Usefulness of humanized cDNA-uPA/SCID mice for the study of hepatitis B virus and hepatitis C virus virology

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Abstract
Urokinase-type plasminogen activator/severe combined immunodeficiency (uPA/SCID) mice transplanted with human hepatocytes are permissive for hepatitis B virus (HBV) and hepatitis C virus (HCV) infection. However, one of the problems affecting uPA transgenic mice is the expansion of mouse hepatocyte colonies due to homologous recombination of the uPA gene. In this study, we attempted to infect HBV and HCV in humanized cDNA-uPA/SCID mice, a novel uPA transgenic mouse model designed to overcome this disadvantage. Three hundred and eighty-six uPA/SCID and 493 cDNA-uPA/SCID mice were transplanted with human hepatocytes and then injected with either HBV- or HCV-positive human serum samples or HBV-transfected cell culture medium. Twelve weeks after human hepatocyte transplantation, the mouse serum concentration of human albumin, which is correlated with the degree of repopulation by human hepatocytes, was significantly higher in cDNA-uPA/SCID mice compared with uPA/SCID mice. HBV-infected cDNA-uPA/SCID mice showed significantly greater and more persistent viraemia, and similar virological effects by entecavir treatment were achieved in both systems. HCV-infected cDNA-uPA/SCID mice developed more frequent and significantly higher viraemia compared with uPA/SCID mice. The present study using a large number of mice showed that cDNA-uPA/SCID mice transplanted with human hepatocytes developed high and long-term persistent viraemia following HBV and HCV infection, and a higher survival rate was observed in cDNA-uPA/SCID compared with uPA/SCID mice. These mice may be a useful animal model for the study of HBV and HCV virology and the analysis of the effect of antiviral drugs.

INTRODUCTION
Hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are serious health problems worldwide. More than 240 and 185 million people are infected with HBV and HCV, respectively [1, 2]. Infected individuals develop acute hepatitis, chronic hepatitis, liver cirrhosis and potentially death due to liver failure and hepatocellular carcinoma [3, 4].

Study of the biology and development of therapies for each virus has long been hampered by the lack of a small animal model capable of supporting hepatitis virus infection. The chimpanzee has been a valuable model for the study of HBV and HCV [5, 6] but its use has become severely hampered by financial and ethical constraints. Immunodeficient mice with a urokinase-type plasminogen activator (uPA) [7, 8] or herpes simplex virus type-1 thymidine kinase (HSVtk) [9] transgene or a targeted disruption of the murine fumaryl acetoacetate hydrolase (FAH) [10, 11] have been shown to be excellent recipients for transplantation of human hepatocytes. These small animal models support infection with hepatitis viruses and are useful for the study of HBV and HCV biology [12, 13]. The human hepatocyte transplanted uPA/severe combined immunodeficiency (uPA/SCID) mouse provided the first really useful animal model of human hepatitis virus acute and chronic infections [7]. Although mouse hepatocytes damaged by overexpression of uPA can be suitable for engrafment and proliferation of human hepatocytes in uPA/SCID mice, partial transgene deletion in mouse hepatocytes may occur, resulting in loss of uPA expression and appearance of colonies of normalized mouse hepatocytes in chimeric mouse livers [14]. Furthermore, the tendency to develop kidney disorders is increased, and body size is...
that mouse livers were more efficiently replaced with human hepatocytes in cDNA-uPA/SCID mice than in uPA/SCID mice.

**HBV infection of BD195 human hepatocyte transplanted uPA/SCID and cDNA-uPA/SCID mice**

Using BD195 mice transplanted with human hepatocytes, we compared serum HBV DNA levels between uPA/SCID and cDNA-uPA/SCID mice. Twelve weeks after hepatocyte transplantation, 52 uPA/SCID chimeric mice and 227 cDNA-uPA/SCID chimeric mice were inoculated with serum samples obtained from patient 1, and 34 uPA/SCID and 5 cDNA-uPA/SCID mice were inoculated with serum samples obtained from patient 2. Serum HBV DNA became positive 2 weeks after inoculation in all mice and reached a plateau at 8 weeks. HBsAg and HBeAg values increased to 47665.1±26386.7 IU ml⁻¹ (n=12) and 2037.1±995.2 signal-to-cutoff ratio (n=10), respectively. Serum HBV levels at week 8 were significantly higher in cDNA-uPA/SCID compared with uPA/SCID (8.8 ±1.1 vs 8.2±0.7 log copies ml⁻¹, P<0.001) mice inoculated with serum from patient 1 (Fig. 4a). Serum HBV DNA levels in cDNA-uPA/SCID mice inoculated with the patient 2 serum sample increased more rapidly and were significantly higher at 4 weeks after inoculation than those in uPA/SCID mice. High titres of HBV viraemia in cDNA-uPA/SCID mice persisted for 34 weeks or more.

Seven BD195 human hepatocyte transplanted uPA/SCID and 16 cDNA-uPA/SCID mice were inoculated with...
HBV-transfected cell culture medium (Fig. 4b). Similar to those of mice inoculated with serum from HBV-infected patients, serum HBV DNA levels at 8 weeks after inoculation were significantly higher in cDNA-uPA/SCID mice compared with uPA/SCID mice (9.0±0.4 vs 8.6±0.2 log copies ml\(^{-1}\), \(P<0.05\)). These mice injected with HBV-transfected cell culture medium were treated with the nucleoside analogue entecavir for 4 weeks. Entecavir treatment resulted in a reduction of mouse serum HBV DNA levels in both uPA/SCID and cDNA-uPA/SCID mice, and similar

Fig. 2. Flowchart of human hepatocyte transplantation and HBV and HCV infection. uPA/SCID and cDNA-uPA/SCID mice were transplanted with either BD85 or BD195 human hepatocytes, then injected with HBV-positive human serum samples obtained from patients 1 or 2 and cell culture medium or HCV-positive human serum samples (patient 3).

Fig. 3. Serum human albumin levels in uPA/SCID and cDNA-uPA/SCID mice. Mice were transplanted with either BD85 or BD195 human hepatocytes. Mice serum human albumin was measured at 12 weeks after hepatocyte transplantation. In these box-and-whisker plots, lines within the boxes represent median values; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively; and the upper and lower bars outside the boxes represent the 90th and 10th percentiles, respectively. **\(P<0.001\).
virological effects were achieved with both systems (1.8±0.4 vs 2.2±0.4 log copies ml⁻¹).

**HCV infection to BD195 human hepatocyte transplanted uPA/SCID and cDNA-uPA/SCID mice**

Two hundred and sixteen uPA/SCID and 164 cDNA-uPA/SCID mice transplanted with BD195 human hepatocytes were inoculated with HCV-positive serum samples obtained from patient 3. Serum HCV RNA became positive until up to 8 weeks after inoculation in 198 uPA/SCID and in all cDNA-uPA/SCID mice (Fig. 5a). No mice became positive for HCV later than 8 weeks after inoculation. The frequency of the development of viraemia was significantly higher in cDNA-uPA/SCID mice compared with uPA/SCID mice (100 and 91.7 %, respectively, P<0.001). In mice that developed viraemia, serum HCV RNA titre increased more rapidly and was significantly higher at 8 weeks after inoculation in cDNA-uPA/SCID mice than in uPA/SCID mice (7.3 ±0.5 vs 7.1±0.6 log copies ml⁻¹, respectively, P<0.001) (Fig. 5b). A prior study showed that cDNA-uPA/SCID chimeric mice have fewer kidney disorders, higher body weights and a higher survival rate than uPA/SCID chimeric mice at week 28 [16]. The present study also showed a higher survival rate in cDNA-uPA/SCID mice than in uPA/SCID mice 8 weeks after HCV infection [92.1 % (164 out of 168) vs 84.8 % (151 out of 198)]. Because of higher titres of viraemia, larger body size and higher survival rates, cDNA-uPA/SCID mice may be preferred over uPA/SCID mice for investigating HBV and HCV virology and analysing the effects of antiviral drugs.

HCV infection induces up-regulation of intrahepatic interferon (IFN)-stimulated genes (ISGs) following activation of innate immune effectors [18]. The induction of intrahepatic ISGs has been shown to be stronger in patients with rs8099917 T/G or G/G genotypes [19]. Using human hepatocyte chimeric mice, we previously reported that HCV replicates more effectively in human hepatocytes with the rs8099917 T/T genotype than that with the T/G genotype [17]. Consistent with this previous report, serum HCV RNA levels at 8 weeks after infection were significantly higher in mice transplanted with BD195 human hepatocytes.
(rs8099917 T/T genotype) than in mice transplanted with BD85 human hepatocytes (rs8099917 T/G genotype) in both uPA/SCID [7.2 (6.7–7.5) and 6.3 (5.9–6.7) log copies ml$^{-1}$, respectively; $P<0.001$] and cDNA-uPA/SCID mice [7.4 (7.0–7.6) and 6.2 (6.1–6.7) log copies ml$^{-1}$, respectively; $P<0.001$] (Fig. 6). The higher HCV RNA levels in BD195 human hepatocyte transplanted mice do not appear to be due to greater repopulation with human hepatocytes because mice serum HSA levels in BD195 human hepatocyte transplanted mice were similar to those in BD85 human hepatocyte transplanted mice in both uPA/SCID and cDNA-uPA/SCID mice.

Although IFN treatment resulted in a significantly greater induction of intrahepatic ISG expression in mice transplanted with rs8099917 T/T genotype hepatocytes [17], we observed no statistically significant differences in ISG expression levels with respect to IL28B single-nucleotide polymorphism genotype before therapy (data not shown). This may result from lower ISG expression levels before therapy.

The relationship between serum HBV DNA levels and transplanted human hepatocyte IL28B genotype was also analysed; however, no significant correlation was observed (data not shown). The correlation between intrahepatic ISG expression and HBV replication is unclear because HBV has been reported to induce no detectable ISG expression by microarray analysis in the chimpanzee model [20]. Further analysis is needed to clarify the correlation between HBV replication and IL28B genotype.

In conclusion, the present study showed using a large number of mice that human hepatocyte transplanted cDNA-uPA/SCID mice developed high and persistent viraemia following HBV and HCV infection. This improved mouse model provides a useful animal model for the study of HBV and HCV virology and the analysis of the effects of antiviral drugs.

**METHODS**

**Animal treatment**

Generation of uPA+/+/SCID+/+ mice (uPA/SCID) and cDNA-uPA/SCID mice and transplantation of human hepatocytes were performed as described previously [8, 16]. All animal protocols described in this study were performed in accordance with the guidelines of the local committee for animal experiments, and all animals received humane care. Mouse serum concentrations of HSA, which serve as a useful marker of the extent of repopulation, were measured as described previously [8]. Mice underwent transplantation with frozen human hepatocytes obtained from two different human donors, BD85 and BD195 (Table 1). Twelve weeks after hepatocyte transplantation, mice were injected intravenously with serum or culture medium including $10^5$ copies of either HBV or HCV. Mouse serum samples were obtained every 2 weeks after infection, and serum HBV DNA or HCV RNA levels were measured.

**Human serum samples**

Human serum samples were obtained from two patients with chronic hepatitis B (patients 1 and 2) and a patient with chronic hepatitis C (patient 3) after obtaining written informed consent. Characteristics of the two patients with chronic hepatitis B are shown in Table 2. Serum obtained from patient 3 contained a high titre of genotype 1b HCV ($2.2 \times 10^6$ copies ml$^{-1}$). The individual serum samples were divided into small aliquots and stored separately in liquid nitrogen until use. The study protocol conforms to the ethical guidelines of the Declaration of Helsinki and was approved a priori by the institutional review committee.

**Culture medium**

HepG2 cells were cultured in Dulbecco’s modified Eagle’s medium [DMEM, high glucose (Gibco 41965)] containing
10% FBS at 37°C and <5% CO₂. Transfection of HepG2 cells with 1.4 genome length HBV DNA was performed as described previously [21]. Three to five days after transfection, the culture supernatant was collected for mouse infection.

**Treatment of mice with entecavir**

Beginning 8 weeks after HBV infection, when the mice had developed stable viraemia, mice were administered food containing 0.3 mg kg⁻¹ day⁻¹ of entecavir (Baraclude Solution; Bristol-Myers Squibb) for 4 weeks.

**Quantitation of HBV and HCV**

DNA and RNA extraction and quantitation of HBV and HCV by real-time reverse transcription PCR were performed as described previously [22]. Briefly, DNA was extracted using SMITEST (Genome Science Laboratories) and dissolved in 20 µl H₂O, and RNA was extracted from

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**Table 1. Characteristics of donors for transplanted human hepatocytes**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>BD85</th>
<th>BD195</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
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<td>Male</td>
</tr>
<tr>
<td>Age</td>
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<td>2</td>
</tr>
<tr>
<td>Ethnic group</td>
<td>African American</td>
<td>Hispanic</td>
</tr>
<tr>
<td>rs8099917</td>
<td>T/G</td>
<td>T/T</td>
</tr>
<tr>
<td>rs12979860</td>
<td>T/T</td>
<td>C/C</td>
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Table 2. Inoculum used for infection experiments

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patient 1</th>
<th>Patient 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBV DNA (log copies ml^{-1})</td>
<td>&gt;9.1</td>
<td>4.7</td>
</tr>
<tr>
<td>ALT (IU l^{-1})</td>
<td>23</td>
<td>37</td>
</tr>
<tr>
<td>HBs antigen (IU l^{-1})</td>
<td>88 109.58</td>
<td>15 348.43</td>
</tr>
<tr>
<td>HBe antigen (S/CO)</td>
<td>1425.51</td>
<td>124.23</td>
</tr>
<tr>
<td>HBe antibody (%)</td>
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<td>0</td>
</tr>
<tr>
<td>Precore</td>
<td>Wild</td>
<td>Wild</td>
</tr>
<tr>
<td>Core promoter</td>
<td>Wild</td>
<td>Mutant</td>
</tr>
</tbody>
</table>

S/CO, signal-to-cutoff ratio.

serum samples using SepaGene RVR (Sankojunyaku) and reverse-transcribed with a random hexamer and a reverse transcriptase (ReverTraAce; TOYOBO) according to manufacturers’ instructions. Quantitation of HBV DNA and HCV RNA was performed using a LightCycler (Roche Diagnostics). The lower detection limits of real-time PCR for HBV DNA and HCV RNA are 4.4 and 3.5 log copies ml^{-1}, respectively.

**Histochemical analysis of mouse liver**

Immunohistochemical staining using antibody against human albumin (Bethyl Laboratories) was performed as described previously [21, 22]. Immunoreactive materials were visualized using a streptavidin–biotin staining kit (VECTASTATIN ABC KIT; Vector Laboratories Inc., Burlingame, CA, USA) and diaminobenzidine.

**Statistical analysis**

Differences in serum HSA levels, HBV DNA and HCV RNA levels were examined for statistical significance using unpaired t-tests. Frequencies of the development of HCV viraemia were compared by the Mann–Whitney U test. A P value less than 0.05 was considered statistically significant.

**Funding information**

This research was partially supported by the Research Program on Hepatitis from the Japan Agency for Medical Research and Development, AMED (grant number, 15fk0210001h0002; www.amed.go.jp). The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript. No additional external funding was received for this study.

**Acknowledgements**

The authors thank Rie Akiyama and Yoko Matsumoto (Department of Gastroenterology and Metabolism, Applied Life Science, Institute of Biomedical & Health Science, Hiroshima University) for their expert technical help and Akemi Sada, Chikako Kanehara and Emi Nishio (Department of Gastroenterology and Metabolism, Applied Life Science, Institute of Biomedical & Health Science, Hiroshima University) for clerical assistance.

**Conflicts of interest**

K.C. received honoraria from MSD K.K., Bristol-Myers Squibb, Gilead Sciences and AbbVie and research funding from Dainippon Sumitomo Pharma, TORYA, Eisai, Otsuka Pharma, Mitsubishi Tanabe Pharma, Daiichi Sankyo and Bristol-Myers Squibb. M.I. received honoraria and research funding from Bristol-Myers Squibb. M.T. received research funding from Bristol-Myers Squibb.

**References**


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