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immune system receptors, including RIG-I. In addition, it is also not clear whether ME3738 has anti-viral effects on genotype 1b HCV, which is the most common and most IFN-resistant genotype in Japan [14].

Recently, HCV-infected mice have been developed by inoculating HCV-infected human serum into urokinase-type plasminogen activator (uPA)-severe combined immunodeficiency (SCID) mice engrafted with human hepatocytes [15,16]. We and other groups had reported that this mouse model is useful for evaluating anti-HCV drugs such as IFN-α and anti-NS3 protease *in vivo* [17–19].

In the present study, we investigated the effects of ME3738 on HCV replication both *in vitro* and *in vivo* using the genotype 1b HCV replicon and HCV-infected human hepatocyte chimeric mice. The results demonstrate that ME3738 itself had an inhibitory effect on HCV replication, and when combined with IFN, ME3738 enhanced the anti-HCV effect of IFN by up-regulation of ISGs, such as oligoadenylate synthetase (*OAS*) *1*, myxovirus resistance protein A (*MxA*), and *ISG15* in HCV replicon cells. We also showed that the combination therapy increased *OAS1*, RNA-dependent protein kinase (*PKR*) and ubiquitin specific peptidase 18 (*USP18*) expression levels, and reduced virus levels effectively without liver cell damage in human hepatocyte chimeric mice.

Material and methods

Cell culture

Cells supporting replication of the genotype 1b-derived subgenomic HCV replicon, ORN/3-5B/KE cells [20] (kindly provided by N. Kato, Okayama University, Japan) and Con-1 cells [21], were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Invitrogen Life Technology, Carlsbad, CA) supplemented with 10% fetal bovine serum, non-essential amino acids, glutamine, penicillin, and streptomycin (complete DMEM) in the presence of G418 (300 μg/ml; Geneticin, Invitrogen, Carlsbad, CA). ORN/3-5B/KE and Con1 replicon cells (2×10^4) were seeded onto 12-well plates and incubated for 3 days with or without ME3738 (Meiji Seika Kaisha, Tokyo, Japan) [9], human IFN-α (Dainippon Sumitomo Pharma Co., Tokyo), or the combination of both drugs.

Quantitation of HCV RNA and ISG mRNAs

RNA extraction and quantitation of HCV by real-time polymerase chain reaction (PCR) were performed as described previously [19]. Briefly, RNA was extracted from mice serum, livers, or cellular lysate 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 HCV RNA was performed using the Real-Time PCR system (Applied Biosystems, Foster City, CA). The primers used for amplification were 5′-GAGTGTCGTGCAGCCTCCA-3′ and 5′-CACTCGCAAGCAC CCTATCA-3′. Quantitation of ISGs (*OAS1*, *MxA*, *PKR*, *USP18* and *ISG15*) was performed using real-time PCR Master Mix (TOYOBO) and TaqMan Gene Expression Assay primer and probe sets (PE Applied Biosystems, Foster City, CA). Thermal cycling conditions were as follows: a precycling period of 1 min at 95 °C followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. Each ISG expression level was expressed relative to the endogenous RNA levels of the housekeeping reference gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

Luciferase reporter assay

After 72 h of IFN and/or ME3738 treatment, ORN/3-5B/KE cells were harvested with Renilla lysis reagent (Promega, Madison, WI) and subjected to the luciferase assay according to the manufacturer's protocol.

Western blotting

The cells were ruptured with 250 μl lysis buffer [10 mM Tris/HCl pH 7.4, 140 mM NaCl and 0.5% (v/v) NP-40] followed by centrifugation for 2 min at 15,000g. Cell lysates were subjected to Western blotting using antibodies against NS3 (Novocastra Laboratories, UK) and β-actin (Sigma, Tokyo, Japana) as described previously [22].

WST assay

Cell viability was determined by employing tetrazolium salt, WST-8, using the WST-8 Cell Proliferation Assay Kit (Dojindo Laboratories., Kumamoto, Japan), according to the instructions provided by the manufacturer.

Human serum samples

Human serum samples containing high titers of genotype 1b HCV (2.2×10^6 copies/ml) were obtained from a patient with chronic hepatitis after obtaining written informed consent. Aliquots were stored in liquid nitrogen until use.

Animal treatment

All animal protocols in this study were in accordance with the guidelines of the local committee for animal experiments and under approval of the Ethics Review Committee for Animal Experimentation of the Graduate School of Biomedical Sciences, Hiroshima University. We transplanted human hepatocytes into uPA$^{+/+}$/SCID$^{+/+}$ mice as described previously [16]. All mice used in this study were transplanted with frozen human hepatocytes obtained from the same donor. Mice were injected intravenously with 50 μl of HCV-positive human serum samples. Six weeks after HCV infection, mice were fed a normal chow containing 0.15% (w/w) ME3738 for 4 weeks, with or without IFN-α. IFN-α-treatment was provided daily by intramuscular injection of diluted IFN solution. Serum samples were collected every week, and human serum albumin (HSA) concentration and HCV RNA were measured. Mouse serum concentrations of HSA, which correlate with the repopulation rates, were measured as described previously [16]. Serum ME3738 concentrations were measured by liquid chromatography/mass spectrometry/mass spectrometry. After the fourth week of treatment, mice were sacrificed, and livers were either fixed with 4% buffered-paraformaldehyde for histological examination or frozen immediately in liquid nitrogen to measure HCV core antigen. To investigate the expression of ISGs in mouse livers, mice were kept for 1 week with or without 0.45% (w/w) ME3738 and then given a single injection of 1500 IU/g IFN-α. Four hours after injection, mice were sacrificed and liver samples were collected.

Quantitation of HCV core antigen in the mouse liver

Livers were homogenized in phosphate-buffered saline with 1% Triton X-100, 0.1% SDS, and 0.5% sodium deoxycholate. The homogenates were centrifuged at 20,000g for 30 min. HCV core antigen levels in the supernatant of liver homogenates were measured using enzyme immunoassay as described previously [23].

Statistical analysis

All data are expressed as mean ± SD. Levels of HCV RNA and ISG mRNAs were compared using the Mann–Whitney *U*-test. A p value less than 0.05 was considered statistically significant. All statistical analyses were performed with SPSS 14.0 software (SPSS, Tokyo, Japan).

Results

Antiviral activity of ME3738 on HCV subgenomic replicon

The effect of ME3738 on HCV replication was analyzed *in vitro* using subgenomic HCV replicon cells possessing the luciferase reporter. ORN/3-5B/KE cells were treated with either IFN-α or ME3738 for 72 h. The luciferase reporter assay demonstrated that the HCV RNA replication level decreased depending on the

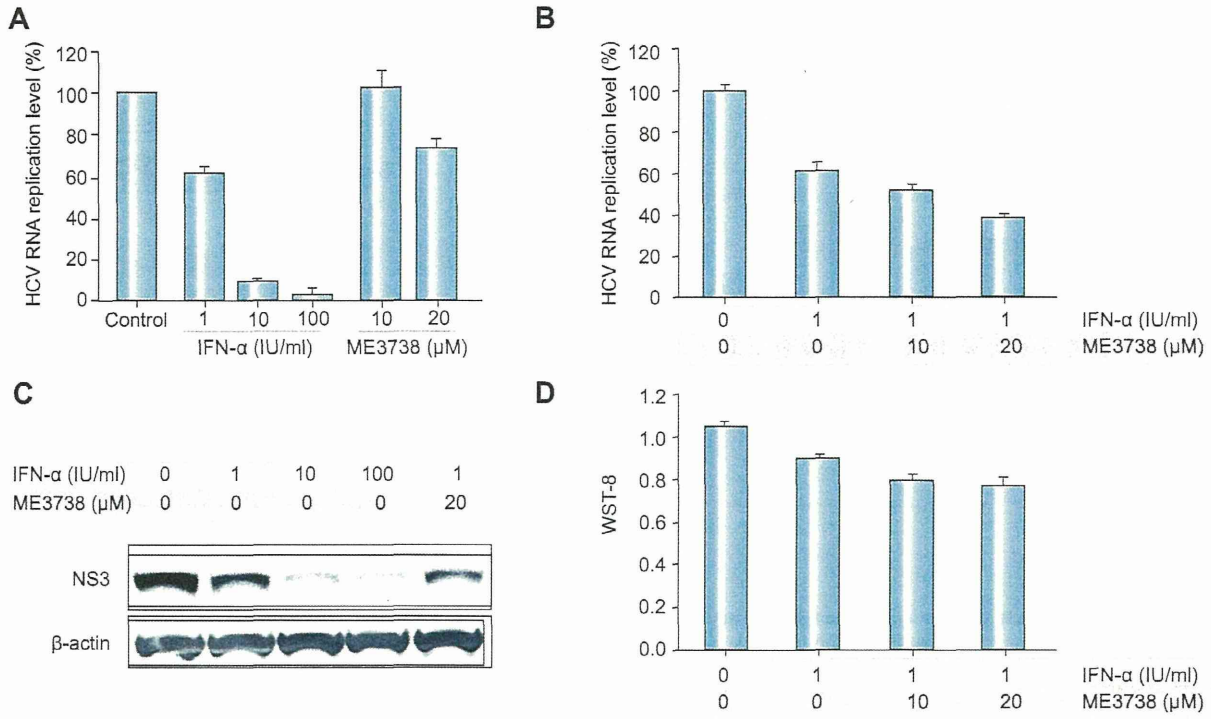

Fig. 1. Effects of ME3738 on HCV replication in the subgenomic HCV replicon, ORN/3-5B/KE cells. ORN/3-5B/KE cells were treated for 72 h with the indicated concentration of interferon (IFN)-α alone, ME3738 alone, or IFN-α plus ME3738. (A and B) Intracellular HCV RNA replication levels were determined as luciferase activity and expressed relative to cellular viability. (C) Cell lysates were analyzed by immunoblotting with antibodies to NS3 and β-actin. (D) Cellular viability was analyzed by WST assay. Data are represented as the mean ± SD of 6 experiments. Control: cells treated with neither ME3738 nor IFN-α.

IFN-treatment dose as reported previously (Fig. 1A) [20]. Treatment with 20 μM of ME3738 also reduced HCV RNA replication. Next, we investigated whether ME3738 enhances the effect of IFN-α. IFN-α (1 IU/ml) plus ME3738 inhibited the HCV RNA replication dose in a dependent manner with ME3738 (Fig. 1B). The level of cellular HCV NS3 protein was reduced depending on IFN-α-treatment and was reduced effectively by IFN-α/ME3738 combination treatment (Fig. 1C). The viability of cells treated with IFN-α/ME3738 combination treatment was lower than that of the control treatment and almost the same as with IFN-α treatment alone (Fig. 1D).

The effect of ME3738 was also tested in a different replicon system, Con-1 cells. ME3738 reduced HCV RNA replication dose dependently in Con-1 cells (Fig. 2A). Similar to ORN/3-5B/KE cells, IFN-α (1 IU/ml) plus ME3738 inhibited HCV RNA replication dose in a dependent manner with ME3738 (Fig. 2A), and the level of cellular HCV NS3 protein was reduced effectively by IFN-α/ME3738 combination treatment (Fig. 2B). The viability of cells treated with IFN-α/ME3738 combination treatment was lower but was not significant with IFN-α treatment alone (Fig. 2C). These results indicate that ME3738 itself has an inhibitory effect on HCV replication and enhances the effect of IFN-α.

Expression of ISGs in ME3738-treated replicon cells

We measured the levels of ISGs in drug-treated ORN/3-5B/KE cells and Con1 cells. IFN-α treatment significantly increased the expression levels of *OAS1*, *MxA*, *PKR*, *USP18* and *ISG15*, which reached maximum levels at 24 h in ORN/3-5B/KE cells (Fig. 3A) and 8 h in Con1 cells (Fig. 3B). ME3738 treatment alone significantly increased the expression of *OAS1* in both cells. IFN-α treatment significantly increased the expression of ISGs; however, IFN-α/ME3738 combination treatment significantly induced the expressions of *OA1S*, *MxA* and *ISG15* to levels higher than IFN-α alone in both cells. These results indicate that ME3738 enhances the effect of IFN-α to increase ISG expression, and this effect may contribute to the inhibition of HCV replication.

Effect of ME3738 on HCV replication in vivo

To further analyze the effects of ME3738, we used genotype 1b HCV-infected human hepatocyte chimeric mice [17,19]. Six weeks after HCV infection, when the mice developed stable viremia (10^6–10^7 copies/ml, data not shown), the animals were treated with ME3738 alone, IFN-α alone, or ME3738/IFN-α for 4 weeks (Fig. 4A). Mouse serum concentrations of ME3738 increased in ME3738- and ME3738 plus IFN-α-treated mice (Table 1). ME3738 alone did not reduce the levels of HCV RNA in mice, while IFN-α-treatment reduced the HCV RNA levels, as reported previously [17]. ME3738 plus IFN-α-treatment significantly reduced HCV to levels lower than that achieved by ME3738 or IFN-α alone. We also measured the HCV core protein level in the livers of treated mice. As shown by replicon experiments, core protein levels were reduced most effectively by the ME3738/IFN-α-combination therapy (Fig. 4B). Since the level of HSA did not decrease in these treatments, it was concluded that

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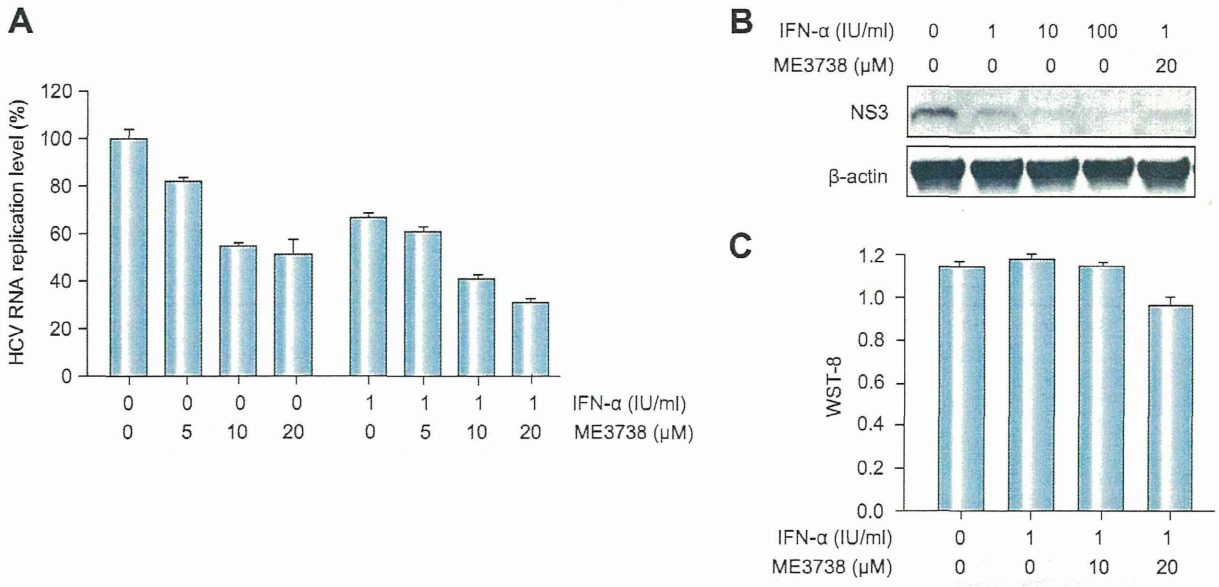

Fig. 2. Effects of ME3738 on HCV replication in the subgenomic HCV replicon, Con1 cells. Con1 cells were treated for 72 h with the indicated concentration of ME3738 alone or IFN-α plus ME3738. (A) Intracellular HCV RNA replication levels were determined via real-time PCR. (B) Cell lysates were analyzed by immunoblotting with antibodies to NS3 and β-actin. (C) Cellular viability was analyzed by WST assay. Data are represented as the mean ± SD of 6 experiments. Control: cells treated with neither ME3738 nor IFN-α.

the reduction of HCV in chimeric mice was not due to toxicity of the drugs (Fig. 4A). This was also supported by histopathological findings, including lack of cytotoxic changes in the livers of all four groups of mice (Fig. 4C). The effect of ME3738 to increase ISG expression was assessed in mouse liver following treatment with a high concentration of ME3738 for 1 week and a single injection of IFN-α. ME3738 alone showed no increase in the expression of ISGs in mouse livers (Fig. 5). IFN-α treatment significantly increased the expression of ISGs; however, IFN-α/ME3738 combination treatment significantly induced the expressions of *OAS1*, *PKR* and *USP18* mRNA levels in mouse livers to levels higher than IFN-α alone. These results indicate that ME3738 inhibits HCV replication, enhancing the effect of IFN-α to increase ISG expression *in vivo*.

Discussion

Although the treatment outcome of chronic HCV infection has improved with the advent of pegylated IFN-α and ribavirin, the eradication rate of HCV is only about 50%. Many patients are unable to receive this therapy because of the harmful side effects or the financial costs. Development of effective, safe and inexpensive therapies should be encouraged.

ME3738 is reported to attenuate various liver pathologies in animals [8–12]. Furthermore, Hiasa et al. reported recently that ME3738 induces IFN-β mRNA expression and inhibits the replication of HCV [13]. We thus attempted in this study to evaluate the effect of ME3738, especially in combination with IFN-α, on HCV.

The results of the present study show that ME3738 induced the gene expression of *OAS* (Fig. 2) and inhibited HCV replication (Fig. 1A). Hiasa et al. reported that ME3738 enhanced the expression of *IFN-β* mRNA and that the enhanced production of IFN-β resulted in the increased expression of ISGs [13]. They showed also that the effect of ME3738 on HCV was abolished following the inhibition of IFN-β expression with siRNA or antibody. Our results are consistent with their findings. The extent of the increase in ISG expression was smaller in Hiasa et al. [13] than in our results. This is probably because they used the T7-genotype 1a-cDNA transient transfection-infection system to produce HCV in HepG2 or Huh7 cells [13,24,25] and assessed the effect of ME3738 by utilizing naturally produced IFN-β. The amount of IFN is likely to be very small in their system compared to that used in our study. We also tried to detect *IFN-β* mRNA in our replicon system but were unable to detect it in our replicon cells (Huh7 based ORN/3-5B/KE cells and Con1 cells). This is probably due to a defect of the innate immune system in producing IFN-β in those cells. This is consistent with their finding that ME3738 had an inferior effect in Huh7 cells than in HepG2 cells to produce ISG products [13].

As we showed in this study, ME3738 enhances the effect of IFN against HCV replication both *in vitro* (Figs. 1B and 2A) and *in vivo* (Figs. 4A and 4B). ME3738 enhanced the effect of IFN-α by increasing the expression levels of ISGs both *in vitro* (Fig. 3) and *in vivo* (Fig. 5). How ME3738 enhances the transcription of ISGs is unknown at this stage. ME3738 was reported initially to protect liver cells against injury through induction of IL-6 [8,9]. IL-6 is reported to provide protection to certain cells [26–28] by preventing apoptosis. In the present study, we tried to detect IL-6 protein in the serum and mRNA in the liver of ME3738-treated mice. However, the levels of both were too low to measure. Further studies should be conducted to elucidate the mechanism by which ME3738 enhances immunity against viral infections.

Our results showed that ME3738 did not reduce cell viability. We also showed that the drug is not hepatotoxic, as inferred by HSA level and liver histology. Since ME3738 is reported to

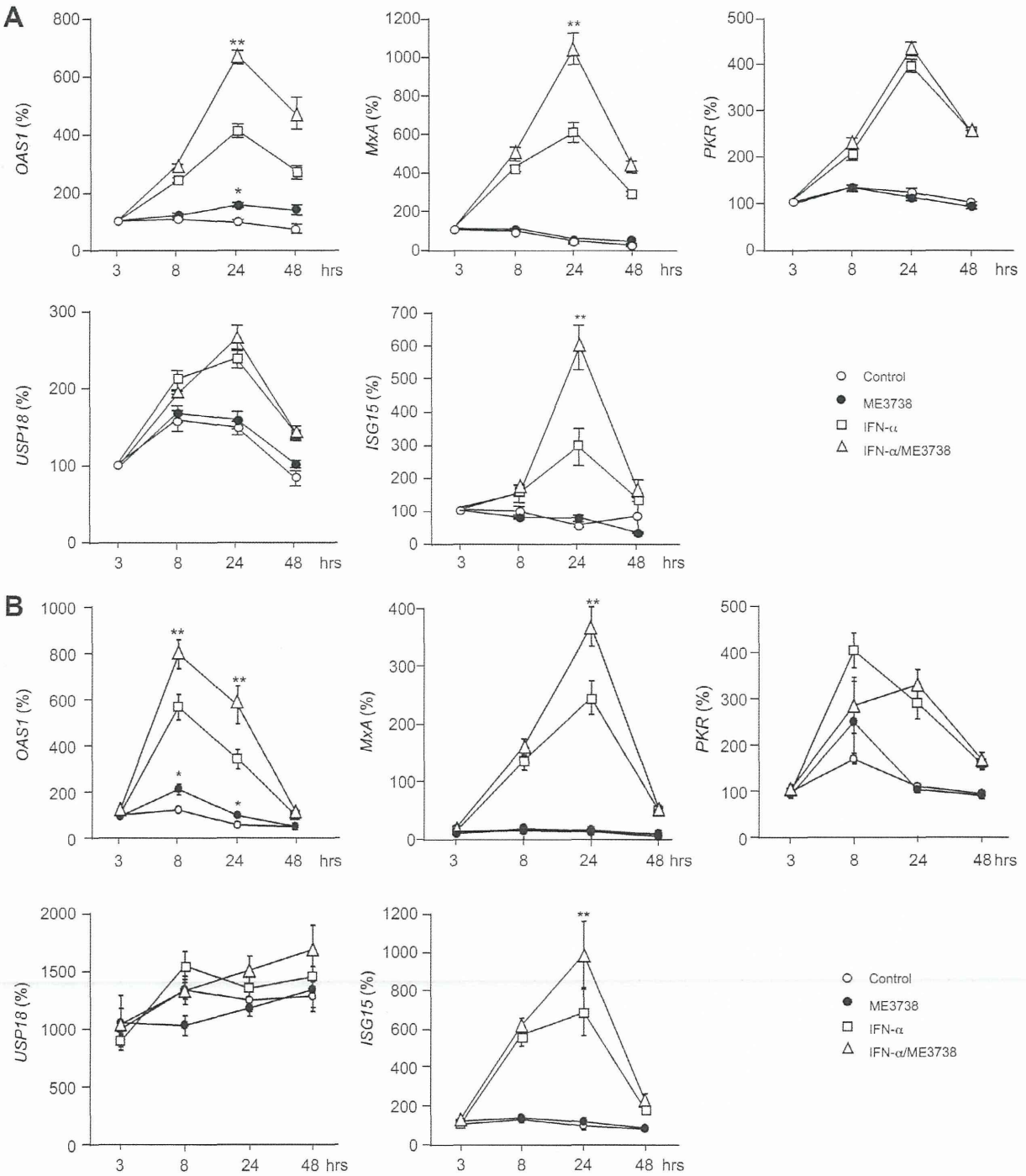

Fig. 3. Effects of ME3738 on the expression of interferon-stimulated genes. ORN/C-5B/KE cells (A) and Con1 cells (B) were treated with 20 μM of ME3738 and/or 1 IU/ml of interferon (IFN)-α for 48 h. Intracellular gene expression levels of oligoadenylate synthetase (*OAS*), myxovirus resistance protein A (*MxA*), double stranded RNA-activated protein kinase (*PKR*), *USP-18* and interferon-stimulated gene (*ISG*) *15* were measured at the indicated times. RNA levels were expressed relative to *GAPDH* mRNA. Data are shown as the mean ± SD of 6 experiments. Control: cells treated with neither ME3738 nor IFN-α. (*p <0.05 compared with Control, "p <0.05 compared with IFN-α treatment).

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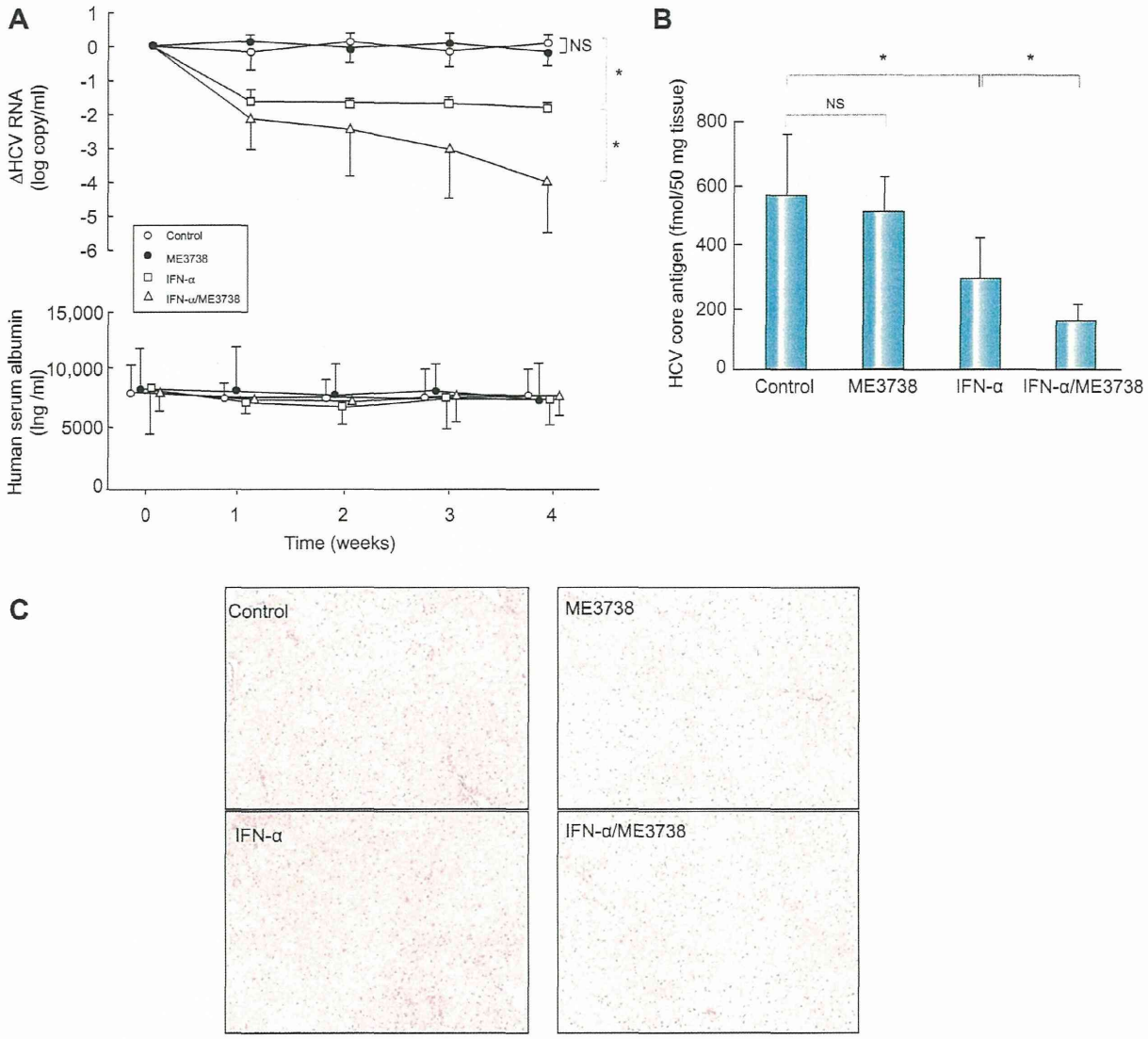

Fig. 4. ME3738 enhances the effect of IFN in mice with HCV infection. Mice were injected intravenously with 50 μl of HCV-positive human serum samples. Six weeks after HCV infection, mice were treated with ME3738 and/or interferon (IFN)-α for 4 weeks. (A) Mouse serum samples were obtained every week, and HCV RNA titer (upper panel) and human serum albumin concentration (lower panel) were analyzed. (B) HCV core antigen was measured in the mouse livers after 4 weeks of treatment. Data are mean ± SD of 6 mice. (*p <0.05; **p <0.01; NS, not significant). (C) Liver samples obtained from mice were stained with hematoxylin-eosin (Original magnification, 100×). Note the lack of specific changes in the mice of each group. Control: HCV-infected mice treated with neither ME3738 nor IFN-α.

Table 1. Concentrations of ME3738 in mouse serum samples.

	Control	ME3738	IFN-α	ME3738/ IFN-α
ME3738 (μM)	<0.01	4.02 ± 0.90	<0.01	2.44 ± 0.21

Concentrations of ME3738 in serum samples obtained from mice after 4 weeks of treatment were measured by liquid chromatography/mass spectrometry/mass spectrometry. Data are shown as mean ± SD of three mice. Control: HCV-infected mice treated with neither ME3738 nor IFN-α.

attenuate liver disease in several animal models of acute and chronic liver injury [8–12], the drug could be suitable for treatment of patients with chronic hepatitis C. In the current regimen of PEG-IFN and ribavirin combination therapy, IFN reduces the replication rate of the virus by inducing expression of ISGs in liver cells. Ribavirin enhances the effect of IFN synergistically through an unknown mechanism. ME3738 also enhances the effect of IFN similarly to ribavirin and may protect liver cells from apoptosis. Combination therapy using these three drugs might yield excellent anti-viral and anti-inflammatory effects. Alternatively, ME3738 could be used instead of ribavirin if the drug shows a superior effect in combination with IFN. Further animal and human studies should be conducted to develop an effective regimen for the treatment of patients with chronic hepatitis C.

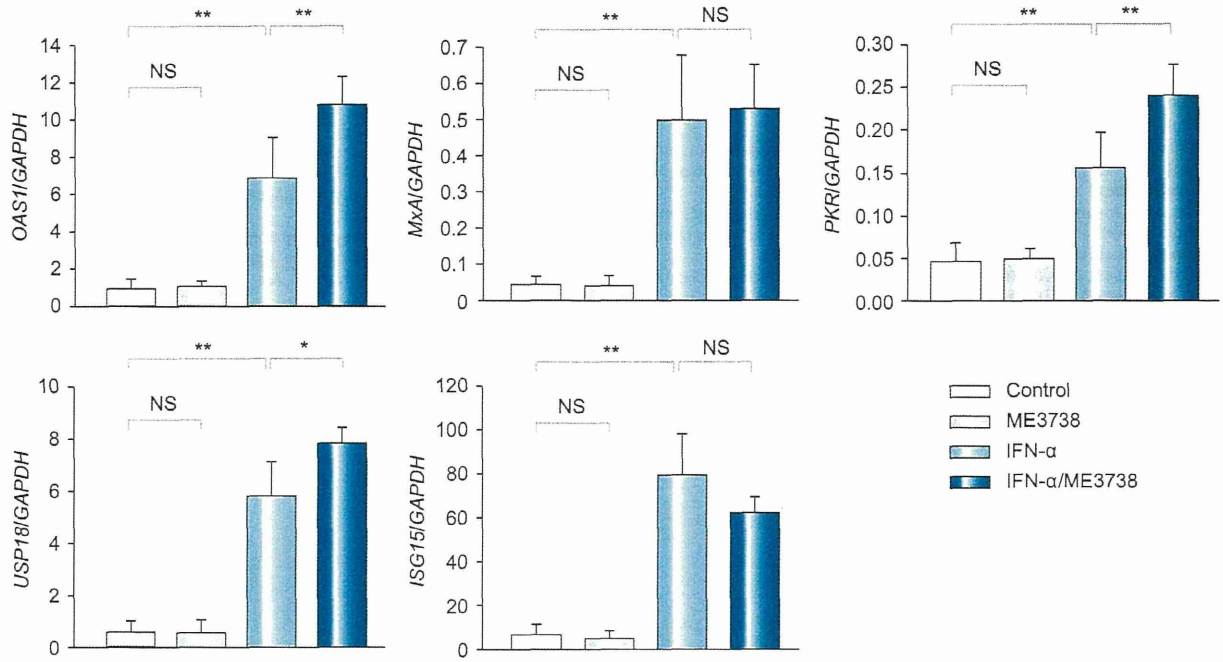

Fig. 5. Interferon-stimulated gene expression in mouse liver samples. Mice were treated with or without 0.45% (w/w) ME3738 for 1 week and then given a single injection of 1500 IU/g IFN-α. Four hours after IFN-α injection, interferon stimulated gene expression in mouse livers was measured. RNA levels are expressed relative to *GAPDH* mRNA. Data are presented as mean ± SD of six mice. Control: Mice treated with neither ME3738 nor IFN-α. (*p <0.05; **p <0.01; NS, not significant).

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Transplantation of Engineered Chimeric Liver With Autologous Hepatocytes and Xenobiotic Scaffold

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Objective: Generation of human livers in pigs might improve the serious shortage of grafts for human liver transplantation, and enable liver transplantation without the need for deceased or living donors. We developed a chimeric liver (CL) by repopulation of rat hepatocytes in a mouse and successfully transplanted it into a rat recipient with vessel reconstruction. This study was designed to investigate the feasibility of CL for supporting the recipient after auxiliary liver grafting.

Methods: Hepatocytes from luciferase transgenic or luciferase/LacZ double-transgenic rats were transplanted into 20- to 30-day-old urokinase-type plasminogen activator/severe-combined immunodeficiency (uPA/SCID) mice (n = 40) to create CLs with rat-origin hepatocytes. After replacement of mouse hepatocytes with those from rats, the CLs were transplanted into wild-type Lewis (n = 30) and analbuminemia (n = 10) rats, followed by immunosuppression using tacrolimus (TAC) with/without cyclophosphamide (CPA) or no immunosuppression. Organ viability was traced by in vivo bioimaging and Doppler ultrasonography in the recipient rats for 4 to 6 months. Rat albumin production was also evaluated in the analbuminemia rats for 4 months. In addition, histological analyses including Ki67 proliferation staining were performed in some recipients.

Results: Both immunosuppressive protocols significantly improved graft survival and histological rejection of CLs as compared to the nonimmunosuppressed group. Although rat albumin production was maintained in the recipients for 4 months after transplantation, ultrasonography revealed patent circulation in the grafts for 6 months. Ki67 staining analysis also revealed the regenerative potential of CLs after a hepatectomy of the host native liver, whereas immune reactions still remained in the mouse-origin structures.

Conclusions: This is the first report showing that engineered CLs have potential as alternative grafts to replace the use of grafts from human donors.

Keywords: alternative organ graft, auxiliary liver transplantation, chimeric liver, engineered organ, liver transplantation

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Liver transplantation is currently regarded as the most effective treatment for end-stage liver diseases. Because the worldwide graft shortage remains unresolved,[1,2] engineered organs are anticipated as alternative grafts to fill the scarcity and ultimately replace those from deceased and living donors. Although regenerative technology has already developed various kinds of regenerative cells and tissues,[3,4] they are still insufficient to cure patients with end-stage liver diseases because of the absence of complicated functions and limited volume. Therefore, a regenerative liver graft for use as an "organ" is required. To achieve an appropriate 3-dimensional structure and differentiation to specific tissues in a single organ, application of native organs as scaffolds has been reported as a possible solution.[5–7] Recent advances in genetic manipulation of animals such as pigs have produced transgenic animals with a lower risk of xenorejection.[8,9] Thus, using their native organogenetic potentials, development of engineered liver "organs" is expected.[10] In such a protocol, human hepatocytes are transplanted to a transgenic pig to replace its native hepatocytes, resulting in development of a chimeric liver (CL) with human parenchyma and swine nonparenchymal components, including vessels, bile ducts, and other connecting tissues. However, it is unclear whether such engineered CLs can be transplanted into recipients, or whether they can maintain their organ structures and functions after transplantation. In this study, we developed a rodent model of chimeric liver transplantation to investigate its feasibility (Fig. 1A). We used mice and rats as substitutes for transgenic pigs and humans, respectively, and created CLs in transgenic mice using hepatocytes derived from transgenic rats. After chimeric liver transplantation into rat recipients with vessel reconstruction, we examined the long-term viability and functions of the transplanted CL grafts.

MATERIALS AND METHODS

Animals

All mice and rats were housed in a temperature-controlled environment under a 12-hour light-dark cycle with free access to water and standard rodent chow diet. Male and female albumin enhancer-/promoter-driven urokinase-type plasminogen activator/severe-combined immunodeficiency (uPA/SCID) mice (Phoenixbio Co, Ltd, Japan) (n = 40), which express and accumulate urokinase-type plasminogen specifically in native hepatocytes, resulting in liver disease,[11,12] were used as the scaffolds for CL regeneration. Male luciferase transgenic Lewis (Luc-LEW) (MHC haplotype; $RT1^l$)[13] (n = 2), and female luciferase and LacZ double-transgenic Lewis (Luc/LacZ-LEW) ($RT1^l$) rats[14] (n = 1) were used as hepatocyte donors. Male wild-LEW rats ($RT1^l$) (Charles River Japan, Japan) (n = 30) and male Nagase analbuminemia rats ($RT1^a$)[15] (Japan SLC, Japan) (n = 10) were used as the recipients of chimeric liver transplantation. All experiments in this study were performed in accordance with the Jichi Medical University Guide for Laboratory Animals after approval by the ethics committee of PhoenixBio Co, Ltd.

Generation of CLs

Isolated hepatocytes were obtained from 10-week-old Luc-LEW and Luc/LacZ LEW rats using a standard 2-step collagenase

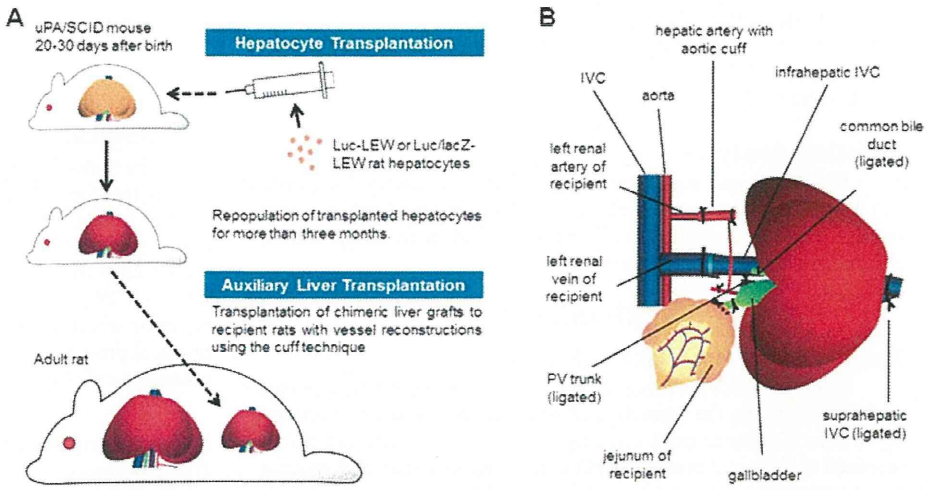

FIGURE 1. A, Experimental protocol used for Chimeric liver transplantation. B, Schema of engraftment of CL into recipient rat.

perfusion method.[16] Collagenase (75 mg/body) (COLLAGENASE S-1; Nitta-gelatin, Japan) was perfused through the portal vein, then 5.0×10^5 hepatocytes were transplanted into 20- to 30-day-old uPA/SCID mice, as previously reported.[12] These mice were kept for 3 months to obtain CLs with satisfactory size (mean ± SEM, 1.69 ± 0.04 g) and chimerism.

Harvesting of CLs

The CLs were harvested from uPA/SCID mice along with the hepatic artery with the aortic cuff and infrahepatic inferior vena cava. The portal vein trunk, common bile duct, and suprahepatic inferior vena cava were ligated. After systemic injection of 300 U of heparin sodium (Novo-Heparin; Mochida, Japan), the graft was harvested and cryo-preserved in 0.9% saline. A 10-mm plastic tube (22G SurfloR, IV catheter; Terumo, Japan) was inserted into the gallbladder, and a plastic cuff (18G SurfloR) was installed onto the end of the infrahepatic inferior vena cava.

Auxiliary Liver Transplantation of CLs

The CLs were engrafted in an auxiliary manner with vessel reconstruction using a cuff technique, as we previously reported.[17] In brief, after a simple left nephrectomy, a plastic cuff (22G SurfloR) was installed on the end of the recipient left renal artery. The graft hepatic artery and infrahepatic inferior vena cava were anastomosed to the recipient left renal artery and vein, respectively (see Video and Figure, Supplemental Digital Contents 1 and 2, demonstrating reperfusion of graft, available at http://links.lww.com/SLA/A254 and http://links.lww.com/SLA/A256, respectively). The graft gallbladder was connected to the recipient jejunum (Fig. 1B) and the recipient liver was left intact. There were no significant differences regarding any of the parameters for the animals and surgical procedures (data not shown). Recipient rats that died within 24 hours after chimeric liver transplantation or showed no luminescent signals on day 1 were excluded from postoperative analyses.

PostTransplant Treatment

Solid immunosuppressive protocols with tacrolimus (TAC) for T cell-mediated rejection and cyclophosphamide (CPA) for antibody-mediated rejection were used. The LEW rat recipients were treated with one of the following protocols: daily tacrolimus [TAC (+)CPA (−)] (n = 15), daily tacrolimus and cyclophosphamide pretreatment [TAC (+)CPA(+)] (n = 6), or no immunosuppression [TAC(−)CPA(−)] (n = 6). In the TAC(+)CPA(−) and TAC(+)CPA(+) groups, tacrolimus (Prograf; Astellas, Japan) was injected intramuscularly into the recipient rats at a dose of 0.64 mg/kg before and every day after transplantation. In TAC (+)CPA(+), candidate recipient rats were prepared with an intraperitoneal injection of cyclophosphamide (Endoxan, Shionogi, Japan) at a dose of 60 mg/kg 10 days before transplantation (dose of cyclophosphamide modified from previous report[18]). For Nagase analbuminemia rat recipients (n = 8), tacrolimus was given every day intramuscularly at a dose of 0.32 mg/kg. The recipient rats were observed daily and those in very poor condition were euthanized.

Evaluation of Luminescence From CLs

In vivo luciferase imaging of CLs was performed in both uPA/SCID mice for 3 months after hepatocyte transplantation and recipient rats throughout their survival after chimeric liver transplantation using a noninvasive bioimaging system (IVIS, Xenogen, CA), and the images were analyzed using a software package (Igor; WaveMetrics, OR, and IVIS Living Image; Xenogen). Before imaging, D-luciferin (potassium salt; Biosynth, Switzerland) (30 mg/kg) was injected into the peritoneal cavity of uPA/SCID mice or the penile vein of recipient rats. Signal intensity was quantified as photon flux in units of photons (s/cm^2/steradian) in the region of interest.

ELISA for Serum Rat Albumin Levels

Blood samples were obtained from uPA/SCID mice for 3 months after hepatocyte transplantation and Nagase analbuminemia rat recipients for 4 months after chimeric liver transplantation. To examine hepatic functions specific to CLs in our model with an intact recipient liver, we measured serum rat albumin using a Rat Albumin ELISA KIT (AKRAL-120, Shibayagi, Japan).

Histological Analyses

Tissue samples were fixed in 10% formalin for hematoxylin-eosin staining, and 10-μm thick sections were used. For X-gal and immunohistochemical staining, 4%-paraformaldehyde-fixed frozen samples were sliced into 4-μm thick sections. X-gal analyses were performed as previously reported.[14] Immunohistochemical analyses of the CL grafts were performed before and 2 days after the host left lobectomy with anti-Ki67 rabbit polyclonal antibody (RB-1510-P, Thermo Fisher Scientific, CA). Fifteen random views of each sample were obtained and Ki67-positive hepatocytes were separately counted in areas (mm^2) with viable hepatocytes using image software (Scion Image; Scion, MD).

Ultrasonographic Analyses

Blood flow velocities were measured using an ultrasound system (Prosound SSD-α5; ALOKA, Japan). Velocity was quantified in unit of cm/second.

Statistical Analyses

We performed statistical analyses using StatView5.0 (SAS, NC). We used the analysis of variance test and Holm-Sidak as a post hoc test. Data are presented as the mean ± SEM, with values of $P < 0.05$ considered to be statistically significant.

RESULTS

Development of CL Grafts in Mice

On the basis of the repopulation of transplanted rat hepatocytes in uPA/SCID mice, the spreading of positive areas was observed using in vivo bioluminescent imaging (Fig. 2A). Luminescent fluxes increased rapidly and reached a plateau for approximately 3 months after hepatocyte transplantation (Fig. 2B). Moreover, serum rat albumin concentration on day 30 was significantly higher than that on day 5, then remained until day 85 (Fig. 2C). Luminescent fluxes and albumin concentrations were strongly correlated (Spearman correlation coefficient $r = 0.86$, $P < 0.0001$), suggesting that luminescence reflected CL function. The appearance was that of a normal mouse liver (Fig. 2D). Although hematoxylin-eosin staining showed normal histological structures, X-gal staining revealed that the mouse hepatic parenchyma was nearly entirely replaced by LacZ-positive rat hepatocytes, except for the other hepatic components, such as the vessels and bile ducts in Glisson capsules (Fig. 2E).

Graft Viability After Auxiliary Transplantation

In the TAC(−)CPA(−) group, luminescence vanished rapidly until day 5 after Chimeric liver transplantation, whereas it was stably maintained for 4 weeks in both TAC(+)CPA(−) and TAC(+)CPA(+). There was no significant difference between those 2 groups (Fig. 3A).

Histological Analyses of Transplanted CL Grafts

Hematoxylin-eosin staining showed massive necrosis and destruction of the arrangement of the hepatic lobules in TAC(−)CPA(−) on day 5 (Fig. 3B). In contrast, TAC(+)CPA(−) on day 7 showed maintenance of those, even with massive cellular infiltration from the Glisson capsules to the central areas (Fig. 3C). Furthermore, in TAC(+)CPA(+), the hepatic structures were maintained with fewer rejection changes (Fig. 3D). Even on day 14, the TAC(+)CPA(+) protocol protected the CL from severe xenobiotic rejection, with only moderate cellular infiltration in the Glisson capsules (Fig. 3E).

Long-Term Patency of Reconstructed Vessel Circulations

Doppler ultrasonography showed the flow patterns of the arterial inflow and venous outflow in the transplanted CLs on days 14 and 188 in the TAC(+)CPA(−) group. Peak velocities on day 14 were 34.2 (artery) and 9.9 (vein) cm/s, whereas those on day 188 were 12.5 and 3.4 cm/s, respectively (See Figure, Supplemental Digital Content 3, available at http://links.lww.com/SLA/A257).

Long-Term Maintenance of Secretion of Rat Albumin in Nagase Analbuminemia Rats

Although serum rat albumin concentrations in Nagase analbuminemia rat recipients were undetectable before Chimeric liver transplantation, the transplanted CLs produced albumin on day 5 and then maintained production for 4 months, with maintenance of luminescence under the TAC(+)CPA(−) protocol (Fig. 4A).

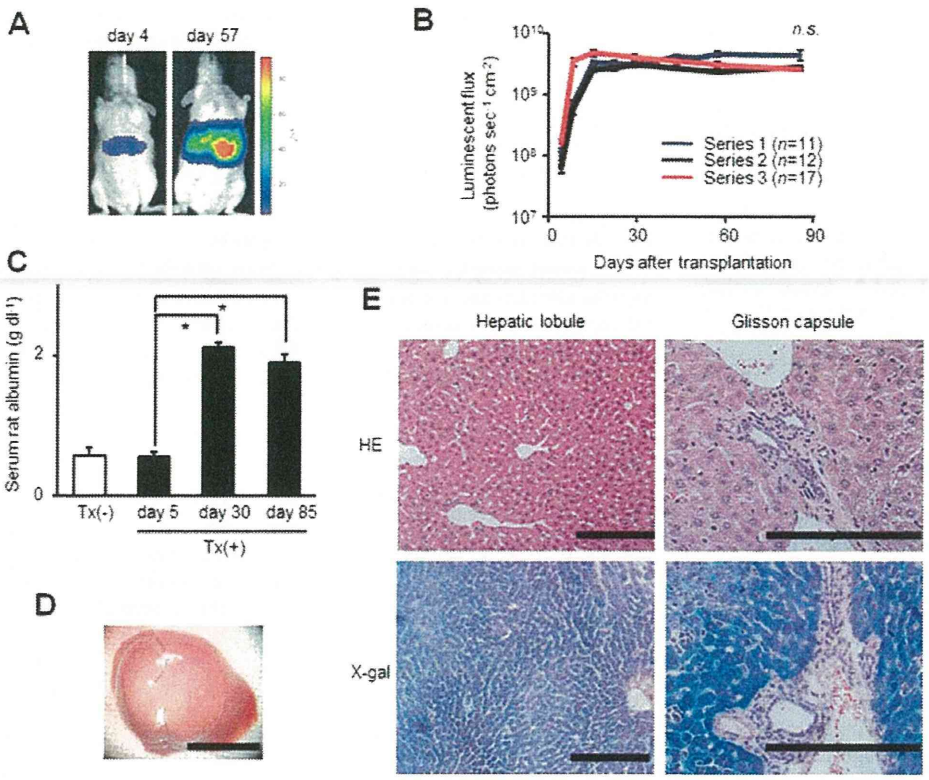

FIGURE 2. Development of CL grafts in uPA/SCID mice. A, Spread of luminescence-positive area. B, Luminescence after hepatocyte transplantation in 3 independent series. *n* as indicated; values are shown as the mean ± SEM; *n.s.*, no significant difference on day 85. C, Production of rat albumin by transplanted rat hepatocytes in mice ($n = 12$). *P <0.01; Values are shown as the mean ± SEM; Tx, hepatocyte transplantation. D, Macroscopic appearance of CL before engraftment. Scale bar = 10 mm. E, hematoxylin-eosin and X-gal staining of intact CLs. The results shown are representative of 5 separate analyses. Scale bars = 200 μm.