Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

The entry inhibitor Myrcludex-B efficiently blocks intrahepatic virus spreading in humanized mice previously infected with hepatitis B virus

Tassilo Volz[1,†], Lena Allweiss[1,†], Mounira Ben ḾBarek[1], Michael Warlich[1], Ansgar W. Lohse[1], Jörg M. Pollok[2], Alexander Alexandrov[3], Stephan Urban[4], Jörg Petersen[5], Marc Lütgehetmann[1,6,††], Maura Dandri[1,*,††]

[1]*Department of Internal Medicine, University Medical Center Hamburg-Eppendorf, Hamburg, Germany;* [2]*Department of Hepatobiliary Surgery, University Medical Center Hamburg-Eppendorf, Hamburg, Germany;* [3]*MYR-GMBH, Bad Homburg, Germany;* [4]*Department of Infectious Diseases, Molecular Virology, University Hospital Heidelberg, Germany;* [5]*IFI Institute for Interdisciplinary Medicine at Asklepios Clinic St. Georg, Hamburg, Germany;* [6]*Department of Medical Microbiology, Virology and Hygiene, University Medical Center Hamburg-Eppendorf, Hamburg, Germany*

Background & Aims: Currently approved antivirals rarely cure hepatitis B virus (HBV) infection. Therefore additional therapeutic strategies interfering with other viral replication steps are needed. Using synthetic lipopeptides derived from the HBV envelope protein, we previously demonstrated prevention of *de novo* HBV infection *in vivo*. We aimed at investigating the ability of the lipopeptide Myrcludex-B to block HBV spreading post-infection.

Methods: uPA/SCID mice reconstituted with human hepatocytes were infected with HBV. Daily subcutaneous Myrcludex-B administration was initiated either 3 days, 3 weeks or 8 weeks post HBV inoculation. Viral loads were quantitated in serum and liver, and visualized by immunohistochemistry.

Results: Myrcludex-B efficiently prevented viral spreading from the initially infected human hepatocytes, as demonstrated by the lack of increase in viremia, antigen levels and amount of HBcAg-positive human hepatocytes determined 6 weeks after treatment. Myrcludex-B efficiently blocked HBV dissemination also when treatment was started in the ramp-up phase of infection, in mice displaying moderate levels of circulating virions (median 3×10^6 HBV DNA copies/ml). Notably, after 6 weeks of treatment, not only the amount of HBcAg-positive hepatocytes, but also intrahepatic cccDNA loads, remained comparable to values found in mice sacrificed 3 weeks post-infection. In none of the experimental settings, drug administration affected human hepatocyte half-life or altered virion productivity.

Conclusions: Myrcludex-B efficiently not only prevented HBV spreading from infected human hepatocytes *in vivo*, but also hindered amplification of the cccDNA pool in initially infected hepatocytes. Administration of an entry inhibitor, possibly used in combination with current HBV drugs, may improve patients' treatment outcome.

Introduction

The hepatitis B virus (HBV) still represents a major health threat with about 350 million chronically infected individuals worldwide. HBV infection leads to a wide spectrum of liver disease ranging from acute to chronic viral hepatitis, which is often associated with the development of liver cirrhosis and hepatocellular carcinoma (HCC). Despite the availability of an effective prophylactic vaccine, about 1 million people die each year due to HBV-associated liver pathologies [1]. Currently approved antiviral treatments based on interferon alpha or nucleos(t)ide analogues that inhibit the viral reverse transcriptase can induce long-term responses, defined as HBsAg loss or seroconversion, only in a minority of patients. Therefore the development of novel therapeutic strategies interfering with other steps of the viral life cycle is needed to improve the treatment outcome.

Viral entry inhibition may represent a rather new and attractive therapeutic concept to combat viral infections both in the acute or chronic phase. Indeed, maintenance of chronic HBV infection is thought to depend on a dynamic turnover of infected hepatocytes that are cleared by the immune system and cells that become newly infected. Moreover, independent reports have shown that cccDNA intrahepatic loads are often below 1 cccDNA copy per cell in human liver biopsies (median 0.1–1 cccDNA

Keywords: HBV; uPA; Humanized mice; Entry inhibitor; cccDNA.
Received 25 April 2012; received in revised form 29 November 2012; accepted 5 December 2012; available online 13 December 2012

* Corresponding author. Address: Medizinische Klinik und Poliklinik, Zentrum für Innere Medizin, Universitätsklinikum Hamburg-Eppendorf, Martinistr. 52, D – 20246 Hamburg, Germany. Tel.: +49 40 7410 52949; fax: +49 40 7410 57232.
E-mail address: m.dandri@uke.de (M. Dandri).
† TV and LA contributed equally to this work.
†† ML and MD contributed equally to this work.

Abbreviations: IFN, interferon; HBV, hepatitis B virus; pgRNA, pregenomic RNA; cccDNA, covalently closed circular DNA; rcDNA, relaxed circular DNA.

Research Article

copy/cell) [8,12,16–19], indicating that the liver of HBV-chronically infected patients often harbors a significant fraction of uninfected hepatocytes. Furthermore, drug resistant variants may eventually emerge under antiviral pressure using nucleos(t)ide analogs. Thus, the use of drugs able to prevent infection of the hepatocytes and spreading of drug resistant variants may represent an important new antiviral concept in the setting of chronic HBV infection.

We have previously shown that Myrcludex-B, which is the GMP version of a synthetic lipopeptide derived from the preS1 domain of the HBV envelope protein, specifically targets the hepatocytes and efficiently blocks *de novo* HBV infection both *in vitro* [5,15] and *in vivo*, after pretreatment of human chimeric uPA/SCID mice [13]. To investigate whether Myrcludex-B administration may also prevent intrahepatic viral spreading in HBV-infected uPA/SCID mice, at a time when only a minority of the human hepatocytes are infected, mice repopulated with primary human hepatocytes were first injected with HBV to permit an initial infection establishment and than treated with the HBV entry inhibitor. We show here that 6 weeks of Myrcludex-B administration, initiated either 3 days or 3 weeks post HBV-injection, efficiently blocked HBV dissemination and cccDNA amplification *in vivo*.

Materials and methods

Generation of human chimeric mice and infection with HBV

UPA/SCID mice were maintained under specific pathogen free conditions in accordance with institutional guidelines, under approved protocols [13]. Humanized homozygous uPA/SCID mice were generated as previously described [10]. All animal experiments were conducted in accordance with the European Communities Council Directive (86/EEC) and approved by the Ethical Committee of the city and state of Hamburg and according to the principles of the Declaration of Helsinki. Human chimeric animals displaying serum albumin (HSA) concentrations between 1 and 2 mg/ml were employed for this study [10]. Mice received a single intraperitoneal injection of human HBV containing serum (5×10^7 HBV DNA copies/mouse; genotype D, HBeAg pos.).

Antiviral treatment

Three days or three weeks post HBV inoculation, humanized mice were injected daily subcutaneously (s.c.) with 2 μg/g body weight Myrcludex-B, the GMP material dissolved in an appropriate formulation developed for s.c. injections. Control mice received mock injections with buffer. Treatment duration was either 3 or 6 weeks. Mice were sacrificed or left untreated to determine viral rebound as indicated in the Results section. For entecavir treatment, mice received drinking water supplemented with 1 μg/ml Baraclude Solution (Bristol-Myers Squibb). Liver specimens removed at sacrifice were snap-frozen in liquid nitrogen for further analyses.

Virological measurements

HBV DNA was extracted from serum samples using the QiAmp MinElute Virus spin kit (Qiagen, Hilden, Germany). HBV-specific primers and hybridization probes were used to determine HBV DNA viremia and cccDNA loads quantitatively as described previously [16]. DNA and RNA were extracted from liver specimens using the Master Pure DNA purification kit (Epicentre, Biozym, Germany) and RNeasy RNA purification kit (Qiagen, Hilden, Germany) [16]. Intrahepatic HBV DNA values were normalized for cellular DNA contents using the beta-globin gene kit (Roche DNA control Kit; Roche Diagnostics) [13]. Levels of rcDNA were estimated by subtracting cccDNA amounts from total HBV DNA. Viral RNAs and genomic RNAs were reverse transcribed using oligo-dT primers and the Transcriptor Kit (Roche Applied Science) and quantified by using primers specific for total viral RNAs [16]. HBV RNA levels were normalized to human specific *GAPDH* RNA [10]. HBsAg quantification was performed using the Abbott Architect platform (quant. HBsAg kit, Abbott, Ireland, Diagnostic Division), as recommended by the manufacture.

Immunohistochemistry

Cryostat sections of chimeric mouse livers were immunostained using human-specific cytokeratin-18 monoclonal (Dako, Glostrup, Denmark) or calnexin antibodies (Cell Signaling, Danvers, MA) to stain human hepatocytes. For the detection of the HBV core antigen (HBcAg), the polyclonal rabbit anti-HBcAg was used. To estimate human cell proliferation, the mouse anti-human Ki67 (Dako, Glostrup, Denmark) was used. Specific signals were visualized by employing the Alexa-labeled secondary antibodies (Invitrogen, Darmstadt, Germany) or TSA-Fluorescein (HBcAg) System (Perkin Elmer, Jügesheim, Germany), while nuclear staining was obtained with Hoechst 33342 (Invitrogen). Stained sections were analyzed by fluorescence microscope [10]. The presence of apoptotic and/or necrotic cells was determined by performing DNA fragmentation labeling (TUNEL assay) following manufacturer's instructions (In situ Cell Death Detection Kit, Roche, Mannheim, Germany).

Statistics

Friedman test was used for non-parametric pair wise and Kruskal–Wallis for independent group wise comparisons, both followed by Dunns comparison of all pairs as post test. The Mann Whitney U test was used for non-parametric pair wise comparisons. p Values <0.05 were considered significant.

Results

Administration of Myrcludex-B starting 3 days post HBV infection prevents viral spreading in humanized uPA mice

To investigate the capacity of the entry inhibitor Myrcludex-B to inhibit infection of the human hepatocytes *in vivo*, by treating mice after the initial infection establishment, 15 naïve humanized uPA/SCID mice were inoculated with 5×10^7 copies of HBV genome equivalents to permit viral entry. As depicted in Fig. 1A, three days after infection, mice received either Myrcludex-B treatment (n = 8; 2 μg/g body weight/day) or saline buffer as control (n = 7) for 6 weeks. Two weeks after viral injection, median levels of viremia (6×10^5 HBV DNA copies/ml *vs.* 2.4×10^5 copies/ml; $p = 0.17$) and HBsAg concentrations (16.5 IU/ml *vs.* 5 IU/ml; $p = 0.17$) did not differ significantly between Myrcludex-B treated and control mice, indicating that the establishment of input-derived HBV infection was achieved with similar efficiency in both groups (Fig. 1B and C). These data are in line with previous *in vitro* experiments showing that administration of preS1-derived peptides 4 h after HBV infection does not hinder viral replication in infected cells [5]. While a significant increase in viremia (median 6.9×10^5 and 2.9×10^6 HBV DNA copies/ml at 4 and 6 weeks, respectively; $p = 0.012$) and HBsAg concentrations (median 100 IU/ml and 616 IU/ml, at 4 and 6 weeks, respectively; $p = 0.003$) was determined in the control group, Myrcludex-B completely blocked the rise of viremia (median 7.5×10^4 HBV DNA copies/ml after 4 weeks and 2.6×10^4 copies/ml after 6 weeks) (Fig. 1B) and HBsAg concentrations (2.7 IU/ml and 2.4 IU/ml at week 4 and 6, respectively) in treated mice (Fig. 1C).

To determine the proportion of HBV-infected human hepatocytes in the liver of treated and control mice, 3 animals from both groups were sacrificed after the six weeks of treatment and analysed intrahepatically. Forty-five days post HBV infection, the great majority of human hepatocytes reconstituting the mouse livers stained HBcAg-positive in the control group (Fig. 1D, lower panels), while very few scattered human hepatocytes stained