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Ⅳ. 研究成果の刊行物・別刷

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Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis C virus and its susceptibility to interferon

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Abstract **We developed a reverse genetics system of hepatitis C virus (HCV) genotypes 1a and 2a using infectious clones and human hepatocyte chimeric mice. We inoculated cell culture-produced genotype 2a (JFH-1) HCV intravenously. We also injected genotype 1a CV-H77C clone RNA intrahepatically. Mice inoculated with HCV by both procedures developed measurable and transmissible viremia. Interferon (IFN) alpha treatment resulted in greater reduction of genotype 2a HCV levels than genotype 1a, as seen in clinical practice. Genetically engineered HCV infection system should be useful for analysis of the mechanisms of resistance of HCV to IFN and other drugs.**
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Keywords: Human hepatocyte chimeric mouse; Human serum albumin; HCV RNA; Interferon

1. Introduction

The hepatitis C virus (HCV) infects an estimated 170 million people worldwide [1]. HCV causes persistent infection in adults leading to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [2,3]. The most effective therapy for viral clearance is a 48-week combination therapy of pegylated interferon (IFN)-alpha and ribavirin. However, the success rate of this combination therapy is only about 50% [4]. Development of new anti-HCV drug had been severely restricted by the absence of a cell culture system that supports the efficient replication of HCV, as well as the lack of a small animal model. A cell culture system has been developed recently using a unique genotype 2a HCV gnome (JFH-1), which does not require adaptive mutations for efficient replication [5–7]. Chimpanzee was the only useful animal for the study of HCV until recently, although the availability of this model is severely restricted [8]. Recently, HCV-infected mice have been developed by inoculating HCV-infected human serum into chimeric urokinase-type plasminogen activator (uPA)-severe combined immunodeficiency (SCID) mice with engrafted human hepatocytes [9]. This HCV-infected mouse model has been reported to be useful for evaluating anti-HCV drugs such as IFN-alpha and anti-NS3 protease [10]. We have generated a human hepatocyte chimeric mouse where mouse hepatocytes were extensively replaced by human hepatocytes [11], and established a genetically engineered hepatitis B virus (HBV) system [12]. Using this mouse, we show in this paper the development of reverse genetics system of genotypes 1a and 2a after intrahepatic injection of transcribed RNA and intravenous injection of cell culture-produced virus, respectively. We also show here that HCV in these mice can be transmitted to naïve mice. Interferon treatment of these mice resulted in a greater reduction of HCV titer in genotype 2a clone infected mice than in genotype 1a infected mice. As these results are consistent with our clinical experience, we consider this model suitable for the study of resistance of HCV against IFN and other drugs.

2. Materials and methods

2.1. Generation of human hepatocyte chimeric mice and quantification of human serum albumin

Generation of the $uPA^{+/+}/SCID^{+/+}$ mice and transplantation of human hepatocytes were performed as described recently by our group [11,12]. All mice used in this study were transplanted with frozen

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Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; HSA, human serum albumin; IFN, interferon; SCID, severe combined immunodeficiency; uPA, urokinase-type plasminogen activator

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human hepatocytes obtained from one donor. Infection, extraction of serum samples, and sacrifice were performed under ether anesthesia. Mouse serum concentrations of human serum albumin (HSA) correlate with the repopulation index [11], and were measured as described previously [12]. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Graduate School of Biomedical Sciences, Hiroshima University.

2.2. HCV RNA transcription and inoculation into chimeric mice

A plasmid containing the full-length genotype 1a HCV cDNA clone, pCV-H77C, was kindly provided by Dr. Robert H. Purcell (National Institutes of Health). Ten micrograms of plasmid DNA, linearized by *Xba*I (Promega, Madison, WI) digestion, was transcribed in a 100-μl reaction volume with T7 RNA polymerase (Promega) at 37 °C for 2 h [13], and analyzed by agarose gel electrophoresis. Each transcription mixture was diluted with 400 μl of phosphate-buffered saline (PBS) and injected into the liver of chimeric mice. Transcripts of plasmid pJFH-1 containing the full-length HCV genotype 2a were transfected into Huh7 cells as described previously [6]. Seventy-two hours after transfection, 200 μl of the culture medium was injected intravenously into the chimeric mice. IFN-treatment was also performed by intramuscular injection of diluted IFN solutions. IFN-alpha was a kind gift from Hayashibara Biochemical Labs, Inc. (Okayama, Japan). Serum samples collected every 2 weeks after inoculation were frozen at −80 °C until further analysis.

2.3. Human serum samples

For control infection experiments, human serum containing a high titer of genotype 1b HCV (2.2×10^6 copies/ml) was obtained from a patient with chronic hepatitis after obtaining a written informed consent. The individual serum samples were divided into small aliquots and separately stored in liquid nitrogen until use.

2.4. RNA extraction and amplification

RNA was extracted from serum samples by Sepa Gene RV-R (Sankojunyaku, Tokyo), dissolved in 8.8 μl RNase-free H_2O, and reverse transcribed by using a random primer (Takara Bio, Inc., Shiga, Japan) and M-MLV reverse transcriptase (ReverTra Ace, TOYOBO Co., Osaka, Japan) in a 20 μl reaction mixture according to the instructions provided by the manufacturer. One microliter of cDNA solution was amplified by Light Cycler (Roche Diagnostic, Japan, Tokyo) for quantitation of HCV. The primers used for amplification were 5′-TTTATCCAAGAAAGGACCC-3′ and 5′-TTCACGCAGAAAGCGTCTAGC-3′. The amplification conditions included initial denaturation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 5 s, and extension at 72 °C for 6 s. The lower detection limit of this assay is 10^3 copies/ml. Nested PCR was used with the outer primers NC1 (5′-CAACACTACTCGGCTAGCAGT-3′) and NC2 (5′-CCTGTGAGGAACTACTGTC-3′) and inner primers cc6 (5′-TTTATCCAAGAAAGGACCC-3′) and cc7 (5′-TTCACGCAGAAAGCGTCTAGCttc-3′). The amplification condition included 35 cycles of 94 °C for 30 s, 58 °C for 1 min 30 s, and 72 °C for 1 min after 5 min of initial denaturation at 94 °C followed by 7 min of final extension using Gene Taq (Wako Pure Chemicals, Tokyo) with anti-Taq high according to the instructions provided by the manufacturer (TOYOBO).

2.5. Histochemical analysis of mouse liver

Histopathological analysis and immunohistochemical staining using an antibody against HSA (Bethyl Laboratories Inc.) were performed as described previously [12].

3. Results

3.1. High serum HCV RNA titer in human hepatocyte chimeric mice after inoculation of serum samples obtained from HCV-infected patient

We inoculated 50 μl of genotype 1b serum samples into five chimeric mice intravenously to test their susceptibility to HCV infection. All mice became positive for HCV RNA by nested PCR at 2 weeks after inoculation (Fig. 1). The viremia reached a plateau level at 6–8 weeks after infection, and persisted for more than 12 weeks.

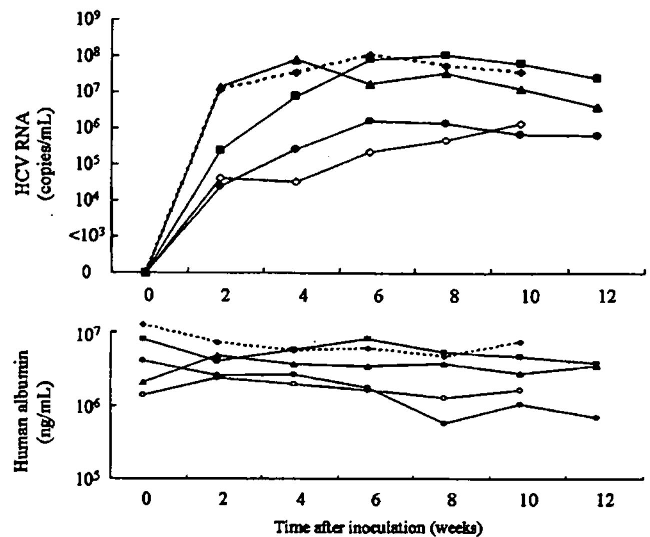

Fig. 1. Serial changes in HCV RNA and human serum albumin in sera of mice inoculated with human serum samples positive for genotype 1b HCV. Fifty microliter serum samples were injected intravenously into each mouse. Mice serum samples were obtained every 2 weeks after injection, and HCV RNA titer was analyzed.

3.2. Infection with in vitro-transcribed genotype 1a HCV RNA and cell culture generated genotype 2a HCV

In the next step, we tried to establish infection of cloned HCV using infectious genotype 1a and genotype 2a clones. In these experiments, we used two different strategies to establish infection using these two clones because genotype 1a has not been confirmed to replicate in cell culture system. We used genotype 1a HCV RNA (CV-H77C), which has been reported to be infectious to chimpanzee [13]. In vitro-transcribed HCV RNA was directly injected intrahepatically in three chimeric mice. We also infected three chimeric mice by intravenous injection of Huh7 cell-produced genotype 2a HCV after transfection of in vitro transcribed RNA from an infectious clone JFH-1. This clone has been shown to be infectious to a chimpanzee [6] and a chimeric mouse [7]. All mice developed measurable viremia 2 weeks after inoculation. At 6 weeks after inoculation, HCV RNA titer was 2.4×10^7 copies/ml (range: 8.8×10^6–2.9×10^7 copies/ml) in genotype 1a HCV-infected mice, and 2.5×10^5 copies/ml (range: 1.4×10^5–3.7×10^5 copies/ml) in genotype 2a HCV-infected mice (Fig. 2).

3.3. Passage experiment of HCV to naïve chimeric mice

We then performed passage experiments using naïve mice. Each of three mice was inoculated intravenously with 10 μl serum samples obtained from the above genotype 1a and genotype 2a HCV-infected mice at week 6. Two weeks after injection, all mice developed measurable viremia, and the titer was 8.5×10^6 copies/ml (range: 1.4×10^6–2.4×10^7 copies/ml) in genotype 1a, and 1.7×10^5 copies/ml (range: 1.5×10^5–2.5×10^5 copies/ml) in genotype 2a HCV-infected mice (Fig. 3).

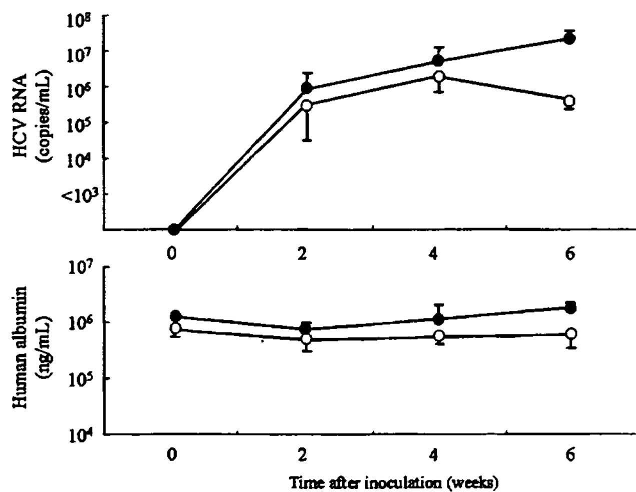

Fig. 2. Changes in HCV RNA and human albumin concentrations in serum of mice infected with clonal HCV. Each of three mice were inoculated intrahepatically with in vitro transcribed genotype 1a HCV RNA (closed circles) or intravenously with a culture medium collected from Huh7 cells transfected with JFH-1 genome intravenously (open circles). Data are mean ± S.D.

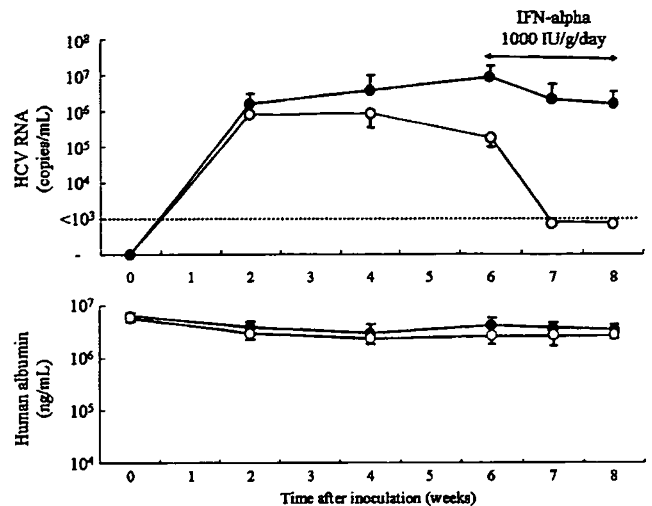

Fig. 3. Passage experiment and response to IFN-alpha therapy in mice infected with HCV genotypes 1a and 2a clones. Serum samples (10 μl) obtained from genotype 1a and 2a clonal HCV-infected mice sera (see Fig. 2) were inoculated intravenously into each of three naïve chimeric mice. Six weeks after infection, all six mice were injected intramuscularly with 1000 IU/g/day of IFN-alpha daily for 2 weeks. Closed circles: genotype 1a HCV-infected mice, open circles: genotype 2a HCV-infected mice. Data are mean ± S.D.

3.4. Variable susceptibility of HCV clones to IFN therapy

We treated each of the three mice infected with genotype 1a and 2a clones by passage experiments with 1000 IU/g of IFN-alpha daily for 2 weeks. Such treatment induced only a slight decrease in HCV in genotype 1a-infected mice; the viral load decreased only 0.6 and 0.7 log after 1 and 2 weeks of treatment, respectively (Fig. 3). In contrast, the same treatment reduced HCV genotype 2a RNA to undetectable levels after 1 and 2 weeks of IFN therapy. During IFN-treatment, serum HSA levels did not decrease in mice infected with genotype 1a or 2a HCV. Histopathological examination showed no morphological changes or apoptotic hepatocytes in replaced

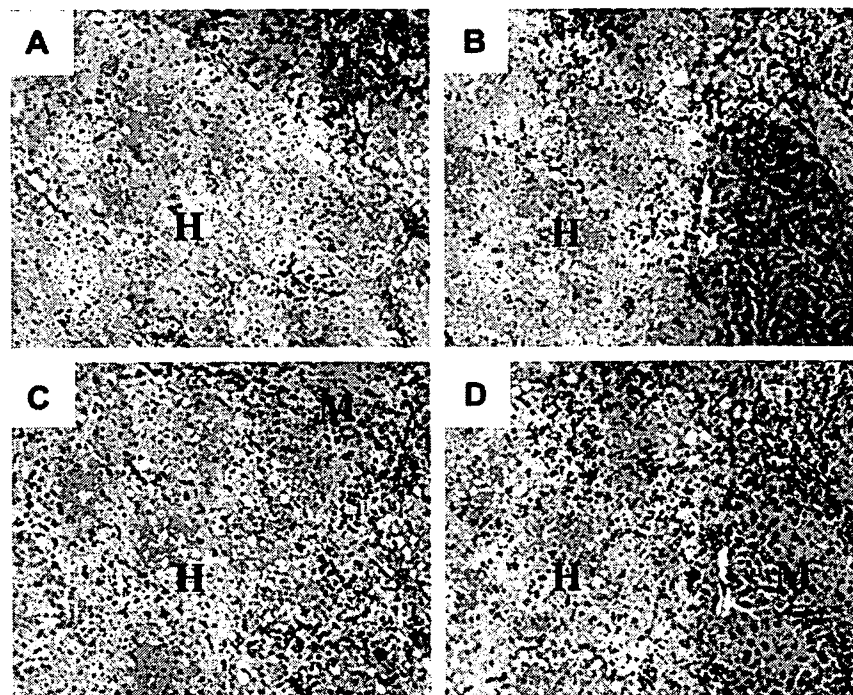

Fig. 4. Histochemical analysis of the tissues of infected chimeric mice. Liver samples obtained from mice infected with genotype 1a (A, C) and genotype 2a (B, D) stained with hematoxylin–eosin staining (A, B) or by immunohistochemical staining with anti-human serum albumin antibody (C, D). Regions are shown as human (H) and mouse (M) hepatocytes, respectively. (Original magnification, ×100.)

human hepatocytes in mice infected with each genotype after 2-week IFN-treatment (Fig. 4). These results suggest that the decrease in HCV is due to the direct anti-viral effect of IFN and not induced by liver cell damage. The difference in the virus titer and susceptibility to IFN are considered to be due to the characteristics of the genotypes.

4. Discussion

In this study, we established a reverse genetics system of HCV genotype 1a and 2a clones using human hepatocyte chimeric mice. The HCV genotype 2a clone, JFH-1, has remarkable features, i.e., infects cultured Huh7 cell line as well as establish infection in chimeric mouse [7]. It has been reported that HCV genotype 1a clone, H77-S, also infects Huh7 cell line and produces infectious virion [14]. In the present study, we intrahepatically inoculated genotype 1a infectious clone, CV-H77C. As reported in chimpanzee [13,15–17], we were able to establish genotype 1a infection using human hepatocyte chimeric mice. Using this technique, it is hoped that we can conduct further experiments in the future using genetically engineered HCV clones. Experiments using chimeric clone described by Lindenbach et al. [7] should also provide further information regarding the variable replication property of HCV genomes. Modifying genomes with nucleotide substitutions allowed examination of the functions of HCV peptides as we showed with HBV [12].

As reported recently by Kneteman et al. [10], the mouse model system is useful for evaluating the effect of anti-HCV drugs such as IFN, protease inhibitors and polymerase inhibitors. As we showed in this study, the response to IFN therapy varied according to HCV genotype. Further experiments are necessary to determine whether differences in response to IFN are due to the different replication ability (replication level of genotype 2a clone was slightly lower than that of genotype 1b, see Figs. 2 and 3) or differences in genotypes, as has been reported in clinical studies [18]. As we showed in this study (Fig. 4), there is no hepatocyte damage or inflammation in the liver of the infected chimeric mouse. Thus, this model is suitable for the study of mechanisms involved in HCV replication and IFN resistance.

The intrahepatic injection method used in this study simplified our experiments using genetically engineered virus. This is particularly important in studies of protease inhibitors and polymerase inhibitors because HCV will easily develop resistance against these small molecule agents.

Previous studies identified amino acid sequences that correlate with different susceptibilities of genotype 1b HCV against IFN therapy, namely, interferon sensitivity determining region [19] and the PKR-eIF2 phosphorylation homology domain [20,21]. To elucidate such issues, we are currently trying to establish genotype 1b infection system using the method described in this paper.

In summary, we showed in the present study the successful application of a genetically engineered HCV in human hepatocyte chimeric mice. Using this mouse model, we showed that genotypes 1a and 2a HCV clones exhibit different susceptibilities to IFN-alpha therapy. Our mouse model seems useful for the study of HCV virology and resistance of HCV against IFN and for the development of new anti-HCV therapy.

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Dual effect of APOBEC3G on *Hepatitis B virus*

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G to A hypermutation of *Hepatitis B virus* (HBV) and retroviruses appears as a result of deamination activities of host APOBEC proteins and is thought to play a role in innate antiviral immunity. Alpha and gamma interferons (IFN-α and -γ) have been reported to upregulate the transcription of APOBEC3G, which is known to reduce the replication of HBV. We investigated the number of hypermutated genomes under various conditions by developing a quantitative measurement. The level of hypermutated HBV in a HepG2 cell line, which is semi-permissive for retrovirus, was 2.3 in 10^4 HBV genomes, but only 0.5 in 10^4 in permissive Huh7 cells. The level of APOBEC3G mRNA was about ten times greater in HepG2 cells than in Huh7 cells. Treatment of HepG2 cells with either IFN-α or -γ increased the transcription of APOBEC3G and hypermutation of HBV. These mRNAs and hypermutation of HBV genomes were induced more prominently by IFN-γ than by IFN-α. Both IFNs decreased the number of replicative intermediate of HBV. Overexpression of APOBEC3G reduced the number of replicative intermediate of HBV and increased hypermutated genomes 334 times, reaching 968 in 10^4 genomes. Deamination-inactive APOBEC3G did not induce hypermutation, but reduced the virus equally. Our results suggest that APOBEC3G, upregulated by IFNs, has a dual effect on HBV: induction of hypermutation and reduction of virus synthesis. The effect of hypermutation on infectivity should be investigated further.

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

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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 upregulate 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 as human APOBEC3F transcript variant 1 (GenBank NM_145298) and cloned into pcDNA3/HA (Invitrogen). APOBEC3G mutants were constructed using a QuikChange mutagenesis kit (Stratagene). The construction of wild-type HBV 1.4 genome length, pTRE-HB-wt, has been described previously (GenBank accession no. AB206816) (Tsuge *et al.*, 2005).

Cell culture and transfection. Huh7 and HepG2 cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal calf serum at 37 °C in 5 % CO_2. Cells were seeded to semi-confluence in six-well tissue culture plates. Transient transfection of the plasmids into HepG2 and Huh7 cell lines was performed using *TransIT*-LT1 (Mirus) according to the instructions provided by the supplier. A plasmid encoding a secreted form of human placental alkaline phosphatase (SEAP) was co-transfected to adjust the transfection efficiency. The SEAP assay in the culture medium was performed using the Great EscAPe SEAP Reporter System 3 (BD Bioscience).

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 α (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′-ACTTCAACCCCAACAMRRATCA-3′ (nt 2978–2999) [numbers are those of HBV subtype C reported by Norder *et al.* (1994)] and #2, 5′-AGAGYTTGKTGGAATGTKGTGGA-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)-TTAGAGGTGGAGAGATGG-(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 H_2O. Hypermutated genomes were detected by modified 3D-PCR using primers #1 and #2 and DNA solution from serum containing 8.0×10^7 or 2.3×10^5 copies of core-associated HBV DNA in 25 μl of 100 mM Tris/HCl pH 8.3, 50 mM KCl, 15 mM $MgCl_2$, 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 % (w/v) 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.*, 1995).

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 10^4 genomes present in HepG2 cells transiently transfected with APOBEC3G. The buffer comprised 10 mM Tris/HCl pH 8.3, 50 mM KCl, 3 mM $MgCl_2$, 10 mM EDTA, 60 nM Passive Reference 1 (Applied Biosystems), 0.2 mM each dNTP, 0.9 μM each primer, 0.25 μM probe, 5×10^6 copies of HBV DNA

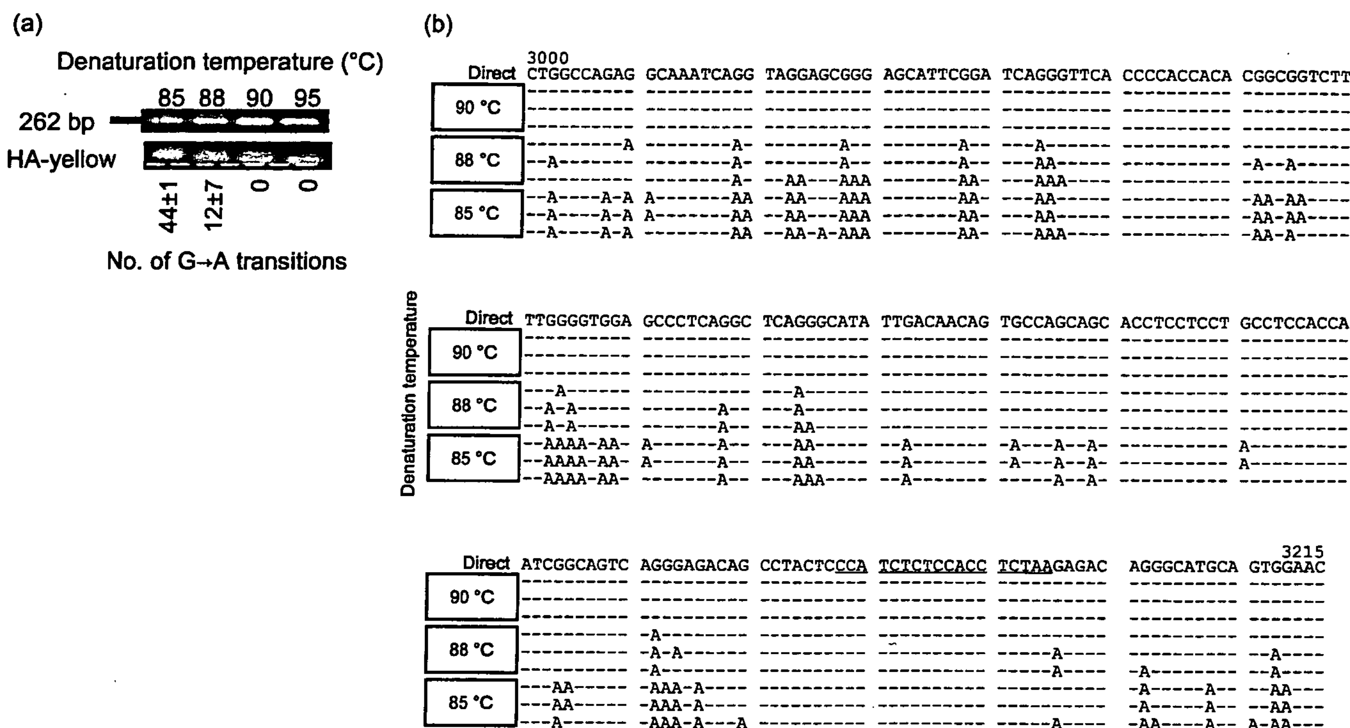

Fig. 1. Amplification of HBV DNA by 3D-PCR. (a) Detection of hypermutated genomes by HA-yellow agarose gel electrophoresis. The numbers of G to A transitions are expressed as means ± SD generated from the sequence analysis of five independent clones from PCR products. The white dotted line was added to help visualize the retardation of AT-rich DNA in HA-yellow agarose gel. (b) Nucleotide sequences of HBV amplified by 3D-PCR. The nucleotide sequences obtained by direct sequencing are 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.

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.

Infectivity of luciferase reporter viruses produced from HepG2 and Huh7 cell lines. Luciferase reporter viruses with or without viral infectivity factor (Vif) were prepared by co-transfection of pNL43/ΔEnv-Luc (wild-type) or pNL43/ΔEnvΔvif-Luc (ΔVif) plus pVSV-G together with a mock vector or expression vectors for A3G by Lipofectamine (Invitrogen) as described previously (Janini *et al.*, 2001; Shindo *et al.*, 2003). Productive infection was measured by luciferase activity. Values were presented as percentage of infectivity relative to the value of each virus without expression of APOBEC3G proteins.

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

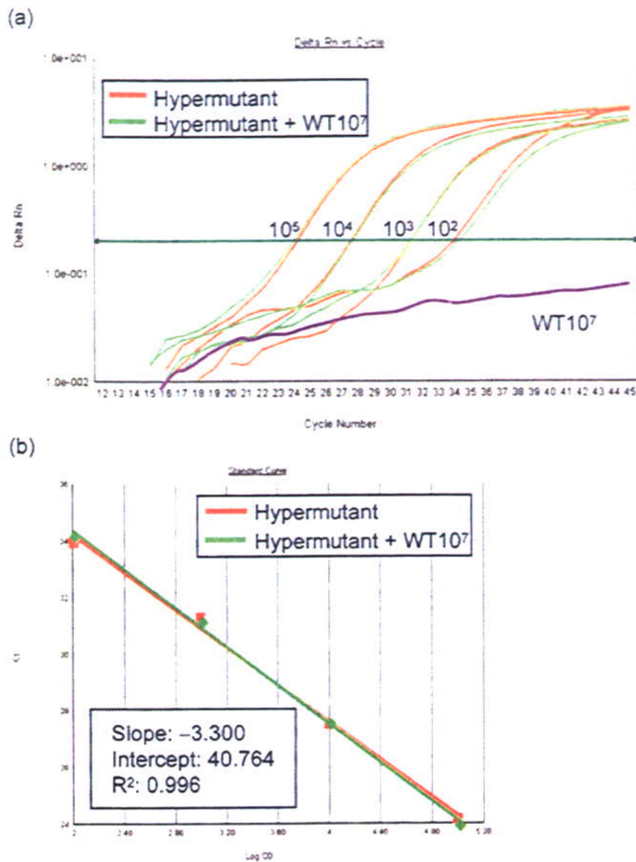

Fig. 2. Quantitative measurement of hypermutated HBV DNA using 3D-PCR combined with real-time PCR. The indicated numbers (10^2–10^5) of hypermutated genomes alone (orange lines) and a mixture of wild-type plus hypermutated genomes (green lines) were amplified by 3D-PCR. 3D-PCR did not result in amplification of wild-type sequence (purple line). Denaturation temperature was 88 °C.

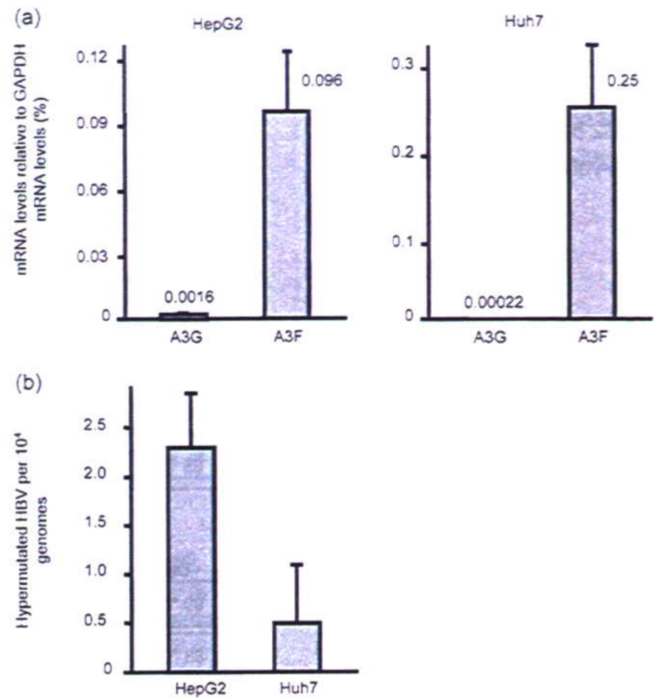

Fig. 3. Expression levels of APOBEC3G and -3F protein mRNAs in HepG2 and Huh7 cell lines. (a) mRNAs were extracted from cultured cell lines and the number of mRNA was quantified by real-time PCR with a probe for APOBEC3G and -3F. The expression levels were expressed as a percentage of GAPDH mRNA. (b) Number of hypermutated HBV genomes measured by real-time 3D-PCR in HepG2 and Huh7 cell lines transiently transfected with pTRE-HBV-wt. Results are means ± SD values of three independent experiments.

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

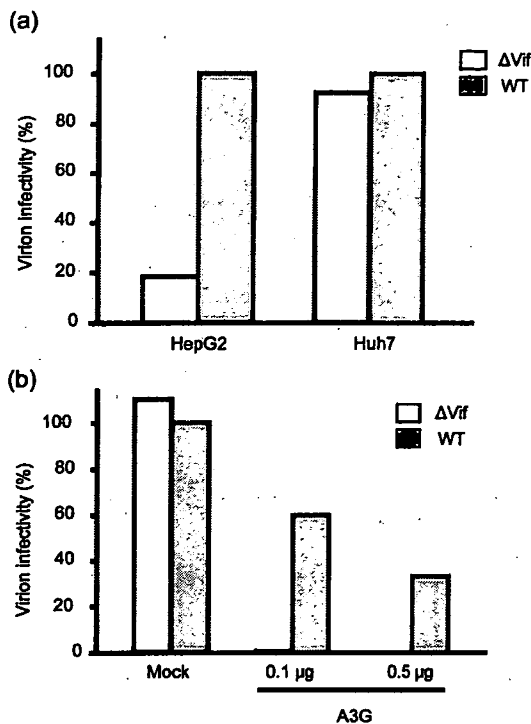

Fig. 4. Infectivity of HIV-1 virions produced from HepG2 and Huh7 cell lines. (a) Wild-type and mutant viruses lacking Vif protein produced from the two cell lines were examined for infectivity as described in Methods. The relative infectivity of the wild-type is shown. (b) Effect of APOBEC3G (A3G) expression on infectivity. HIV-1 virions produced by Huh7 cells co-transfected with the indicated number of APOBEC3G expression plasmid were used for measurement of infectivity.

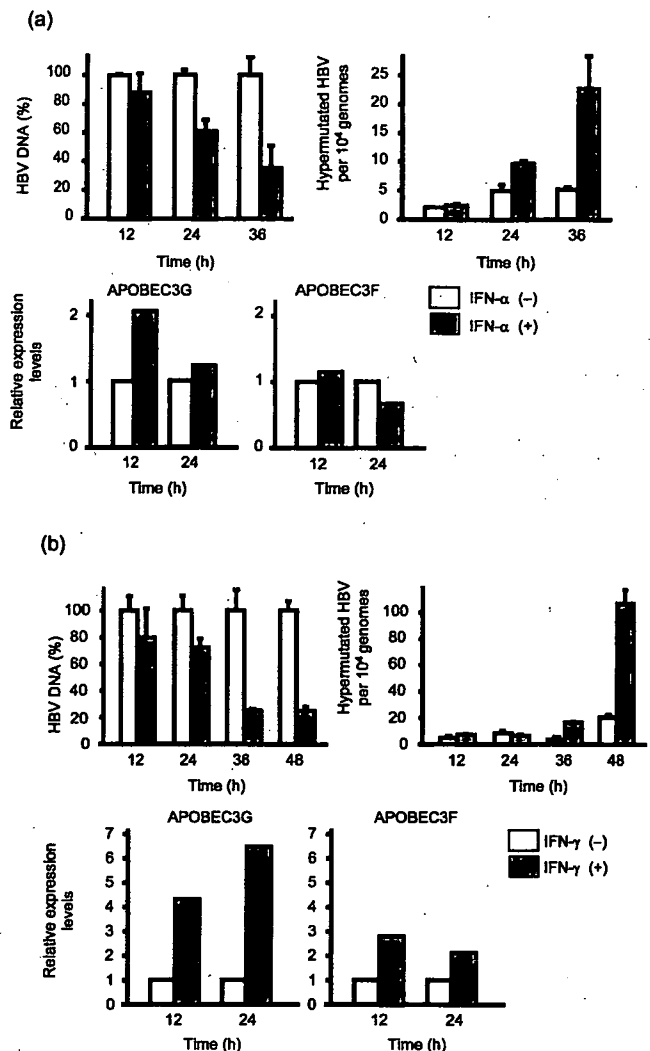

Fig. 5. Effects of IFN-α and -γ on HBV-producing cells. (a) The IFN-α-treated and -untreated HBV-producing T23 cell line was harvested at the indicated time after IFN treatment and examined for the number of core-associated HBV DNA, the number of hypermutated genome and mRNAs of APOBEC3G and APOBEC3F. (b) IFN-γ-treated and -untreated HBV-producing T23 cell line was examined as described in (a). Results are means ± SD values of three independent experiments.

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, increased 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

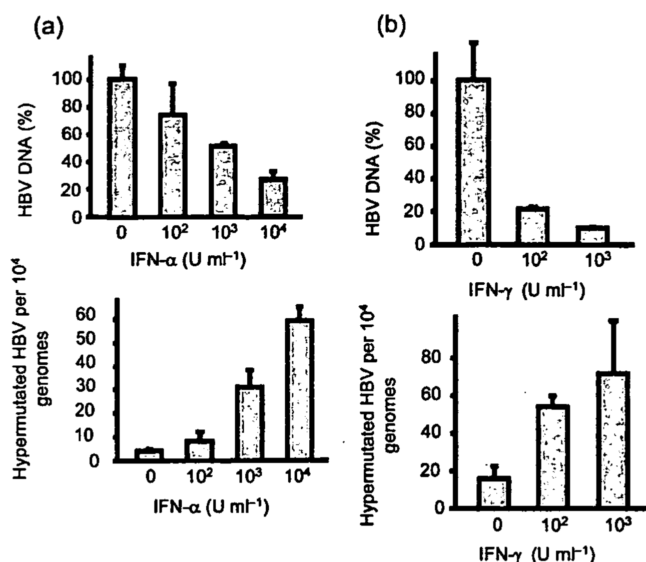

Fig. 6. Dose-dependent reduction of HBV replication and hypermutation of genomic sequences. HBV-producing cell line T23 was harvested after (a) IFN-α and (b) IFN-γ treatment for 72 h. The number of core-associated HBV DNA and the number of hypermutated genomes were measured. Results are means ± SD values of three independent experiments.

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

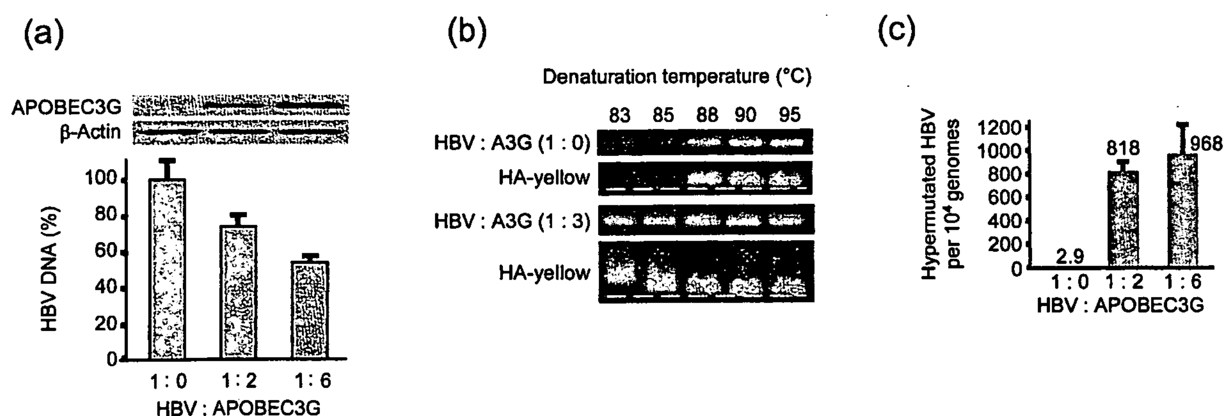

Fig. 7. Effects of APOBEC3G expression on HBV hypermutation. A plasmid containing 1.4 genome length HBV DNA was co-transfected with pcDNA3/HA-A3G into HepG2 cells. At 72 h after transfection, the cells were harvested. (a) Quantification of core-associated HBV DNA and Western blot analysis of cytoplasmic extracts with anti-HA or anti-β-actin antibody. (b) Detection of hypermutated genomes by HA-yellow agarose gel electrophoresis. Hypermutated genomes in the presence or absence of APOBEC3G-HA were amplified by 3D-PCR. The white dotted line was added to help visualize the retardation of AT-rich DNA in HA-yellow agarose gel. (c) Quantification analysis of hypermutated genomes by real-time 3D-PCR. Results are means ± SD values of three independent experiments.

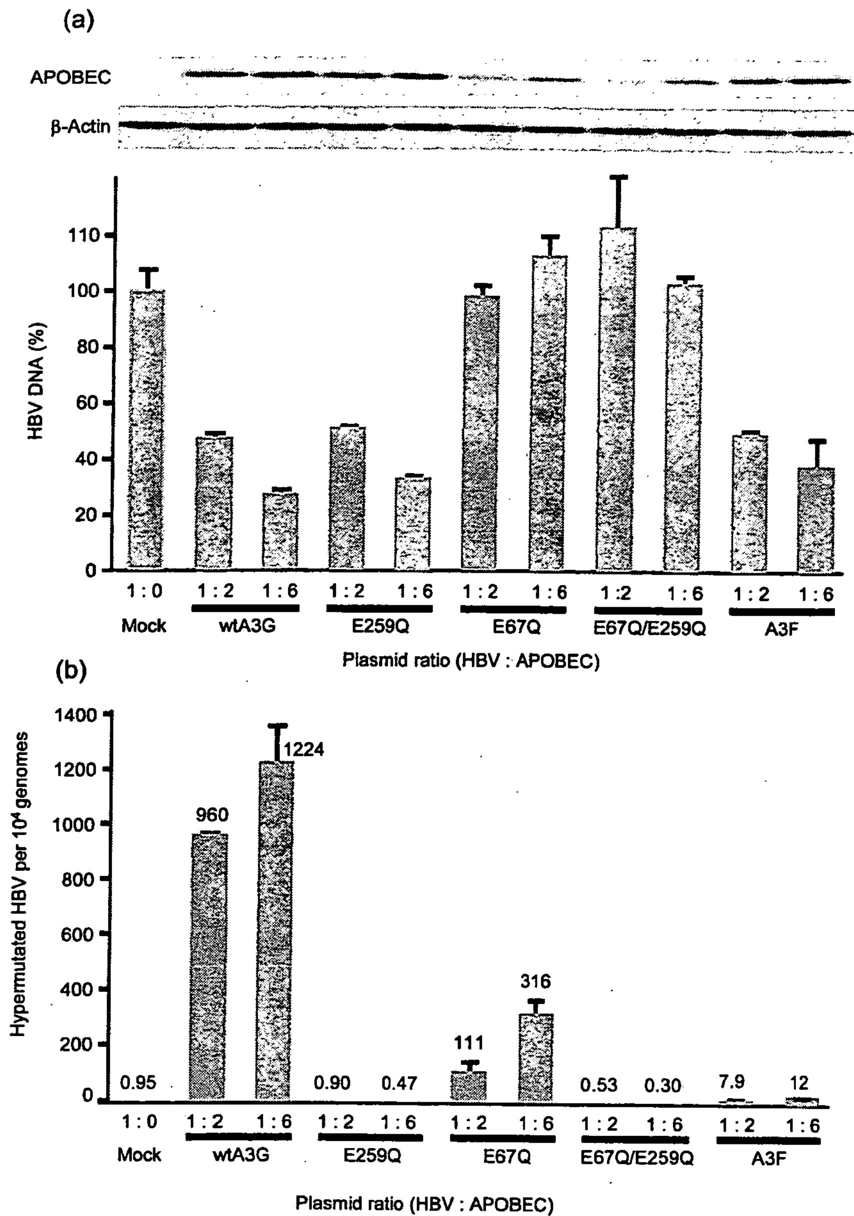

Fig. 8. Effect of APOBEC proteins on HBV hypermutation. A plasmid containing 1.4 genome length HBV DNA was co-transfected with wild-type, enzymically impaired APOBEC3G mutants (E67Q, E259Q, E67Q/E259Q) and APOBEC3F into Huh7 cells (plasmid ratio HBV:APOBEC=1:2 or 1:6). The cells were harvested at 96 h after transfection. (a) Quantification of core-associated HBV DNA and Western blot analysis of cytoplasmic extracts with anti-HA or anti-β-actin antibody. (b) Quantification of hypermutated genomes by real-time 3D-PCR. Results are means ± SD values of three independent experiments.

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 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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Pretreatment predictor of response, time to progression, and survival to intraarterial 5-fluorouracil/interferon combination therapy in patients with advanced hepatocellular carcinoma

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Background. Several studies have reported survival benefits of combination therapy with intraarterial 5-fluorouracil (5-FU) and subcutaneous interferon (IFN) α for advanced hepatocellular carcinoma (HCC) with portal vein tumor thrombosis (PVTT). We investigated the pretreatment predictive factors of early response, time to progression (TTP), and survival in response to intraarterial 5-FU/IFN combination therapy. ***Methods.*** Patients with nonresectable HCC and variable PVTT grades (without PVTT to PVTT in the trunk) received intraarterial 5-FU/IFN combination therapy (n = 55). ***Results.*** After two courses of the combination therapy, 1 (2%), 15 (27%), 16 (29%), 12 (22%), and 11 (20%) of 55 patients showed complete response (CR), partial response (PR), stable disease (SD), progressive disease (PD), or had dropped out (DO), respectively, when their early response to treatment was assessed. Univariate analysis identified only hepatitis C virus (HCV) antibody positivity as having significantly influenced the early response ($P = 0.028$) and TTP ($P = 0.021$). Multivariate analysis identified performance status ($P = 0.003$) and HCV antibody positivity ($P = 0.007$) as significant and independent determinants of survival. PVTT grade did not influence early response, TTP, or survival. The survival rate was significantly higher in patients who achieved CR or PR than in those that assessed as SD or PD, or DO ($P < 0.0001$, each). ***Conclusions.*** HCV antibody positivity may be a significant pretreatment predictor of early response, TTP, and survival of patients with advanced HCC treated with 5-FU/IFN. CR or PR as the early response to the combination therapy might indicate a more favorable prognosis in patients with advanced HCC. PVTT grade did not seem to influence the efficacy of combination therapy.

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Introduction

Hepatocellular carcinoma (HCC) is a life-threatening neoplasm and one of the most common neoplasms in Africa and Asia, including Japan. Deaths due to HCC are increasing worldwide.[1–3] Advances in biotechnology have resulted in new diagnostic techniques, such as ultrasonography, computed tomography (CT), magnetic resonance imaging, and angiography. Similarly, new treatment options have become available, such as surgical resection, radiofrequency ablation (RFA), percutaneous ethanol injection (PEI), and transcatheter arterial chemoembolization (TACE). As a result, the prognosis of HCC patients has gradually improved. Nevertheless, the survival rates of patients with advanced HCC and complications such as portal vein tumor thrombosis (PVTT) or distant metastasis remains extremely poor.[4–8]

Advances in implantable drug delivery systems have allowed repeated arterial infusions of anticancer agents. First, monotherapy with intraarterial 5-fluorouracil (5-FU) for unresectable HCC was reported.[9,10] However, such treatment resulted in a low response rate (13.0% and 22.0%). Next, several authors reported favorable results with low-dose cisplatin and 5-FU for advanced HCC with PVTT, with a response rate ranging from 33.0% to 48.0%.[11–13] Recently, several studies have reported survival benefits of combination therapy with intraarterial 5-FU and subcutaneous interferon (IFN) α for advanced HCC with PVTT, with a response rate ranging from 43.6% to 72.7%.[14–17] In these studies, only HCC patients with PVTT (in the main trunk or first branch) without distant metastases were treated. The