Dual effect of APOBEC3G on Hepatitis B virus

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INTRODUCTION

Hepatitis B virus (HBV) is a small, enveloped DNA virus with partially double-stranded DNA as a genome (Ganem & Schneider, 2001; Seeger & Mason, 2000). The virus replicates through transcription of pregenome RNA and reverse transcription, like retroviruses (Skalka & Goff, 1993; Summers & Mason, 1982). Infection with HBV causes chronic hepatitis and often leads to liver cirrhosis and hepatocellular carcinoma (Wright & Lau, 1993; Bruix & Llovet, 2003; Ganem & Prince, 2004).

Recent reports have shown that a cytidine deaminase, APOBEC3G, which is packaged in human immunodeficiency virus (HIV) virions in non-permissive cells, induces G to A hypermutation to a nascent reverse transcript of HIV and serves as part of the innate antiviral activity (Mangeat et al., 2003; Zhang et al., 2003; Lecossier et al., 2003; Harris et al., 2003). Recent studies have demonstrated that a small number of HBV DNA in serum samples of patients with chronic HBV infection contains hypermutated genomes (Gunther et al., 1997; Suspene et al., 2005a; Noguchi et al., 2005). We reported previously that there are small numbers of hypermutated genomes in serum samples of the majority of patients with chronic HBV infection and that G to A hypermutation could be induced in cultured liver cells derived from HepG2 cell lines (Noguchi et al., 2005) using a peptide nucleic acid-mediated PCR clamping method. Suspene et al. (2005a) developed the more sensitive differential DNA denaturation (3D)-PCR method to detect hypermutated genomes and found that some APOBEC proteins induce G to A, and in some cases C to T, hypermutations in HBV DNA (Suspene et al., 2005a). Why only a very small proportion of the HBV genome is hypermutated is unknown at present. Furthermore, the
mechanism that controls the level of APOBEC protein expression and degree of hypermutation has not been fully investigated. Recently, Tanaka et al. (2006) identified an interferon (IFN)-stimulated response element (ISRE) in the promoter region of APOBEC3G and showed that IFN-α upregulates transcription of APOBEC3G. Peng et al. (2006) also reported that IFN-α and γ reduce uracil mRNA transcription of APOBEC proteins. However, these reports did not analyse whether increased numbers of APOBEC proteins actually increase hypermutation. More recently, Bonvin et al. (2006) demonstrated that IFN induces transcription of APOBEC proteins and increases hypermutation of HBV.

IFNs are cytokines that play a major role against many pathogens (Samuel, 2001; Colonna et al., 2002; Grandvaux et al., 2002). We also reported in a previous study that both IFN-α and γ reduce virus replication in stably HBV-transfected cell lines without inducing a remarkable increase in G to A hypermutation (Noguchi et al., 2005). However, the method used in previous experiments for detection of hypermutation was not as sensitive as the method of Suspene et al. (2005a, b) and not quantitative. To assess the level of hypermutation, a reliable measurement of hypermutated genome is needed. In the present study, we developed a new and sensitive method for the measurement of hypermutated genome levels. Using this method, we show here that both IFN-α and γ increased the levels of hypermutated genomes in cultured cell lines. Furthermore, both IFNs increased the mRNA level of APOBEC3G. We also performed overexpression experiments to examine whether APOBEC3G and its inactive mutants increase the levels of hypermutation and reduce HBV replication.

METHODS

Plasmid constructs. The expression vector for haemagglutinin (HA)-tagged human APOBEC3G, pcDNA3/HA-A3G, was constructed as described previously (Kobayashi et al., 2004). APOBEC3F cDNA was obtained by modifying APOBEC3F like IMAGE clones from Open Biosystems) to have the same sequence. APOBEC3F cDNA was obtained by modifying APOBEC3F like IMAGE clones from Open Biosystems) to have the same sequence. APOBEC3F cDNA was obtained by modifying APOBEC3F like IMAGE clones from Open Biosystems) to have the same sequence. APOBEC3F cDNA was obtained by modifying APOBEC3F like IMAGE clones from Open Biosystems) to have the same sequence. APOBEC3F cDNA was obtained by modifying APOBEC3F like IMAGE clones from Open Biosystems) to have the same sequence.

Cell culture and transfection. Hep7 and HepG2 cell lines were cultured using a method described previously. T23 cells are HepG2 cells stably transfected with the plasmid pTRE-HB-wt. They were cultured using a method described previously (Tsuge et al., 2005). Cells were seeded to semi-confluence in six-well tissue culture plates and then treated with medium containing either IFN-α (Hayashibara Biochemical Laboratories) or IFN-γ (Shionogi & Co.). The cells were harvested 12–72 h after IFN treatment. Core-associated HBV DNA was extracted from the cells for HBV DNA quantification and quantitative analysis of G to A hypermutated genomes (Noguchi et al., 2005).

Analysis of core-associated HBV DNA. The cells were harvested 4 days after transfection and lysed with 250 μl lysis buffer [10 mM Tris/ HCl pH 7.4, 140 mM NaCl and 0.5 % (v/v) NP-40] followed by centrifugation for 2 min at 15 000 g. The core-associated HBV genome was immunoprecipitated from the supernatant by mouse anti-core monoclonal antibody anti-HBc determinant z (Institute of Immunology, Tokyo, Japan) and subjected to quantitative analysis after SDS/proteinase K digestion followed by phenol extraction and ethanol precipitation. Quantitative analysis was performed by real-time PCR using the 7300 Real-Time PCR system (Applied Biosystems). The primers used for amplification were 1, 5'-ACTTCAACCCCAACAMMRAC-3' (nt 2978–2999) [numbers are those of HBV subtype C reported by Norder et al. (1994)] and 2, 5'-AGATTTGKGAATGTGGGA-3' (nt 24–1), where M is A/C, R is G/A, Y is T/C and K is G/T. The probe was a 6-carboxy-fluorescein (FAM)-labelled minor-groove binder (MGB) probe, 5'-FAM-TTAGAGTTGAGAGATGGG-MGB-3' (nt 3184–3167). Real-time PCRs were set up in 25 μl TaqMan Universal Master Mix with 1 μl DNA solution, 0.9 μM each primer and 0.25 μM probe. The amplification conditions were 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of amplification (denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s and extension at 62 °C for 90 s).

Amplification and analysis of hypermutated HBV genomes by 3D-PCR. HBV DNA was extracted from 100 μl serum obtained from a chronic HBV carrier (genotype C) by SMITEST (MBL International) and was dissolved in 20 μl H2O. Hypermutated genomes were detected by modified 3D-PCR using primers #1 and #2 and DNA solution from serum containing 8.0 × 106 or 2.3 × 105 copies of core-associated HBV DNA in 25 μl of 100 mM Tris/HCl pH 8.3, 50 mM KCl, 15 mM MgCl2, 0.2 mM each dNTP, 10 pmol each primer and 1.25 U Taq DNA polymerase (Gene Taq, Nippon Gene Co.,) together with 0.25 μg anti-Taq high (TOYOBO Co.). The amplification conditions included an initial denaturation step at 83–95 °C for 5 min, followed by 45 cycles of denaturation at 83–95 °C for 1 min, annealing at 50 °C for 30 s, extension at 72 °C for 30 s followed by 10 min of final extension. Amplicons were separated by electrophoresis on 2 % agarose gel, cloned and sequenced in an ABI PRISM 3130 Genetic Analyzer with a BigDye Terminator version 3.1 cycle sequencing ready reaction kit (Applied Biosystems). The PCR products were also analysed on Hanse Analytik (HA)-yellow gel as described previously (Suspene et al., 2005b; Tsuge et al., 2005; Abu-Daya et al., 1993).

Quantitative analysis of hypermutated genomes by real-time PCR. Hypermutated genomes were quantified by real-time PCR using the 7300 Real-Time PCR system (Applied Biosystems) and the above primers and probes. The amplification conditions included activation at 95 °C for 10 min followed by initial denaturation at 88 °C for 20 min and 45 cycles of amplification (denaturation at 88 °C for 15 s, annealing at 50 °C for 30 s and extension at 62 °C for 90 s). We chose 88 °C as this temperature is appropriate for detection of about 20 % hypermutated genomes. There are 200–300 such hypermutated genomes in 104 genomes present in HepG2 cells transiently transfected with APOBEC3G. The primer comprised 10 mM Tris/HCl pH 8.3, 50 mM KCl, 3 mM MgCl2, 10 mM EDTA, 60 mM Passive Reference 1 (Applied Biosystems), 0.2 mM each dNTP, 0.9 μM each primer, 0.25 μM probe, 5 × 105 copies of HBV DNA.
and 0.625 U AmpliTaq Gold DNA polymerase (Applied Biosystems) in a final volume of 25 μl. A standard curve was constructed by the simultaneous amplification of serial dilutions of the 3D-PCR products.

**Western blot analysis.** Cell lysates were prepared as described above, resolved on 10% (w/v) SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Whatman) via electro-blotting. The membranes were incubated with anti-haemagglutinin fusion epitope monoclonal antibody (Roche) or with anti-β-actin monoclonal antibody (Sigma-Aldrich) followed by incubation with horseradish peroxidase-conjugated donkey anti-rabbit antibody or sheep anti-mouse immunoglobulin (Amersham Biosciences). Proteins were visualized via the ECL system (Amersham Biosciences).

**Quantification of mRNA of APOBEC3G or APOBEC3F by reverse transcription and real-time PCR.** Total RNA was extracted from HepG2 cell lines by using an RNeasy Mini kit (Qiagen). The RNA was reverse transcribed with random primers and Moloney murine leukemia virus reverse transcriptase (ReverTra Ace, TOYOBO Co.) at 42 °C for 60 min according to the instructions provided by the manufacturer. Quantitative analysis of APOBEC3G and APOBEC3F cDNA was performed by real-time PCR using TaqMan Gene Expression assays (Applied Biosystems). To confirm that the APOBEC3G and -3F PCR primers specifically amplify the target genes, quantitative PCR on the expression plasmids encoding human APOBEC3G and -3F, used as templates, was performed. No cross amplification was observed, even when we used 10^7 copies of APOBEC3G plasmid in the amplification reaction of APOBEC3F and vice versa. A standard curve was constructed by the amplification of serial dilutions of the known number of plasmids containing human APOBEC3G and APOBEC3F. The target cDNA was normalized to the endogenous RNA level of the housekeeping reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers and FAM-labelled probe used to quantify GAPDH were purchased from Applied Biosystems.

**RESULTS**

**Quantitative analysis of hypermutated genome by real-time PCR**

Using serum samples from a patient with a high viral load, we amplified a large number of hypermutated genomes by 3D-PCR and detected them by HA-yellow agarose gel electrophoresis (Fig. 1a). Nucleotide sequence analysis of the amplified genomes by direct sequencing was used as a reference sequence. The nucleotide sequences where the probe hybridizes are underlined. Note that the number of G to A mutations correlates with denaturation temperature.
showed detection of more heavily hypermutated genomes at lower denaturation temperatures (Fig. 1b). To develop quantitative measurement, we selected sequences with many G residues, designed primers that contained only a small number of G residues and used degenerate primers. A probe sequence was designed without a G residue. Using this primer and probe set, we could amplify only hypermutated genomes (Fig. 2). When hypermutated and non-mutated genomes were co-amplified, only hypermutated genomes were successfully amplified using the above primer and probe set (Fig. 2b). Non-hypermutated genomes (10^7 copies) were not amplified, although conventional PCR amplified both mutated and non-mutated genomes equally (data not shown). We also tried to detect only slightly (four of the 58 G residues) mutated genomes by 3D-PCR, but could not detect such genomes. It should thus be noted that the quantitative measurement we developed in this study detects only hypermutated genomes.

Detection of APOBEC3G mRNA and hypermutated genomes in semi-permissive and permissive cell lines

In retrovirus studies, it is known that some cell lines allow production of infectious retrovirus virions with Vif deficiency (permissive cells) while others do not. The difference between semi-permissive and permissive cell lines is the expression of APOBEC3G (Mangeat et al., 2003; Zhang et al., 2003; Lecossier et al., 2003; Harris et al., 2003; Shirakawa et al., 2006). Thus, we examined the expression of APOBEC3G in both HepG2 and Huh7 cell lines. The APOBEC3G mRNA level detected by real-time PCR was very low (approx. 0.002 % relative to GAPDH mRNA) and about ten times greater in HepG2 cells than in Huh7 cells (Fig. 3a).

The number of hypermutated genomes in HepG2 cells transiently transfected with pTRE-HB-wt was about five times that in Huh7 cells (Fig. 3b). Vif-deficient HIV-1 virions produced from HepG2 cell exhibited very low infectivity compared with wild-type (Fig. 4a). In contrast, the infectivity of HIV-1 virions produced by Huh7 was...
similar to that of the wild-type virus (Fig. 4a). Transient expression experiments showed that the expression of APOBEC3G in Huh7 cell lines reduced infectivity of wild-type HIV-1 produced in these cell lines in a dose-dependent manner (Fig. 4b). Infectivity of Vif-deficient HIV-1 was reduced to almost undetectable levels (Fig. 4b). Thus, APOBEC3G effectively suppressed the production of infectious HIV in these cell lines.

**Both IFN-α and -γ induce APOBEC3G mRNA expression and hypermutation of HBV genomes and reduce replication of HBV**

We treated HepG2 cell lines stably transfected with 1.4 genome length construct HBV (Tsuge et al., 2005) with either IFN-α or -γ to examine their influence on the expression of APOBEC3G mRNA and G to A hypermutation of HBV genomes. Chronological studies showed that the core-associated HBV DNA in the stably HBV-producing cell line gradually decreased until 36 h after IFN-α treatment (Fig. 5a). Expression levels of APOBEC3G mRNA, but not those of APOBEC3F, in this cell line at 12 h after the IFN treatment (Fig. 5a). Hypermutated genomes in this cell line increased with time until 36 h after IFN-α treatment. Similarly, the core-associated HBV DNA decreased gradually to about 20% of the levels in untreated cells after IFN-γ treatment (Fig. 5b). The increase in APOBEC3G mRNA expression was more prominent after IFN-γ than after IFN-α treatment. The level of APOBEC3F mRNA was also about double that of untreated cells. G to A hypermutation of HBV genomes increased markedly with time after IFN-γ treatment (Fig. 5b).

We further examined the effect of IFN on reduction of HBV replication and induction of hypermutation by comparing the effects of different doses of IFN-α and -γ. Both IFN-α...
and γ treatment decreased core-associated HBV DNA in a dose-dependent manner (Fig. 6). Hypermutation of HBV genomes also increased with higher doses of IFN (Fig. 6).

Expression of APOBEC3G increases hypermutation of the HBV genome

To confirm that the increase in hypermutation of the HBV genome is induced by the effect of APOBEC3G, we performed expression experiments of APOBEC3G and its deaminase function-deficient mutants into HepG2 cell lines and measured the number of hypermutated HBV genomes. Transient expression experiments showed that the number of HBV DNA was decreased by co-transfection of APOBEC3G in HepG2 cells (Fig. 7a). 3D-PCR and detection with HA-yellow agarose gel electrophoresis showed the presence of heavily hypermutated genomes (Fig. 7b). No amplification was observed at the 81°C denaturation temperature (data not shown). Quantitative analysis showed an about 334-fold increase in hypermutated genomes compared with mock-transfected control cells (Fig. 7c). However, the proportion of hypermutated genomes was 9.68% (968 in 10^4 genomes).

To confirm the effect of APOBEC3G on HBV hypermutation, we transfected wild-type and inactive mutants of APOBEC3G (Fig. 8a, b) into Huh7 cells. Wild-type APOBEC3G effectively induced hypermutation of HBV genomes and reduced the replication of HBV. In contrast, insufficient deaminase activity in the E67Q mutant induced less hypermutation of HBV genomes than in the wild-type. No increase in hypermutation was observed in cell lines transfected with deamination-defective E259Q and E67Q/E259Q mutants, although the number of HBV replication was reduced in these cells (Fig. 8a). We observed similar reduction in HBV replication by transient transfection of APOBEC3F. Induction of hypermutation by APOBEC3F was less efficient than by wild-type and the E67Q mutant of APOBEC3G.
APOBEC3G. These results suggest that hypermutation of HBV contributes very little to reduce the number of replicative intermediate.

**DISCUSSION**

Induction of G to A hypermutation in HIV has been reported as part of host innate immunity against virus infection (Mangeat et al., 2003; Zhang et al., 2003; Lecossier et al., 2003; Harris et al., 2003; Sheehy et al., 2002). We and others have reported the presence of hypermutated genomes of HBV in serum samples of chronically infected patients and in HepG2 cell lines (Gunther et al., 1997; Suspene et al., 2005a; Noguchi et al., 2005; Rosler et al., 2004). Hypermutation of HBV was induced in hepatocytes (Noguchi et al., 2005), and expression of APOBEC proteins in liver cell-derived cell lines increased hypermutation (Suspene et al., 2005b; Rosler et al., 2004). However, the estimated number of hypermutated genomes in chronically infected patients is very low (Noguchi et al., 2005; Suspene et al., 2005b). The reason for the partial hypermutation of HBV remains an enigma. It might be due to the low expression levels of APOBEC proteins in liver cells (Jarmuz et al., 2002). Alternatively, rapid packaging of pregenome RNA into capsid might prevent access of APOBEC3G to the first strand DNA. Furthermore, rapid degradation of edited HBV genomes by uracil DNA glycosylase in liver cells might also explain the low number of hypermutated genomes.

The mechanism that controls the activities of APOBEC proteins to cause hypermutation has not been analysed until
recently, Tanaka et al. (2006) reported that IFN-α increases the expression levels of APOBEC3G mRNA. They reported the presence of ISRE elements in the promoter region of APOBEC3G and that the promoter was activated by IFN-α. However, they did not examine the occurrence of G to A hypermutation in their experiments. Moreover, Peng et al. (2006) showed that IFN-α and -γ cooperatively induce APOBEC3G expression and that the inhibition of HIV production by a small number of IFN is cancelled by a small interfering RNA (siRNA) against APOBEC3G. More recently, Bonvin et al. (2006) demonstrated that IFN-α induces transcription of APOBEC proteins. They showed that IFN treatment increased APOBEC3B, -3C, -3F and -3G mRNAs, particularly when they were used primary cultured hepatocytes. They also reported that they were able to detect hypermutated genomes after transfection of APOBEC3 plasmids, but did not measure the direct effect of IFN on G to A hypermutation.

These studies did not analyse quantitatively the increase in hypermutation of viral genomes. The studies that analysed the expression of APOBEC protein and reduction of HBV DNA also did not analyse quantitatively the number of hypermutated genome (Suspene et al., 2005a; Noguchi et al., 2005; Turelli et al., 2004a, b; Rosler et al., 2005). In the present study, we developed a method that accurately measures the level of hypermutation using real-time PCR. It is often difficult to design a primer set and a probe to detect G to A hypermutation because they are located in a region with many G residues, but the primer and probe sequences should not contain any. It is thus possible that we did not see any C to T substitution because we did not design a primer–probe set to detect this substitution. We also tried to select such a primer–probe set applicable for all genotypes of HBV, but were able to select only one suitable for genotype C.

Using this method, we demonstrated that both IFN-α and -γ increased G to A hypermutation of the HBV genome. Although the expression levels of APOBEC3G increased after IFN treatment, we did not observe an apparent shift of preferred dinucleotide sequence of APOBEC proteins from 3F to 3G. This is probably because the increase in APOBEC3G is only slight (Fig. 5).

The exact mechanism by which IFNs activate the transcription of APOBEC3G is unknown. Furthermore, what kind of sensor(s) detects HBV infection and how the signal is communicated for the production of IFNs and subsequent induction of effector molecules have not been analysed yet. Although the importance of the IFN system in eliminating HBV and its possible mechanism have been reported (Wieland et al., 2004a, b, 2005), further studies are needed to fully describe the mechanism of action of IFNs including the activation of APOBEC3G.

We also demonstrated that the number of hypermutated genomes increased with the expression of APOBEC3G and APOBEC3F (Fig. 8), but not in deaminase-inactive mutants, as demonstrated previously in HIV studies (Shindo et al., 2003; Newman et al., 2005). However, these mutants also reduced the replication of HBV almost to the wild-type level. This suggests that the contribution of hypermutation of HBV to the reduction of virus replication is only minimal and supports the previous report that showed that APOBEC3G reduced the replication of HBV through inhibition of packaging of the pregenome (Turelli et al., 2004a). However, the effect of hypermutation on infectivity of the virus should be investigated further. The effects of APOBEC proteins, including other family members, especially under physiological conditions, should also be examined further. Whether any HBV protein inhibits deamination of the genomic DNA awaits further investigation. Furthermore, the mechanism that enables HBV to cause chronic infection, especially escape from innate antiviral immunity, should also be clarified in order to control chronic HBV infection and reduce HBV-related morbidity.

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