Hypermutation of hepatitis B virus genomes by APOBEC3G, APOBEC3C and APOBEC3H

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Hepatitis B virus (HBV) is a DNA virus that causes liver disease and replicates by reverse transcription of an RNA template. Previous studies have reported that HBV genomes bearing G→A hypermutation are present at low frequency in human serum. These mutations are most likely due to the activity of apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like (APOBEC) cytosine deaminases, cellular proteins known to confer innate immunity against retroviruses by generating lethal hypermutations in viral genomes. This study assessed APOBEC3G, APOBEC3C and APOBEC3H, three members of this protein family present in human liver, for their ability to edit HBV genomes. Transfection of human HepG2 hepatoma cells with a plasmid encoding the APOBEC3C protein resulted in abundant G→A mutations in the majority of newly formed HBV genomes. By contrast, transfection of APOBEC3G- and APOBEC3H-encoding plasmids only marginally increased hypermutation rates above the level caused by the cytosine deaminases naturally present in HepG2 cells. APOBEC3G- and APOBEC3H-mediated hypermutation, however, was clearly revealed by transfection of chicken LMH hepatoma cells, which lack endogenous cytosine deaminases. These results indicate that APOBEC3G, APOBEC3C and APOBEC3H have the ability to edit HBV DNA and that each protein is likely to contribute to various degrees to the generation of modified genomes in human liver cells.

INTRODUCTION

Over the past few years, a novel intracellular defence mechanism has been discovered that protects humans from retroviral infection by inducing an error catastrophe in the genome of the pathogenic agent. It was found that human cells produce a particular set of enzymes, the apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like (APOBEC) proteins, which, if incorporated into viral capsids, deaminate cytidine bases in single-stranded DNA formed during reverse transcription of the RNA template. Deaminated cytidine forms base pairs equivalent to thymidine. Consequently, guanosine (G) is replaced by adenosine (A) during synthesis of complementary second-strand DNA and the virus fails to propagate in the infected host organism because of abundant mutations in the viral genome (Cullen, 2006; Harris & Liddament, 2004; KewalRamani & Coffin, 2003; Turelli & Trono, 2005; Wiegand & Cullen, 2007).

Hepatitis B virus (HBV) causes a broad spectrum of liver diseases in humans, and is a DNA virus that shares some molecular features of retroviruses (Ganem & Prince, 2004; Kao & Chen, 2002; Wright & Lau, 1993). Analogous to retroviral replication, synthesis of the HBV genome is preceded by reverse transcription of a pre-genomic RNA template. Elongation of HBV DNA, however, is almost completed in the producer cell and viral particles circulating in the blood contain a circular, almost fully double-stranded DNA genome (Nassal & Schaller, 1993).

In earlier studies, it was discovered that HBV genomes with G→A hypermutation were present at low frequency in human serum (Gunther et al., 1997; Suspene et al., 2005a). These findings suggest that APOBEC proteins can be trapped in HBV particles where they deaminate cytosine residues of the nascent viral DNA. In the present study, we addressed this issue and investigated which members of the APOBEC protein family potentially generate these hyper-mutated genomes in natural HBV infection.

METHODS

Plasmids and transfection. The HBV construct used for transfection of HepG2 cells was derived from HBV subtype ayw and harboured a 5′-terminal redundancy ranging from the NsiI site at position 2346 to the BglII site at position 84 relative to the core protein start codon. This redundancy enabled transcription of a pre-genomic RNA molecule encoding all viral proteins. A different HBV plasmid construct with the natural core promoter substituted by the cytomegalovirus (CMV) promoter was used for transfection of chicken hepatoma LMH cells (Nassal, 1992). The CMV promoter enabled efficient transcription of pre-genomic RNA and subsequent viral replication in the chicken LMH cells. The duck HBV (DHBV) plasmid construct used to transfect LMH cells had a redundancy...
ranging from the BamHI site at position 1658 to the SphI site at position 2850 relative to the EcoRI restriction site in the DHV core reading frame (Schlicht et al., 1989).

The APOBEC3C expression plasmid was derived from an RT-PCR fragment, which was amplified from RNA of human peripheral blood mononuclear cells (PBMCs). A HindIII site was introduced 23 nt upstream from the APOBEC3C start codon (forward primer 5’-GGGACAAACCTATCTAAAGACCTG-3’) and a XhoI restriction site was introduced 25 nt downstream from the APOBEC3C stop codon (reverse primer 5’-GACCTCAGGACCCAGGAGACCC-3’). Following amplification, the fragment was ligated into pcDNA3.1 cloning vector (Invitrogen) and the accuracy of the construct was verified by sequencing of the recombinant plasmid. The APOBEC3H expression plasmid was generated essentially the same way by cloning PCR-amplified cDNA from human PBMCs into the HindIII and XhoI sites of pcDNA3.1. The restriction sites were introduced 30 nt upstream of the start codon (forward primer 5’-CTAAGGAAGCTTGGCCAGAACGACAG-3’) and 80 nt downstream of the stop codon (reverse primer 5’-GTCATTCTGTTGTCTGATTAATGAG-3’). The APOBEC3G-specific fragment was generated from the cDNA of PBMCs with forward primer 5’-TAAGCGGAATTCATGGAATCTCCATCTGTGAAAGAGATGG-3’ and reverse primer 5’-TCTTCCGTACAGTGGGCCGATCCCTCC-3’). The amplification product was digested with EcoRI and XhoI and ligated into the pcDNA3.1 vector.

A plasmid encoding a modified APOBEC3H with the influenza haemagglutinin (HA) epitope at the C-terminal end was created to enable Western blot detection of the protein. The APOBEC3H-coding sequence was amplified with forward primer 5’-ATGGCTCTGTTAACGACCCGAACATCCTTC-3’ and reverse primer 5’-TGACGTAACGTTGGCCAGAACGACAG-3’. The amplification product was digested with EcoRI and XhoI and ligated into the pcDNA3.1 vector.

Extraction and analysis of viral DNA. A 0.4 ml aliquot of the supernatant was digested with 30 U micrococcal nuclease (Abersham Biosciences) for 1 h at 37 °C in the presence of 2 mM CaCl₂ to remove the transfected plasmid DNA. The nuclease was inactivated by addition of EDTA to a final concentration of 5 mM. DNA protected in viral capsids was extracted using a QiAamp DNA Mini kit (Qiagen) with RNase A treatment included as recommended by the manufacturer. The absence of transfected plasmid DNA was verified by PCR amplification using vector-specific primers. DNA samples were loaded onto a 1.3% agarose gel next to HBV or DHBV size marker fragments. The DNA was blotted onto nylon membranes (Abersham Biosciences) and probed with a 32P-labelled full-length HBV or DHBV genome in QuikHyb hybridization solution (Stratagene).

HBV DNA was amplified with Taq polymerase using forward primer HBV F949 (5’-TGCGGCAAATCTCTTCACC-3’) and reverse primer HBV R2855 (5’-CAGGCGATGAGAAGCGCAGAC-3’). The nomenclature of the primers is according to their position in the HBV genome relative to the core protein start codon. Initial denaturation was for 5 min at 94 or 88 °C, followed by 22 cycles of 30 s at 94 or 88 °C, 30 s at 55 °C and 2 min at 72 °C, with a final extension for 7 min at 72 °C. The PCR fragments were visualized on an ethidium bromide-stained agarose gel along with a 1 kb DNA ladder from Invitrogen. Alternatively, the PCR fragments were ligated into a TOPO TA cloning vector (Invitrogen). Sequencing of plasmid DNA from individual colonies was performed by a commercial sequencing service facility (SeqLab). About 300 nt of plus-strand DNA were routinely read using forward primer HBV FI276 (5’-AGTTGAAATCCCAACCTC-3’).

The DHBV genome was amplified with primers DHBV F350 (5’-CAGGCGAAGCTTCCACCC-3’) and DHBV R2445 (5’-CTGGAGAAGGCGCCGTACAGTGGATAGGACTG-3’). The APOBEC3G transcript was detected with forward primer A3G F537 (5’-ATGGCTCTGTTAACGACCCGAACATCCTTC-3’) and reverse primer A3G R669 (5’-GACTGACCCAGGAGACCCGACAG-3’). Cycling conditions were 25 cycles of 30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C. The PCR products were visualized on an ethidium bromide-stained agarose gel. Alternatively, PCR fragments obtained using a denaturation temperature of 94 °C were ligated into a TOPO TA cloning vector (Invitrogen) and the recombinant plasmids were sequenced with primer DHBV F680 (5’-TAAACCCGCTCTATGAAGCA-3’).

Detection of cellular transcripts. HepG2 cells were cultured and harvested as described above. PBMCs were isolated from human blood by Ficoll gradient centrifugation. Total RNA from HepG2 and PBMCs was prepared using an RNeasy protocol (Qiagen). Cell lysates were passed through QIAshredder columns (Qiagen) to reduce viscosity and the RNA samples were digested with RNase-free DNase I (Roche) to remove chromosomal DNA.

Oligo(dT)-primed cDNA was synthesized with Moloney murine leukemia virus reverse transcriptase (Invitrogen) and amplified with APOBEC3G-specific forward primer A3G F65 (5’-AAAAACCTATGGGGAAGGCAACG-3’) and reverse primer A3G R357 (5’-ACTGGAGAAGCTTCCCTCCGCC-3’) using cycling conditions of 27 cycles of 30 s at 94 °C, 30 s at 55 °C and 1 min at 72 °C. The APOBEC3H transcript was detected with forward primer A3H F1 (5’-ATGGCTCTGTGATACGGAAGAAACATTCCG-3’) and reverse primer A3H R353 (5’-CTTCTCAAGCCGGCTGGTTATGAGC-3’) using 30 amplification cycles. The PCR products were visualized on 1.5% standard agarose gels.

The APOBEC3G transcript was detected with forward primer A3G F537 (5’-GAGGCGTACGATTAACTCTGCC-3’) and reverse primer A3G R669 (5’-CATTGGCAGTACGACCCTC-3’). Cycling conditions were 25 cycles of 30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping transcript was detected with forward primer GAPDH F6 (5’-GAGGCTGGAAGTAGCTGAGTC-3’) and reverse primer GAPDH R229 (5’-GAAGATGGTGATGAGGATTTC-3’) and 22 cycles were carried out as described above. The APOBEC3G- and GAPDH-specific amplification products were analysed on 2.5% MetaPhor (Bioproducts) agarose gels.

A human liver cDNA library was obtained from Invitrogen. The transformed Escherichia coli cells were serially diluted, inoculated in Terrific Broth containing ampicillin and kept at 37 °C for 12 h with constant shaking. Plasmid DNA was purified from the E. coli cells by ion-exchange chromatography. DNA (10 ng) from each library sample was amplified with the APOBEC3G-, APOBEC3C-, APOBEC3H- and GAPDH-specific primers as described above.
Western blot detection of APOBEC proteins. LMH and HepG2 hepatoma cells were transfected with plasmids encoding the HA-tagged APOBEC proteins as described above. Cytoplasmic lysates were separated on 12% polyacrylamide gels and the proteins were transferred to Immobilon-P membranes (Millipore) and visualized with a monoclonal antibody specific to the HA epitope (Cell Signalling) using an ECL Plus Western blotting detection system (GE Healthcare).

RESULTS

Endogenous APOBEC activity in human liver cells

Recently, a novel PCR technique has been introduced that allows selective amplification of hypermutated DNA (Suspene et al., 2005a). The technique is based on lowering the denaturation temperature in the PCR protocol, thereby melting the DNA template depending on its AT content (Suspene et al., 2005b). As seen in Fig. 1, amplification of viral DNA with a low denaturation temperature of 88 °C resulted in weakly positive signals. These PCR products were derived from hypermutated HBV genomes as verified by sequence analysis of the cloned fragments (data not shown). The hypermutated genomes are formed by endogenous cytosine deaminases in virus-producing hepatoma cells and are also found in serum from HBV-infected individuals (Gunther et al., 1997; Noguchi et al., 2005; Suspene et al., 2005a).

To identify the enzymes potentially generating the modified HBV genomes, we first investigated transcription of APOBEC protein-coding genes in HepG2 cells and human liver tissue. Positive PCR signals were obtained for the APOBEC3G, APOBEC3C and APOBEC3H transcripts with cDNA from HepG2 cells (Fig. 2a) and human liver tissue (Fig. 2b). Gene expression in the liver was highest for APOBEC3G, followed by APOBEC3C and APOBEC3H. HepG2 cells but not liver cDNAs were also positive for APOBEC3B and APOBEC3F transcripts (data not shown). Amplification experiments with APOBEC2- or APOBEC3A-specific primers resulted in very faint signals, and APOBEC1- or APOBEC3DE-specific sequences were not detectable (data not shown).

APOBEC-mediated hypermutation of HBV genomes in HepG2 cells

Our subsequent studies focused on APOBEC3G, APOBEC3C and APOBEC3H because the corresponding genes were found to be transcribed in human liver.

Fig. 1. Selective amplification of hypermutated viral DNA. HBV-encoding plasmid and viral DNA from HBV-producing HepG2 cells were amplified using denaturation temperatures of 94, 90, 88 or 86 °C. Lane H2O is the negative control without template DNA. M, DNA size marker. A faint signal was visible for viral DNA amplified using a denaturation temperature of 88 °C, which was due to hypermutated genomes formed by endogenous cytosine deaminases in HepG2 cells.

Fig. 2. Detection of APOBEC protein-coding transcripts. (a) RNA from HepG2 cells was amplified by RT-PCR with primers specific for APOBEC3G (A3G), APOBEC3C (A3C), APOBEC3H (A3H) or GAPDH. RNA from PBMCs was included as a positive control, as expression of APOBEC genes in PBMCs has been reported previously (Wedekind et al., 2003). Reverse transcriptase (RT) was omitted as a negative control, as indicated. (b) DNA from a human liver cDNA library was amplified with APOBEC- or GAPDH-specific primers. The relative number of individual cDNA clones present in each library sample is indicated below. Accordingly, APOBEC3G-coding clones were present at least 30 times less frequently in the library than those encoding GAPDH. The APOBEC3G-positive clones were present three and 30 times more frequently than those encoding APOBEC3C and APOBEC3H, respectively. M, DNA size marker.
Expression plasmids encoding the respective proteins were generated and transfected into HepG2 cells, together with a construct harbouring the HBV genome. Viral DNA formed in the co-transfected cells was prepared and visualized by Southern blot analysis. As shown in Fig. 3(a), co-transfection of the APOBEC3G-coding plasmid drastically reduced the amount of viral DNA. Side-by-side comparison of serially diluted DNA samples revealed an approximate 30-fold decrease (data not shown). This finding confirmed previous reports describing a strong inhibitory effect of APOBEC3G on HBV replication (Nguyen et al., 2007; Rosler et al., 2005; Turelli et al., 2004).

A second aliquot of the viral DNA was analysed by PCR using denaturation temperatures of 94 and 88 °C. For the APOBEC3C co-transfections, strong signals were obtained with a low denaturation temperature of 88 °C (Fig. 3b). This finding indicated that overexpression of APOBEC3C in HepG2 cells resulted in high levels of mutated viral DNA. The PCR fragments obtained with a denaturation temperature of 94 °C were further ligated into a cloning vector for sequence analysis. G→A mutations were found at high frequency in most of the clones tested (Table 1). This result confirmed the PCR analysis presented in Fig. 3(b) and clearly demonstrated that APOBEC3C mediates HBV genome editing in the majority of viral particles.

Amplification products obtained from APOBEC3G and APOBEC3H co-transfected cells were also analysed by cloning and sequencing. Mutated genomes, however, were not detected among 15 individual clones tested for each transfection. Nonetheless, weak PCR signals were visible for the corresponding DNA samples if amplified at a low denaturation temperature of 88 °C (see Fig. 3b, lower panels). This result indicated that transfection of the APOBEC3G and APOBEC3H plasmids may have created hypermutated genomes to a minor extent. Assessment of low-level editing activity, however, is limited by the cytosine deaminases naturally present in HepG2 cells, which cause hypermutation in almost 2 % of viral genomes (Rosler et al., 2004). The APOBEC3 gene cluster is known to be absent in birds (Conticello et al., 2005). We therefore decided to perform further investigations with chicken hepatoma LMH cells to avoid the background effect of endogenous APOBEC activity in HepG2 cells.

**APOBEC-mediated hypermutation of HBV genomes in LMH cells**

LMH cells were co-transfected with an HBV genome construct and the respective APOBEC expression plasmids. Viral DNA formed in the transfected cells was visualized by Southern blot analysis. As shown in Fig. 4(a), the amount
of viral DNA was drastically reduced by co-transfection of the APOBEC3G plasmid, indicating that the protein strongly inhibits HBV replication in LMH cells, similar to in HepG2 cells. Amplification of viral DNA using a low denaturation temperature of 88 °C resulted in strong PCR signals for APOBEC3C-transfected cells, suggesting that the human APOBEC3C protein efficiently hypermutates HBV genomes in LMH cells (Fig. 4b). Positive, albeit faint, PCR signals were seen for APOBEC3G and APOBEC3H co-transfection, indicating the presence of some mutated viral DNA. For a more detailed examination, amplification products obtained using a denaturation temperature of 94 °C were ligated into a cloning vector and analysed by sequencing. Screening of 15 individual clones revealed three strongly mutated genomes formed in APOBEC3G-transfected LMH cells (Fig. 5). Among 30 clones tested for the APOBEC3H co-transfection experiment, three clones carried G→A mutations. Thus, the human APOBEC3G and APOBEC3H proteins indeed produce hypermutated HBV genomes in transfected LMH cells.

### Table 1. APOBEC3C-mediated hypermutation of HBV genomes in HepG2 cells

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APOBEC-mediated hypermutation of DHBV genomes in LMH cells

The LMH cell line is commonly used for research on DHBV, an avian hepadnavirus that naturally infects ducks. DHBV and HBV share the same replication strategy, but differ significantly in the primary structure of their viral proteins. We addressed the question of whether the human APOBEC proteins were potentially active in DHBV particles. Thus, LMH cells were co-transfected with a DHBV genome construct and the APOBEC protein-coding plasmids. As shown in Fig. 6(a), DHBV replication was reduced by co-transfection of APOBEC expression plasmids, with APOBEC3G again having the strongest effect.

Amplification of viral DNA using a low denaturation temperature of 84 °C generated strong PCR signals for all
three APOBEC co-transfection experiments (Fig. 6b). Sequence analysis of cloned PCR fragments, which were obtained using a high denaturation temperature of 94 °C revealed abundant G→A mutations in almost every plasmid tested (Fig. 6c). This result clearly showed that the human APOBEC3G, APOBEC3C and APOBEC3H proteins generate hypermutated DHBV genomes upon expression in chicken LMH cells.

Synthesis of APOBEC proteins in transfected cells was visualized by Western blot analysis. As shown in Fig. 7, APOBEC3G and APOBEC3C proteins were strongly expressed in both HepG2 and LMH cells. Two signals were obtained for the APOBEC3C polypeptide in HepG2 cells, suggesting that the protein is modified by ubiquitination to some extent. Expression levels of APOBEC3H were low, especially in HepG2 cells, which may account for the lack of significant antiviral activity of the protein in these cells.

**DISCUSSION**

Strong inhibition of HBV replication by APOBEC3G has been reported previously in several studies. This inhibitory effect is presumably due to interference of the protein with encapsidation of pre-genomic RNA and inhibition of viral DNA synthesis in the respective particles (Nguyen et al., 2007; Rosler et al., 2005; Turelli et al., 2004). Further investigations revealed hypermutation of HBV genomes in some of the viral particles that were still formed in the presence of APOBEC3G (Noguchi et al., 2007; Rosler et al., 2004). Our data confirm these earlier reports. In addition, the results obtained with LMH cells unambiguously prove that APOBEC3G is indeed capable of hypermutating HBV genomes. As APOBEC3G is clearly expressed in human liver, it is reasonable to assume that this protein contributes to HBV hypermutation during natural infection.

APOBEC3C is another member of the protein family, for which liver-specific transcription has been observed in our study and previously (Bonvin et al., 2006; Noguchi et al., 2005). In contrast to APOBEC3G, the APOBEC3C protein only slightly inhibits HBV replication but produces hypermutated DNA molecules at high frequency in transfected HepG2 cells. Similar results have also been obtained with the Huh7 hepatoma cell line (Baumert et al., 2007). Therefore, APOBEC3C most likely generates the most significant number of mutated HBV genomes in human hepatocytes.

Low amounts of APOBEC3H-specific transcripts were detectable in HepG2 cells and liver tissue. APOBEC3H was found to edit HBV genomes at moderate frequency, probably due to low protein expression levels, especially
in transfected HepG2 cells. Therefore, APOBEC3H presumably plays a minor role in the production of mutated HBV genomes in the liver. The human APOBEC3H protein has been reported previously to lack significant effects on primate lentiviruses in mammalian cells (Dang et al., 2006; OhAinle et al., 2006). Our findings, however, demonstrated that APOBEC3H is very efficient at editing DHBV genomes, suggesting that the viral target has a major effect on the activity of the protein.

Our studies so far have focused on three APOBEC proteins that are most likely present in human liver. Further investigations are needed to determine the extent to which other members of the protein family are able to modify hepadnaviral genomes. These studies will need to include experiments with DHBV and HBV in LMH cells to detect enzymatic activities that may remain unnoticed in human cells and to investigate more specifically the properties of the respective proteins in the context of different virus targets.

Finally, it remains to be established whether APOBEC-mediated hypermutation of HBV genomes is of relevance during the natural course of infection. Interestingly, expression of APOBEC-coding genes in primary human hepatocytes has recently been reported to be stimulated by interferon (IFN)-α treatment (Bonvin et al., 2006; Tanaka et al., 2006). More recently published data, however, indicate that blocking of APOBEC proteins does not eliminate the inhibitory effect of IFN on viral DNA synthesis (Jost et al., 2007). Nevertheless, the APOBEC proteins may contribute to the clearance of infection by enhancing viral genome editing. Therefore, further studies are needed to examine whether the relative abundance of hypermutated DNA in human serum actually increases during IFN-α therapy.

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