G-to-A Hypermutation in Hepatitis B Virus (HBV) and Clinical Course of Patients with Chronic HBV Infection

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Background. The apolipoprotein B messenger RNA editing enzyme, catalytic polypeptide–like family of cytidine deaminases induce G-to-A hypermutation in hepatitis B virus (HBV) genomes and play a role in innate antiviral immunity. The clinical relevance of this protein family is unknown.

Methods. We analyzed 33 instances in which 17 patients with chronic HBV infection experienced >2 increases of >100 IU/L in alanine aminotransferase (ALT) level; we used a quantitative differential DNA denaturation polymerase chain reaction assay to quantify the hypermutated HBV genomes observed during 21 of these 33 increases in ALT level.

Results. Of the 9 increases in ALT level that involved a >5-fold increase (relative to basal levels) in the number of hypermutated genomes observed, 8 were associated with a >2-log reduction in plasma HBV DNA level. In contrast, a corresponding decrease in plasma HBV DNA level was observed for only 1 of the 12 increases in ALT level that did not involve an increase in the number of hypermutated genomes ($P < .001$). Hepatitis B e antigen clearance was often observed in patients who experienced an increase in the number of hypermutated genomes. Interferon treatment induced hypermutation in HBV genomes in an animal model. However, there was no apparent increase in the number of hypermutated genomes among the majority of patients who received interferon therapy, probably because the number of hypermutated genomes had already increased prior to the initiation of therapy.

Conclusion. Our results suggest that a marked increase in the number of hypermutated genomes represents a strong immunological host response against the virus and is predictive of hepatitis B e antigen clearance and plasma HBV DNA level reduction.

Despite the availability of safe and effective vaccines for >2 decades, hepatitis B virus (HBV) infection is still a global health problem. Worldwide, >2 billion people are infected with HBV, and chronic HBV infection affects ~400 million people [1, 2]. It is estimated that >500,000 people die annually because of cirrhosis and/or hepatocellular carcinoma due to HBV infection [3].

Recent reports have shown that cellular cytosine deaminase (apolipoprotein B messenger RNA [mRNA] editing enzyme, catalytic polypeptide–like 3G [APOBEC3G]), packaged in human immunodeficiency virus type 1 (HIV-1), induces G-to-A hypermutation to a nascent reverse transcript of HIV-1 and reduces the infectivity of HIV, thus contributing in part to innate antiviral activity [4–8]. HIV-1 overcomes this innate defense barrier in T cells with HIV virion infectivity factor, a protein that specifically targets APOBEC3G to proteasomal degradation [9–12]. HIV-1 can infect resting CD4 T cells in lymphoid tissues but not those circulating in peripheral blood [13–16]. Resting CD4 T cells in peripheral blood are protected from HIV infection through the action of the deaminase-active
APOBEC3G [17]. Recent reports have shown that interferon (IFN)-α is a potent inducer of APOBEC3G [18–21]. It has also been reported that some of the HIV restriction exerted by APOBEC3G may be independent of its cytidine deaminase activity [17, 22–24].

We and others have reported the presence of small numbers of hypermutated genomes in serum samples obtained from HBV-infected patients [25–27]. Studies using HepG2 cell lines and primary human hepatocytes showed that such hypermutation is induced by the cytidine deaminase activity of the APOBEC family of proteins [27]. In our previous study, IFN induced little hypermutation in the HBV genome [27]. However, after extensive investigation supported by development of a quantitative analysis of hypermutation, we showed that both IFN-α and IFN-γ actually increase transcription of APOBEC3G mRNA in HepG2 cell lines and induce an increase in the number of hypermutated genomes [28]. We also showed that APOBEC3G induces hypermutation in HBV and reduces HBV replication levels in the absence of the deaminase activity. Thus, APOBEC3G has dual antiviral actions against HBV and is thought to be part of the host defense mechanisms, as has been shown for HIV infection. Although it is assumed that APOBEC3G is important in the host anti-HBV defense system, little is known about the clinical importance of this enzyme, because there are no methods available for the precise quantification of small amounts of hypermutated genomes.

Using a method that can measure small amounts of hypermutated genomes (differential DNA denaturation polymerase chain reaction [3D-PCR] combined with TaqMan PCR [28]), we analyzed fluctuations in the number of hypermutated genomes observed in patients with chronic HBV infection who experienced increased alanine aminotransferase (ALT) level. The study group included patients who received IFN treatment and patients who did not.

### METHODS

**Patients.** From 2002 through 2006 at Hiroshima University Hospital (Hiroshima, Japan), there were 17 consecutive patients with chronic hepatitis B who experienced >2 increases of >100 IU/L in ALT level and for whom stored serum samples were available. These 17 patients were enrolled in this study, among whom 33 such increases in ALT level were observed. Thirteen of 17 patients received IFN treatment, usually during an increase in ALT level. The clinical profiles of these 17 patients are shown in table 1. Written informed consent was obtained from all patients, and the study was approved by the Hiroshima University Ethics Committee.

### HBV markers.

Hepatitis B e antigen and antibody against e antigen were quantified by use of enzyme immunoassay kits (Abbott Diagnostics). HBV DNA was measured by use of real-time PCR performed with the 7300 Real-Time PCR System (Applied Biosystems), in accordance with the manufacturer’s instructions. The primers used for amplification were 5'-TT-
serum samples by use of the SMITEST DNA Extraction Kit (Gene-Science Laboratories) and dissolved in 20 μL of water. Hypermutated genomes were quantified by use of TaqMan 3D-PCR performed with the 7300 Real-Time PCR System (Applied Biosystems); we used a procedure described elsewhere [28], with slight modifications. In brief, the HBV DNA fragments were amplified by use of 3D-PCR in which the denaturation temperature was set lower than usual so that only G-to-A hypermutated genomes would be amplified. The amplification conditions were as follows: activation at 95°C for 10 min; followed by initial denaturation at 95°C for 20 min, to allow nonhypermutated genomes reanneal; and 45 cycles of amplification (denaturation at 95°C for 15 s and annealing and extension at 62°C for 1 min). The lower detection limit of this assay was 10^3 copies/mL.

**Extraction of HBV DNA and quantitative analysis of hypermutated genomes.** HBV DNA was extracted from 100-μL serum samples by use of the SMITEST DNA Extraction Kit (Gene-Science Laboratories) and dissolved in 20 μL of water. Hypermutated genomes were quantified by use of TaqMan 3D-PCR in which the denaturation temperature was set lower than usual so that only G-to-A hypermutated genomes would be amplified. The amplification conditions were as follows: activation at 95°C for 10 min; followed by initial denaturation at 95°C for 20 min, to allow nonhypermutated genomes reanneal; and 45 cycles of amplification (denaturation at 95°C for 20 s, annealing at 50°C for 30 s, and extension at 62°C for 20 s). TaqMan PCR was performed using the following primers: 5’-ACTTCAACCCACAACAMRATCA-3’ (nt 2978–2999) and 5’-AGAGTTCGGAATGTGTTGGGA-3’ (nt 24–1), where M is A or C, R is G or A, Y is T or C, and K is G or T. The probe was a 6-carboxyfluorescein (FAM)–labeled MGB probe, 5’-(FAM)-TTAGAGGTGGAGATGGG-(MGB)-3’ (nt 3184–3167). The detection limit of hypermutated genomes was 10^2 copies/mL, and nonhypermutated genomes were not amplified by 3D-PCR [28]. The reproducibility of the assay was quite high (as indicated by the small standard deviation relative to the results of the quantitative PCR control reaction), as reported in our previous study [28].

**Cell culture and transfection.** HepG2 cell lines were grown in Dulbecco’s modified Eagle medium supplemented with 10% (vol/vol) fetal calf serum at 37°C in 5% CO₂. Cells were seeded to semiconfluence in 6-well tissue culture plates and transfected with the plasmid pTRE-HBV-wt, which contained 1.4–genome length wild-type HBV genomes [31], by calcium phosphate precipitation. Seventy-two hours after transfection, the supernatant was collected for HBV DNA quantification by real-time PCR and for quantitative analysis of G-to-A hypermutated genomes [28]. The remaining supernatant was stored at −80°C for infection experiments using human hepatocyte–chimeric mice.

**Quantitative analysis of G-to-A hypermutated genomes with human hepatocyte–chimeric mice.** A human hepatocyte–chimeric mouse model was developed, as described previously [32], and used in infection and IFN-treatment experiments. The human hepatocytes progressively repopulated the murine host liver and were susceptible to HBV produced in cultured cell lines [31]. All animal protocols were in accordance with the guidelines of the local animal experimentation committee. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of the Graduate School of Biomedical Sciences, Hiroshima University. Hepatocyte-chimeric mice were inoculated with 500 μL of the supernatant produced by transiently transfected cell lines. After confirmation of high-level HBV viremia, the mice were treated with 7000 IU/g/day of IFN-α, injected intramuscularly, for 14 days (the IFN-α was a gift from Hayashibara Biochemical Labs in Okayama, Japan). Human serum albumin in mouse serum was measured with the Human Albumin ELISA Quantitation Kit (Bethyl Laboratories), used in accordance with the manufacturer’s instructions.

**Statistical analysis.** Differences between clinical groups with respect to HBV DNA and e antigen levels were examined for statistical significance, using the Mann-Whitney U test. A P value <.05 was considered to indicate a statistically significant difference. All statistical analyses were performed with StatView (version 5.0; SAS Institute).

**RESULTS**

**Clinical course of disease in patients with increased ALT levels and fluctuations in the number of hypermutated genomes.** Figure 1A–1D shows clinical courses for 4 representative patients (patients 1–4 in Table 1) with chronic HBV infection who experienced increases in ALT level. We observed marked decreases in HBV DNA level in association with marked increases in hypermutated genomes (figure 1A–1C, black arrows). In contrast, there was no apparent reduction in HBV level in the absence of an increase in hypermutated genomes (1A–1D, white arrows). We also analyzed the effect of IFN therapy on the number of hypermutated genomes. In some patients, we observed an increase in the number of hypermutated genomes during IFN therapy (figure 1B and 1C) as well as a marked increase in the number of hypermutated genomes and a reduction of the virus accompanied by an increase in ALT level just after cessation of IFN therapy (1A–1C, black arrows). However, in some patients, such as patient 1 (figure 1A), we observed no apparent increase in the number of hypermutated genomes in response to IFN therapy. However, the number of hypermutated genomes observed in samples from this patient obtained just before the initiation of IFN therapy (996/10^6 genomes) was already higher than the baseline level (157/10^6 genomes). Samples from patient 4 (figure 1D) showed an increase in the number of hypermutated genomes during IFN therapy (1907/10^6 genomes), though this is less than the increase observed during natural exacerbation (12,404/10^6 genomes). In fact, there was no significant difference between IFN-treated patients and untreated patients with respect to the number of hypermutated genomes observed (data not shown). These results suggest that the host’s antiviral immunity level was higher at baseline than it was after
Figure 1. Clinical courses for 4 patients (A–D) with chronic hepatitis B virus (HBV) infection who experienced exacerbation of infection. Black arrows, exacerbation associated with an increase in the number of hypermutated genomes (>5 times basal levels); white arrows, exacerbation not associated with an increase in the number of hypermutated genomes; horizontal dotted lines, upper normal limit of alanine aminotransferase (ALT) (40 IU/mL; upper panel, A–D) and the detection limit for antibody against e antigen (HBeAb) (35%; lower panel, A–D). HBeAb, antibody against HBV e antigen; HBeAg, HBV e antigen; IFN, interferon.
IFN or that the feedback system for IFN signaling was already active before initiation of therapy.

We also compared the degree of reduction in the plasma HBV DNA level for exacerbations (i.e., increases in ALT level) associated with a marked increase in the number of hypermutated genomes (i.e., those in which the peak number was \( > 5 \) times the number observed prior to exacerbation) and for exacerbations not associated with such an increase. As shown in figure 2, 8 of 9 exacerbations that were coupled with a marked increase in the number of hypermutated genomes (group A) were associated with a \( > 2 \)-log reduction in the HBV DNA level. In contrast, only 1 of the 12 exacerbations not associated with a marked increase in the number of hypermutated genomes (group B) was associated with a \( > 2 \)-log reduction in plasma HBV DNA level. The median serum HBV DNA level decreased from 8.8 to 5.0 log copies/mL among the patients in group A (\( P < .001 \)) but did not decrease for patients in group B (figure 2).

In addition, we compared the reduction in e antigen level for these 2 groups. Levels were reduced in both groups, but the median reduction was more prominent for patients in group A than for those in group B (figure 3). All 4 exacerbations coupled with e antigen seroconversion (from positive to negative) were associated with marked increase in hypermutated genomes (figure 3).

Effect of IFN treatment on the rate of HBV hypermutation in chimeric mice. Next, we examined the effect of IFN treatment on G-to-A hypermutation in HBV genomes in human hepatocyte–chimeric mice. Two mice were intravenously injected with supernatant produced by HepG2 cells transiently transfected with a plasmid containing 1.4–genome length wild-type HBV genomes. Ten weeks later, after confirmation of high-level HBV viremia, the mice were treated with 7000 IU/g/day of IFN-\( \alpha \), injected intramuscularly, for 14 days. We observed a \( > 1.5 \)-log reduction in plasma HBV DNA level accompanied by an increase in the number of hypermutated genomes in both mice (figure 4A). In a mouse inoculated with HBV but treated with phosphate-buffered saline, no increase of hypermutated genomes was observed (figure 4B). We also observed a 36-fold increase in the level of APOBEC3G mRNA, as determined by human oligonucleotide microarray (data not shown).

Infectivity of hypermutated genomes. To study the biological significance of hypermutated genomes, culture supernatant from HepG2 cells transfected with both HBV and APOBEC3G (5 \( \mu \)g each) was injected into a chimeric mouse. As shown in figure 5, the culture supernatant contained a large number of hypermutated genomes. In contrast, we could not detect hypermutated genomes in the chimeric mouse inoculated with this.
supernatant (figure 5A and 5B). These results suggest that the infectivity (or replication ability) of HBV with hypermutated genomes is very poor. It is possible that the inoculum contained less abundantly mutated genomes. To test this, we cloned and sequenced 72 clones of 217-bp DNA fragments amplified at a denaturation temperature of 95°C. Of 72 clones obtained from the inoculum, we found 1 clone with 8 G-to-A substitutions, 1 clone with 5 substitutions, 2 clones with 3 substitutions, and 1 clone with 1 substitution (figure 5C). In contrast, 1 of the 72 clones obtained from the mouse serum had 1 G-to-A substitution. If G-to-A substitutions were excluded, the only other nucleotide substitution observed in the 144 clones sequenced was a single C-to-T substitution.

DISCUSSION

In a previous study, we found that the majority of serum samples obtained from HBV-infected patients contained a small number of hypermutated genomes [27]. Recently, we developed a method (TaqMan 3D-PCR) to measure small numbers of hypermutated genomes [28]. Using this method, we reported dual antiviral effects for APOBEC3G, namely induction of hypermutation and reduction of viral replication. We also reported that IFN increased the transcription of APOBEC3G and enhanced the effect of the protein in vitro [28]. Other investigators also showed that IFN enhances the action of APOBEC proteins against HIV [18–21]. It is thus assumed that the antiviral effect of APOBEC proteins should be enhanced by IFN and other cytokines in vivo.

In the present study, we showed that an increase in ALT level accompanied by an increase in the number of hypermutated genomes was associated with reduction in the plasma HBV DNA level. In contrast, no decrease in HBV DNA level was observed if the increase in ALT level occurred in the absence of an increase in the number of hypermutated genomes. It is difficult to know which of the two antiviral effects of APOBEC3G (or other APOBEC proteins) reduced the viral level. It is also impossible to estimate the importance of APOBEC proteins in this reduction. However, it is clear that the increase in the number of hypermutated genomes of HBV correlates with activation of the host antiviral defense against HBV.

We also demonstrated that exacerbations of HBV infection associated with a marked increase in the number of hypermutated genomes were associated not only with a decrease in the plasma HBV DNA level but also with clearance of e antigen.
Furthermore, all exacerbations followed by seroconversion to positivity for antibody against e antigen were associated with a marked increase in the number of hypermutated genomes. Clearance of e antigen often results from a G-to-A nucleotide substitution at the first position of a 5'-GGGG stretch in the precore coding sequence (the G1896A mutation). Because this substitution (changing TGGGG to TAGGG) is in agreement with the dinucleotide pattern preferentially edited by APOBEC3G, one might assume that G-to-A substitution in this region could be caused by this enzyme and is related to the clearance of e antigen. However, we observed that hypermutation was induced in only some genomes, whereas the majority of genomes were unaffected. Thus, it seems unlikely that APOBEC proteins play a role in seroconversion to positivity for antibody against e antigen, although it is still possible that the 5'-GGGG stretch in the precore region is the preferred editing site for the enzyme. Importantly, such substitution of the 5'-GGGG stretch should result in the occurrence of multiple stop codons (TAG, TGA, and TAA) in HBV genomes, as we observed and reported in our previous study [28], which makes the replication of mutated genomes impossible.

In the present study, we did not observe any increase in the number of hypermutated genomes during IFN therapy in some patients. This finding is discrepant from the results of previous in vitro experiments that showed increased numbers of hypermutated genomes after the application of IFN [28]. Interestingly, our experimental results also showed the induction of APOBEC3G gene expression, an increase in the number of hypermutated genomes, and a reduction of plasma HBV DNA level in 2 human hepatocyte–chimeric mice treated with IFN (figure 4). What is the reason for the lack of increase in hypermutation in some IFN-treated patients? We usually administer IFN to patients who have high ALT levels. The patients in this study had abnormal ALT levels prior to treatment with IFN—that is, their livers were inflamed, and the levels of many cytokines produced by the immune cells in the liver were already high. We presume that the effect of these elevated cytokine levels masked the effect of the IFN we administered. It could also be argued that the effect

Figure 4. Effect of interferon (IFN)–α therapy on hepatitis B virus (HBV) hypermutation in HBV-infected, human hepatocyte–chimeric mice. Two chimeric mice (A) were inoculated with recombinant wild-type HBV produced by transfected HepG2 cells; 10 weeks later, after confirmation of high-level HBV viremia, they were treated with IFN-α at 7000 IU/g/day for 14 days, by intramuscular injection. Upper panels in both parts of A, serum HBV DNA levels and the number of hypermutated genomes; lower panels in both parts of A, human serum albumin concentrations. Note that the albumin levels are stable during IFN-α therapy. A control mouse (B) was inoculated with recombinant wild-type HBV produced by transfected HepG2 cells and treated with phosphate-buffered saline (PBS). Upper and lower panels of B show the same information as in A.
observed in mice represents the absence of the immune response in mice, whereas the lack of a clear response to IFN in the study patients was the result of the complex immune response in human beings. Alternatively, the concentrations of IFN in treated patients might be lower than those used for the cell culture or the chimeric mice. Although we did not perform this analysis in the present study, it would be interesting to determine the expression levels of APOBEC proteins and IFN-stimulated genes in the liver of IFN-treated patients.

The present study showed that the number of hypermutated genomes increased during some increases in ALT level, probably as a result of IFN-activated APOBEC proteins and other cytokines in patients with chronic hepatitis B. However, the number of hypermutated genomes was very small, only 28,378 in 10^6 HBV genomes at most (figure 1A). Because it was possible that the less abundantly hypermutated genomes were not detected (i.e., that genomes with only 1 or 2 G-to-A substitutions were not amplified by 3D-PCR), cloning and sequencing were performed to detect such genomes. However, the number of genomes containing G-to-A substitutions was still low (5 [6.9%] of 72 clones), even in the culture medium of HepG2 cells cotransfected with APOBEC3G and HBV (figure 5C). This means that the number of genomes with only a small number of G-to-A substitution was not high, suggesting that only selected DNA molecules were heavily mutated while the remaining DNA was not. Does this mean that the effect of APOBEC proteins in antiviral defense is trivial in patients with chronic HBV infection? It is a possible that the heavily deaminated genomes are an easy target for uracil DNA glycosylase. Although the dual antiviral effects of APOBEC proteins are currently known to reduce the amount of HBV, the importance and magnitude of APOBEC proteins with respect to in vivo virus reduction should be investigated further.

Treatment of patients with chronic HBV infection has improved with the advent of new nucleoside and nucleotide analogues. However, reactivation of HBV and flare-ups of hepatitis are often seen in patients who stop such therapy. Furthermore, hepatitis B surface antigen clearance is rare in patients treated

Figure 5. Results for a human hepatocyte–chimeric mouse inoculated with hepatitis B virus (HBV) produced by HepG2 cells transfected with an equal amount (5 μg each) of HBV and apolipoprotein B messenger RNA editing enzyme, catalytic polypeptide–like 3G plasmids. The inoculum contained ~6.25% hypermutated genomes. A serum sample was obtained 10 weeks after the inoculation. A, HBV DNA was amplified by polymerase chain reaction (PCR) that used different denaturation temperatures and run on 2% agarose gel. B, Quantitative measurement of HBV DNA and hypermutated DNA in the inoculum and mouse serum. C, Number of G-to-A substitutions found in each of 72 clones obtained from products of PCR of culture supernatant or mouse serum.
with these antiviral drugs. On the other hand, most patients with chronic HBV infection achieve sufficient viral suppression and disease quiescence through immunological suppression of the virus. As we showed in this study, the immunological suppression of HBV is much stronger than that achieved with IFN therapy, but it is often transient. It is thus necessary to clarify the mechanism of transient immune response and to develop treatment that produces persistent suppression of HBV. Quantitative measurement of hypermutated genomes should be useful in monitoring the immune response in this context.

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References