Research Article

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Human liver chimeric mice as a new model of chronic hepatitis E virus infection and preclinical drug evaluation

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Background & Aims: Hepatitis E virus (HEV) is a major cause of acute hepatitis as well as chronic infection in immunocompromised individuals; however, *in vivo* infection models are limited. The aim of this study was to establish a small animal model to improve our understanding of HEV replication mechanisms and permit the development of effective therapeutics.

Methods: UPA/SCID/beige mice repopulated with primary human hepatocytes were used for infection experiments with HEV genotype (GT) 1 and 3. Virological parameters were determined at the serological and intrahepatic level by real time PCR, immunohistochemistry and RNA *in situ* hybridization.

Results: Establishment of HEV infection was achieved after intravenous injection of stool-derived virions and following co-housing with HEV-infected animals but not via inoculation of serum-derived HEV. GT 1 infection resulted in a rapid rise of viremia and high stable titres in serum, liver, bile and faeces of infected mice for more than 25 weeks. In contrast, viremia in GT 3 infected mice developed more slowly and displayed lower titres in all analysed tissues as compared to GT 1. HEV-infected human hepatocytes could be visualized using HEV ORF2 and ORF3 specific antibodies and HEV RNA *in situ* hybridization probes. Finally, six-week administration of ribavirin led to a strong reduction of viral replication in the serum and liver of GT 1 infected mice.

Conclusion: We established an efficient model of HEV infection to test the efficacy of antiviral agents and to exploit mechanisms of HEV replication and interaction with human hepatocytes *in vivo*.

Introduction

The hepatitis E virus (HEV) is a small non-enveloped virus (size 27–34 nm) with a positive-sense, single-stranded RNA genome (7.2 kb) belonging to the family of *Hepeviridae* [1]. The capped and polyadenylated RNA genome comprises a 5′ UTR, three open reading frames (ORF1-3) and a 3′ UTR [2]. ORF 1 encodes non-structural proteins such as the helicase and the RNA-dependent RNA polymerase. ORF2 protein is the viral capsid protein inducing neutralizing antibodies during infection [3], while ORF3 encodes a small phosphorylated protein that is essential for virion release [4,5]. Four major human pathogenic HEV genotypes (GT 1-4) have been described [6]. HEV GT 1 and 2 are restricted to humans, are mainly transmitted via the faecal-oral route through contaminated drinking water and are responsible for large epidemics in developing countries in Asia (HEV1) and Africa/Mexico (HEV2). In contrast, HEV GT 3 and 4 are found in humans, pigs and other mammalian species leading to sporadic cases of HEV infections in industrialized nations. GT 3 and 4 are mainly acquired through zoonotic transmission through ingestion or contact with infected animals but transmission through contaminated blood products has also been reported [7]. Every year approximately 20 million people are newly infected with HEV worldwide. Among them 3.4 million patients develop an acute symptomatic infection and 70,000 people die due to the development of fulminant hepatitis [8]. In Germany, seroprevalence is estimated to be between 4 and 20% and HEV RNA is detected in 0.04–0.08% of healthy blood donors [7]. Acute HEV GT 3 infection occurs in Europe and the U.S. and is usually a self-limited disease with an asymptomatic course in the vast

Keywords: Hepatitis E Virus (HEV); Humanized mice; uPA/SCID/beige; Chronic viral hepatitis; Preclinical drug testing; Ribavirin.

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Abbreviations: HEV, hepatitis E virus; uPA, urokinase-type plasminogen activator; SCID, severe combined immunodeficiency; PCR, polymerase chain reaction; ORF, open reading frame; UTR, untranslated region, USB, uPA/SCID/beige; i.v., intravenous; GT, genotype; ELISA, enzyme-linked immunosorbent assay; LLoD, lower limit of detection; IU, international unit; p.i., post infection.

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majority of cases, while GT 1 and 2 infections are restricted to the tropics and tend to take a more severe course [9]. Furthermore, immunosuppressed patients develop a chronic course of HEV GT3 infection in up to 60% of the cases which is associated with life-threatening complications caused by cirrhosis [10]. Therapeutic options are restricted to ribavirin and interferon-alpha, which were successfully applied to terminate viral replication in patients with acute or chronic HEV infection [11]. However, the lack of suitable *in vivo* infection models has limited the knowledge of the molecular biology of HEV and thus the possibility to find new treatment options. To date *in vivo* infection with all four human pathogenic HEV GTs is only supported by chimpanzees and monkey species such as rhesus or cynomolgus macaque [12,13], but high costs and ethical restrains make these models unavailable for many research projects. Furthermore, none of these models can mimic chronic HEV infections, which are a severe clinical problem in immunocompromised patients especially after solid organ transplantation [10]. In this study, we employed humanized uPA/SCID/beige mice [14,15] (shortly termed USB) to establish a small *in vivo* HEV infection model allowing new insights into HEV pathogenicity and serving as a preclinical model to evaluate potential treatment strategies.

Material and methods

Generation of humanized mice

Homozygous USB (uPA/SCID/beige) mice were housed and maintained under specific pathogen-free conditions according to institutional guidelines under authorized protocols. All procedures were approved by the ethical committee of the city and state of Hamburg and were in accordance with the principles of the Declaration of Helsinki. Animal experiments were in accordance with the European Union directive 86/609/EEC. The generation of human liver chimeric mice was conducted as described previously [16,17]. Briefly, 1×10^6 thawed human hepatocytes were injected intrasplenically into 3-week-old USB mice anesthetized with isofluoran. Levels of human chimerism were determined by measuring human serum albumin in mouse serum with the Human Albumin ELISA kit (Immunology Consultants Lab, Portland, USA). USB mice with serum albumin concentrations between 1 and 7 mg/ml corresponding to 30 to 90% human chimerism were employed for the study.

HEV infection and treatment

To establish an HEV infection, humanized mice were inoculated with patient-derived HEV-positive serum (1×10^5 IU/mouse, GT 1) or with cleaned faecal suspensions (1×10^5 IU/mouse, GT 1 and 3). Patient samples used for infection were obtained from one acutely GT1-infected patient, one acutely GT3-infected and one chronically GT3-infected patient. Clinical characteristics of these patients are displayed in Supplementary Table 1. The biological samples were obtained for diagnostic purpose. The surplus material and patient's characteristics were used in accordance to the principles of the Declaration of Helsinki and approved by the ethical committee of the city and state of Hamburg (PV4081).

Isolated viral RNA from patient stool samples were sequenced using the nested primer set MJ-C as previously described [18]. Sequences are available in the Supplementary Table 2 and can be downloaded at the European Nucleotide Archive (http://www.ebi.ac.uk/ena/data/view/LN997653-LN997655). A phylogenetic analysis is provided in Supplementary Fig. 1. Different routes of infection were tested in this study: intraperitoneal (i.p.) injection, intravenous (i.v.) injection, faecal-oral route (through co-housing) and oral administration. For GT 1, various combinations of infection routes and infectious preparations were applied as summarized in Table 1. HEV-infected mice were sampled for blood and stool for at least 8 weeks and euthanized at different time points as indicated in the results. Liver specimens removed at the time of sacrifice were snap-frozen in 2-methylbutane for histological and molecular analyses. Some HEV-infected mice received ribavirin (Rebetol, MSD, United Kingdom) supplemented in the drinking water (50 mg/kg daily, equivalent to a 5-fold human dose) [19] for two (n = 2) or six weeks (n = 2).

Virological measurements

Viral RNA was extracted from serum and bile samples (5 µl) using the QiAmp MinElute Virus Spin Kit (Qiagen, Hilden, Germany). Stool suspensions were prepared by dissolving approximately 25 mg stool in 1 ml of PBS. RNA was subsequently purified using the automated QIAsymphony system (Qiagen). HEV RNA levels were quantified by one-step quantitative real time PCR (qRT-PCR) (ViiA™ 7 Real-Time PCR System Life Technologies, Carlsbad, CA) according to published procedures [20]. Briefly, 5 µl of the eluate was added to the TaqMan® Fast Virus 1-Step Master Mix (Life Technologies, Carlsbad, CA) with HEV-specific primers and probe (JVHEVF 5′GGTGGTTTCTGGGGTGAC; JVHEVR 5′AGGGGTTGGTTGGATGAA, TaqMan minor grove binding probe JVHEVPh 5′FAM-TGATTCTCAGCCCTTCGC-MGB). Cycling conditions were as follows: initiating step of 5 min at 50 °C, 45 cycles of amplification with each cycle consisting of 10 s at 95 °C and 30 s at 60 °C. Serial dilutions of an HEV containing plasmid were used as a standard for quantification. The World Health Organization 1st International Standard (Paul Ehrlich Institute, Langen, Germany) was used for the conversion from copy number to international units (IU). The lower limit of detection (LLoD) was determined to be at 6000 IU/25 mg for stool samples and at 2400 IU/ml for serum and bile samples. To increase the sensitivity for GT3 infected mice, 100 µl of serum obtained at sacrifice was used for viral RNA extraction and quantification, thus reducing the LLoD to 120 IU/ml.

To quantify intrahepatic HEV RNA levels, total RNA was extracted from mouse liver specimens as previously described [17] using the RNeasy RNA purification kit (Qiagen). HEV RNA quantification was performed in a one-step qRT-PCR as aforementioned while the expression of the human housekeeping gene GAPDH (TaqMan® Gene Expression assay ID: Hs99999905_m1) was used for normalization.

Immunofluorescence

HEV proteins were visualized on acetone- or paraformaldehyde-fixed, cryo-preserved liver sections by using HEV ORF2 (1:4000, Millipore, Darmstadt, Germany) and ORF3 specific antibodies (1:1600, Bioss, Woburn, USA). Human hepatocytes were co-stained with anti-SP100 (1:1000, Sigma, St. Louis, USA), anti-CNX (dilution 1:50, Cell Signaling, Leiden, Netherlands) or anti-KRT18 (1:400, Santa Cruz, Heidelberg, Germany) antibodies, which do not cross-react with murine hepatocytes. Specific signals were visualized with Alexa Fluor 546-labeled secondary antibodies (dilution 1:800, Invitrogen, Carlsbad, CA, USA) or the TSA Fluorescein System (Perkin Elmer, Jügesheim, Germany) for amplification of HEV-derived signals. Nuclear staining was achieved with Hoechst 33258 (dilution 1:20,000, Invitrogen). Stained sections were analysed by fluorescence microscopy (Biorevo BZ-9000, Keyence, Osaka, Japan) using the same settings for all groups.

Table 1. Modes of HEV infection.

n	GT	Administration	Material	Source	Infection
12	1	Intraperitoneal injection	Serum	Human	Negative
3	1	Intraperitoneal injection	Faecal extract	Human	Negative
7	1	Oral (contaminated food)	Faecal extract	Human	Negative
4	1	Intravenous injection	Serum	Infected mouse	Negative
6	1	Intravenous injection	Faecal extract	Human	Positive (n = 6/6)
3	1	Co-housing	Faeces	Infected mouse	Positive (n = 3/3)
7	3	Intravenous injection	Faecal extract	Human	Positive (n = 7/7)

Infection routes. To establish an HEV infection in humanized USB mice, different routes of infection (administration) and different infectious materials were tested. Successful HEV infection was achieved only by i.v. injection of faecal extract (HEV GT1, GT3) and by co-housing (HEV GT1).