UNIVERSITÄTSKLINIKUM HAMBURG-EPPENDORF

Aus der I. Medizinischen Klinik und Poliklinik
des Zentrums für Innere Medizin

Direktor: Prof. Dr. Ansgar W. Lohse

Humanized chimeric uPA mouse model for the study of Hepatitis B and D virus interactions and preclinical drug evaluation

Dissertation

zur Erlangung des Grades eines Doktors der Medizin
an der Medizinischen Fakultät der Universität Hamburg.

vorgelegt von:

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aus Kiel

Hamburg 2012

Angenommen von der
Medizinischen Fakultät der Universität Hamburg am: 29.01.2013

Veröffentlicht mit Genehmigung der
Medizinischen Fakultät der Universität Hamburg.

Prüfungsausschuss, der/die Vorsitzende: PD Dr. M. Dandri

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Humanized Chimeric uPA Mouse Model for the Study of Hepatitis B and D Virus Interactions and Preclinical Drug Evaluation

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No specific drugs are currently available against hepatitis delta virus (HDV), a defective virus leading to the most severe form of chronic viral hepatitis in man. The lack of convenient HDV infection models has hampered the development of effective therapeutics. In this study, naïve and hepatitis B virus (HBV) chronically infected humanized uPA/SCID mice were employed to establish a small animal model of HBV/HDV coinfection and superinfection. For preclinical antiviral drug evaluation, the GMP version of the myristoylated preS-peptide (Myrcludex-B), a lipopeptide derived from the pre-S1 domain of the HBV envelope, was applied to prevent *de novo* HBV/HDV coinfection *in vivo*. Virological parameters were determined at serological and intrahepatic level both by real-time polymerase chain reaction (PCR) and by immunohistochemistry. Establishment of HDV infection was highly efficient in both HBV-infected and naïve chimeric mice with HDV titers rising up to 1 × 10E9 copies/mL. Notably, HDV superinfection led to a median 0.6log reduction of HBV viremia, which although not statistically significant suggests that HDV may hinder HBV replication. In the setting of HBV/HDV simultaneous infection, a majority of human hepatocytes stained HDAg-positive long before HBV spreading was completed, confirming that HDV can replicate intrahepatically also in the absence of HBV infection. Furthermore, the increase of HBV viremia and intrahepatic cccDNA loads was significantly slower than in HBV mono-infected mice. Treatment with the HBV entry inhibitor Myrcludex-B, efficiently hindered the establishment of HDV infection *in vivo*. *Conclusion*: We established an efficient model of HBV/HDV infection to exploit mechanisms of viral interference in human hepatocytes and to test the efficacy of an HDV-entry inhibitor *in vivo*. (HEPATOLOGY 2012;55:685-694)

Approximately 20 million individuals are chronically infected with hepatitis delta virus (HDV) worldwide. Chronic HDV infection is considered the most severe form of viral hepatitis, because it is associated with an accelerated course of fibrosis progression and liver cirrhosis in up to 70% of patients, and the highest risk for liver decompensation and development of hepatocellular carcinoma.[1-4]

The virion is a hepatotropic assembly–deficient virus composed of an envelope, provided by the hepatitis B virus (HBV), surrounding the RNA genome and the delta antigen (HDAg). Because HBV and HDV particles share the same coat, it is assumed that the early steps of viral entry, and particularly the binding to cellular receptors, are identical.[5,6] Apart from the entry step and the need for highly differentiated human hepatocytes to propagate,[7] the genome organizations and the life cycles of these two viruses differ completely.

HDV replication occurs in the nucleus in a rolling circle process, during which the negative single-

Abbreviations: cccDNA, covalently closed circular DNA; DAPI, 4′,6-diamidino-2-phenylindole; FRET, fluorescence resonance energy transfer; HBcAg, HBV core protein antigen; HBsAg, hepatitis B virus surface antigen; HBV, hepatitis B virus; HDAg, hepatitis delta antigen; HDV, hepatitis D virus; HSA, human serum albumin; PCR, polymerase chain reaction; pgRNA, pregenomic RNA; rcDNA, relaxed circular DNA.

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Received June 1, 2011; accepted October 4, 2011.

Supported by the Bundesministerium für Bildung und Forschung (BMBF), Innovative Therapieverfahren, Contract grant number: 01GU0702. M.D. is supported by the Deutsche Forschungsgemeinschaft (SFB 481; DA 1063/2-1). M.L. is supported by the Deutsche Forschungsgemeinschaft (LU 1707/1-1).

**These authors contributed equally to this study.*

stranded circular RNA genome is copied into a full-length, complementary RNA.[8,9] Three processed HDV RNAs accumulate in infected cells: the RNA genome, the antigenome, and a 5′-capped and 3′-polyadenylated mRNA translating into the only viral protein, the delta antigen, that is essential for replication. An RNA editing event, mediated by host cellular proteins, modifies the antigenomic open reading frame and permits the production of a longer form of the delta antigen, which is essential for HDV assembly.[10,11] *In vitro* experiments have shown that HDV can redirect cellular DNA-dependent RNA-polymerases to replicate its viral RNA genome. However, the production of envelope proteins provided by HBV is essential for the secretion of HDV virions and hence to complete its life cycle.[6]

Thus, HDV needs the presence of the HBV minichromosome, the covalently closed circular DNA (cccDNA), which is the template for all HBV RNAs, in the same infected hepatocyte.[12] However, only the HBV envelope proteins (HBsAgs) are required for HDV secretion, and previous clinical studies have indicated that the coexistence of both viruses is generally associated with lower levels of HBV viremia and intrahepatic viral loads in HBV/HDV-infected patients,[13,14] suggesting that HDV infection can interfere with the HBV replicative pathway. *In vitro* experiments have also shownd that the large form of the delta antigen (p27) has transsuppressive effects on HBV transcription.[15]

Because of the lack of convenient animal models of HDV infection,[1,16] and hence of information on HDV infection efficiency, spreading kinetics, and consequences for the already established intrahepatic HBV replication, it is unclear whether HDV directly interferes with HBV productivity in infected human hepatocytes and if so, at which step of the virus life cycle. Clinical studies have shown that nucleos(t)ide analogues are not effective against HDV replication and, to date, no HDV-specific antiviral drugs are available.[4] Furthermore, the lack of robust HBV/HDV infection models permitting investigation of the full life cycle of HDV has hampered our understanding of the dynamics of HBV/HDV coinfection, as well as the development of more efficient therapeutic strategies.

In vitro studies have identified acylated pre-S peptides, derived from the large envelope protein of HBV, as highly potent inhibitors of HBV entry into hepatocytes.[17-19] Using human chimeric uPA mice, we previously demonstrated the efficacy of these entry inhibitors, and in particular of Myrcludex-B, the myristoylated pre-S/2-48^{myr} peptide representing the leading substance in the preclinical pipeline, to prevent HBV infection *in vivo*.[20] Furthermore, cell culture studies have indicated that HDV entry could also be inhibited by myristoylated HBVpreS peptides.[5]

In this study, naïve and HBV chronically infected humanized uPA/SCID mice were employed to establish a small animal model of HBV/HDV coinfection and superinfection. We thereby gained insights into the kinetics of HDV spreading *in vivo*, and observed HBV/HDV interferences in the absence of adaptive immune responses. Successful establishment of this new model of HDV infection permitted us to test the ability of the HBV envelope protein–derived entry inhibitor, Myrcludex-B, to block *de novo* HDV infection *in vivo*.

Materials and Methods

Generation of human chimeric mice: HBV infection experiments. UPA transgenic mice (Jackson Laboratories, Bar Harbor, ME) crossed with SCID/beige mice (Taconic Farms, Hallingore, Denmark) were housed and maintained under specific pathogen-free conditions in accordance with institutional guidelines under approved protocols. The presence of the uPA transgene and maintenance of the SCID phenotype were determined as reported.[20,21] Three- to 4-week-old homozygous uPA SCID/beige mice were anesthetized with isofluorane and injected intrasplenically with 1×10^6 viable thawed human hepatocytes isolated from liver specimens obtained from a reduced size liver transplant. Procedures were approved by the Ethical Committee of the city and state of Hamburg and accorded with the principles of the Declaration of Helsinki. Informed consent was obtained from donors.

Cells were cryopreserved and thawed as described.[22] Human hepatocyte repopulation levels were determined by measuring human serum albumin (HSA) concentrations in mouse serum by using the Human

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DOI 10.1002/hep.24758

Potential conflict of interest: Nothing to report.

Additional Supporting Information may be found in the online version of this article.

Albumin ELISA Quantitation Set (Bethyl Laboratories, Biomol GmbH, Hamburg, Germany) as recommended by the manufacturer. Human chimeric animals displaying HSA concentrations ranging from 1 to 2 mg/mL were used for the study. To establish chronic HBV infection, animals received a single peritoneal injection of HBV-positive serum (5×10^7 HBV DNA copies/mouse; genotype D) obtained from HBV chronically infected humanized mice. Eight to 10 weeks after HBV inoculation, HBV chronically infected mice were superinfected with an HDV-positive patient serum (1×10^6 HDV RNA copies/mouse, genotype 1). For coinfection experiments, human chimeric naïve mice received a mixture of HBV/HDV-coinfected serum containing 10^8 HBV DNA copies (genotype D) and 10^6 HDV-RNA copies (genotype 1). Mice were sacrificed at given time points as indicated in the Results section. Liver specimens removed at sacrifice were snap-frozen in liquid nitrogen for further histological and molecular analyses. All animal experiments were conducted in accordance with the European Communities Council Directive (86/EEC) and were approved by the City of Hamburg, Germany.

Virological measurements. Viral DNA and RNA were extracted from serum samples (5 μl) using the QiAmp MinElute Virus Spin Kit (Qiagen, Hilden, Germany). To determine HDV viremia, cDNA was synthesized with the Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany). For the reverse transcription, 10 μl extracted RNA was mixed with random hexamer primers (60 μM) and denatured for 10 minutes at 65°C. Denatured RNA was added to the reverse transcription mixture (total volume 20 μl) supplied by the manufacturer. The reaction was incubated for 10 minutes at 25°C, followed by 30 minutes at 65°C, and stopped by incubation at 85°C for 5 minutes. Real-time PCR was performed in a LightCycler (Roche) using 10 μl of cDNA with HDV-specific primers and TaqMan probes as reported.[23] The reaction consisted of an initiating step of 10 minutes at 95°C, followed by 45 cycles of amplification, with each cycle consisting of 10 seconds at 95°C, 30 seconds at 62°C, and 1 second at 72°C.

Published HBV-specific primers and hybridization probes were used to determine HBV DNA.[24] Known references of cloned HBV DNA were amplified in parallel to establish a standard curve for quantification. The plasmid pBluescript II SK(+), containing one copy of the HDV genome (genotype 1), was used as a standard for HDV cDNA quantification. DNA and RNA were extracted from mouse liver specimens using the MasterPure DNA Purification Kit (Epicentre, Biozym, Oldendorf, Germany) and the RNeasy RNA Mini Kit (Qiagen).[24] Intrahepatic HBV DNA values were normalized for cellular DNA contents using the beta-globin gene kit (LightCycler® Control Kit DNA; Roche Diagnostics), which specifically recognizes sequences of human origin.[20,21]

After purified DNA was treated with 20 U plasmid-safe DNase I (Epicentre) to enrich the cccDNA fraction, intrahepatic cccDNA amounts were determined using cccDNA-specific primers and fluorescence resonance energy transfer (FRET) probes as reported.[24] RcDNA levels were estimated by subtracting cccDNA amounts from the total HBV DNA. Viral RNA was reverse-transcribed from 1 μg total RNA using oligo-dT primers and the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science) and quantified by using primers specific for pregenomic RNA (pgRNA).[24] Steady-state levels of intrahepatic pgRNA amounts were normalized using, as an internal control, human-specific GAPDH primers (QuantiTect Primer Assay; Qiagen).[20] To carry out HDV intrahepatic measurements, 1 μg of liver RNA was used for reverse transcription, and HDV RNA loads were quantified using the same protocol described for the serological HDV RNA measurements. HDV RNA amounts were normalized to human-specific GAPDH expression levels. HBsAg quantification was performed using the Architect HBsAg assay (Abbott Ireland Diagnostics, Sligo, Ireland). Briefly, the mouse serum was diluted 1:150-1:500 in manual dilution serum (Abbott). HBsAg was quantified after assay calibration (HBsAg calibrator; Abbott), as recommended by the manufacture for the testing of reactive human serum.

Immunohistochemistry. Cryostat sections of chimeric mouse livers were immunostained[20,21] with a cytokeratin 18 monoclonal antibody (Dako, Glostrup, Denmark) to specifically stain for the presence of human hepatocytes. HDAg was detected with a high diluted patient serum (diluted 1:8000), or with a polyclonal rabbit anti-HDV antibody (diluted 1:1000; kindly provided by J. Taylor, Fox Chase Cancer Center, Philadelphia, Pennsylvania, United States of America). For detection of the HBV core antigen, the polyclonal rabbit anti-HBcAg antibody (Dako; 1:1000 diluted) was used. Specific signals were visualized with secondary antibodies labeled with Alexa Fluor 488, 546, or 633 (Invitrogen, Darmstadt, Germany) or the TSA Fluorescein System (PerkinElmer, Jügesheim, Germany), and nuclear staining was achieved by DRAQ5 (diluted 1:2000; Axxora, Lörrach, Germany). Stained sections were then mounted with fluorescent mounting media (Dako) and analyzed by fluorescence

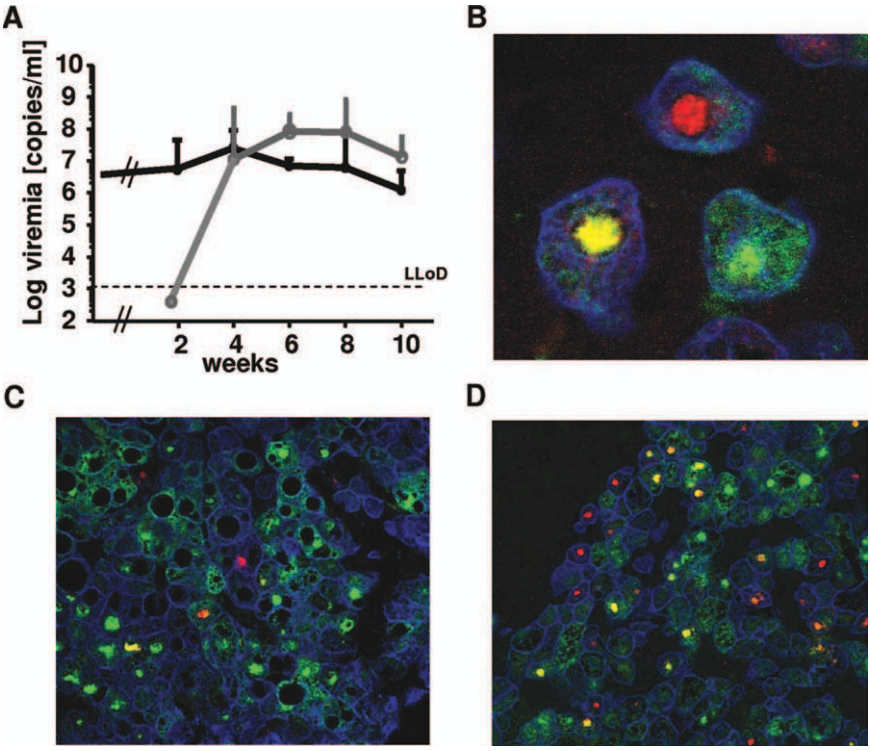

Fig. 1. Establishment of HDV superinfection in HBV-infected humanized uPA/SCID mice. (A) Infection with HDV was successful in 5/5 HBV-positive mice. All mice already displayed high levels of circulating HDV virions at 4 weeks after HDV inoculation (gray line). High levels of HDV viremia were generally accompanied by a small decrease in HBV viremia (black line). (B-D) Immunofluorescence staining of HDV superinfected chimeric uPA mouse livers. Human hepatocytes were visualized using human-specific cytokeratin 18 staining (blue). HDV infection (HDAg staining) is shown in red and HBV infection (HBcAg staining) in green. Pictures were taken with a confocal microscope (Leika) using the z-stack function. Eight weeks after HDV superinfection, expression levels of HBV and HDV proteins strongly differed among human hepatocytes, although the peak of HDV viremia was already achieved (B). The lower number of costained nuclei (yellow) determined at week 4 (C) compared with week 8 after HDV inoculation (D) confirmed that HDV spreading was successful among human hepatocytes.

microscopy and confocal laser scanning microscopy (Leika, Wetzlar, Germany) using the same settings for the different experimental groups. By performing quadruple staining (4′,6-diamidino-2-phenylindole [DAPI], Hu-CK-18, HDAg, and HBcAg) and immunofluorescence analysis, the percentage of HBcAg-positive, HDAg-positive, and HBcAg/HDAg-positive human hepatocytes was estimated using eight visual fields from each mouse that was sacrificed at a given time as specified in the Results section.

Treatment with myristoylated HBV-preS peptides. Humanized uPA mice were injected daily subcutaneously with 2 mg/kg body weight of the lyophilized GMP version of the myristoylated preS-peptide (Myrcludex-B) dissolved in 1X buffer (25 mM $NaHCO_3$/Na_2CO_3 and 50 mg/mL mannitol) at a concentration of 200 μg/mL. Injections were performed once 1 hour before virus inoculation and were repeated daily for the following 4 days. Control mice received mock injections with 1X buffer (100 μL/10 g body weight).

Statistics. The Mann-Whitney test was used for unpaired and the Wilcoxon signed rank test for matched pairs nonparametric comparisons. P values $<$ 0.05 were considered significant.

Results

Serological and histological profiles of HBV/HDV superinfected human chimeric mice. HBV chronically infected human chimeric mice were used to establish a model of HDV superinfection. Animals that were HBV DNA positive for at least 10 weeks and displayed stable viral loads ranging from 1×10^6 to 1×10^8 HBV copies per mL were inoculated intraperitoneally (1×10^6 HDV RNA copies per mouse) with HDV-positive serum. The development of HDV viremia was monitored in blood samples every 2 weeks (Fig. 1A). Four weeks after infection, the detection of HDV RNA in all mice (n = 5) demonstrated that HDV infection was highly efficient in HBV-infected chimeric mice. Indeed, median HDV viremia increased to 1×10^8 HDV RNA copies/mL within 6 weeks after HDV inoculation.

However, confocal microscopy analysis indicated that even at the peak of viremia HDV expression levels varied drastically among neighboring HBV-infected human hepatocytes (Fig. 1B-D). The amount of HDV-positive human hepatocytes clearly increased between week 4 and 8 from a mean of 2% to 46% (Fig. 1C,D), whereas 94% of human hepatocytes appeared HBcAg-positive at both time points, thus demonstrating spreading of HDV infection among HBV-infected human hepatocytes reconstituting the mouse livers. Notably, a median 0.6 log decrease of HBV viremia was determined in the setting of HDV superinfection between weeks 2 and 10 (Fig. 1A), yet HBV reduction did not reach statistical significance.

Establishment of HBV/HDV coinfection in human chimeric mice. Thirteen naïve humanized uPA/SCID mice displaying comparable human repopulation indexes were injected with 1×10^6 HDV and 1×10^8 HBV genome equivalents obtained from a clinical isolate to investigate the efficiency of simultaneous HBV/HDV infection. As expected, the amount of HDV circulating virions was very low in the first 3 weeks after infection, but increased rapidly thereafter up to 1×10^9 HDV RNA copies/mL and reached a plateau within 8 weeks after virus inoculation in nearly all HBV/HDV coinfected animals (Fig. 2A), indicating that HBV spreading supported HDV assembly and secretion in this phase of the infection.

Notably, comparative analyses performed with HBV-monoinfected (n = 11) and HBV/HDV-coinfected mice (n = 13) revealed that during the spreading phase median levels of circulating HBV virions were lower in HBV/HDV-coinfected animals than in HBV-monoinfected mice (Fig. 2A), even though all mice displayed comparable levels of human liver repopulation and were injected with equal amounts of HBV virions.

To shed some light on the mechanisms underlying the lower levels of HBV viremia observed in the setting of HBV/HDV coinfection and, in particular, to investigate whether HDV infection may affect the kinetics of HBV spreading in uPA/SCID mice, intrahepatic cccDNA loads were determined at two different time points in HBV-monoinfected and in HBV/HDV-coinfected mice that had been inoculated with comparable amounts of HBV genome equivalents. The formation of at least one copy of cccDNA is the key event in the replication cycle of HBV and defines the establishment of infection in the target cell. The average amount of intrahepatic cccDNA copies determined per human hepatocytes 8 weeks after virus inoculation was significantly lower in HBV/HDV-coinfected mice (mean 0.3 cccDNA copies/human cell; n = 6) than in HBV monoinfected animals (mean 1.2 cccDNA copies/human cell; n = 5; Fig. 2B). At 12 weeks, cccDNA loads continued to diverge between HBV/HDV-coinfected mice (mean 1 cccDNA copy/cell; n = 4) and HBV-monoinfected animals (mean 4.6 cccDNA copies/cell; n = 3), suggesting that intrahepatic cccDNA accumulation was significantly less efficient ($P = 0.03$) in the context of HBV/HDV coinfection.

HDV infection restrains HBV spreading in human chimeric mice. To investigate whether the lower HBV DNA viremia levels and cccDNA loads determined in HBV/HDV-coinfected mice compared with HBV-monoinfected animals also reflected differences in the amount of HBV-infected hepatocytes, liver sections from mice sacrificed at 4, 8, and 12 weeks after infection were analyzed by immunofluorescence to estimate the different proportions of HBV-positive, HDV-positive, and HBV/HDV-coinfected human hepatocytes over time.

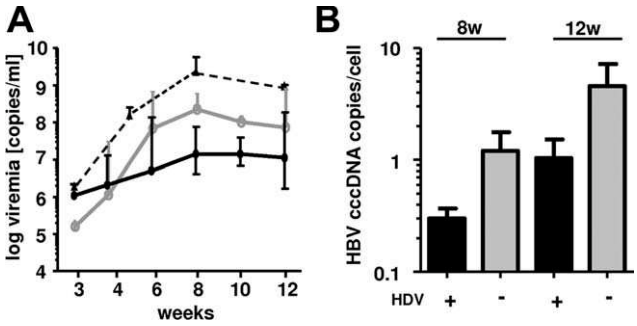

Fig. 2. Establishment of simultaneous HBV/HDV coinfection in naïve humanized uPA/SCID mice. (A) The lines (median values) show the development of both HDV (gray) and HBV (black) viremia determined over time in coinfected chimeric mice (n = 13). Notably, HBV viremia increased more rapidly and achieved higher levels in the setting of HBV monoinfection (dotted line) in mice harboring comparable human repopulation levels (n = 10). (B) Quantitative measurements of intrahepatic cccDNA loads performed by real-time PCR both in HBV/HDV coinfected (black boxes) and in HBV monoinfected (gray boxes) mice sacrificed at 8 or 12 weeks after infection showed that intrahepatic cccDNA accumulation was strongly restrained in the presence of HDV infection.

Four weeks after HBV/HDV coinfection, a remarkable proportion of HDAg-positive human hepatocytes (mean 28%) was detected by immunofluorescence (Fig. 3A,E), although only a few human hepatocytes stained HBcAg-positive (4%) or displayed both viral markers (0.6%). The higher number of HDAg-positive cells indicated that intracellular HDV replication preceded the establishment of HBV infection in a majority of human hepatocytes. The proportion of human hepatocytes staining positive for both viruses was strongly increased after 8 and 12 weeks of HBV/HDV coinfection (mean 48% and 80%, respectively), compared with the amount of monoinfected hepatocytes positive for either HBcAg (15% and 8%, at 8 and 12 weeks, respectively), or HDAg (7% and 3%, respectively), demonstrating that both viruses disseminated in the human chimeric mouse livers (Fig. 3B,C and summarized schematically in E).

However, and in line with the cccDNA measurements, nearly all human hepatocytes (95%) present in HBV-monoinfected mice appeared HBcAg-positive at 8 weeks after infection (Fig. 3D), thus providing further evidence that a lesser number of cells appeared to be HBV-infected after 8 weeks in the setting of coinfection (mean 63%). Analysis of human hepatocyte

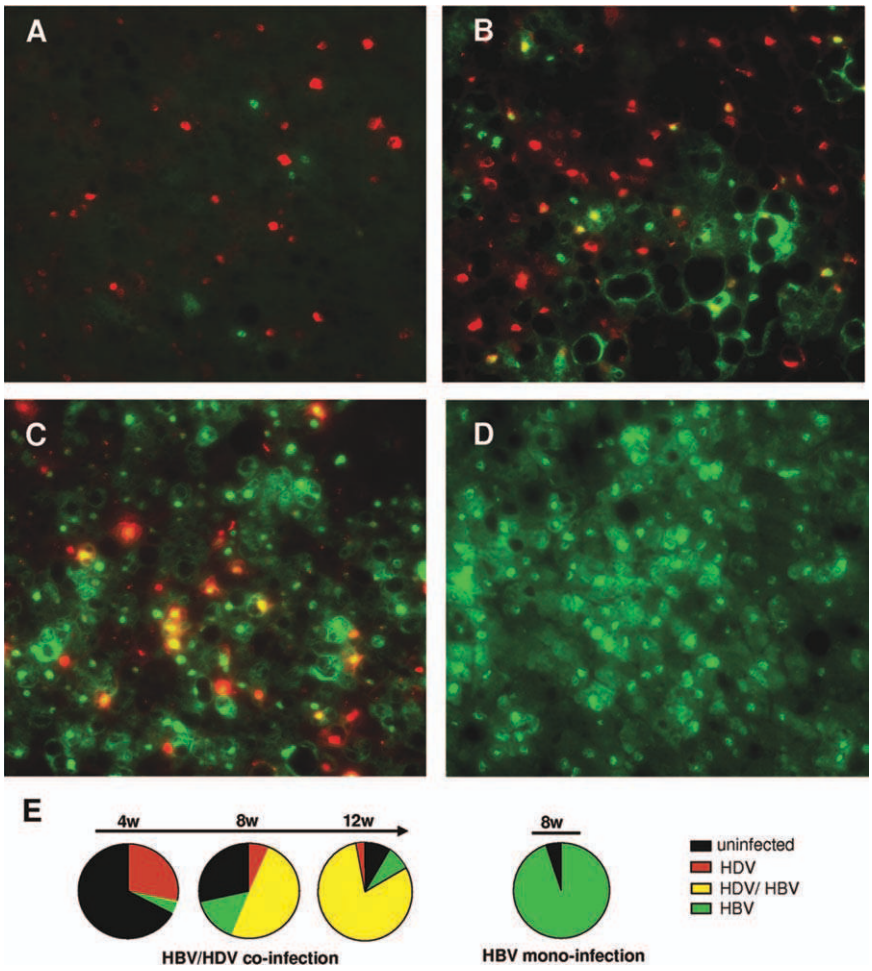

Fig. 3. Estimation of the kinetics of HBV and HDV simultaneous infection within the liver of humanized mice by immunofluorescence. (A) HDAg-positive staining (red) is detected in various human hepatocytes repopulating the liver of a representative mouse sacrificed after 4 weeks of coinfection, whereas only scattered human hepatocytes appear HBcAg positive (green). (B,C) Eight weeks (B) and 12 weeks (C) after HBV/HDV inoculation, both HDAg (red) and HBcAg (green) monoinfected cells, as well as coinfected hepatocytes (yellow), can be detected throughout the area of human hepatocytes present in liver sections, confirming the spreading of both viruses in the setting of simultaneous HBV/HDV infection. (D) A more diffuse HBcAg staining was determined in the setting of HBV monoinfection compared with HBV/HDV coinfection in mice sacrificed after 8 weeks of virus inoculation. (E) The diagram shows the mean proportion of HBcAg-positive (green), HDAg-positive (red), and coinfected (yellow) human hepatocytes observed by immunofluorescence after 4, 8, and 12 weeks of infection. The percentage of infection was calculated by determining the amount of human cells displaying the different virological markers using eight visual fields from each mouse sacrificed at the given time points.

proliferation (by proliferating cell nuclear antigen) and cell death rates (by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling [TUNEL] assay) performed both at the time of infection and in the weeks thereafter showed an absence of signs of human hepatocyte death in HBV/HDV-infected chimeric livers, as well as the fact that cell proliferation rates remained low and comparable to uninfected controls. This further indicates that viral spread kinetics determined in the weeks after infection predominantly reflected virus propagation and not cell division (Supporting Fig. 1).

Sustained viral propagation and serial passage of HDV in human chimeric mice.

High levels of HBV and HDV circulating virions were determined in animals that had been coinfected with both viruses. Both viruses propagated efficiently within the chimeric mouse liver, as shown by measuring the development of both HBV and HDV viremia over 18 weeks in a representative animal (Fig. 4A). To determine whether HDV virions were infectious and hence could be passaged from mouse to mouse, we sacrificed the mouse shown in Fig. 4A and inoculated 12 human chimeric mice with 10 μL of the mouse-derived inoculum (2×10^8 HBV DNA and 5×10^8 HDV RNA genome equivalents/mL). The development of both HBV and HDV viremia was confirmed in all animals, although an approximately 10-fold lower amount of HBV inoculum was used for serial viral passage than for previous experiments and this may have delayed viral spreading in recipient mice (Fig. 4B).

Inhibition of* de novo *HDV infection by treatment with the HBV-derived entry inhibitor Myrcludex-B. Because HBV and HDV particles share the same envelope proteins, it is assumed that both viruses use very similar if not identical entry mechanisms. Previous studies showed that externally applied acylated peptides derived from the preS1 envelope of HBV can block the entry of both HBV and HDV in cell cultures.[19,20] Furthermore, efficient inhibition of HBV infection was also demonstrated *in vivo* by pretreating

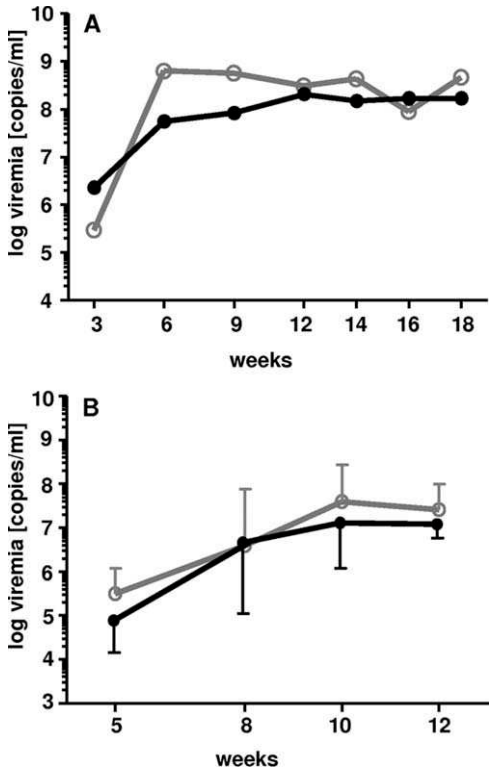

Fig. 4. Establishment of *de novo* HBV/HDV infection in mice injected with mouse-derived HBV/HDV-positive serum. (A) HBV (black line) and HDV (gray line) viremia in an individual mouse that was used to passage HDV infection to naïve chimeric mice. (B) Development of HBV (black) and HDV (gray) titers was confirmed in all mice that had been inoculated with mouse-derived infectious serum (2×10^6 HBV and 5×10^6 HDV GE/mouse) obtained 18 weeks after virus inoculation.

chimeric uPA mice with acylated preS1 peptides.[18] To explore the suitability of this animal model for preclinical anti-HDV drug evaluations, we tested the ability of the HBV entry inhibitor Myrcludex-B to block the establishment of HDV infection in human hepatocytes reconstituting the livers of the uPA/SCID mice.

One hour before injecting the mice with 1×10^6 HDV and 5×10^7 HBV genome equivalents obtained from a patient serum, three naive chimeric mice received a subcutaneous injection of Myrcludex-B, and three control mice received saline. Antiviral treatment was repeated once per day during the following 4 days to inhibit infection induced by virions still present in the circulation. Four weeks after HBV/HDV inoculation, treated and control mice were sacrificed to perform serological and intrahepatic analyses. HBV and HDV viremia was clearly detectable in untreated mice, whereas in all three mice treated with the entry inhibitor, HDV RNA levels remained below the detection limit (Fig. 5A). Quantitative measurements of HBsAg concentrations in mouse serum samples further confirmed that Myrcludex-B administration hindered the establishment of both HBV and HDV infection *in vivo* with high efficiency (Fig. 5B). The block of productive HDV infection *in vivo* was also confirmed by immunohistochemistry, by which HDAg-positive human hepatocytes were detected exclusively in untreated animals (Fig. 5C).

Discussion

The lack of small animal models susceptible to HDV infection has impeded our understanding of HDV biology and the *in vivo* kinetics of HDV infection, as well as of the consequences for the already established intrahepatic HBV replication. In this study, we generated uPA/SCID mice with high rates of human liver chimerism to establish a new model of HBV/HDV infection.

The HDV superinfection studies performed in mice stably infected with HBV showed that the establishment of HDV infection was highly efficient (100%), because the presence of both HBV and HDV circulating virions and coinfected human hepatocytes was demonstrated in all animals. Although nearly all human hepatocytes were already HBV-infected at the time of HDV inoculation, our analyses revealed that the expression levels of HBcAg and HDAg differed considerably among neighboring human hepatocytes (Fig. 1), suggesting that viral interferences may exist at the single cell level. Furthermore, we detected a small, even though statistically not significant, decline in HBV viremia over time in HDV-superinfected mice. Although the molecular mechanisms underlying this virus interplay are not elucidated, this is not entirely surprising. Indeed, previous *in vitro* studies showed that the large form of the delta antigen (p27) has transsuppressive effects on HBV transcription,[15] and cross-sectional studies in patients reported that HDV infection is frequently associated with suppressed HBV replication,[13,14] even if a clear HDV predominance does not hold true for all chronic HBV/HDV patients, indicating that the mechanisms of HBV/HDV interactions might be much more complex than previously thought.[25] In the setting of simultaneous HBV/HDV infection, all naïve human chimeric mice developed HDV and HBV viremia within 6 weeks. Furthermore, HDV particles derived from these mice were successfully propagated into naive human chimeric recipients, demonstrating the *in vivo* infectivity of HDV virions derived from previously infected human chimeric mice.

Some interesting differences related to the ability of these two viruses to launch intracellular viral replication are emerging from our studies in humanized mice. Intrahepatic analyses of HBV-monoinfected

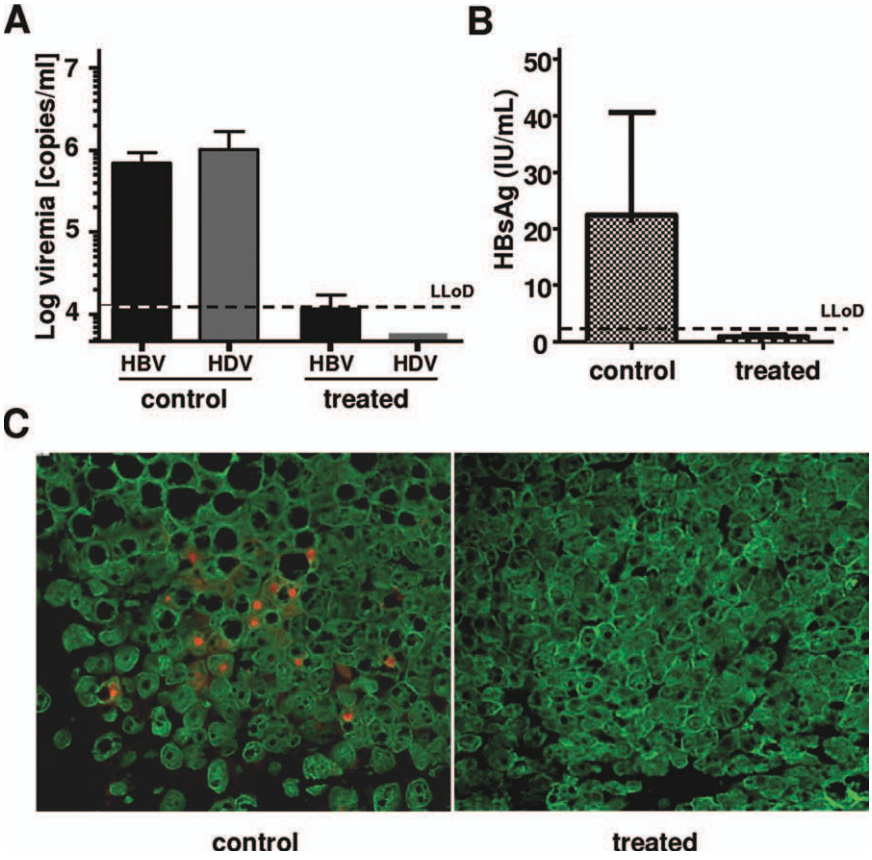

Fig. 5. Inhibition of *de novo* HDV infection in mice pretreated with the HBV entry inhibitor Myrcludex B. (A,B) Four weeks after infection, HBV and HDV titers (A) and serum HBsAg levels (B) remained below the lower limit of detection (LLoD) in mice that received Myrcludex-B (2 mg/kg) 1 hour before HBV/HDV infection and once per day in the first 4 days after infection. (C) Block of HDV infection in mice treated with Myrcludex-B was also confirmed by immunohistochemistry. HDAg-positive (orange) human hepatocytes (green) were found exclusively in liver sections of untreated mice sacrificed 4 weeks after HBV/HDV infection.

chimeric mice showed that only very few human hepatocytes stain HBcAg-positive (approximately 1%-2%[26]) in the first 3 weeks of infection, even if nearly all human hepatocytes become HBcAg-positive within 8 weeks (Fig. 3).[27] In contrast, a remarkably higher proportion (28%) of HDAg-positive human hepatocytes was promptly detected in liver sections of mice sacrificed as early as 4 weeks after HBV/HDV inoculation, whereas only 4% of human hepatocytes stained HBcAg-positive in the same livers. Given that HDV cannot leave the infected hepatocytes and spread throughout the liver in the absence of HBV, our coinfection experiments demonstrated that intracellular HDV replication took place also in the absence of HBV, whereas the establishment of productive HBV infection appeared to proceed at a significantly slower pace. Keeping in mind that both viruses share the same envelope proteins and hence are supposed to use the same route of entry,[5] it is plausible that not the initial cellular entry, but rather downstream steps, such as nuclear entry and cccDNA formation, may be more critical in determining the establishment of HBV infection within the human hepatocytes.

Apart from these initial differences in establishing viral replication in target cells, our studies revealed that HBV spreading was further slowed down in the setting of HBV/HDV simultaneous infection, as indicated by the lower amounts of HBcAg-positive cells, intrahepatic cccDNA loads, and reduced levels of HBV viremia determined between coinfected and HBV-monoinfected animals (Figs. 2, 3). As for the mechanisms, we currently do not know how HDV may influence HBV infection and replication. However, both the superinfection and coinfection experiments indicated that HDV infection and accumulation of delta proteins occurred with high efficiency both in HBV-infected and in naive human hepatocytes. Because HDV nuclear proteins are expected to compete with the HBV capsids for the same HBV envelope proteins, HBV productivity may be affected, and hence, both the reduction of HBV viremia in the setting of superinfection and the slower HBV expansion kinetics determined in the setting of coinfection are plausible.[28] Nevertheless, both viruses could disseminate throughout the chimeric mouse livers over time, so that after 12 weeks of HBV/HDV infection, 80% of the human hepatocytes stained positive for both viruses.

Patients with chronic HDV infection generally have elevated liver enzymes (alanine aminotransferase) with histological aggressive hepatitis, but display low to undetectable HBV viremia. Although immune-

mediated mechanisms may play a major role in suppressing HBV replication in HBV/HDV chronically infected patients, our infection experiments performed in chimeric mice unable to mount an adaptive immune response support the assumption that HDV infection can affect HBV productivity even in the absence of adaptive immune responses.

HDV relies on cellular polymerases to replicate its genome[8]; thus the typical antiviral therapeutic approaches, such as the nucleoside analogues that are used for HBV treatment, are ineffective against HDV, and no HDV-specific antiviral drugs are currently available. Interferon-α remains the only treatment for patients with chronic HDV, although such treatment has limited efficacy and long-term benefit is achieved in only 25% of the patients.[1,2,29] Therefore, new therapeutic strategies directly targeting the HDV life cycle are urgently needed.

We previously demonstrated prevention of *de novo* HBV infection *in vivo* by pretreating human chimeric mice with acylated preS1 peptides derived from the HBV envelope proteins.[20] Because maintenance of chronic HBV and HDV infections is supposed to depend on a dynamic turnover between infected hepatocytes that are cleared by the immune system and cells that become newly infected, the use of drugs able to prevent reinfection of the hepatocytes, possibly in combination with interferon-α or viral polymerase inhibitors, may represent new effective antiviral concepts also in the setting of chronic infection.

In this study we carried out a proof-of-principle preclinical study to test the ability of Myrcludex B, which is the most potent and best characterized HBV entry inhibitor currently available, to inhibit the establishment of *de novo* HDV infection *in vivo*. Our experiments showed that 4 weeks after virus inoculation all control mice developed HBV/HDV viremia and displayed detectable HBsAg in their plasma, whereas mice that had been treated with Myrcludex-B shortly before infection and in the first 4 days after virus inoculation failed to develop detectable HDV and HBV serological markers. Prevention of HDV infection in mice pretreated with Myrcludex B was also demonstrated intrahepatically by HDAg staining. However, further studies aiming at determining the antiviral efficacy of this drug in other experimental settings, are needed.

In summary, we established an efficient small animal model of HDV infection permitting propagation of both HBV and HDV. This system will expand our ability to study HDV infection and interactions with the human hepatocytes, as well as possible mechanisms of mutual interference between these human hepatotropic viruses, and also to perform preclinical antiviral drug evaluations.

Acknowledgments: We are grateful to Drs. John Taylor and A. Alexandrov for kindly providing the antibodies against HDAg and Myrcludex-B (GMP version), respectively; to Roswitha Reusch and Anne Groth for their excellent work in the animal facility; and to Gerlinde Apitzsch and Bernd Zobiak (UKE Microscopic Imaging Facility, Hamburg, Germany) for important technical support.

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ZUSAMMENFASSUNG

Über die Etablierung eines für das Hepatitis-Delta-Virus geeigneten uPA Mausmodells mit humanen Hepatozyten zur Erforschung von Interaktionen zwischen dem Hepatitis-B- und D-Virus sowie präklinische Medikamentenevaluation.

Hintergrund

Weltweit sind etwa 20 Millionen Menschen chronisch mit dem Hepatitis- Delta-Virus (HDV) infiziert. Die Prävalenz in Nord- und Zentraleuropa ist sogar zunehmend, was auf verstärkte Migrationsbewegungen zurückzuführen ist. Die chronische HDV-Infektion gilt als die schwerste Form der viralen Hepatitis. Als leberspezifische Erkrankung ist sie für eine rasche Fibroseentstehung und eine in 70% der Fälle entstehende Leberzirrhose bekannt. In der Folge besteht das Risiko einer dekompensierten Leberinsuffizienz und der Entwicklung eines hepatozellulären Karzinoms, was ein Gesundheitsproblem globaler Bedeutung darstellt (Rizetto 2009, Wedemeyer 2010, Romeo 2009, Hughes 2011).

Virusstruktur

Das Deltavirus, ein negativ gerichtetes Einzelstrang-RNA und hepatotrophes Satellitenvirus, kann sich nur mit Hilfe des vom Hepatitis-B-Virus (HBV) stammenden Oberflächenproteins (HBsAg) vermehren. Das HBsAg umhüllt die HDV-RNA sowie das einzige viruseigene Protein, das Hepatitis-Delta-Antigen (HDAg). Da beide Viren die gleiche Hülle tragen, geht man von einer einheitlichen Zellaufnahme in die Hepatozyten aus, bei der, den Beobachtungen nach, der zwar noch unbekannte, jedoch erwartungsgemäß identische Rezeptor verwendet wird (Engelke 2006, Sureau 2006).

Trotz der Gemeinsamkeiten, wie der Virusaufnahme und der für den viralen Lebenszyklus benötigten differenzierten humanen Hepatozyten (Sureau 2010), unterscheiden sich die beiden Viren deutlich.

Die Produktion des HBsAg ist jedoch für die Sekretion der HDV-Virionen und somit für den Lebenszyklus des Delta-Virus obligatorisch (Sureau 2006). Demzufolge benötigt HDV in derselben infizierten Zelle die Anwesenheit des HBV-

Minichromosoms, die Covalently-Closed-Circular-DNA (cccDNA), welche als Matrize für alle HBV-RNAs dient (Levrero 2009).

Aktueller Stand der Forschung

Aufgrund des Mangels an einem geeigneten Tiermodell für HDV-Infektionen (Rizetto 2009, Casey 2006) fehlen Informationen von HDV-Infektionseffizienz, HDV-Verteilungskinetiken und entstehende Konsequenzen für eine schon etablierte HBV-Infektion. Dieser Umstand erschwert die Beurteilung, ob HDV direkt die HBV-Produktivität in den humanen Hepatozyten hemmt und um welchen Schritt des HBV-Lebenszyklus es sich dabei handeln könnte.
Wie in klinischen Studien gezeigt werden konnte, scheinen Nukleos(t)id-Analoga keinen Effekt auf die HDV-Replikation zu haben und auch die Therapie mit Interferon ist nicht zufriedenstellend. So sind zurzeit keine HDV-spezifischen antiviralen Medikamente verfügbar (Hughes 2011). Darüber hinaus gäbe ein zuverlässiges HBV/HDV-Modell die Möglichkeit, den vollständigen HDV-Lebenszyklus zu erforschen und sowohl die Dynamiken einer HBV/HDV-Infektion zu erkennen, als auch weitere effiziente Therapieansätze zu entwickeln.

Neue Therapiekonzepte

In in-vitro-Studien konnte man ein vom HBV-Hüllprotein stammendes, acyliertes präS-Peptid identifizieren, welches einen potenten Inhibitor für die HBV-Aufnahme in Hepatozyten darstellt (Glebe 2007, Gripon 2005, Schulze 2010). Mit Hilfe von humanen chimären uPA Mäusen konnte man bereits die Wirksamkeit dieses Aufnahmeinhibitors demonstrieren. Vor allem Myrcludex-B, das myristolierte präS-Peptid, zeigte sich als führende Substanz in der präklinischen Forschung zur Verhinderung einer HBV Infektion (Petersen 2008).
Weiterhin gaben Zellkulturversuche mit den myristolierten HBV-präS-Peptiden den Anhalt für einen ebenfalls hemmenden Einfluss auf die HDV-Aufnahme (Engelke 2006).

Ergebnisse

Die Grundlage: das uPA Mausmodell

Für das Tiermodell wurden uPA/SCID Mäuse verwendet. Dabei ermöglicht das hepatotoxische uPA - (Uroplasminogenaktivator) Transgen eine Rekonstruktion der

Leber mit Fremdhepatozyten. Aus nicht zu verwendenden Teilen von Lebertransplantaten konnten humane Hepatozyten gewonnen werden und über die Milz den Mäusen injiziert werden. Das Einkreuzen des SCID- (Severe Combined Immunodeficiency) Transgens erzeugt eine Immundefizienz, die das Überleben der humanen Heptozyten gewährleistet. Die humanen Hepatozyten in der rekonstruierten Mausleber konnten bereits erfolgreich mit HBV infiziert werden (Dandri, 2001, Petersen 2008, Dandri 2012).

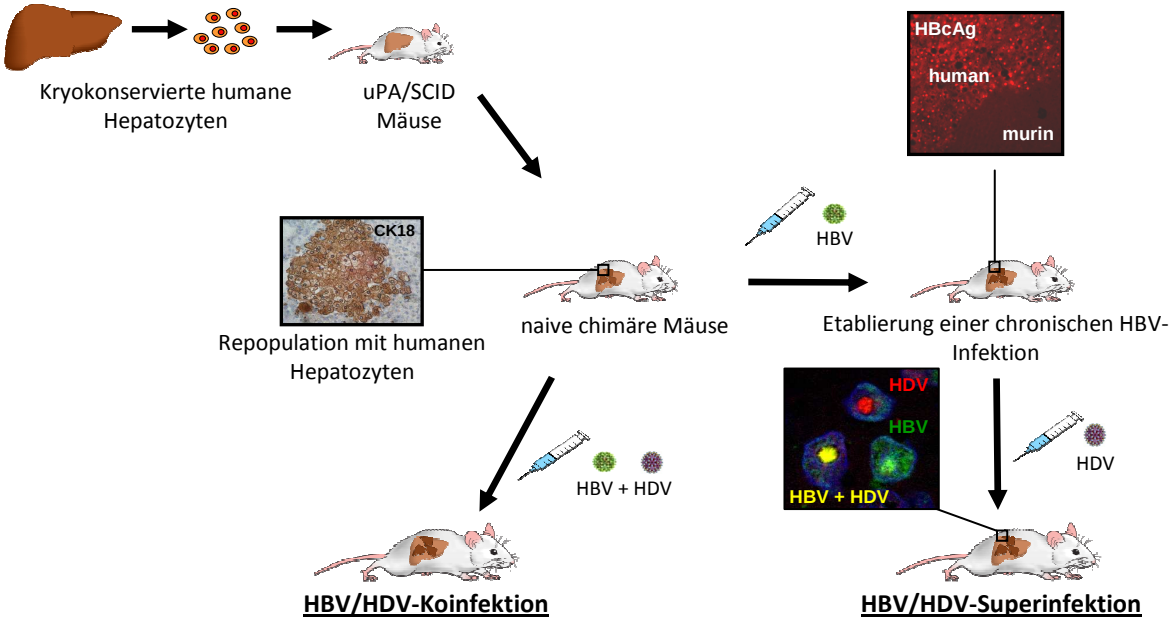

Fig.1 Versuchsaufbau zur Etablierung einer HBV/HDV-Infektion in humanen chimären Mäusen
HBV = Hepatitis-B-Virus, HBcAg = HBV-Core-Antigen, HDV = Hepatitis-D-Virus,
uPA = Uroplasminogenaktivator, SCID = Severe Combined Immunodeficiency

Resultate der HBV/HDV-Superinfektion

Zur Etablierung einer HDV-Superinfektion wurden chronisch HBV-infizierte humane chimäre Mäuse verwendet. Den Mäusen wurde ein HDV-positives Patientenserum (1x10E6 HDV-RNA Kopien pro Maus) intraperitoneal injiziert und in folgendem zweiwöchigen Abstand Blutproben entnommen. Das gewonnene Serum wurde aufgereinigt und mittels quantitativer Real-Time-PCR ausgewertet. Die Entwicklung einer HDV-Virämie konnte in allen Mäusen (n=5) bereits nach vier Wochen erfasst werden, was auf eine effiziente HDV-Infektion in chronisch HBV-infizierten chimären Mäusen schließen ließ.

Auffallend war dabei die Beobachtung einer leichten Reduktion der HBV-Virämie in den mit HDV-superinfizierten Mäusen.
Die erfolgreiche HBV/HDV-Superinfektion in der human repopulierten Mausleber konnte immunohistochemisch zur 4. und 8. Woche nach Superinfektion bestätigt werden.

Resultate der HBV/HDV-Koinfektion

Zur Etablierung einer HBV/HDV-Koinfektion wurden 13 naive humanisierte uPA/SCID Mäuse mit HBV (1x10E8 HBV genomischen Äquivalents) und HDV (1x10E6 HDV genomischen Äquivalents) enthaltendem Serum infiziert.
Innerhalb von 8 Wochen nach der Virusinokulation konnte in allen HBV/HDV-infizierten Tieren ein stabiler Titer für beide Viren nachgewiesen werden. Interessanterweise zeigten vergleichbare Analysen mit HBV-monoinfizierten (n=11) Mäusen in der anfänglichen Ausbreitungsphase durchschnittlich geringere Mengen an zirkulierenden HBV-Virionen als in den HBV/HDV-koinfizierten Mäusen.

Um den Beobachtungen der niedrigeren Werte für die HBV-Virämie in koinfizierten Mäusen weiterhin nachzugehen, wurden intrahepatisch gemessene cccDNA-Werte zu zwei verschiedenen Zeitpunkten in HBV-monoinfizierten und in HBV/HDV-koinfizierten Mäusen verglichen. Dabei ist die Anwesenheit einer einzigen Kopie der cccDNA in der Zielzelle für eine HBV-Replikation ausreichend. Der durchschnittliche Wert für intrahepatische cccDNA war in den HBV/HDV-koinfizierten Mäusen im Vergleich zu den HBV-monoinfizierten Mäusen signifikant niedriger. Um diesen Beobachtungen weiter nachzugehen, wurden HBV/HDV-koinfizierte Mäuse nach 4, 8 und 12 Wochen getötet und deren Leber immunohistochemisch auf unterschiedliche virale Verteilungsmuster analysiert. Bereits vier Wochen nach der HBV/HDV-Koinfektion konnte ein beachtlicher Teil der humanen Hepatozyten HDAg-positiv angefärbt werden und nur wenige Zellen waren zu diesem Zeitpunkt HBcAg-positiv. Die intrazelluläre HDV-Replikation scheint der HBV-Besiedlung in der Mehrzahl der humanen Hepatozyten vorauszugehen. Unter der Annahme, dass HDV ohne die Anwesenheit von HBV die infizierten Hepatozyten nicht verlassen und sich innerhalb der Leber nicht verbreiten kann, demonstrieren unsere Versuche jedoch die autonome intrazelluläre Replikation von HDV.

Ebenfalls macht diese Arbeit die verlangsamte HBV-Ausbreitung im Rahmen einer HBV/HDV-Koinfektion deutlich, was an weniger HBcAg-positiv anfärbbaren Zellen, geringeren intrahepatisch gemessenen cccDNA-Werten, sowie den im Vergleich zu HBV-monoinfizierten Tieren niedrigeren Werten der HBV Virämie zu erkennen ist. Beide Viren können sich erfolgreich in den chimären Mauslebern vermehren, was deutlich an der Entwicklung einer HBV- und HDV- Virämie über 18 Wochen eines repräsentativen Tieres zu erkennen ist.

Außerdem konnten die von diesen Mäusen stammenden Delta-Viren erfolgreich dafür genutzt werden, weitere chimäre Mäuse zu infizieren, was die Infektiösität der in dieser Arbeit erstandenen HDV-Virionen deutlich macht.

Hemmung einer HDV-Neuinfektion durch die Behandlung mit dem bei HBV-Infektion eingesetzten Aufnahmeinhibitors Myrcludex-B

Da HBV und HDV das gleiche Hüllprotein benutzen, nimmt man an, es handle sich bei beiden Viren auch um einen ähnlichen oder sogar identischen Aufnahmemechanismus (Engelke 2006). Vorherige in-vitro- Untersuchungen zeigten, dass ein acyliertes Peptid, abgeleitet von der präS1-Domäne des HBVs, die Aufnahme von HBV und HDV in Zellkultur verhindern konnte (Gripon 2005, Schulze 2010). Weiterhin konnte eine HBV-Infektion bei mit acylierten präS1-Peptiden vorbehandelten chimären uPA Mäusen ebenfalls in vivo erfolgreich verhindert werden (Petersen, 2008). Infolgedessen wurde die Wirksamkeit des HBV-Aufnahmeinhibitors Myrcludex-B in Bezug auf eine Blockierung einer HDV-Neuinfektion getestet. Bevor die Mäuse mit dem von Patienten stammenden HBV und HDV enthaltendem Serum infiziert wurden, bekamen drei naive chimäre Mäuse eine subkutane Injektion mit Myrcludex-B und drei Kontrolltieren wurden Kochsalzlösung injiziert. Die antivirale Behandlung wurde einmal täglich für vier Tage fortgeführt. Vier Wochen nach der HBV/HDV-Inokulation wurden die Behandlungs- und Kontrollmäuse getötet, um die serologischen und intrahepatischen Analysen durchzuführen. Die HBV- und HDV-Virämie war in den unbehandelten Kontrolltieren eindeutig messbar. In den drei Mäusen, welche mit dem Aufnahmeinhibitor behandelt wurden, verblieben die Messungen für HDV-RNA hingegen unter der Nachweisgrenze. Weiterhin konnte die hemmende Wirkung von Myrcludex-B sowohl auf eine HBV- als auch auf eine HDV-Infektion mit den quantitativen Messungen der

HBsAg-Konzentrationen bestätigt werden. Immunohistochemisch wurde die Blockade einer produktiven HDV-Infektion ebenfalls bestätigt, bei der HDAg-positive humane Hepatozyten nur in den unbehandelten Tieren detektierbar waren.

Resümee

Im Rahmen dieser Arbeit wurde eine Versuchreihe entwickelt, bei der naive und mit Hepatitis-B-Virus chronisch infizierte uPA/SCID Mäuse eingesetzt wurden, um eine Ko- bzw. Superinfektion mit HDV zu etablieren.
Dadurch konnten Einblicke in die Verteilungskinetiken von HDV in vivo gewonnen, sowie Beeinflussungen zwischen beiden Viren bei fehlender adaptiver Immunantwort beobachtet werden.
Diese erfolgreiche Etablierung ermöglichte uns ebenfalls den Einsatz des vom HBV-Hüllprotein abgeleiteten Aufnahmeinhibitors Myrcludex B und die Beurteilung bezüglich der Blockierung einer HDV-Neuinfektion in vivo.
In weiteren Analysen können mögliche Interaktionen zwischen dem Delta-Virus und der angeborenen Immunabwehr humaner Hepatozyten untersucht werden. Ebenfalls wird es möglich sein, den Einfluss der derzeitigen Standardtherapie, dem pegyliertem Interferon-alpha, auf die Virus-Wirtszell-Dynamik zu beurteilen

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ERKLÄRUNG DES EIGENANTEILS DER PUBLIKATION

Das Ziel dieser Arbeit war es, eine HDV-Infektion in chimären Mäusen zu etablieren. In Zusammenarbeit mit PD Dr. Maura Dandri und Dr. Marc Lütgehetmann habe ich das Konzept zu dieser Etablierung erarbeitet und verschiedene Denkansätze integriert.
Die Grundlagen, wie die Vorbereitung der humanen Zellen, deren Transplantation und das Infizieren der Mäuse wurden mit Hilfe von Lena Allweiss und Dr. Tassilo Volz umgesetzt.
Die molekularen Analysen, die im speziellen die Ergebnisse dieser Arbeit erbrachten, wurden größtenteils eigenständig von mir durchgeführt. Anregungen und weitere Vorschläge kamen darüber hinaus vor allem von Dr. Marc Lütgehetmann, sowie ergänzendes Zuarbeiten von Martina Helbig.
Nach Anleitung von Till Bornscheuer habe ich die immunohistochemischen Färbungen angefertigt.
Die Überarbeitung der endgültigen Analysen, sowie das Erstellen des Manuskriptes wurden vor allem von PD Dr. Maura Dandri und mir durchgeführt.
Bei der Diskussion zur Fertigstellung der Publikation haben alle Co-Autoren mitgewirkt. Eine konstruktive Teilnahme nahm insbesondere Prof. Dr. Jörg Petersen dabei ein. Die finanzielle Unterstützung, die dieses Projekt ermöglichte, wurde von Prof. Dr. Ansgar W. Lohse gewährleistet.

DANKSAGUNG

Mein Dank gilt all den Menschen, die mich beim Erstellen dieser Arbeit begleitet und unterstützt haben.

Insbesondere möchte ich mich bei PD. Dr. Maura Dandri und Dr. Marc Lütgehetmann für die Bereitstellung meines Promotionsthemas und ihre freundliche Unterstützung bedanken. Vor allem bedanke ich mich für das Vertrauen, meine Arbeit beim monothematischen Hepatitis-Delta-Kongress in Istanbul präsentieren zu dürfen. Dies war eine sehr interessante und spannende Erfahrung für mich, auf die ich gerne zurückblicke.
Ebenfalls bedanke ich mich bei Dr. Tassilo Volz, der mir vor allem bei der experimentellen Arbeit mit Rat und Tat zur Seite stand und mir viele Einblicke in die wissenschaftliche Tätigkeit ermöglichte.
Bei Mounira Ben M'Barek, Martina Helbig, Gerlinde Apitzsch, Lena Allweiss und Till Bornscheuer bedanke ich mich für die gemeinsame Unterstützung, sowie für die freudige und kollegiale Zusammenarbeit.
Ich bin froh und dankbar, dass ich meine Arbeit in dieser Arbeitsgruppe durchführen durfte.

Meiner Familie und meinem Freund Frieder bin ich für den unabdingbaren Beistand und der konstruktiven Hilfe ebenfalls sehr dankbar.

EIDESSTATTLICHE ERKLÄRUNG

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.
Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.
Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

Unterschrift: Lida Mancke