Response to Comment on “Specific and nonhepatotoxic degradation of nuclear hepatitis B virus cccDNA”

Yuchen Xia, Julie Lucifora, Florian Reisinger, Mathias Heikenwalder, Ulrike Protzer

Chisari et al. challenge our central conclusion that the hepatitis B virus (HBV) persistent form, the covalently closed circular DNA (cccDNA), is degraded in a noncytotoxic and specific fashion in the nucleus of infected hepatocytes. Specificity of the assays used, exclusion of cell division or death, and activity of APOBEC3 deaminases in the nucleus, however, were addressed in the paper.

Our work reported in (1) on hepatitis B virus (HBV), and how cure of hepatitis B can occur, was stimulated by the findings of Guidotti et al. and Wieland et al., who observed that clearance of replicating HBV and HBV covalently closed circular DNA (cccDNA) occurs before full-blown cytotoxicity of T cells affects the liver in acute hepatitis B (2,3), and aimed at understanding the underlying mechanisms of clearance. We are convinced that APOBEC editing of cccDNA is one important mechanism for how HBV infection can be controlled and cured, in addition to T cell and natural killer–cell mediated killing of infected hepatocytes (2,3); control of HBV transcription and replication by interferon (IFN)-α, IFN-γ, and tumor necrosis factor (4,5); and loss of cccDNA during cell division (6).

We would like to address the specific questions raised by Chisari et al. (7) as follows:

1) Was the quantitative polymerase chain reaction (qPCR) assay used in this study cccDNA-specific? For cccDNA PCR we used primers that selectively detect HBV cccDNA by spanning the nick and gap in the viral genomic DNA (rcDNA), as well as by a Light Cycler protocol with annealing conditions selecting against amplification of incompletely double-stranded forms of HBV DNA. As a result, PCR was selective at more than 10^-5 for amplification of cccDNA over cytoplasmic or extracellular rcDNA. We refrained from using Hirt extraction in favor of total DNA extraction because the remaining high salt concentration from Hirt extraction interferes with reliable linear amplification in qPCR and thus exact quantification of cccDNA. cccDNA qPCR followed published protocols (6,8), with slight modifications in primer design, and underwent extensive inter- and intra-assay validation. Quantification was confirmed by Southern blot quantification using HepaRG cells (figures 2C, SID, and S1B of (1) and the HepG2-HL3 cell line. Use of these cell lines provided easily detectable amounts of HBV cccDNA and allowed quantification relative to integrated HBV-DNA.

Selection of the cccDNA qPCR was confirmed using serum and plasma from HBV-infected patients, purified HBV virions, and tail and liver DNA from HBV-transgenic mice, because cccDNA is not generated in mice (10,11). Most important, experiments were repeated in the presence of, or after pretreatment with, different nucleoside analogs at doses that inhibited the formation of non-cccDNA replicative intermediates (figures 1D, F, and 2B of (1)). Identical effects in the presence of nucleotide analogs confirmed the effect of IFN-α and lymphotxin β receptor (LTβR) agonists on cccDNA.

2) Was IFN-α or LTβR-mediated cccDNA loss noncytotoxic, or due to cell death or regeneration? We did not detect any signs of cytotoxicity (cell death, reduced metabolic activity, caspase activation, or aminotransferase release) nor of cell proliferation at the doses of IFN-α or LTβR agonists used in our experiments (figures 2A, S1B, and S2H in (1)). To minimize the risk that cell proliferation influences the loss of cccDNA (6), we changed our test systems from hepatoma cell lines to either HepaRG cells differentiated for 4 weeks or to freshly isolated primary human hepatocytes. Experiments using these cells were more tedious but conferred the advantage that neither differentiated HepaRG cells nor primary hepatocytes proliferate considerably. Further, they generate little new cccDNA by rcDNA recycling once HBV infection is established. Specific degradation of cccDNA, but not of an HBV-sequence containing episomal replicon, strongly indicated noncytotoxic cccDNA degradation (figure S16 in (1)). Most important, complete restoration of cccDNA levels after HIV-IV overexpression and APOBEC3A or APOBEC3B siRNA knock-down (despite ongoing IFN-α or LTβR activation, respectively) verified that cccDNA loss was noncytotoxic (figure 4, A to C, in (1)).

3) Did APOBEC3s edit cccDNA (nuclear) or just replicative intermediate (cytoplasmic) HBV DNA? As elaborated above, the qPCR used amplifies cccDNA over other forms of HBV DNA with a specificity of at least 10^-5. Differential DNA denaturation (3D) PCR was based on the cccDNA-selective first PCR, and results obtained by sequencing of 3D PCR products are confirmed by next-generation sequencing of cccDNA PCR products. Owing to space restrictions, however, not all the data could be presented in the manuscript. To show that degradation occurs in the nucleus, on established cccDNA, we pretreated HepaRG cells infected with HBV for 10 days with high-dose (1000 times the median effective concentration) entecavir (ETV) to block reverse transcription and synthesis of cytoplasmic HBV-DNA, to deplete the cells of cytoplasmic HBV-DNA, and to prevent replenishment of cccDNA by the recycling of newly formed rcDNA. Despite ETV pretreatment, IFN-α induced cccDNA degradation (figure S7D in (1)) and degradation (figure 1F in (1)), whereas ETV treatment alone did not show cccDNA quantitation or its integrity. These experiments led us to conclude that cytidine degradation occurred on established nuclear cccDNA.

4) Is therapeutic LTβR activation tenable in the context of chronic HBV infection? We are, as stated in (1), well aware of potential side effects that long-term (6 to 12 months) stimulation of LTβR may have (12,13). Short-term trigger of the LTβR, however, was well tolerated by HepaRG cells and primary hepatocytes (no detectable cell death and no release of transaminases were observed) and showed no obvious toxicity in HBV transgenic mice. Thus, one of several leads for HBV therapy may be to induce nuclear deaminases as we proposed in our manuscript: “inducing nuclear deaminases...allows development of new therapeutics that, combined with existing antivirals, may cure hepatitis B” (1).

REFERENCES AND NOTES


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