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Suppression of hepatitis B virus antigen production and replication by wild-type HBV dependently replicating HBV shRNA vectors in vitro and in vivo

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ABSTRACT

Chronic infection with hepatitis B virus (HBV), a small DNA virus that replicates by reverse transcription of a pregenomic (pg) RNA precursor, greatly increases the risk for terminal liver disease. RNA interference (RNAi) based therapy approaches have shown potential to overcome the limited efficacy of current treatments. However, synthetic siRNAs as well as small hairpin (sh) RNAs expressed from non-integrating vectors require repeated applications; integrating vectors suffer from safety concerns. We pursue a new concept by which HBV itself is engineered into a conditionally replicating, wild-type HBV dependent anti-HBV shRNA vector. Beyond sharing HBV's hepatocyte tropism, such a vector would be self-renewing, but only as long as wild-type HBV is present. Here, we realized several important aspects of this concept. We identified two distinct regions in the 3.2 kb HBV genome which tolerate replacement by shRNA expression cassettes without compromising reverse transcription when complemented in vitro by HBV helper constructs or by wild-type HBV; a representative HBV shRNA vector was infectious in cell culture. The vector-encoded shRNAs were active, including on HBV as target. A dual anti-HBV shRNA vector delivered into HBV transgenic mice, which are not susceptible to HBV infection, by a chimeric adenovirus-HBV shuttle reduced serum hepatitis B surface antigen (HBsAg) up to ~4-fold, and virus particles up to ~20-fold. Importantly, a fraction of the circulating particles contained vector-derived DNA, indicating successful complementation in vivo. These data encourage further investigations to prove antiviral efficacy and the predicted self-limiting vector spread in a small animal HBV infection model.

1. Introduction

Infection with hepatitis B virus (HBV), a small DNA virus that replicates through reverse transcription (Nassal, 2008), is a main cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) (Gish et al., 2015a; Trepo et al., 2014). Current therapies with type I interferon (IFN) and nucleos(t)ide analogues (NAs) have limited efficacy (Kang et al., 2015; Skupsky and Hu, 2014). IFN therapy is associated with frequent adverse effects while NAs suffer, apart from drug resistance, from the need for life-long

Abbreviations: HBV, hepatitis B virus; cccDNA, covalently closed circular DNA; CMV-IE, cytomegalovirus immediate early; dsL, double-stranded linear; FACS, fluorescence-activated cell sorting; NTCP, Na^+-taurocholate cotransporting polypeptide; ORF, open reading frame; RC-DNA, relaxed circular DNA; RNAi, RNA interference; shRNA, small hairpin RNA; siRNA, small interfering RNA; vge, viral genome equivalent; ss, single-stranded.

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treatment (Chevaliez et al., 2013), caused by the persistence in the host cell nucleus of the covalently closed circular (ccc) DNA form of the viral genome (Nassal, 2015). cccDNA serves as template for all viral transcripts. These include the pregenomic (pg) RNA that acts as mRNA for the viral core and polymerase proteins and as precursor for new DNA genomes, plus several subgenomic RNAs giving rise to the other viral antigens (Beck and Nassal, 2007). NAs can potently inhibit reverse transcription of pgRNA and thus formation of new virions, but RNA and antigen production remain largely unaffected. As large amounts of viral antigens may impair adaptive immune responses (Kondo et al., 2013) reduction of viral antigens is considered a desirable therapeutic goal.

RNA interference (RNAi) is a powerful technology for gene silencing and numerous reports have described the application of synthetic small interfering (si) RNAs or vector-expressed small hairpin (sh) RNAs to inhibit various viruses including HBV (recently reviewed in (Gish et al., 2015b; Marimani et al., 2013)). HBV is particularly vulnerable to RNAi because its replication depends directly on the pgRNA and, moreover, all its transcripts share common 3′ sequences such that a single RNAi effector may target more than one viral RNA. However, clinical application of anti-HBV RNAi still faces major challenges. Apart from off-target effects and potential undesired immune stimulation, synthetic siRNAs, despite recent advances in chemical stabilization and target-organ specific delivery (Gish et al., 2015b), require multiply repeated applications, as long as the cccDNA reservoir persists or until immunological control is achieved. Expression of stable hairpin (sh) RNAs also faces the problem of liver-specific delivery and the need to reach virtually all hepatocytes. Efficient delivery can be achieved by viral vectors (Appaiahgari and Vrati, 2015; Borel et al., 2014; Couto and High, 2010; Hutson et al., 2014). However, nonintegrating vectors will only transiently express the shRNA and require repeated administration which is hampered by anti-vector immunity (Hareendran et al., 2013; Lopez-Gordo et al., 2014). Integrating vectors pose their own risks (Rothe et al., 2013).

Most of these problems should be overcome by using HBV itself, in a conditionally replication-competent form, as shRNA delivery vehicle. Like naturally occurring defective HBV particles (Günther et al., 2000; Märschenz et al., 2008) such an HBV vector would have the identical tissue-specificity as genuine HBV and, if properly designed, be itself replication-defective but be trans-complementable by resident virus to form new vector particles that can spread; however, vector propagation would cease once wild-type HBV is silenced. Though attractive, several hurdles have to be overcome to prove this concept, foremostly the compact genetic organization of HBV. All nucleotides (nt) have coding function in one if not two open reading frame (ORFs; see Fig. 1A). Moreover, all regulatory elements and numerous replication cis-elements overlap with the ORFs (Beck and Nassal, 2007). Hence improper sequence modifications can easily preclude vector replication even if all trans-complementation factors are present. Second, the shRNA(s) delivered must per se be effective in targeting HBV and be produced in sufficient quantity to exert the desired RNAi effects, with minimal off-target effects. Third, the vector must not itself be targeted by the shRNA(s). Not the least, superinfection of HBV infected cells by an HBV-derived vector is likely inefficient, although the eventual takeover by drug resistant virus variants under NA therapy indicates that it does occur; moreover, in the woodchuck hepatitis virus model superinfection was recently directly shown (Rodrigues et al., 2015).

In this study, we addressed these issues by constructing a series of HBV vectors carrying at different genome positions one or two expression cassettes for shRNAs targeting humanized *Renilla* (hr) GFP as a model substrate, or for anti-HBV shRNAs (Sun et al., 2010). When delivered by a replication-defective chimeric adenovirus-HBV shuttle (Liu et al., 2013; Ren and Nassal, 2001) into HBV transgenic (tg) mice (Iannacone and Guidotti, 2015), an optimized dual anti-HBV shRNA vector, but not an analogous anti-hrGFP vector, caused a significant reduction in viral antigens, RNAs and circulating enveloped virus particles, a fraction of which contained vector-derived DNA. Neither vector exerted any overt toxicity.

Hence these data provide proof-of-principle for key steps towards conditionally self-renewing HBV vectors as novel anti-HBV shRNA agents, and they strongly encourage evaluation of the predicted self-limiting vector spread in a small animal HBV infection model.

2. Material and methods

2.1. Plasmid constructs

All HBV constructs were based on plasmid pCH-9/3093 (Li et al., 2011; Sun and Nassal, 2006) which harbors a CMV-IE enhancer/promoter controlled 1.05× unit length genotype D HBV isolate (Genbank accession: **V01460.1**) of proven infectivity (Galibert et al., 1979). Replication-defective helper plasmid pCH-9/3142 (Protzer et al., 1999) lacks part of the 5′ RNA encapsidation signal. Vector pTRUF-HBc183, derived from GFP vector pTR-UF5 (Zolotukhin et al., 1996), expresses only core protein from a synthetic HBV C gene (Nassal, 1988). H1 and U6 promoter-driven shRNA expression cassettes were assembled by PCR amplification on appropriate template plasmids using primers adding an Afl II restriction site upstream, and Apa I and Hind III sites downstream of short versions of the human H1 (Myslinski et al., 2001) and U6 promoters (Kunkel and Hixson, 1998); shRNA sequences ending with six T residues were then inserted between the Apa I and Hind III sites as previously described for pBlueScript II (pBSII) based vectors (Sun et al., 2010). For translationally silent HBV vectors, premature stop codons were introduced into the ORFs for core (G28T), polymerase (T444A), preS1 (C954T), preS2 (C1275T), S (C1482T) and X (C2677T). To prevent self-targeting of anti-HBV shRNA encoding vectors, the target sites for sh6 and sh8 (Sun et al., 2010) were mutated from CTGCTATGCCTCATCTTCTT to CaGCaATGCCcCActTaCTa (sh6; mutated residues in lower case) and from CTCAGTTTACTAGTGCCAT to CTCAGTTcACgtcaGCgAT (sh8). All constructs were verified by Sanger sequencing.

2.2. Chimeric adenovirus-HBV vectors

Chimeric vectors were constructed using the AdMax system (Microbix Biosystems) as previously described (Liu et al., 2013) except that in the shuttle plasmid pDC–CH–tMMP8 the HBV-tMMP part was replaced by the respective HBV-shRNA sequences from pCH-203H1shG-1715U6shG and pCH-203H1sh6-1715U6sh8. Production, purification and titer determination were done as previously described (Liu et al., 2013).

2.3. Cell culture and transfection

Culturing of HepG2 and Huh7 human hepatoma cells, transfection with Fugene 6 reagent (Roche) and preparation of cytoplasmic lysates were all done as described (Sun and Nassal, 2006).

2.4. Isolation of viral nucleic acids, southern and northern blotting, quantitative PCR (qPCR)

Viral DNAs from cytoplasmic nucleocapsids were isolated by sequential nuclease and SDS plus proteinase K treatment followed by phenol/chloroform extraction, and detected by Southern blotting using ^{32}P labeled DNA probes as described (Sun et al., 2010;