/mL, 125 IU/mL, 250 IU/mL, 500 IU/mL, 1000 IU/mL, 2000 IU/mL, 4000 IU/mL, 8000 IU/mL) were added and cell medium was collected 3 d after transfection. As shown in Figure 4, different concentrations of interferon-α had different effects on HBV antigen expression and viral replication. Along with increase of interferon-α concentration, HBsAg and HBeAg secretion decreased almost in the same extent in general. The results showed that interferon-α with 2 000 IU/mL medium in vitro could inhibit HBV antigen expression to the highest extent. For the viral DNA secreted to the medium, the real-time PCR was used to detect HBV DNA. With different concentrations of interferon-α in cell medium, the amounts of secreted viral DNA were also different. HBV DNA decreased most in the medium with 1 000 IU/mL interferon-α as the concentration of experiments in vitro.

Precore mutation decreased HBV replication

All the different recombinants were introduced into hepatoma cell Huh7 by calcium phosphate transient transfection method. Cell culture medium was collected 3 d after transfection and HBV DNA was detected by fluorescent real-time quantitative PCR. HBV DNA in the cell medium was given as viral copies per milliliter. As shown in Table 3 and Figure 4, all the recombinants could produce and secrete HBV DNA to the cell medium. When the amounts of HBV DNA in the medium were compared, replication efficiency was different in different recombinants. Recombinants containing precore mutations had lower replication efficiency than wild type. The recombinant containing the A1896 and A1899 double mutations had lower replication efficiency than the recombinant containing A1899 or A1896 single mutations.

Table 3 HBsAg and HBV DNA secreted into cell culture medium with or without IFN (mean±SD)

| Recombinants           | HBsAg (Aso) Without IFN | HBsAg (Aso) With IFN | HBV DNA (realtime PCR, log10) Without IFN | HBV DNA (realtime PCR, log10) With IFN |
|------------------------|-------------------------|----------------------|------------------------------------------|---------------------------------------|
| Puc18-HBV1.2-WT        | 2.63±0.13               | 1.96±0.07            | 5.02±0.06                               | 4.86±0.07                             |
| Puc18-HBV1.2-A1896     | 1.43±0.05               | 1.46±0.07            | 4.43±0.09                               | 5.53±0.07                             |
| Puc18-HBV1.2-A1899     | 1.97±0.15               | 1.96±0.12            | 4.80±0.05                               | 4.90±0.06                             |
| Puc18-HBV1.2-AA        | 2.6±0.05                | 2.5±0.06             | 4.06±0.07                               | 4.46±0.05                             |

n = 3, *p<0.05 (WT in comparison with MT without IFN), †p<0.05 (without IFN in comparison with with IFN). WT: wild type, MT: mutated type.

Figure 3 Sketch map of structure of plasmid pUC-HBV1.2 and the mutation sites of all constructed plasmids. Panel A: Upper line and box figure indicate the 1.2 copy HBV genome cloned into pUC18 EcoRI and Hind III sites. Different open reading frames are indicated with corresponding box. Lower letters indicate HBV BCP and precore region sequence, TA rich region and DR region are underlined. Scheduled nucleosides to be mutated are indicated with numeral; Panel B: Recombinant constructs and their corresponding mutation sites. The constructs are listed at left, long gray box indicate consensus sequences, mutation nucleosides and their sites are indicated.

Figure 4 Detection of HBsAg, HBeAg, HBV DNA secreted to the cell medium with different concentrations of interferon.
Precore mutation exerted no effect on expression of HBsAg
Cell culture medium was collected 3 d after transfection with recombinant constructs. The amount of hepatitis B virus surface antigen (HBsAg) secreted into the cell culture medium was determined by ELISA. All the recombinants could express and secrete HBsAg into cell medium. When the relative amount of HBsAg was compared (Table 3), a slight difference was found among all the recombinants containing different precore mutation combinations. But there was no significant difference by statistic analysis \((P>0.05)\). The result indicated that precore mutation might have no effect on expression of HBsAg.

Recombinants containing precore mutation were resistant to interferon
Sixteen hours after transfection, Huh7 cells were shocked with 15% glycerol solution, and then incubated with fresh culture medium for 24 h. Fresh complete medium with and without 1 000 IU/mL IFN-α was added and the culture medium was harvested 3 d after transfection. HBV DNA in the cell medium was detected by fluorescent real-time quantitative PCR. By comparing HBV DNA in the cell medium with or without addition of IFN, we could know the sensitivity to IFN of different recombinants. The level of HBV DNA was decreased in pUC18-HBV1.2-WT recombinants after IFN was added while others with precore mutations were not. HBV DNA in the medium from the recombinants containing HBV precore mutations such as A1896 and/or A1899 did not decrease in amount after IFN was added. Thus recombinants containing precore mutations were resistant to interferon, precore mutations might be one indicator of HBV resistance to IFN.

Effect of IFN on secretion of HBsAg
As shown in Table 3, HBsAg secreted to the medium was slightly decreased in recombinants with HBV wild type after IFN was added. While after interferon was added, HBsAg in the medium of recombinants with HBV precore mutations did not decrease.

DISCUSSION
Chronic HBV infection remains a major public health problem worldwide as well as a therapeutic challenge. Despite a high rate of viral clearance in immunocompetent adults and the availability of efficient vaccines, a large proportion of the world’s population (400 million) are chronically infected with HBV. This is due to the fact that vertical transmission of HBV in neonates leads to a chronic infection in 90% of cases. Chronic HBV infection may result in a wide spectrum of liver diseases including acute self-limiting hepatitis or chronic infection. There are still 250 000 deaths each year resulted from hepatitis B. While HBV is generally regarded as noncytopathic, HBV-induced liver diseases are mediated by cytotoxic T-lymphocyte (CTL) lysis of infected hepatocytes\(^{[16,17]}\). Nevertheless, activity of HBV-induced liver diseases may also relate to viral factors. The coexistence of repeated cycles of HBV replication and immune lysis of infected hepatocytes are associated with fibrosis, cirrhosis, and hepatocellular carcinoma.

Current strategies for treating hepatitis B are focused on clearance of active HBV infection through suppression of viral replication. Interferon-α (IFN-α) and nucleoside analogs (lamivudine and adefovir) have been approved for their clinical use by FDA. The sustained virological response rate to IFN-α, however, ranges between 20% and 40\(^{[3,4]}\). The actual rate of sustained response in non selected patients is supposed to be lower. The resistance to antiviral treatment by some viruses is due to positive selection of variants harboring mutations that confer resistance\(^{[18]}\). Variations at the precore region have been reported to be associated with the response to IFN therapy\(^{[19]}\). The mutation G to A switch at position 1896 of the pre-core region of the HBV genome that leads to a translational stop codon in the leader sequence of HBsAg protein could result in the inhibition of protein synthesis\(^{[20]}\). Such variants selected during seroconversion from HBsAg to anti-HBe are responsible for HBsAg-negative chronic hepatitis B, which represents up to 50-95% of the chronic hepatitis B cases followed up in liver units in Europe and has an increasing prevalence from north to south\(^{[21]}\). Recently a total of 694 consecutive chronic HBV-infected patients seen in 17 liver centers in the US during a one-year period were enrolled\(^{[22]}\). Precore variants are found in 27% of chronic hepatitis B (CHB) patients and more common in hepatitis B e antigen (HBeAg)-negative patients than in HBsAg-positive patients (38% vs 9%, \(P<0.001\)). The precore stop codon variants are detected in a median of 60% (range 0-100%) of HBeAg-negative patients, 92% in the Mediterranean, 50% in Asia Pacific and 24% in the USA and Northern Europe\(^{[23]}\). Anti-hepatitis e antigen-positive chronic hepatitis B is a progressive liver disease associated with precore mutants and poor response to interferon. It is still unknown why this variant is related to IFN responsiveness, and whether it is IFN-sensitive or resistant, or whether it is directly or causally related to responsiveness. Previous studies have not fully answered these questions and the results are contradictory\(^{[24]}\).

Some studies have shown that mutant-type diseases (anti-HBe-positive/HBeAg-negative) are less responsive to IFN given for 6-12 mo while wild-type responds relatively well to IFN treatment. Naoumov et al\(^{[24]}\) sequenced precore/core region in 46 serum samples obtained before, during, and after interferon treatment of 12 patients. No significant changes occurred in the precore/core regions in responders after seroconversion to anti-hepatitis B e antigen, but multiple variations persisted in nonresponders during treatment and new mutations occurred with the relapse of hepatitis. Other studies had contradictory results. Shindo et al\(^{[25]}\) investigated 93 patients with chronic hepatitis B treated with IFN alpha. The results suggest that the precore wild type and mutant have similar sensitivity to IFN. Schepis et al\(^{[26]}\) found core gene variability did not seem to be involved either in the outcome of infection or in the responses to IFN treatment in children with chronic HBV infection. Zampino et al\(^{[27]}\) showed that G1896A precore stop codon mutation was not detected before, during and after interferon treatment in young Caucasian cancer survivors who acquired HBV infection during chemotherapy for malignancies. Some researchers\(^{[28]}\) have found that precore mutant HBV could influence the response to interferon when it reaches significant serum levels (> 20% of total viremia).

For the study of HBV infection, no permissive cell line or small animals are available. Stable cell lines with integrated HBV genomes, e.g., HepG2.2.15 cells\(^{[29]}\), are commonly used for assessing the action of drugs on HBV replication. HBV-transgenic mice have been proved to be very useful for immunological studies\(^{[29]}\). However, stable cell lines as well as transgenic mice have the disadvantage that, unlike in natural infection, HBV replicates from an integrated genome which cannot be eliminated. In contrast to almost all previous studies with cell lines containing chromosomally integrated HBV DNA or transfection of cells with subgenomic HBV fragments\(^{[30-32]}\), we developed recombinant constructs in which 1.2-fold-overlength HBV containing precore mutations were inserted. All the different recombinants were introduced into hepatoma cell line Huh7 by calcium phosphate transient transfection method. The constructs efficiently initiated virus replication and antigen expression in transiently transfected hepatoma cell line Huh7, which could be demonstrated by measuring...
HBSAg and HBV DNA secreted to the cell culture medium. All the recombinant constructs were replication-competent. By comparing the amount of HBV DNA into the medium, we found that the replication efficiency was different in different recombinants. Whether HBV harboring single A1896 and A1899 precore mutations affects replication efficiency or not is still uncertain.

Interferons exert their cellular activities by binding to specific membrane receptors on the cell surface. Once bound to the cell membrane, interferons initiate a complex sequence of intracellular events including induction of certain enzymes, suppression of cell proliferation, immunomodulating activities such as enhancement of the phagocytic activity of macrophages and augmentation of the specific cytotoxicity of lymphocytes for target cells, and inhibition of virus replication in virus-infected cells. It has been reported that type I interferons have immunomodulatory properties as well as direct antiviral activity \cite{33}. Previous results obtained in \textit{in vivo} studies could not show whether precore mutation of HBV is directly related to between IFN responsiveness. We used an \textit{in vitro} system by hepatoma cell lines (as described before) to investigate the relationship between precore mutation and IFN responsiveness. By comparing HBV DNA in the cell medium, we found that the production of HBV DNA was decreased in HBV wild type after adding IFN but not in HBV precore mutations. It demonstrates that IFN could directly suppress viral replication in Huh7 cells transiently transfected with wild type HBV DNA \textit{in vitro}. These \textit{in vitro} experiment results indicate that the mechanism of IFN's antiviral effect is not only by immune regulation but also by direct or indirect suppression of viral replication. IFN cannot suppress viral replication in Huh7 cells transiently transfected with mutant type HBV DNA \textit{in vitro} while it suppresses wild type. Meanwhile our results imply that the antiviral effect of IFN \textit{in vitro} is dose-dependent. Based on the above results, we conclude that interferon has direct anti-viral effects, HBV harboring precore mutations influences the sensitivity of IFN. Therefore precore mutations might at least partly affect the response of IFN \textit{in vivo}.

As discussed before, IFN might have direct antiviral effects on HBV. HBSAg secreted to the medium was slightly decreased in recombinants with HBV wild type after adding IFN while HBSAg did not decrease in recombinants with precore mutations. The results imply that the antiviral mechanism of IFN is not only by affecting viral replication but also by affecting viral antigen expression of HBV wild type. Precore mutations might hamper this mechanism. These results are similar to previous studies. Recent studies in chimpanzees have shown that in the initial phase of acute HBV-infection a non-cytolytic downregulation of HBV-replication takes place before cytotoxic T-cell mediated destruction of HBV-antigen \cite{20}. Various \textit{in vitro} studies have examined the inhibitory effects of IFN-α/β on HBV replication. Rang et \textit{al.} \cite{33} showed that IFN-α may reduce the stability of HBV RNA in transiently transfected Huh7-cells and Caselmann et \textit{al.} \cite{10} observed a decrease of pregenomic HBV RNA in HepG2 cells stably transfected with the HBV-genome when they were treated with IFN-β. Hamaasaki et \textit{al.} \cite{34} demonstrated a reduction of HBs-antigen mRNA-levels by IFN-α in hepatoma cells with integrated HBV-DNA. Thus type I IFNs inhibit HBV replication and protein production by a variety of mechanisms.

Our experiments indicate that HBV with precore stop mutation is resistant to IFN treatment \textit{in vitro}. The result has been ascertained by some studies in vivo. The anti-viral mechanisms of IFN are very complicated. Further studies are needed to explore the mechanisms of HBV precore mutation resistance to IFN therapy. The pathogenicity and the treatment of HBV precore mutation remain to be investigated.

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