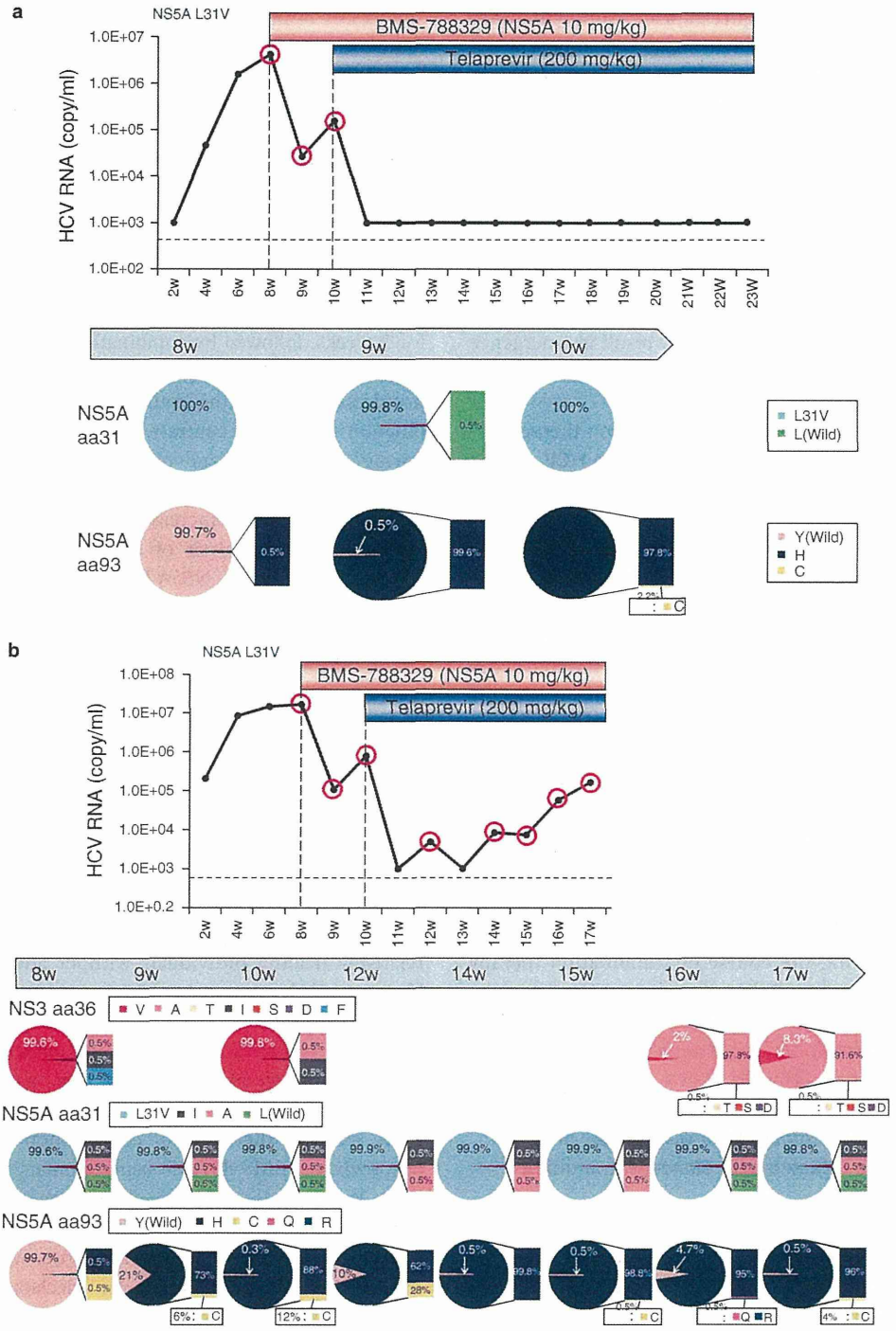

Figure 3. Effect of telaprevir and BMS-788329 combination therapy in human hepatocyte chimeric mice infected with an HCV clone containing NS5A L31V resistance mutation. We infected mice with an infectious clone harboring an NS5A inhibitor-resistant NS5A L31V mutation. (**a**, **b**) Mice received 200 mg/kg (mouse body weight) of telaprevir and 10 mg/kg (mouse body weight) of BMS-788329 (NS5A inhibitor). HCV, hepatitis C virus; w, weeks.

with *in vitro* experiments showing higher ED50 levels of these drugs against genotype 2 (30,31). The combination of BMS-788329 and telaprevir thus might be a good candidate for clinical trial in patients with genotype 1b infection. Higher dosage or a next-generation NS3 protease inhibitor should be considered for treatment of patients infected with genotype 2.

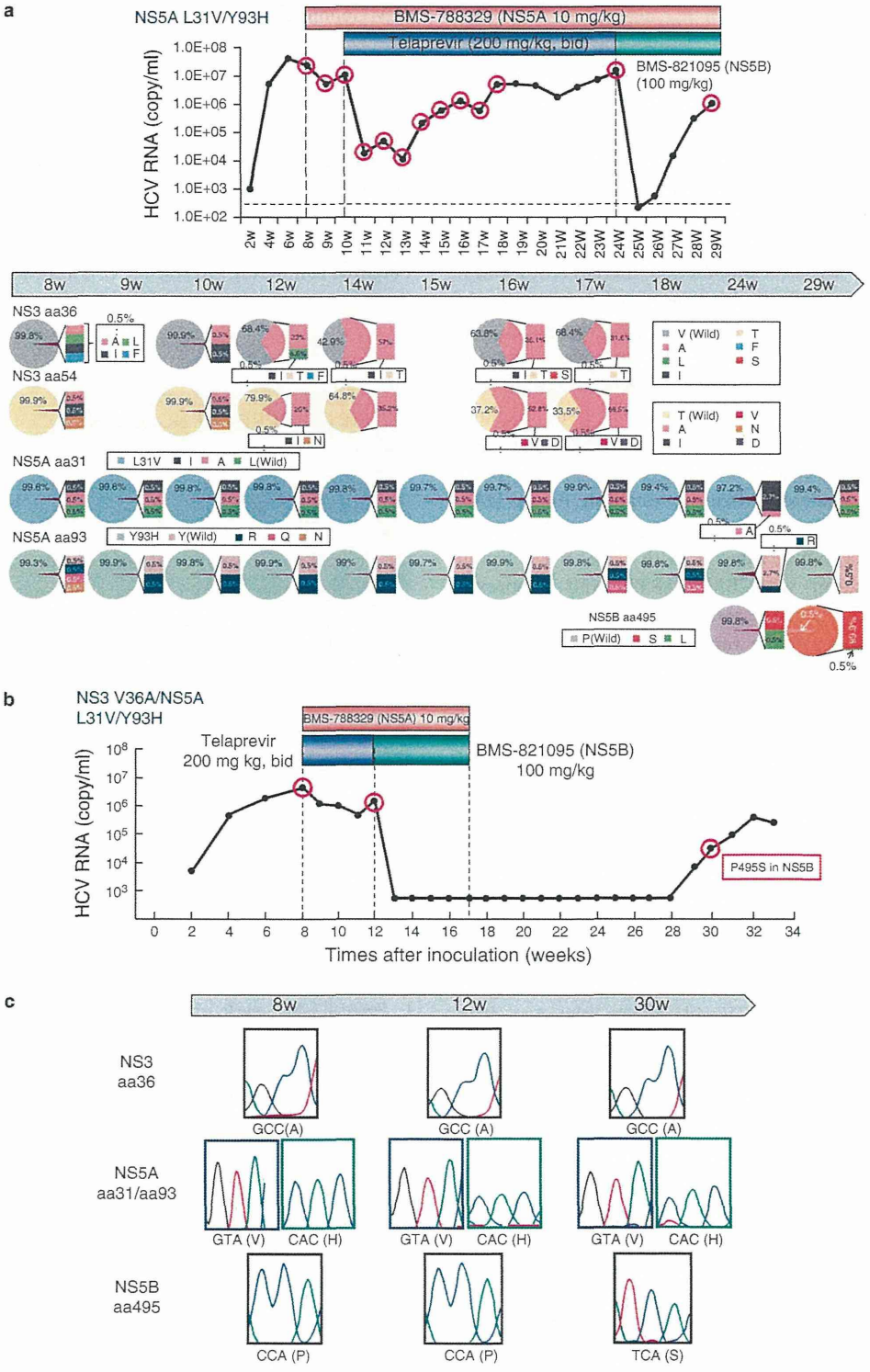

Figure 4. Effect of BMS-788329 in combination with telaprevir or NS5B inhibitor in mice infected with clones with multiple drug-resistant mutations. (**a**) We infected a mouse with an infectious clone harboring NS5A inhibitor-resistant NS5A L31V and Y93H mutations. The mouse received 200 mg/kg (mouse body weight) of telaprevir and 10 mg/kg (mouse body weight) of BMS-788329 (NS5A inhibitor). After 14 weeks of telaprevir plus BMS-788329 combination therapy, we replaced telaprevir with 100 mg/kg (mouse body weight) of NS5B inhibitor and continued combination therapy with BMS-788329 for an additional 5 weeks. (**b**, **c**) We infected a mouse with an infectious clone harboring resistance mutations against both telaprevir (NS3 V36A) and NS5A inhibitor (NS5A L31V and Y93H). The mouse received 200 mg/kg (mouse body weight) of telaprevir and 10 mg/kg (mouse body weight) of BMS-788329 (NS5A inhibitor) for 2 weeks, followed by combination therapy with BMS-788329 and 100 mg/kg (mouse body weight) of NS5B inhibitor. W, weeks.

As one of the mice treated with the combination of BMS-788329 and telaprevir showed poor response followed by relapse, we decided to analyze the effect of pre-existing resistance mutations on response to therapy. We infected mice with HCV clones having introduced mutations known to be associated with resistance to specific DAAs. Combination therapy with BMS-788329 and telaprevir effectively suppressed replication of HCV BMS-788329-resistant NS3 V36A HCV (**Figure 2**). This combination might be useful for patients who have a naturally occurring drug resistance profile. In contrast, two mice with a BMS-788329-resistant NS5A L31V mutation easily acquired an additional Y93C mutation, which has been reported to confer very strong resistance against the drug (**Figure 3**) (28). These factors should be considered when we establish future DAA combination therapies.

When we treated a mouse infected with a NS5A L31V and Y93H double mutation with BMS-788329 and telaprevir, the mice rapidly developed resistance against telaprevir. Furthermore, we observed the rapid emergence of an NS5B P495S mutant during combination therapy with BMS-788329 and BMS-821095 (NS5B inhibitor) (**Figure 4**). Such mutant strains with triple resistance features were also observed when the virus reappeared after cessation of a similar treatment (**Figure 4**). These results imply that mutant strains resistant to all three drugs can emerge after sequential use of these DAAs.

DAA combination therapy without interferon and ribavirin is expected to become a primary treatment option in the near future. As we showed in this study, however, multidrug-resistant strains may appear after incomplete, sequential use of DAAs (**Figure 4**). Although simultaneous use of three drugs is the strongest therapy against HCV, side effects related to drug interactions may occur. Therefore, we should further examine possible combinations of DAAs to establish the best combination therapy to eradicate HCV from all treated patients without incurring serious side effect.

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CONFLICT OF INTEREST

Guarantor of the article: Kazuaki Chayama, MD, PhD.

Specific author contributions: H. Abe, N. Hiraga, and M. Imamura designed and performed the experiments. C. N. Hayes analyzed the data. H. Abe and C. N. Hayes wrote the manuscript. M. Tsuge, D. Miki, S Takahashi, and H. Ochi participated in data analysis and discussion. K. Chayama initiated and directed the entire study, designed experiments and wrote the manuscript.

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Potential competing interests: K.C. is a speaker for BMS, MSD and Roche.

Study Highlights

WHAT IS CURRENT KNOWLEDGE

- ✓ DAAs against HCV have recently been developed.
- ✓ DAAs have been recommended to be used as interferon-based regimen.
- ✓ DAAs without interferon must be used in combination because of development of resistant strain.
- ✓ Development of multidrug-resistant strains remains to be characterized.

WHAT IS NEW HERE

- ✓ Resistant strains easily develop from cloned virus strains after sequential DAAs combination therapy.
- ✓ Sequential use of DAAs must be avoided to prevent a development of resistant strains.

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A novel TK-NOG based humanized mouse model for the study of HBV and HCV infections

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ABSTRACT

The immunodeficient mice transplanted with human hepatocytes are available for the study of the human hepatitis viruses. Recently, human hepatocytes were also successfully transplanted in herpes simplex virus type-1 thymidine kinase (TK)-NOG mice. In this study, we attempted to infect hepatitis virus in humanized TK-NOG mice and urokinase-type plasminogen activator-severe combined immunodeficiency (uPA–SCID) mice. TK-NOG mice were injected intraperitoneally with 6 mg/kg of ganciclovir (GCV), and transplanted with human hepatocytes. Humanized TK-NOG mice and uPA/SCID mice were injected with hepatitis B virus (HBV)- or hepatitis C virus (HCV)-positive human serum samples. Human hepatocyte repopulation index (RI) estimated from human serum albumin levels in TK-NOG mice correlated well with pre-transplantation serum ALT levels induced by ganciclovir treatment. All humanized TK-NOG and uPA–SCID mice injected with HBV infected serum developed viremia irrespective of lower replacement index. In contrast, establishment of HCV viremia was significantly more frequent in TK-NOG mice with low human hepatocyte RI (<70%) than uPA–SCID mice with similar RI. Frequency of mice spontaneously in early stage of viral infection experiment (8 weeks after injection) was similar in both TK-NOG mice and uPA–SCID mice. Effects of drug treatment with entecavir or interferon were similar in both mouse models. TK-NOG mice thus useful for study of hepatitis virus virology and evaluation of anti-viral drugs.

1. Introduction

Hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are serious health problems worldwide. More than 350 and 170 million people are infected with HBV and HCV, respectively [1,2]. Both types of hepatitis viruses result in the development of chronic liver infection and potentially death due to liver failure and hepatocellular carcinoma [3]. Although the chimpanzee is a useful animal model for the study of HBV and HCV infection, there are ethical restrictions and hampered by the high financial cost on the use of this animal. The immunodeficient mice with a urokinase-type plasminogen activator (uPA) transgene [4,5] or a targeted disruption of the murine fumaryl acetoacetate hydrolase (FAH) [6–10] were shown to be excellent recipients for human hepatocyte. These small animal models are available for hepatitis viruses infection [4,11], and are useful for the study of HBV and HCV biology [12–14]. However, there are disadvantages that limit the utility of this model for many applications, including excessive mortality [9].

Recently, human hepatocytes were successfully transplanted into severely immunodeficient NOG mice with the herpes simplex virus type-1 thymidine kinase (HSVtk) expressing in mouse hepatocytes (TK-NOG) [15]. Mouse liver cells expressing HSVtk

Abbreviations: ALT, alanine aminotransferase; GCV, ganciclovir; HBV, hepatitis B virus; HCV, hepatitis C virus; HSA, human serum albumin; HSVtk, herpes simplex virus type-1 thymidine kinase; IFN, interferon; PegIFN-alpha, pegylated interferon-alpha; RI, repopulation index; RT-PCR, reverse transcript-polymerase chain reaction; SCID, severe combined immunodeficiency; uPA, urokinase-type plasminogen activator.

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were ablated after a brief exposure to ganciclovir (GCV), and transplanted human hepatocytes were stably maintained within the mouse liver without exogenous drug administration [15]. The analyses of drug interactions and pharmacokinetics have previously been reported using TK-NOG mice transplanted with human hepatocytes [15–18]. In the present study, we succeeded in infecting human hepatocyte-transplanted TK-NOG mice with HBV and HCV and showed that this mouse model is as useful as the uPA/SCID model for the study of hepatitis viruses.

2. Materials and methods

2.1. Animal treatment

TK-NOG mice were purchased from Central Institute for Experimental Animals (CIEA, Kawasaki, Japan). Eight-weeks-old mice were injected intraperitoneally with 6 mg/kg of GCV twice a day. After two days, mice were re-injected with the same amount of GCV. Seven days after 1st GCV injection, mice were transplanted with 1 or 2 $\times$ 10^6 of human hepatocytes obtained from human hepatocyte transplanted uPA–SCID chimeric mice by collagenase perfusion method by intra-splenic injection. Transplanted human hepatocytes used in this study were obtained from a same donor. One week after the first GCV treatment, serum alanine aminotransferase (ALT) levels were measured (Fuji DRI-CHEM, Fuji Film, Tokyo, Japan). Infection, extraction of serum samples, and sacrifice were performed under ether anesthesia. Mouse serum concentration of human serum albumin (HSA), which correlated with the human hepatocyte repopulation index (RI) [15], was measured as previously described [5]. Generation of the uPA/SCID mice and transplantation of human hepatocytes were performed as described previously [5,12,19]. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of the Graduate School of Biomedical Sciences, Hiroshima University.

2.2. Human serum samples

Human serum samples containing high titers of either genotype C HBV (5.3 $\times$ 10^6 copies/mL) or genotype 1b HCV (2.2 $\times$ 10^6 copies/mL) were obtained from patients with chronic hepatitis who provided written informed consent. The individual serum samples were divided into small aliquots and stored separately in liquid nitrogen until use. Mice were injected intravenously with 50 μL of either HBV- or HCV-positive human serum. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved a priori by the institutional review committee.

2.3. Quantitation of HBV and HCV

DNA and RNA extraction and quantitation of HBV and HCV by real-time polymerase chain reaction (RT-PCR) were performed as described previously [12,13,19]. Briefly, DNA was extracted using SMITEST (Genome Science Laboratories, Tokyo, Japan) and dissolved in 20 μL H_2O, and RNA was extracted from serum samples using SepaGene RVR (Sankojunyaku, Tokyo, Japan) and reverse transcribed with a random hexamer and a reverse transcriptase (ReverTraAce; TOYOBO, Osaka, Japan) according to the instructions provided by the manufacturer. Quantitation of HBV DNA and HCV RNA was performed using Light Cycler (Roche Diagnostic, Japan, Tokyo). The lower detection limits of real-time PCR for HBV DNA and HCV RNA are 4.4 and 3.5 log copies/mL, respectively.

2.4. Histochemical analysis of mouse liver

Liver specimens of HBV-infected TK-NOG mice were fixed with 10% buffered-paraformaldehyde and embedded in paraffin blocks for histological examination. Hematoxylin-eosin and immunohistochemical staining using antibodies against HSA (Bethyl Laboratories Inc., Montgomery, TX) and hepatitis B core antigen (HBc-Ag) (DAKO Diagnostika, Hamburg, Germany) were performed as described previously [12].

2.5. Treatment with antiviral agents

Mice were treated with antiviral agents eight weeks after HBV or HCV infection, by which time stable viremia had developed. HBV-infected mice were administered either food containing 0.3 mg of entecavir/kg of body weight/day or daily intramuscular injections with 7000 IU/kg of IFN-alpha (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan). HCV-infected mice were administered intramuscular injection with either 1000 IU/kg of IFN-alpha daily or 10 μg/kg of PegIFN-alpha-2a (Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) twice a week for three weeks.

2.6. Statistical analysis

Differences in HSA levels between TK-NOG mice and uPA–SCID mice, and incidence of infection between highly and poorly repopulated mice were examined for statistical significance using the Mann–Whitney *U*-test.

3. Results

3.1. Correlation between serum ALT level after GCV administration and the human hepatocyte index in TK-NOG mice

We analyzed the correlation between serum ALT levels after GCV injection and the human hepatocyte RI using 194 TK-NOG mice. Seven days after GCV injection when serum ALT levels had reached maximum levels [15], mice were transplanted with human hepatocytes. After transplantation of human hepatocytes, serum concentrations of HSA increased and reached plateau at 6–8 weeks. Serum ALT levels one week after GCV administration and HSA levels 8 weeks after hepatocyte transplantation showed a positive correlation, indicating that the higher serum ALT level, the higher the RI (Fig. 1A). HSA levels 8 weeks after human hepatocyte transplantation in TK-NOG mice were lower than in uPA–SCID mice (Fig 1B), which indicates that mice livers were more efficiently replaced with human hepatocytes in uPA–SCID mice than in TK-NOG mice.

3.2. Infection with hepatitis viruses in humanized TK-NOG mice and uPA–SCID mice

Eight weeks after human hepatocyte transplantation, TK-NOG mice and uPA–SCID mice with HSA levels over 1.0 mg/mL were inoculated with either HBV- or HCV-positive human serum samples. Eight weeks after injection, the frequency of the development of viremia was compared between the mice with lower (<70%) and higher ($\geqslant$70%) human hepatocyte RI. 70% of RI corresponds to 5.4 and 6.3 mg/dl of serum HAS in TK-NOG mice and uPA–SCID mice, respectively [5,15]. All humanized TK-NOG and uPA–SCID mice inoculated with HBV developed viremia 8 weeks after injection, irrespective of the RI (Fig. 2A). Incidence of HCV viremia was also high in TK-NOG mice regardless of the RI. In contrast, the frequency of HCV viremia was much lower in uPA–SCID mice with the RI. Only 20% (1 of 5) of uPA–SCID mice with low RI became

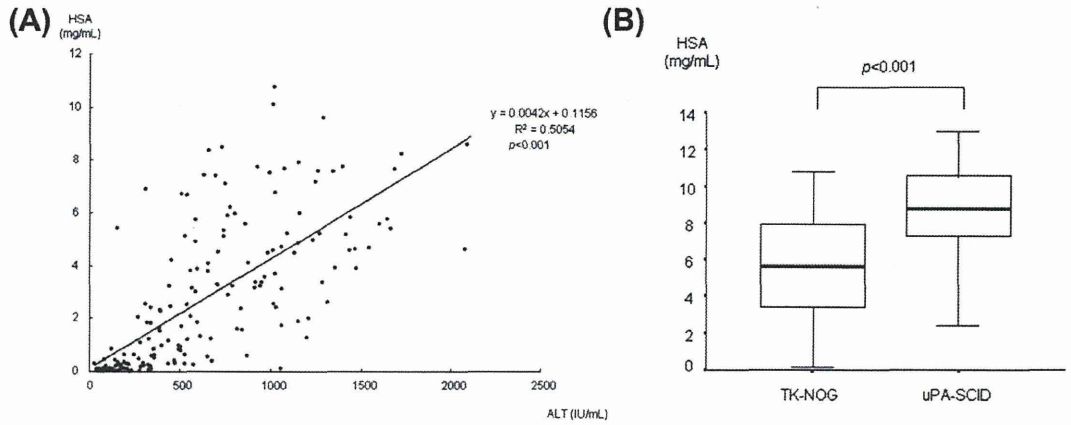

Fig. 1. Human hepatocyte repopulation index in humanized mice. Serum alaninaminotransferase (ALT) levels in TK-NOG mice were measured one week after ganciclovir treatment. Human serum albumin (HSA) levels were measured eight weeks after transplantation of human hepatocytes. (A) Correlation between serum ALT level after ganciclovir administration and human hepatocyte repopulation index in TK-NOG mice. Points represent single mouse measurements. *r* (Spearman rank) and *P* value are shown. (B) HSA levels in TK-NOG mice and uPA–SCID mice. 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.

positive for HCV, whereas 94.3% (50 of 53) of mice with high RI became positive ($p = 1.07 \times 10^{-6}$). Serum viral titers gradually increased in mice that developed viremia. Eight weeks after infection, HBV DNA and HCV RNA titers increased to approximately 8 and 6 log copies/mL, respectively in both TK-NOG and uPA–SCID mice (Fig. 2B). Viremia levels were slightly higher in uPA–SCID mice than TK-NOG mice, probably due to higher human hepatocyte RI (HSA levels) in uPA–SCID mice. In HBV-infected TK-NOG mice, histological analysis showed that hepatocytes positive for HSA were also positive for HB core antigen (Fig. 2C), which is in line with our previous findings using uPA–SCID mice [12].

3.3. The effect of antiviral agents on hepatitis virus-infected humanized mice

We analyzed the effect of antiviral agents on HBV- and HCV-infected humanized mice. Eight weeks after HBV-infection, 2 humanized TK-NOG mice were orally administrated 0.3 mg/kg day of entecavir, and 2 other mice received intramuscular injections with 7000 IU/g of IFN-alpha daily for 3 weeks. Both treatments resulted in a rapid reduction of mouse serum HBV DNA titers (Fig. 3A). Two HCV-infected humanized TK-NOG mice were administrated IFN-alpha daily, and 2 other mice received PegIFN-alpha-2a injections twice a week for 3 weeks. Both treatments resulted in a reduction of HCV RNA titers in mouse serum. The effects of these antiviral agents on HBV and HCV in TK-NOG mice were similar to those in uPA–SCID mice (Fig. 3B).

3.4. Incidence of unexpected death

The incidence of unexpected death is high in human hepatocyte chimeric uPA–SCID mice [20]. Incidence of unexpected death in the early stages of viral infection (within 8 weeks of viral infection) was similar between TK-NOG mice and uPA–SCID mice (6.3% vs 10.6%, $p = 0.465$) (Fig. 4).

4. Discussion

Human hepatocyte chimeric mice are valuable tool for hepatitis virology and drug assessment [12–14]. To establish human hepatocyte chimerism, two conditions are necessary: immunodeficiency and mouse-specific liver cell damage. For immune deficiency, SCID mice [4,5,12–14,20], NOG mice [8,21] and RAG-2 deficient mice [6,9,10] have been reported. We previously reported that the level of immunodeficiency in SCID mice, which are the most weakly immunodeficient of the three types, is sufficient to prevent rejection of transplanted human hepatocytes [5]. However, preventive treatments for human liver cell rejection via mice NK cells, such as an anti-asialo GM1 antibody, are necessary in SCID mice [5].

To evoke mouse liver cell injury, uPA and FAH transgene techniques were used [4–10]. Recently, successful human liver cell transplantation to TK-NOG mice in the absence of ongoing drug treatment after a brief exposure to a non-toxic dose of GCV has been reported [15]. We thus attempted to use TK-NOG mice to establish high levels of replacement with human hepatocytes and tried to infect hepatitis viruses.

In this study, we transplanted human hepatocytes to 194 TK-NOG mice and analyzed whether elevated serum ALT levels, which results from liver damage caused by GCV exposure, reflects HSA levels, as it is known that HSA levels are correlated with the human hepatocyte RI and can serve as a surrogate measure [15]. We found a positive correlation between ALT and HSA levels (Fig. 1A), indicating that higher levels of liver damage are associated with establishment of higher levels of repopulation of the liver with human hepatocytes. As the human hepatocyte RI obtained in this study using TK-NOG mice is lower than in uPA–SCID mice (Fig 1B), dose escalation of GCV or alternative treatment timing might result in more highly repopulated mice.

We infected humanized TK-NOG mice with hepatitis viruses and compared infection rates and serum viral titers with humanized uPA–SCID mice. HBV inoculation resulted in development of viremia without regard for the human hepatocyte replacement index in both TK-NOG mice and uPA–SCID mice (Fig. 2A). Incidence of HCV viremia was also high in TK-NOG mice regardless of HSA levels, whereas HCV viremia was infrequent in uPA–SCID mice with low HSA levels. These results are consistent with those of Vanwolleghem et al. [20] who showed, using a large number of human hepatocyte chimeric uPA–SCID mice, that an HSA level well above 1 mg/mL is important for successful HCV infection. The reason for the higher infection rate in TK-NOG mice with low human hepatocyte RI in this study is unknown. Although the level of immunodeficiency is higher in TK-NOG mice, it is difficult to conclude that this difference in immunodeficiency alone is responsible for the enhanced HCV infection rate. Although some studies have

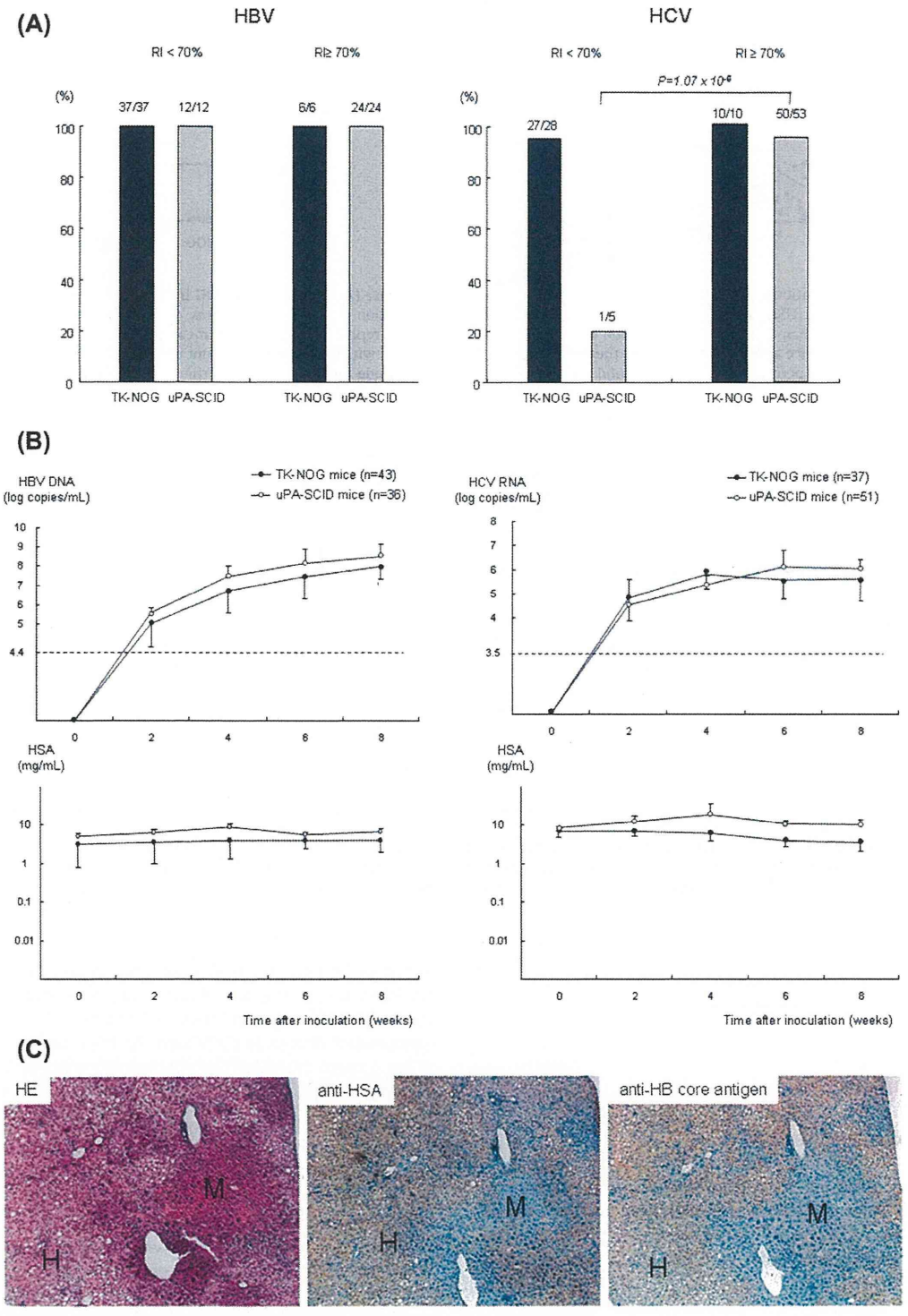

Fig. 2. Hepatitis viruses infection in chimeric mice. (A) Eight weeks after human hepatocyte transplantation, mice with serum HSA level over 1 mg/mL were inoculated with HBV- or HCV-positive human serum samples. Percentages of mice that became positive for HBV DNA (left panel) or HCV RNA (right panel) 8 weeks after inoculation according to human hepatocyte repopulation index (RI) in TK-NOG mice and uPA–SCID mice are shown. 70% of RI corresponds to 5.4 and 6.3 mg/dl of serum HAS in TK-NOG mice and uPA–SCID mice, respectively. (B) Changes in serum titers of HBV DNA (left panel) and HCV RNA (right panel) (upper panels) and HSA levels (lower panels) of TK-NOG mice and uPA–SCID mice. The horizontal dashed lines represent the lower detection limit of HBV DNA and HCV RNA (4.4 and 3.5 log copies/mL, respectively). (C) Histochemical analysis of liver samples obtained from HBV-infected TK-NOG mice. Hematoxylin-eosin staining (HE) and immunohistochemical staining using monoclonal antibodies against HSA and HB core antigen are shown. Regions are shown as human (H) and mouse (M) hepatocytes, respectively (Original magnification 100×).

reported structural differences between wild type and chimeric mice [22,23], the influence of such structural differences on HCV infectivity remains to be determined.

Human hepatocyte transplanted uPA–SCID mice are useful for evaluating antiviral agents [12–14]. In this study, we analyzed the efficacy of antiviral agents such as entecavir, IFN-alpha and

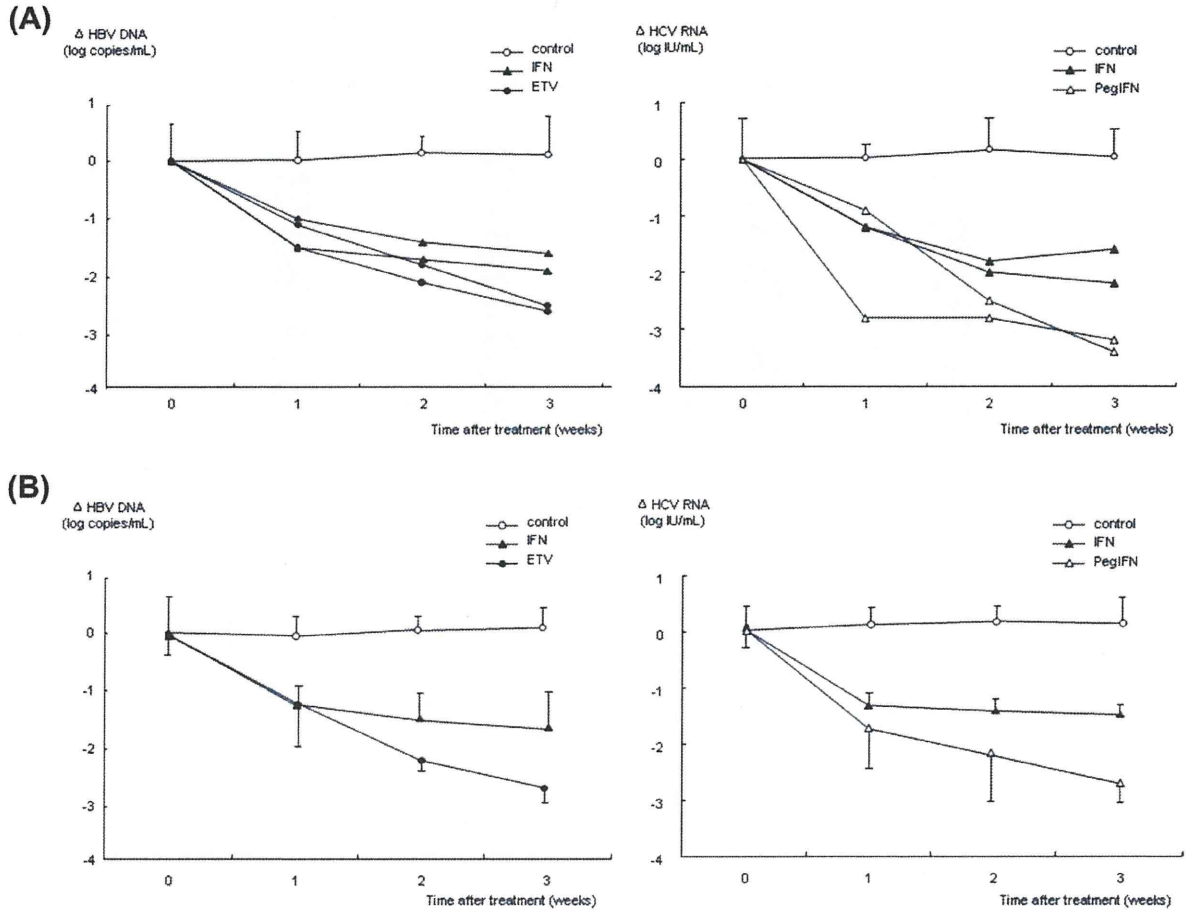

Fig. 3. Reduction of serum viral titers in mice treated with anti-viral agents. (A) HBV- (left panel) or HCV-infected (right panel) TK-NOG mice were treated with entecavir, interferon (IFN)-alpha or PegIFN-alpha-2a. Control: HBV- and HCV-infected mice without antiviral treatment. (B) HBV- (left panel) or HCV-infected (right panel) uPA–SCID mice were treated with entecavir, IFN-alpha or PegIFN-alpha-2a. Data are shown using the mean ± SD ($n = 4$).

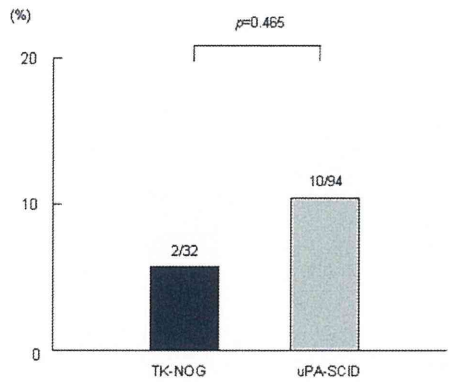

Fig. 4. Frequency of unexpected death within 8 weeks in mice. The numbers of sudden deaths occurring within 8 weeks of viral infection in TK-NOG mice and uPA–SCID mice are shown as bars.

PegIFN-alpha using HBV- and HCV-infected TK-NOG mice and compared them with uPA–SCID mice (Fig. 3). The results showed that both mouse models are equally useful for evaluation of antiviral drugs.

Human hepatocyte chimeric uPA–SCID mice are weak and prone to unexpected death [20], and this limitation appears to apply to TK-NOG mice as well. Incidence of unexpected death in the early stages of viral infection was not significantly different between TK-NOG mice and uPA–SCID mice (Fig. 4). The cause of these unexpected deaths is unknown. Further study is necessary to develop a more robust and easy to manipulate animal model.

In summary, we established a hepatitis virus infection mouse model using the human hepatocyte transplanted TK-NOG mouse. This model is useful for the study of hepatitis virology and evaluation of antiviral agents.

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