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

Francis V. Chisari, William S. Mason, Christoph Seeger

Lucifora et al. (Research Articles, 14 March 2014, p. 1221) report that the hepatitis B virus (HBV) transcriptional template, a long-lived covalently closed circular DNA (cccDNA) molecule, is degraded noncytolytically by agents that up-regulate APOBEC3A and 3B. If these results can be independently confirmed, they would represent a critical first step toward development of a cure for the 400 million patients who are chronically infected by HBV.

Viral clearance during acute hepatitis B virus (HBV) infection is mediated by CD8-positive cytotoxic T lymphocytes (CTL) that kill infected cells and secrete antiviral cytokines that noncytolytically inhibit HBV gene expression and replication. In contrast, the CTL-induced mechanisms that mediate clearance of the nuclear pool of viral covalently closed circular DNA (cccDNA), the long-lived transcriptional template of HBV, have been difficult to resolve, largely because of the absence of a cell-based HBV-infection system. Based on in vivo experiments in duck, woodchuck, mouse, and chimpanzee animal models, two competing, but not mutually exclusive, hypotheses of cccDNA clearance have emerged.

On one hand, there is ample evidence that cccDNA elimination requires the turnover of infected hepatocytes in ducks infected with duck hepatitis B virus (DHBV) and in woodchucks infected with woodchuck hepatitis virus (WHV), in which a large fraction of the infected hepatocyte population is killed and replaced by hepatocyte cell division (7). On the other hand, CTL-induced, interferon gamma (IFN-γ)-mediated clearance of HBV replicative DNA intermediates is well documented in HBV transgenic mice (2), and clearance of HBV in acutely infected chimpanzees is initiated by the influx of IFN-γ-producing CD8 T cells and characterized by a disproportionate loss of both cytoplasmic HBV replicative DNA intermediates and nuclear cccDNA relative to multiple simultaneous markers of cell death (3, 4).

The latter results suggest that cccDNA may be susceptible to noncytolytic clearance by antiviral cytokines; that is, clearance not requiring the death or proliferation of infected cells. Confirmation of noncytolytic cccDNA clearance in experimentally tractable systems would be a major step forward in finding antiviral therapies for chronic hepatitis B. We were therefore very encouraged by the paper by Lucifora et al. (5) reporting that high-dose interferon alpha (IFN-α) and lymphopoxin β receptor (LTβR)-mediated stimulation of APOBEC3A or 3B expression noncytopathically reduces cccDNA levels in differentiated HepaRG cells and in primary human hepatocytes. We were also encouraged by evidence suggesting that this loss might include, as an intermediate step, a detectable level of cccDNA depurination. However, after a careful reading of this Report, which entails a wealth of technically difficult experiments, we believe several critical points warrant further investigation before these conclusions can be exploited.

1) Was the quantitative polymerase chain reaction (qPCR) assay used in this study cccDNA-specific? Quantifying HBV cccDNA in infected cells is extremely challenging because of the vast excess of non-cccDNA replicative forms in total DNA extracts like those that were used for qPCR analysis in this study. However, cccDNA can be physically separated from other viral DNA forms by Hirt extraction, allowing specific qPCR amplification. Also, it coisolates with mitochondrial DNA that can be used to normalize results. Although Hirt extraction was used to prepare cccDNA before Southern blot analysis shown in figure 2C and SID of (5), the results were not normalized against mitochondrial DNA, so the small lane-to-lane differences observed in those figures could reflect differences in extraction efficiency or loading rather than true differences in cccDNA. Moreover, qPCR assays for cccDNA did not employ Hirt extraction, raising concerns about their specificity. Hopefully, future studies employing both Hirt extraction and mitochondrial DNA normalization for qPCR analysis will confirm that cccDNA can be noncytopathically degraded by IFN-α and LTβR-mediated APOBEC3 editing, as reported by Lucifora et al. (5).

2) Was IFN-α- or LTβR-mediated cccDNA loss noncytopathic, or due to cell death or regeneration? This study is based on specialized cell culture systems where, typically, only a fraction of cells are productively infected. Hence, these cells likely differ from the bulk of cells in the culture and may be more sensitive to high-dose IFN-α- or LTβR-mediated toxicity. Because infection levels in this study were not reported, it is a formal possibility that some or all of the cccDNA decline is due to cell death. This might not have been apparent if very few of the cells are infected. cccDNA loss at division could also be a factor if infected cells divided. LTβR agonists have been shown to trigger tumor cell apoptosis (6) and proliferation (7), both of which might explain the observed loss of cccDNA. LTβR-induced hepatocellular proliferation almost certainly occurred in some of their experiments, because it is the only known mechanism that can explain the disappearance of nuclear HBcAg in the HBV transgenic mouse hepatocytes shown in figure S6E of Lucifora et al. (7, 8). Thus, induced cell death and proliferation must be ruled out before the noncytopathic nature of the cccDNA loss can be accepted.

3) Did APOBEC3s edit cccDNA (nuclear) or just replicative intermediate (cytoplasmic) HBV DNAs? Members of the APOBEC3 family can deaminate the nascent, reverse transcribed (minus) DNA strand of HBV, leading to G to A hypermutation in plus strands (9). In this Report, hypermutation of the plus strand of cccDNA involved almost exclusively G to A mutations, suggesting that mutation preceded cccDNA formation from replication intermediates or was minus-strand specific on a cccDNA target. Distinguishing these possibilities, or even showing that the minus strand of cccDNA was the APOBEC3 target is difficult, because cccDNA accumulates at much lower levels (~0.1 to 1.0%) than the relaxed circular minus- and plus-strand DNA replicative forms in the cytoplasm of infected hepatocytes. The authors stated that their qPCR assay amplified cccDNA with 1000-fold higher specificity than the cytoplasmic DNA forms, but they did not offer evidence of that specificity. Moreover, the subsequent three-dimensional (3D) PCR, used to selectively amplify edited DNA for sequencing, did not have cccDNA specificity. Rather, this method would amplify any low-melting point, AT-enriched DNA present in the products of the first PCR. Hence, the possibility that the 3D PCR products actually originated from cytoplasmic DNA, not from cccDNA, has not been excluded. Quantitative differences between treated and untreated cells were also unclear.

4) Is therapeutic LTβR activation tenable in the context of chronic HBV infection? Because LTβR agonists have been shown to trigger apoptosis (6), hepatocellular proliferation (7), inflammation, and hepatocellular carcinoma (10, 11), safety considerations are likely to preclude regulatory approval of LTβR activation for the “development of new therapeutics” as suggested by Lucifora et al.

REFERENCES AND NOTES

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28 March 2014; accepted 15 May 2014
10.1126/science.1254082
Editor's Summary

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Science 344 (6189), 1237. [doi: 10.1126/science.1254082]

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