Lipoprotein profiles in SCID/uPA mice transplanted with human hepatocytes become human-like and correlate with HCV infection success

Rineke H. G. Steenbergen,1,4,5 Michael A. Joyce,1,5 Garry Lund,3 Jamie Lewis,2,5 Ran Chen,1,4,5 Nicola Barsby,1,5 Lin Fu Zhu,2 D. Lorne J. Tyrrell,1,3,4,5* and Norman M. Kneteman2,3,4,5*

1Department of Medical Microbiology and Immunology and 3Li Ka Shing Institute of Virology, Katz Centre for Pharmacy and Health Research, 2Department of Surgery, University of Alberta, 3KMT Hepatech, Inc., Edmonton, Alberta; and 4National Canadian Research Training Program in Hepatitis C, Centre de Recherche du Centre Hospitalier de l’Université de Montréal, Hopital Saint-Luc, Montreal, Quebec, Canada

Submitted 28 April 2010; accepted in final form 19 July 2010

HEPATITIS C VIRUS (HCV) is a small enveloped, positive-strand RNA virus of the family of Flaviviridae and the only member of the genus Hepacivirus. Another genera in the family are Flavivirus, such as West Nile and dengue virus, and Pestivirus, such as bovine diarrhea virus. These viruses have a positive-strand RNA genome in common. Their genome consists of a single open reading frame, encoding a polyprotein that is cleaved co- and posttranslationally (11). Most Flaviviruses can infect a large range of hosts (11), but tropism of HCV infection is limited to humans and chimpanzees, and it remains unclear why other species are not susceptible to HCV infection. Possibly, the lack of entry receptors on the surface of liver cells of other species prevents infection, and recent evidence may support that idea: expression of human occludin, together with human CD81, allows for infection of mouse cells with hepatitis C pseudoparticles (18). However, replication of HCV in, for example, mouse cells is inefficient (23, 28), and there is no evidence that assembly and release of virions occur in murine hepatocytes. It is therefore likely that several other proteins or processes contribute to the tight association of HCV infection to humans cells.

In the chimeric mouse model, all the factors needed for entry and replication of HCV are present on or in the transplanted human hepatocytes. Nonetheless, we observed that infection is reliable only in mice with a high degree of human chimerism, despite the presence of considerable amounts (20–30%) of human hepatocytes in mice with lower engraftment successes. This led us to believe that another human, liver-derived factor is essential for efficient infection. We hypothesize that HCV infection also depends on a humanized lipoprotein profile.

The liver plays a key role in cholesterol metabolism and lipoprotein synthesis (5, 8), as illustrated in Fig. 1. Hepatocytes synthesize apolipoprotein B-100 (ApoB) in the endoplasmic reticulum. Lipids are added to the particle, which is then secreted into the plasma as VLDL (Fig. 1A). The formed particles are rich in triglycerides (TG); ~80% of the lipid content is TG, and the remainder is mostly cholesterol and phospholipids. Most of the VLDL is further processed by endothelial lipases and hepatic lipases, which gradually remove TG, resulting in the formation of intermediate-density lipoprotein and, eventually, LDL.

LDL delivers cholesterol to the liver and peripheral tissues through the LDL receptor (LDL-R) pathway. The liver expresses LDL-R at very high levels and, like all other cells, can utilize LDL to acquire cholesterol. In addition, the liver also removes LDL remnants from the circulation and degrades them by converting the lipids to bile acids. In the circulation, the exchange of other apolipoproteins further regulates the metabolism of VLDL (Fig. 1B). ApoC-II functions as a cofactor for endothelial lipoprotein lipases (LPL) that hydrolyze TG in VLDL and make the released fatty acids available for energy production. VLDL additionally acquires ApoE in the circulation, which facilitates uptake of the VLDL remnant by the liver. A third exchangeable
secreted by human liver cells (Fig. 1A). CETP is another lipoprotein metabolism marker that is involved in the exchange of cholesterol esters (CE) from HDL to VLDL and LDL by cholesterol ester transfer protein (CETP). As a result of CETP activity, a much smaller fraction of total cholesterol is associated with HDL in humans than in mice. LDL-R, LDL receptor; SR-B1, scavenger receptor B1.

The liver secretes VLDL, a particle rich in triacylglycerol (TG), which is subsequently processed into intermediate-density lipoprotein (IDL) and LDL by progressive hydrolysis of TG. Additionally, the liver secretes apolipoprotein A1 (A1), which is the precursor to HDL. In the circulation, HDL accumulates cholesterol and, to a lesser extent, TG. In humans, but not mice, cholesterol ester transfer protein (CETP) shuttles cholesterol esters (CE) from HDL to VLDL and LDL. Therefore, VLDL and LDL contain more cholesterol in humans than in mice. LDL-R, LDL receptor; SR-B1, scavenger receptor B1. B: in the bloodstream, association and dissociation of exchangeable apolipoproteins, mainly ApoC-II (C-II), ApoC-III (C-III), and ApoE (E), further regulate VLDL processing. ApoC-II is a cofactor for endothelial lipoprotein lipases, which are involved in hydrolysis of TG. ApoC-III prevents binding to lipoprotein receptors, thereby promoting further processing of the particles. Association of ApoE to the lipoprotein particles promotes uptake by lipoprotein receptors. C and D: cholesterol- and TG-based lipoprotein profiles of fasted mouse and human serum. Lipoproteins were separated on a size-exclusion fast-protein liquid chromatography column. As a result, large particles (VLDL) elute first. After separation, cholesterol (C) or TG (D) was measured in-line. Lipoprotein profiles therefore simultaneously provide information about size, amount, and lipid composition of lipoproteins. Murine serum, unlike human serum, contains only low levels of the ApoB-containing lipoproteins VLDL and LDL. AU, arbitrary units.

Apolipoprotein, ApoC-III, inhibits hepatic lipolysis of TG and is an inhibitor of interaction with the LDL-R, thereby preventing the uptake of these particles by the liver and promoting further processing (5, 8).

Hepatocytes also secrete ApoA1, as disk-like particles. In the process known as reverse cholesterol transport, these particles continuously take up excess cholesterol and, to a lesser extent, TG from peripheral tissues and transport it back to the liver (Fig. 1A). The resulting HDL and its content are then recycled by the liver, mostly through interaction with scavenger receptor B1 (SR-B1).

Different species rely, to a greater or lesser extent, on each of these pathways for hepatic cholesterol acquisition, as is reflected in their lipoprotein profiles. Mice rely mostly on HDL for their hepatic cholesterol uptake, and mouse serum contains only low levels of ApoB-containing lipoproteins (Fig. 1, C and D). In humans, hepatocytes rely more on VLDL and LDL for cholesterol acquisition, and their serum contains much higher levels of LDL and VLDL (Fig. 1, C and D). Hepatic uptake of cholesterol from HDL is less significant in humans, because cholesterol is shuttled from HDL to VLDL and LDL by cholesterol ester transfer protein (CETP). CETP is another lipoprotein metabolism marker that is secreted by human liver cells (Fig. 1A). Mice do not produce CETP. As a result of CETP activity, a much smaller fraction of total cholesterol is associated with HDL in humans than in mice (Fig. 1C).

Several studies have shown that the HCV life cycle is intrinsically linked to, and dependent on, cholesterol metabolism and VLDL synthesis (6, 27). Unlike humans, mice naturally have low circulating levels of the ApoB-containing VLDL and LDL (Fig. 1, C and D), and in this study we investigated if the secretion of human lipoprotein synthesis markers and humanization of lipoprotein profiles are positively associated with infection success in mice with liver that consisted of varying degrees of human engrafted hepatocytes.

We have shown in this study that the lipoprotein profiles of chimeric mice become more human-like at high levels of engraftment of human hepatocytes. This and expression of human markers of lipoprotein biosynthesis, human ApoB (hApoB) and CETP, show a strong positive correlation with successful infection. We show that chimeric mice, which are hard to infect with HCV, have large groups of human hepatocytes that are readily infected with another hepatotropic virus, hepatitis B virus (HBV). Thus it is unlikely that the lack of infection can simply be attributed to low hepatocyte numbers or a hepatic architecture that is not supportive of infection. Association of HCV in the blood of chimeric mice with ApoB-containing lipoproteins is comparable to the association in patient serum and provides further support for a critical role for ApoB-containing lipoproteins in the infectious cycle of HCV.

MATERIALS AND METHODS

Transplantation of SCID/Alb-uPA mice with human hepatocytes. Animals were transplanted with freshly isolated human hepatocytes as described previously (12). Recipient animals were cared for in accordance with Canadian Council on Animal Care guidelines. Experimen-
The level of human engraftment was determined by ELISA, detecting human α1-antitrypsin (hAAT) or human albumin (hAlb) in serum, as described previously (12, 14). Serum samples were taken 6 and 8 wk after transplantation. hAAT values of successfully transplanted mice increase rapidly over the first 6–8 wk; thereafter they typically continue to rise, albeit at a slower rate. All mice were infected with the serum from a single HCV-positive patient 8 wk after transplantation. HCV titers were determined 2 wk after inoculation.

PCR of human and mouse genomic DNA. We estimated the number of human compared with murine cells in chimeric livers by measuring the amounts of human and mouse succinyl-CoA synthetase (SCS-a) genes as described previously (25).

**Histology and immunohistochemistry.** Hematoxylin and eosin staining and methyl green counterstaining on paraffin-embedded liver sections were performed according to standard procedures (7). Briefly, sections of the livers of chimeric mice were fixed in formalin and embedded in paraffin, and 4-μm sections were cut. Immunohistochemical analysis was performed on paraffin-embedded sections that were deparaffinized by incubation in xylene for 5 min, sequentially rehydrated by two immersions in 50%, 95%, and 70% ethanol, and incubated for 5 min in distilled water. Antigen retrieval was then performed by boiling in pH 6.0 10 mM citrate buffer for 15 min following by cooling for an additional 15 min. Immunohistochemical detection of HBV infection was outsourced to HistoBest (Edmonton, AB, Canada). Hepatitis B core antigen (HBcAg) and hepatitis B surface antigen (HBsAg) were detected using rabbit anti-HBcAg antibody (1:1,000 dilution; B0586, Dako) and rabbit anti-HBsAg antibody (1:1,000 dilution; Mu364-UC, BioGenex). Purified rabbit IgG was used as an isotype control. Slides were blocked in normal goat serum, washed, incubated with the primary antibodies, washed, incubated with 3% peroxide, and incubated with goat anti-rabbit horseradish peroxidase (HRP)-conjugated polyclonal antibodies (Dako). The peroxidase reaction was developed using a high-sensitivity diaminobenzidine-positive liquid substrate chromagen system (Dako). In situ hybridization using FITC-labeled Ala DNA probes (InnoGenex, San Ramon, CA) was performed according to the manufacturer’s specifications and developed using the supersensitive polymer HRP-in situ hybridization system (BioGenex).

Infection of chimeric mice with HCV and HBV. Mice were inoculated with serum from a single HCV-infected patient (genotype 1a) by intraperitoneal injection of 100 μl of patient serum. Titers of the inocula were ~105 RNA copies/ml. Cell culture-derived HCV (HCVcc) virus was produced by electroporating JFH RNA into HuH7.5 cells as described previously (24), and mice were inoculated with 100 μl of the cell supernatant (~1 × 106 RNA copies/ml). Infectious H77c virus was produced, as described recently (10), through intrahepatic injection of 50 μg of in vitro-transcribed H77c RNA.

HBV infection of mice was achieved by intraperitoneal injection of 100 μl of serum obtained from a HBV-infected patient, with a viral titer of 5 × 106 viral genome equivalents (vge)/ml. Mouse blood was then collected weekly for 6–7 wk and monitored for the presence of HBV.

**HBV isolation and quantitation.** HBV DNA was quantitated as described previously (19). The standard curve for quantitation was calculated using serial 10-fold dilutions (10–3–10–10 vge/ml) of a plasmid containing the HBV genome.

**HCV isolation and quantitation.** Murine serum was analyzed in blinded fashion at KMT Hepatech. Viral RNA was extracted using the methods described by Boom et al. (4a). The RNA was transcribed to cDNA with a HCV-specific primer (5′-aggttaggatcggtgctc) with a high-capacity RNA-to-cDNA kit (catalog no. 4369016, Applied Biosystems) according to the manufacturer’s directions. RT-PCR was performed using a real-time PCR system (model 7300, Applied Biosystems) and TaqMan chemistry, with all our measurements in duplicate. We used 6-FAM-CACCCCTATCAGGGCATACCAAAG-GCC-TAMRA as the HCV-specific detection probe and a primer set detecting the conserved 5′-untranslated region of HCV (5′-TGGCG-GAACCGGTGAGTACA, 5′-aggttaggatcggtgctc). For absolute quantitation, we created a standard curve of known dilutions of a plasmid containing the sequence for HCV variant H77c (pCV-H77c), alongside a HCV RNA high control (Optiquant).

**Calculation of median titers and geometric averages of titers.** The median titer is defined as the “intermediate” value of all viral titers and is useful when little importance is attached to outliers. It allows inclusion of all data, including zeros. The geometric mean is calculated by taking the nth square root of the product of all positive values. Like median titers, it also depicts a central tendency or typical titer and is generally useful for numbers that are exponential in nature. The “normal” arithmetic mean under such circumstances is influenced strongly by the higher values on an exponential scale.

**Quantification of CETP and ApoB.** Serum levels of CETP and human ApoB were determined by quantitative dot-blot. hApoB and CETP levels were determined in serum samples of known hAAT.

Blood was drawn from animals, and 1 μl of serum was serially diluted in PBS and applied to a dot-blot (Bio-dot microfiltration apparatus, Bio-Rad) according to the instructions provided by the manufacturer. Blots were first incubated with a monoclonal antibody against CETP (TP2, Ottawa Heart Institute) or a monoclonal antibody directed against human ApoB (1D1, Ottawa Heart Institute; no cross-reactivity with murine or bovine ApoB). We used HRP-coupled anti-mouse IgG as the detecting antibody. A standard curve was generated by application of known dilutions of human serum in mouse serum to the same dot-blot. Optical density of the dots was determined with ImageJ software, which can be downloaded from the website of the National Institutes of Health.

**Analysis of lipoprotein profiles.** Lipoprotein analysis was performed at the Lipid Analysis Core of the Women and Children’s Health Research Institute at the University of Alberta (Edmonton, AB, Canada), as described previously (26). Lipoprotein profiles are determined by separating serum particles on a size-exclusion column (large particles, such as VLDL, elute first) and in-line measurement of cholesterol or TG associated with the different fractions after separation. VLDL, containing mostly TG and little cholesterol, is prominent on TG profiles but does not show up on cholesterol profiles, and the reverse is true for HDL (high cholesterol amount, little or no TG). Lipoprotein profiles simultaneously give information on amount, particle size, and particle composition, and the particle size changes when the lipid composition changes.

**Immunoprecipitation of ApoB-containing particles.** Immunoprecipitation experiments were essentially performed as previously described (22). Serum samples (50 μl) with titers of ≥104 RNA copies/ml were incubated overnight with 7.5 μl of anti-ApoB antibody [goat anti-human ApoB (Ab742, Chemicon); cross-reacts with human, mouse, and bovine ApoB] at 4°C while rotating. Protein G slurry (20 μl; Protein-Sepharose 4 fast flow, GE Healthcare) was added to each sample, which was then incubated for ≥1 h on a rotator. The protein G complexes were precipitated at 14,000 g in a microfuge. The supernatants were collected and assayed by ApoB dot-blot for the absence of ApoB to ensure complete precipitation of ApoB. The amount of HCV in the supernatants was also determined. The pellets were washed once with PBS, and viral RNA was isolated directly from the pellets (QiaAmp viral RNA kit, Qiagen) and analyzed by quantitative RT-PCR. Samples containing high amounts of immunoglobulins (e.g., patient serum) were preclreated with protein G beads, prior to immunoprecipitation, to ensure quantitative immunoprecipitation of the ApoB complexes. To ensure that the protein G...
beads did not bind HCV or HCV complexes directly, we incubated HCV-containing mouse serum with protein G-Sepharose beads in the absence of anti-ApoB antibodies. The HCV titers in the precipitate of these samples were negligible.

RESULTS

Estimation of the degree of human chimerism. We noticed that, in chimeric mice with low hAAT values (<200 µg/ml), the infection with HCV was unreliable (~35%), and we wanted to determine if this was due to low hepatocyte numbers. We use serum levels of hAAT to estimate engraftment success, while most other groups use hAlb for this purpose. We therefore compared hAlb levels with hAAT levels in 190 transplanted mice. Circulating hAlb levels were approximately eight times higher than hAAT levels (Fig. 2A). Also, two-thirds of the mice without detectable hAAT were positive for hAlb. We can therefore conclude that, by using hAAT values, we actually underestimate the quality and number of transplanted mice. However, we have never achieved infection in mice with hAAT <20 µg/ml, and we can also conclude that the hAAT assay is sufficiently sensitive for our purposes.

To estimate the relative amount of human and mouse cells, we also determined the relative amount of human and mouse SCS-α genes (25). Even at low hAAT values (25–50 µg/ml), an estimated 20–30% of the chimeric liver consists of human cells (Fig. 2B). Finally, we examined histological sections of low-hAAT mice for the presence of human cells. Human cells are easy to distinguish from mouse cells, since they are less eosinophilic and show as lighter clusters (3, 14). Even in mice with hAAT <15 µg/ml, large clusters of human cells are evident and easy to find (Fig. 2C).

In summary, we do not believe that the number of human hepatocytes in mice with hAAT <200 µg/ml should be a limiting factor for HCV infection.

HBV infection in “low-hAAT” mice. To show that the human hepatocytes that are present in low-hAAT mice are sufficient to support infection with other hepatotropic viruses, we inoculated 19 mice with HBV. The hAAT values of these mice ranged from 19 to 230 µg/ml. All inoculated mice had high levels of HBV in their blood (10^5–10^9 vge/ml) as early as 1 wk after infection (Fig. 3A). Typically, titers further increased 10-fold over the next 6–8 wk. It is unlikely that the observed viral titers, especially the higher values, are the result of longevity of the initial inoculum: although HBV was still detectable in the serum of inoculated mice 6 h postinjection, at 2 days postinfection viral titers were <1,000 vge/ml (not shown). Additionally, in all but two mice, the measured titers exceeded the initial amount of virus injected.

We further examined the accessibility of human hepatocytes to HBV and numbers of cells infected by immunohistochemical detection. In Fig. 3B, we used Alu staining to distinguish human cells from mouse cells (hAAT = 192 µg/ml). The

Fig. 2. Degree of human chimerism. A: comparison of human α1-antitrypsin (hAAT) levels with human albumin (hAlb) levels in 190 chimeric mice. hAAT and hAlb concentrations were determined by ELISA. hAlb levels are ~8 times higher than hAAT levels, and two-thirds of the mice that were negative for hAAT were positive for hAlb; these values are plotted on the y-axis at their corresponding hAlb level. hAAT: range 0–686 µg/ml, median 8 µg/ml, average 39–153 µg/ml; hAlb: range 0–5,280 µg/ml, median 8 µg/ml, average 153 µg/ml. B: relative amount of human and mouse succinyl CoA-synthetase-α (SCS-α) genes. Entire liver of the mouse was dissected into 4–6 pieces. Genomic DNA isolated from each section was amplified by PCR with a single primer set amplifying murine and human SCS-α. Human and mouse amplification products can be distinguished on the basis of their size. To determine the relative amount of human SCS-α gene in each section, PCR products were separated by gel electrophoresis, and bands were quantified. Relative contribution of mouse and human cells in each section was calculated using a standard curve generated by plasmid controls, and weight correction was made for the relative contribution of each section to the entire liver. We analyzed livers of 30 mice, with each point representing 1 mouse. hAAT: range 27–1,002 µg/ml, median 340 µg/ml, average 357 µg/ml. C: hematoxylin-eosin-stained histological section of livers of chimeric mice with circulating hAAT concentration of 7 µg/ml (top) and 11 µg/ml (bottom). Large groups of human cells, which typically are less eosinophilic than mouse cells and, therefore, show as lighter clusters (⋆), are evident, even in mice with very low hAAT values.
corresponding regions of adjacent sections show HBsAg and HBcAg in a large percentage of the human cells (Fig. 3, C and D). This region also further demonstrates the high degree of humanization of the liver and visible vascularization.

On the basis of these data, it is not likely that the variable infection of mice with hAAT II/II >200 μg/ml can be attributed to low amounts of human hepatocytes or inaccessibility of the engrafted cells to pathogens.

**HCV infection vs. degree of human chimerism: infection success and HCV titers.** To determine the degree of human chimerism necessary to reliably infect chimeric mice, we retrospectively analyzed HCV infection in relation to hAAT levels in 455 mice. The mice in this study were transplanted with hepatocytes from 25 different human donors. Figure 4A shows HCV titers of all 455 mice as a function of their hAAT value. HCV titers are undetectable in the serum of the mice 1 day postinfection (not shown); therefore, we consider it highly unlikely that the observed viral titers are due to longevity of the initial inoculum. Mice with hAAT II/II >50 μg/ml can only sporadically be infected with HCV (~12% infection success), and only in mice with very high hAAT values (>500 μg/ml) does infection success appear to reach a plateau, with ~90–95% of the mice infected (Fig. 4B). We have no solid explanation for the lack of infection in the remaining 5–10% of mice: it possibly represents the unexpected clearance of the virus, but it also may be the result of a technical error, e.g., accidental injection into the intestines, instead of into the peritoneum, at the time of infection.

Viral titers also increase with increasing hAAT values. We determined the median titers, as well as the geometric averages of HCV titers (as further explained in MATERIALS AND METHODS), and both show similar trends: titers gradually increase with hAAT, and maximal titers are reached in mice with hAAT >500 μg/ml (Fig. 4C).

We have analyzed infection success and viral titers according to liver tissue donor. We found that once engrafted, there are no significant differences in infection rates between hepatocytes from different donors, and infection success only changes with engraftment success. Also, there are essentially no differences in viral titers in relation to hAAT values due to donor variability, with the exception of two donors. The viral titers in these two groups were lower than expected, but infection rates were not altered. These data are responsible for the majority of the outliers. We have not excluded these data from our analysis. We have no explanation for these lower viral titers, since the indication for these donors (tumor resection) was not unusual compared with the other donors.

These data show that reliable infection, with maximal titers, is reached at hAAT of 500 μg/ml, corresponding to chimeric livers that consist of 70–80% of human cells. However, even in mice with very low hAAT values (<50 μg/ml), ~20–30% of the livers consist of human cells. Also, clusters of human hepatocytes are easy to detect in histological slides, and nodules of human cells are visible macroscopically when hAAT values have reached 100 μg/ml. Since the cells from the same donors are infectable in mice with higher engraftment rates, we can assume that all the entry receptors and machinery for replication are present in these cells. We therefore believe that at least one other human and liver-derived factor is essential for successful infection with HCV. Since this factor appears not to
be present on or in the hepatocytes, we hypothesized that it is a secreted factor and that it must reach a critical level before infection is fully reliable and titers reach their maximums.

Circulating levels of human ApoB and CETP in chimeric mice. On the basis of the reported tight link between HCV and lipoprotein metabolism (reviewed in Refs. 6, 27), we further analyzed the secretion of markers of human lipoprotein synthesis, CETP and hApoB, into the blood of chimeric mice. As shown in Fig. 5A, hApoB increases gradually in relation to hAAT values and reaches a plateau at hAAT of \(\sim 500\) \(\mu\)g/ml. At these levels, the amount of hApoB is similar to the amount of ApoB found in human serum. As shown in the overlay plot (Fig. 5C), this coincides with the transition point where infection becomes reliable and maximal titers are reached. CETP is secreted into the blood of chimeric mice in a similar fashion: CETP increases gradually with increasing hAAT and also reaches a plateau at hAAT of 500 \(\mu\)g/ml (Fig. 5B).

**Humanization of lipoprotein profiles in chimeric mice.** Determination of lipoprotein profiles offers an alternative approach to look at changes in lipoprotein synthesis and maintenance. Since the lipoprotein profiles of mice are very different

---

**Fig. 4.** Infection of chimeric mice with HCV. **A:** HCV infection vs. hAAT value. We infected 455 chimeric mice with positive hAAT values with serum from a HCV-infected patient and determined HCV titers in mouse blood. All samples that were negative for HCV are plotted on the x-axis according to their hAAT value. We also analyzed these data according to liver tissue donor. We did not find significant differences between donors, with the exception of 2 donors. Viral titers in the mice transplanted with these hepatocytes were lower, although infection rates were not affected. These values are responsible for a major part of the outliers. These differences were significant in mice with hAAT \(>500\) \(\mu\)g/ml \((P = 0.002\) for both, Student’s t-test), but not in mice with hAAT \(<500\) \(\mu\)g/ml. **B:** infection success was calculated in mice with different hAAT ranges. Group size was based on number of mice in each group, as well as (absence of) obvious jumps in infection success in each range. Group sizes varied from 7 mice (hAAT 700–800 \(\mu\)g/ml) to 81 mice (hAAT 50–100 \(\mu\)g/ml), median group size was 26 mice, and average group size was 32 mice. **C:** geometric averages of HCV titers (●, positive values only) and median HCV titers (bars, all values, including zeros) in mice with different hAAT ranges.

---

**Fig. 5.** Serum levels of human ApoB and CETP in blood of chimeric mice. **A:** serum levels of hApoB were determined by quantitative analysis of dot-blot, with an antibody that specifically recognizes human (and not murine) ApoB. hApoB amounts were determined by comparison with a standard curve consisting of known ratios of human serum mixed with murine serum. ApoB amounts in unmixed human serum from 3 different human donors are depicted at far right. **B:** quantitative analysis of human CETP in blood of chimeric mice as determined by dot-blot using a monoclonal CETP antibody. CETP amounts in 3 different human serum samples are depicted at far right. **C:** overlay plot of hApoB amounts (Fig. 4A) and infection and viral titer data (Fig. 3A).
from the lipoprotein profiles of humans, as explained in Fig. 1, we determined whether triacylglycerol (TG) and cholesterol profiles gradually become more human in relation to transplantation success.

TG and cholesterol lipoprotein profiles change gradually from typical mouse lipoprotein profiles (Fig. 6, A and B, left) to lipoprotein profiles that closely resemble human lipoprotein profiles (Fig. 6, A and B, right) at high hAAT values. Since the amount of ApoB-containing lipoproteins (VLDL + LDL) relative to the amount of HDL in mice is different from humans, we used this ratio as a measure of the humanization of lipoprotein profiles. We analyzed 23 cholesterol profiles in mice with hAAT values ranging from 0 to 900 µg/ml (Fig. 6C) and 17 TG profiles in mice with hAAT values ranging from 0 to 550 µg/ml (Fig. 6D). The ratios based on cholesterol and TG show a similar trend: with increasing hAAT value, the ratios of the ApoB-containing lipoproteins compared with HDL approach the ratios found in human blood samples.

In summary, the lipoprotein profiles (TG and cholesterol) of chimeric mice appear increasingly human with increasing hAAT values and, typically, reach maximal humanization at hAAT of 500 µg/ml. This coincides with the point where maximal, and “human-like,” amounts of human ApoB and CETP are present. It also coincides with the point of maximal HCV infection success and maximal HCV titers.

**Correlations.** Finally, we wanted to determine the correlation between hApoB, CETP, or hAAT contents and infection success or viral titers. We grouped mice in appropriate ranges based on their hAAT and corresponding hApoB and CETP and used the data above to determine the infection success and geometric average of HCV titers in each of these groups. Infection success increases linearly when each of these factors increases exponentially (Fig. 7). The exponential increase of the viral titers shows a linear correlation to the exponential increase of hApoB, CETP, or hAAT (Fig. 8).

HCV associates to ApoB-containing lipoproteins in blood of patients and chimeric mice. HCV in patient serum is partly associated with ApoB-containing lipoproteins, which has been linked to high infectivity. We wanted to investigate if HCV in the blood of chimeric mice also is associated with ApoB-containing particles. Mice were infected with HCV in three different ways: by infection with patient serum (genotype 1a, 4 mice), by infection with HCVcc (JFH, 4 mice), and by intrahepatic injection of H77c RNA (2 mice). We determined the percentage of HCV that can be immunoprecipitated with anti-ApoB antibodies in the serum of infected mice. For comparison, we also analyzed ApoB association in the serum of four different HCV patients (all genotype 1a). Our values [54 ± 27% (SD), range 29–82%] are within the (wide) ranges reported for patient sera by other groups (1, 22). In chimeric mouse serum samples, ApoB association levels are comparable to those in humans [Fig. 9; 58 ± 17% (SD), combined data for all infection methods] and, similarly, varied considerably (range 37–87%). Individual measurements of different mice or patients are depicted in Fig. 9, with the horizontal bar representing the average. No obvious differences were found in the

---

**Fig. 6. Triacylglycerol (TG)- and cholesterol-based lipoprotein profiles in normal mice, chimeric mice, and humans.**

A: TG-based lipoprotein profiles of normal mice (left), humans (right), and chimeric mice with progressive human engraftment (middle panels, left to right). Lipoprotein profiles were determined as described in MATERIALS AND METHODS. Numbers in middle panels represent hAAT values of the mice.

B: cholesterol-based lipoprotein profiles of normal mice (left), humans (right), and chimeric mice with progressive human engraftment (middle panels, left to right). Numbers in middle panels represent hAAT values of the mice.

C and D: as a measure for humanization of lipoprotein profiles in relation to hAAT values, we determined the ratio of area under peaks of VLDL + LDL compared with area under HDL peak. C: ratios calculated from TG profiles. D: ratios calculated from cholesterol profiles. Ratios in normal human serum are depicted at far right.
mice between the different infection methods. The percentage of HCV that can be coimmunoprecipitated with ApoB is also independent of hAAT value. Association of HCV with ApoB-containing particles further supports a role for lipoproteins in the infectious cycle of HCV in chimeric mice.

**DISCUSSION**

Although multiple determinants for HCV infection are known, it remains partly unclear what determines the human specificity of HCV infection. Conceivably, presence of the appropriate entry receptors is essential, and this may partly explain why HCV is unable to infect cells of nonhuman origin. However, it appears that many other factors contribute to human-specific tropism. For example, although HCV replicons are capable of replicating in mouse cells (23, 28), this process is inefficient, and there is no evidence that assembly of functional viral particles occurs in murine cells.

We show in this study that the presence of relatively high numbers of human hepatocytes in successfully transplanted SCID/Alb-uPA mice is not necessarily sufficient for infection. Consistent with other studies (3, 4, 14), even at hAAT values of 20–50 μg/ml, an estimated 20–30% of the liver consists of human cells, and significant clusters of human cells are easy to find on histological sections of the livers from these mice. Nevertheless, we were only able to infect these mice at a rate of ~15%. To eliminate the possibility that we missed infection because of the lack of sensitivity of our quantitative PCR assay (theoretical limit 255 copies/ml), we also performed a qualitative assay that could detect ~10 copies/ml, and still we found low infection rates in mice with low hAAT values (data not shown). Importantly, early studies using different amounts of virus for infection showed that inocula with ≥50,000 copies of virus did not increase infection rates. In this study, all mice were infected with ~100,000 copies of virus.

Surprisingly, even when an estimated 50% of the liver consists of human cells (hAAT of 150–200 μg/ml), we could infect only 40–50% of these mice with HCV. However, these
mice can reliably be infected with another hepatotropic virus, HBV (13; present study) or with the malaria parasite (20). Therefore, it is unlikely that hepatic malfunction or organization can explain the lack of infection. This is consistent with other studies into the organization of human hepatocytes in chimeric mice: human hepatocytes repopulate the SCID/Alb-uPA mouse livers in a highly organized fashion, with preservation of normal cell function and microstructure (3, 14).

These observations led us to believe that at least one additional human factor, likely a secreted product, is essential for infection with HCV. Our data indeed show an excellent correlation between infection success or viral titers and the exponential increase in serum levels of all secreted human products that we measured (hAAT, hApoB, or CETP).

HCV circulates in patient blood in a very heterogeneous population, a major portion of which has an unusual low buoyant density compared with other members of the Flaviviridae and can be attributed to its association with ApoB-containing particles (1, 22). Importantly, the low-density, lipoprotein-associated fractions are associated with high infectivity in chimpanzees and in cell cultures (reviewed in Refs. 6, 27). Since the majority of HCV in mouse serum is bound to ApoB-containing particles (our data), similar to patient sera (1, 22; present study), we hypothesized that the humanization of lipoproteins is a cofactor in HCV infection.

CETP and hApoB reach maximal concentrations when hAAT in the chimeric mice reaches 500 µg/ml. This coincides with the transition point where viral titers reach a maximum and infection success approaches 100%. Human ApoB and CETP levels reach concentrations similar to those in human blood, and cholesterol and TG profiles at this point closely resemble human profiles. Although we did not investigate serum levels of other apolipoproteins, previous studies showed that, for example, human ApoE and human ApoA1 are also secreted by human/mouse chimeric livers (14), providing further support for the idea that the lipoprotein composition in chimeric mice indeed becomes human-like.

HCV is linked to lipoprotein synthesis and metabolism during viral assembly and egress in the circulation, as well as during viral entry, as has been extensively reviewed (6, 27). It is likely that this association of HCV with lipoproteins occurs intracellularly, since several studies have shown that HCV assembly and secretion are linked to, and dependent on, lipoprotein assembly and secretion (reviewed in Refs. 6, 27). The early steps of viral entry are also dependent on ApoB or lipoproteins (6) through interactions with scavenger receptor B1, LDL-R, and highly sulfated glycosaminoglycans (reviewed in Refs. 6, 27; also see Refs. 16, 17).

Since murine cells are capable, albeit inefficiently, of replicating HCV virus (23, 28), we tested whether the secretion of human lipoproteins by murine cells is sufficient to support assembly of the virus and enables a successful infection cycle. We infected mice that are double-transgenic for hApoB and CETP and that have a human-like lipoprotein profile (9) with HCVcc and patient-derived virus without success. We also tried to bypass the viral entry steps by intrahepatically injecting H77c RNA or JFH RNA; we still could not detect mature HCV in the circulation of these mice (unpublished results). Although this pilot study is not exhaustive, we can conclude that secretion of human ApoB and CETP by murine cells and humanization of lipoprotein profiles alone are insufficient to support infection.

Possibly, mouse physiology contributes to the prevention of infection, for example, by the secretion of (unknown) inhibitors of infection, processing that is unsupportive for infection, and/or removal of HCV-containing lipoproteins from the circulation. This may be especially noticeable in mice with suboptimal engraftment for HCV infection. One example of a factor that potentially could prevent infection is LPL. The main sources of LPL, in vivo, are endothelial cells outside the liver. In the chimeric mouse model, LPL would therefore be produced by murine cells, independent of the degree of human engraftment. It has been shown, in vitro, that externally added LPL (albeit at hyperphysiological concentrations) possibly targets lipoproteins for degradation in cell cultures (2) and has virolytic activity in patient sera (21). Whether these processes are relevant in vivo remains unclear, since the association and dissociation of exchangeable apolipoproteins in the circulation (Fig. 1) differentially regulate whether LPL targets lipoproteins for degradation or has lipolytic activity, a process that is absent in cell culture systems. Although we cannot fully exclude the possibility that LPL may be involved in the prevention of infection at lower engraftment rates, inhibition of LPL for the duration of the infection is unfeasible, since it would prevent the processing of larger lipoproteins into smaller particles in the circulation and results in a milky appearance of serum because of the accumulation of chylomicrons and VLDL, which likely has many secondary effects on HCV infection.

It is likely that murine hepatocytes function as competitors with human hepatocytes in our mouse model, and high engraftment may partly be needed to overcome this competition. Mouse hepatocytes are capable of efficiently removing human LDL from the circulation (15), as is also supported by our own studies. We injected high amounts of human lipoproteins (VLDL and LDL) in mice, which resulted in a human-like lipoprotein profile shortly after injection (4 h; not shown).

**Fig. 9.** Association of HCV to ApoB-containing particles in serum of chimeric mice and human serum. Fraction of HCV in circulation associated with ApoB-containing lipoproteins was determined by quantitative immunoprecipitation of ApoB followed by quantitative RT-PCR of pelleted fractions and unprecipitated fractions. Relative contribution of ApoB-associated fraction compared with total HCV titer is depicted. Serum sample from a different infected mouse (1, 10 mice total) or human (1, 4 total) is shown, with duplicate measurements for each (negligible differences). Human values are consistent with those reported in other studies. Horizontal bars represent means ± SD for each group: 59 ± 23 for JFH (n = 4), 57 (SD not determined) for H77c (n = 2), 59 ± 17 for patient serum-infected mice (n = 4), 58 ± 17 for all mice combined (n = 10), and 53 ± 27 for sera from HCV-infected patients (n = 4).
However, in nontransplanted SCID/Alb-uPA mice and in successfully transplanted mice, these lipoproteins are essentially undetectable as early as 24 h after injection (unpublished results). Hence, when the majority of the chimeric liver is murine in nature, part of the lipoproteins, HCV associated or not, may be removed from the circulation by mouse hepatocytes. The same applies to other (unknown) factors that may be essential for infection and may be removed by murine cells.

In summary, the data presented in this study support the notion that HCV infection in chimeric mice is a complex interplay of processes. The weakest link in the chain does not appear to be the presence of human hepatocytes per se, although hepatocytes not are omissible in the infectious cycle of HCV. We envision that infection success and/or viral titers will remain suboptimal, until hepatocyte numbers are high enough to balance production of lipoprotein-associated virus, with the continuous lipoprotein processing in the circulation and viral entry vs. nonproductive removal of viral particles. Our data indicate that this point is not reached until the chimeric livers, and lipoprotein profiles, are almost completely humanized. Vice versa, when fairly high numbers of human cells are present, but lipoprotein profiles remain mouse-like, infection does not occur. Although we have only been able to show correlations between humanization of lipoprotein profiles, infection success, and viral titers, we believe that these data provide important insights into the dynamics of in vivo HCV infection in the context of the gradual humanization of mouse livers and subsequent humanization of lipoprotein profiles. Additionally, given the uncertainty of infection with HCV at lower hAAT/hAlb values, these data provide an important guideline for interpretation of future studies in chimeric mouse models, especially those utilizing smaller numbers of mice with suboptimal engraftment for HCV infection. Finally, with the data presented in this study, we have established an excellent platform to determine experimentally the role of lipoproteins in infection with HCV in vivo.

ACKNOWLEDGMENTS

We thank Dr. T. Wakita for his kind gift of JFH plasmid, Dr. C. Rice for HuH7.5 cells, and Dr. J. Bukh for the H77c plasmid. Audric Moses for expert technical help with the determination and interpretation of lipoprotein profiles, and Dr. Gordon A. Francis for helpful discussions. We also thank Dr. J. Vance and Dr. W. A. Addison for critical review of the manuscript.

GRANTS

R. H. G. Steenbergen and R. Chen received funding from the National Canadian Research Training Program for Hepatitis C. R. H. G. Steenbergen was funded in part by a Canadian Institutes for Health Research/Wyeth Pharmaceuticals research fellowship. D. L. J. Tyrrell received financial support from the Canadian Institutes for Health Research for Studies in HBV (MOP 57824) and HCV (EOP 64705).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES


