

Fig. 2. Cre-mediated genomic DNA recombination and HCV core protein expression in transgenic mouse livers. (A) Structure of the Cre-mediated activation transgene unit CALNCN2 (Wakita et al., 1998). pCAG, CAG promoter; neo, neomycin-resistance gene; pA, poly(A) signal. The R6CN2 HCV cDNA (nucleotides 294–3435) contains the core, E1, E2, and NS2 regions. This construct does not allow HCV mRNA transcription prior to Cre-mediated DNA recombination, detected with the primer pair CAG-1688-S20 and neo-2020-R16. When Cre-expressing AdV is injected, the *neo* gene and poly(A) signal are removed by recombination between two *loxP* sequences. The recombinant HCV transgene is detected with the primer pair CAG-1688-S20 and 6-450-R20. (B) Determination of Cre-mediated DNA recombination. CN2-29 transgenic mice were injected with 1.0×10^9 PFU for each AdV, and liver samples were harvested 7 days post-injection. Genomic DNA was extracted from the livers, and the numbers of copies of the recombinant HCV transgenes were determined using quantitative RTD-PCR with specific probes (6-294-S20FT) and a primer pair (CAG-1688-S20 and 6-450-R20). The values shown are means \pm S.D. of more than three individual specimens. (C) Measurements of HCV core protein concentration in liver samples obtained from CN2-29 transgenic mice 7 days after injection of 1.0×10^9 PFU for each AdV. The samples were homogenized and the concentrations of HCV core protein were determined by EIA. The values shown are means \pm S.D. of three individual specimens. (D) Immunofluorescence analysis of HCV core proteins. Liver sections of CN2-29 transgenic mice 7 days after injection of 1.0×10^9 PFU for each AdV were fixed and co-stained with rabbit anti-core polyclonal antibody (green) and DAPI (blue). Scale bar, 50 μ m.

2.7. Determination of Cre-mediated HCV transgene recombination in mouse livers

The transgenic mouse livers were digested at 37 °C overnight in lysis buffer [50 mM Tris-HCl (pH 8.0), 0.1 M NaCl, 20 mM EDTA, 1% SDS] containing 1 mg/mL proteinase K. Total genomic DNA was then extracted using the phenol–chloroform extraction method. The copy numbers of the recombinant HCV transgene in the livers were assessed via quantitative RTD-PCR (Takeuchi et al., 1999) with the specific probe 6-294-S20FT (5'-[FAM]-TGATAGGGTGCTTGGCAGTG-[TAMRA]-3') and the primer pair CAG-1688-S20 (5'-GGTTGTTGTGCTGTCTCATC-3') and 6-450-R20 (5'-ACAGGTAAACTCCACCAACG-3') (Fig. 2A). The standard curve was generated using pCALNCN2/59-2 (Wakita et al., 1998) and quantitative RTD-PCR with the specific probe neo-1801-S23FT (5'-[FAM]-TCAAGAGACAGGATGAGGATCGT-[TAMRA]-3') and the primer pair CAG-1688-S20 (5'-GGTTGTTGTGCTGTCTCATC-3') and neo-2020-R16 (5'-TGCCTCGCTGCAGT-3') (Fig. 2A). The *GAPDH* gene was used as an internal control for all samples. Analyses were carried out on an ABI PRISM 7700 Sequence Detection System with TaqMan Universal PCR Master Mix (Applied Biosystems).

2.8. Quantitation of HCV core proteins in mouse liver lysates

The transgenic mouse livers were homogenized in 0.5 mL RIPA buffer, and centrifuged at 15,000 rpm for 10 min at 4 °C. The protein concentrations of the supernatants were measured using the Bradford method (DC protein assay; Bio-Rad). The concentrations of HCV core proteins in the liver samples were determined using the Ortho HCV core protein ELISA kit (Eiken Chemical).

2.9. Biochemical analyses of mouse sera

Sequential blood samples were obtained by orbital bleeding after each AdV administration, and the sera were isolated by centrifugation at 10,000 rpm for 3 min at 4 °C. Serum ALT levels were determined using the Transaminase-CII Test A (Wako Pure Chemicals).

2.10. Histology and immunohistochemical staining

The liver samples were fixed with 4% paraformaldehyde in PBS, paraffin-embedded, sectioned at 4- μ m thickness, and stained

with hematoxylin and eosin (H&E). Liver histology was evaluated according to modified Histology Activity Index (HAI) scores in three categories: piecemeal necrosis, spotty necrosis, and portal inflammation (Knodell et al., 1981; Yang et al., 1994).

The liver tissues were frozen in OCT compound (Tissue Tech) for immunohistochemical staining of HCV core proteins. The sections were fixed with a 1:1 solution of acetone:methanol at -20°C for 10 min and then washed with PBS. Subsequently, the sections were incubated with the IgG fraction of an anti-HCV core rabbit polyclonal antibody (RR8) (Wakita et al., 1998) labeled with biotin in blocking buffer for 1 h at 4°C . The sections were incubated with strept-avidin-conjugated horseradish peroxidase for 30 min at room temperature. Immunohistochemical staining was conducted using the Tyramide Signal Amplification Kit (Molecular Probes). Fluorescently labeled sections were stained with 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes) to stain the cell nuclei at room temperature before cover slipping. Fluorescence was observed under a fluorescence microscope (Carl Zeiss).

2.11. Statistical analysis

Data are shown as the mean \pm S.D. Statistical analyses were performed using analysis of variance (ANOVA) followed by the Student–Newman–Keuls (SNK) test or analyzed using the unpaired Student's *t*-test. Statistical significance was established at $p < 0.05$.

3. Results

3.1. Generation of Cre-expressing AdVs

To enable HCV transgenic mice using the Cre/loxP system to express HCV protein persistently without severe inflammatory responses to AdV, we first constructed AdVs that expressed Cre with or without a nuclear localization signal (NLS) tag (AxEFNCre or AxEFCre, respectively) together with *LacZ* under the control of the EF1 α promoter (AxEFLacZ) (Fig. 1A). AxCANCre and AxCACre were also generated to compare the impacts of using the CAG promoter and the EF1 α promoter (Fig. 1A). Expression of Cre proteins from various AdVs was confirmed in human liver-derived HepG2 cells by Western blot analysis (Fig. 1B). Cre protein expression levels were not significantly different whether the gene was expressed under the control of the CAG or the EF1 α promoter in the HepG2 cells (Fig. 1B). Next, we examined the recombination activities of Cre expressed via the AdVs using the Hep-CALNLZ cell line, HepG2 cells that express CALNLZ (Fig. 1C). When the Cre-expressing AdVs bearing the CAG or EF1 α promoters infected these cells, the blue color produced by *LacZ* activation was observed for MOIs of 0.15–160. The cells infected with AxEFLacZ showed the blue staining in an MOI-dependent manner (Fig. 1D, lane AxEFLacZ). In contrast, the color faded for MOIs >40 when the Cre-expressing AdVs were used (Fig. 1D, lanes AxCANCre, AxCACre, AxEFNCre, and AxEFCre). At an MOI of 160, all of the Cre-expressing AdVs resulted in cytotoxicity, while the LacZ-expressing AdV did not affect cell viability (Fig. 1E).

AdV-induced immune responses are partly caused by co-expression of Ad-pIX (Nakai et al., 2007). To confirm the protein expression levels of Ad-pIX due to the AdVs, we performed Western blotting with anti-Ad-pIX sera (Fig. 1F). When HepG2 cells were infected with AdVs bearing the CAG promoter, significant amounts of Ad-pIX were detected as 14-kDa bands (Fig. 1F, lanes AxCANCre and AxCACre). In contrast, when using AdVs bearing the EF1 α promoter, the 14-kDa band representing Ad-pIX was undetectable, as was the case for mock-infected HepG2 cells (Fig. 1F, lanes AxEFN-Cre, AxEFCre, AxEFLacZ, and mock). We also examined the mRNA expression levels of Ad-pIX and obtained similar results that correlated with the protein expression levels (Fig. 1G).

3.2. HCV gene expression and core protein production mediated by various Cre-expressing AdVs in transgenic mouse livers

The HCV transgenic mouse CN2-29 contains a reporter unit (CALNCN2) that is activated by Cre and conditionally expresses the HCV gene (Fig. 2A; Wakita et al., 1998). To assess the efficiency of Cre-expressing AdVs in promoting HCV gene expression, we intravenously injected the CN2-29 transgenic mice with various AdVs. At 7 days post-injection, Cre protein expression was confirmed by Western blot analysis of liver lysates (data not shown). The recombinant HCV transgene levels in the livers were determined by quantitative RTD-PCR using specific probes and primer pairs, as described in Section 2 (Fig. 2A and B). When each Cre-expressing AdV was injected, the respective recombinant HCV transgene was detectable; AxCANCre-injected CN2-29 transgenic mice expressed the highest levels of the recombinant HCV transgene in their livers (Fig. 2B). CN2-29 transgenic mice injected with AdVs expressing NLS-tagged Cre had higher levels of the recombinant HCV transgene in their livers (Fig. 2B, AxCANCre and AxEFNCre). This result suggests that NLS-tagged Cre efficiently translocated to the cell nucleus, which is consistent with our previous data (Baba et al., 2005). However, the levels of the recombinant HCV transgene were not correlated with the expression level of HCV core protein (Fig. 2C).

The core protein levels in the livers were measured by enzyme immunoassay (EIA) as described in Section 2. The expression of the E1 and E2 proteins in the CN2-29 transgenic mouse livers has been shown previously (Wakita et al., 1998). The mean core protein level was 1.3 ng/mg total protein in the CN2-29 transgenic mouse livers 7 days after administration of AxCANCre (Fig. 2C). AxCACre- and AxEFNCre-injected mice expressed approximately one-half of the core protein levels resulting from AxCANCre injection (Fig. 2C).

Expression of core proteins in AdV-injected CN2-29 transgenic mouse livers was confirmed through immunofluorescence staining. Core proteins were expressed in the hepatocytes in the lobules of liver sections from Cre-expressing AdV-injected mice (Fig. 2D). In contrast, AxEFLacZ-injected transgenic mice did not express core proteins (Fig. 2C and D).

3.3. Liver injury and Ad-pIX expression in HCV transgenic mice injected with AdVs

To evaluate hepatocellular injury caused by expression of HCV proteins in CN2-29 transgenic mice injected with Cre-expressing AdVs, we serially estimated the serum ALT levels (Fig. 3A). For AxCANCre, the serum ALT level was elevated on day 5 and peaked 1–2 weeks post-injection (Fig. 3A, open triangle). ALT levels in AxCACre-injected transgenic mice were also elevated, although these levels declined over time (Fig. 3A, open circle). When AxEFN-Cre or AxEFCre was injected, ALT levels did not immediately increase, although they gradually increased after day 5 (Fig. 3A, closed triangle and closed circle, respectively). Injection of AxEFLacZ did not increase serum ALT levels in the CN2-29 transgenic mice (Fig. 3A, closed rectangle).

We also performed histological analyses of liver sections from CN2-29 transgenic mice 7 days after AdV injection (Fig. 3B). We found that severe inflammation with lymphocyte infiltration and spotty necrosis were diffusely observed in the livers of mice injected with the AdVs bearing the CAG promoter (AxCACre and AxCANCre) (Fig. 3B, a,b). In contrast, AxEFNCre-injected and AxEFCre-injected transgenic mouse livers exhibited mild inflammation without massive piecemeal necrosis on day 7 (Fig. 3B, c,d). No inflammation was observed in the AxEFLacZ-injected mice (Fig. 3B, e).

To confirm the expression levels of Ad-pIX in AdV-injected transgenic mice, we determined Ad-pIX mRNA in the liver using

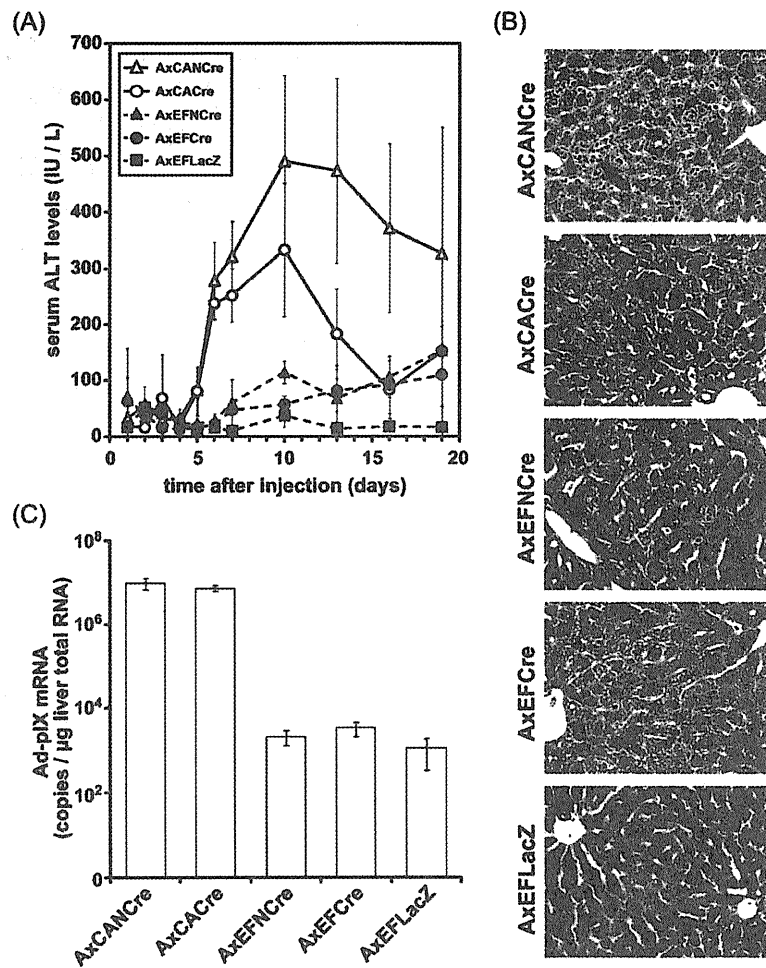


Fig. 3. Effects of AdVs on liver injuries in HCV transgenic mice. (A) Serum ALT levels were measured at the indicated time-points in CN2-29 transgenic mice injected with 1.0×10^9 PFU AxCANCre (open triangle), AxCACre (open circle), AxEFNCre (closed triangle), AxEFCre (closed circle), and AxEFLacZ (closed rectangle). The ALT levels are shown as means \pm S.D. of three individual specimens. (B) Histopathologic changes in the livers of transgenic mice 7 days after injection of each AdV. The liver sections were stained with H&E. The arrows represent lymphocyte infiltrations. Scale bar, 50 μ m. (C) mRNA expression of Ad-pIX in the livers. CN2-29 transgenic mice were injected with 1.0×10^9 PFU of the AdVs. After 12 h, the livers were harvested. The total RNA extracts from the livers were subjected to reverse transcription and RTD-PCR with an Ad-pIX-specific probe and a primer pair, as described in Section 2. The numbers of copies of Ad-pIX mRNA are shown as means \pm S.D. of three individual specimens.

reverse transcription and quantitative RTD-PCR, as described under Section 2. The copy numbers of Ad-pIX mRNA were quite high in transgenic mice that were injected with AdV bearing the CAG promoter (Fig. 3C). The observed inflammation levels were consistent with the expression levels of Ad-pIX.

3.4. Liver inflammatory responses to the HCV protein inducibly expressed by AdVs in transgenic mice

Because our results indicated that severe liver injuries were caused by AdVs bearing the CAG promoter, we evaluated liver inflammatory responses to the HCV protein inducibly expressed by AdVs in transgenic mice 7 days post-injection according to the modified HAI scoring system (Fig. 4A) (Knodell et al., 1981; Yang et al., 1994). Among the transgenic mice, more severe liver damage was observed in those that were injected with Cre-expressing AdVs bearing the CAG promoter (Fig. 4A, AxCANCre and AxCACre) compared to those injected with Cre-expressing AdVs bearing the EF1 α promoter (Fig. 4A, AxEFNCre and AxEFCre).

Because AxEFCre more efficiently expressed HCV proteins than AxEFNCre (Fig. 2C and D), we injected AxEFCre into transgenic mice and wild-type mice to examine the effects of HCV protein expression. The severity of liver inflammation in the AxEFCre-injected transgenic mice was significantly greater than in the AxEFCre-injected wild-type mice or the AxEFLacZ-injected transgenic mice (Fig. 4A and B).

Seven days after AdV administration, serum ALT levels of AxCANCre-injected wild-type mice were significantly higher than those of AxEFCre-injected wild-type mice (Fig. 4C). This ALT elevation was observed in both transgenic and wild-type mice injected with AxCANCre (Fig. 4C). In contrast, AxEFCre was injected into the two groups, transgenic mice expressing HCV proteins exhibited more severe liver injury than wild-type mice (Fig. 4C and D).

3.5. Effects of Cre-expressing AdV bearing the EF1 α promoter on HCV protein expression in transgenic mouse livers

To investigate whether CN2-29 transgenic mice injected with AdVs bearing the EF1 α promoter showed liver inflammation caused

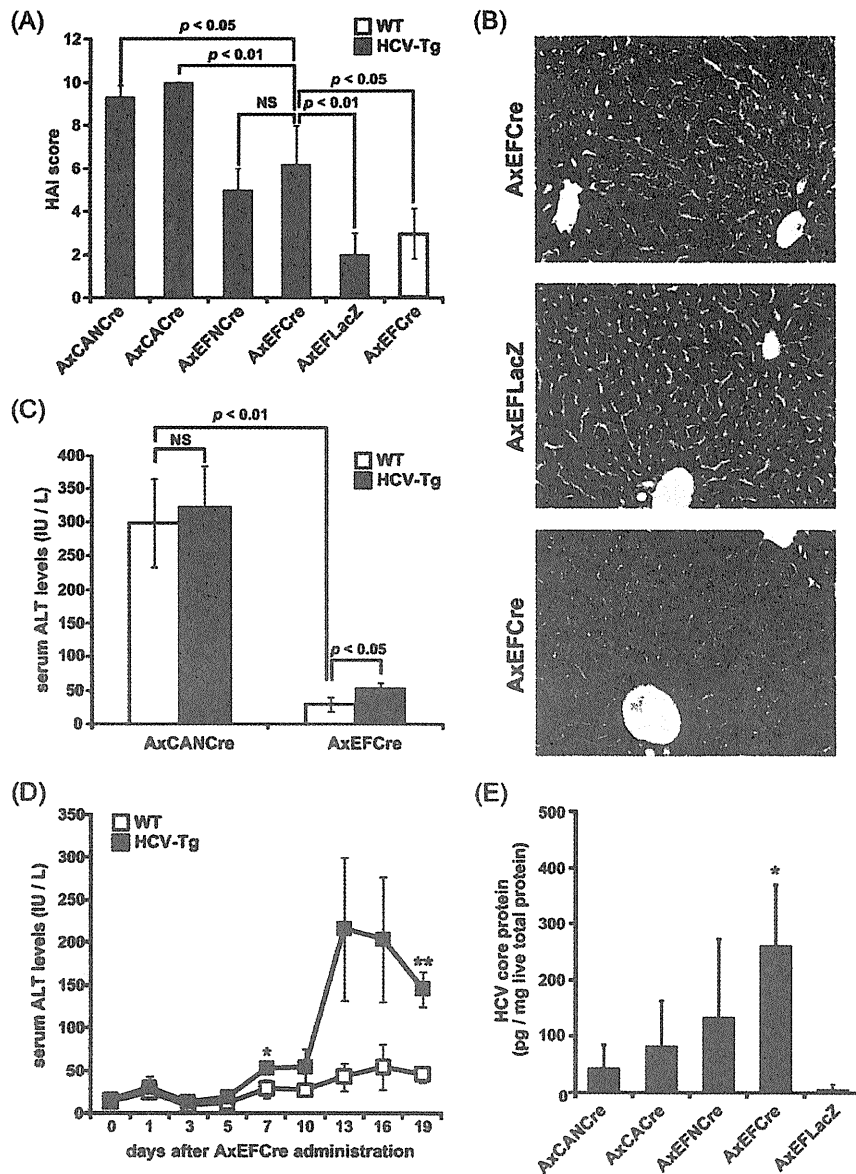


Fig. 4. Liver inflammatory responses due to HCV protein expression induced by AxEFCre. (A) Histopathology of mouse livers after injection of AdVs. Histopathologic features of the livers of CN2-29 transgenic mice (HCV-Tg, closed bars) injected with 1.0×10^9 PFU of AxCANCre, AxCACre, AxEFNCre, AxEFCre, or AxEFLacZ, and wild-type mice that were injected with 1.0×10^9 PFU of AxEFCre at day 7 post-injection (WT, opened bar). Pathologic changes were evaluated by light microscopy of H&E-stained sections of the mouse livers using the modified HAI scoring system. The extent of pathology was scored on a scale from 0 (none) to 12 (severe). All of the scores are means \pm S.D. of more than three individual specimens. Statistical analysis was performed using an unpaired Student's *t*-test. NS, not significant. (B) Histopathologic changes resulting from HCV protein expression in mouse livers. CN2-29 transgenic mice (HCV-Tg) were injected with AxEFCre or AxEFLacZ and wild-type mice (WT) were injected with AxEFCre 7 days post-injection. The liver sections were stained with H&E. The arrows represent piecemeal necrosis. Scale bars, 50 μ m. (C) Serum ALT levels with or without HCV protein expression 7 days after administration of the AdVs. Statistical analysis was performed using an unpaired Student's *t*-test between CN2-29 transgenic mice (HCV-Tg) and wild-type mice (WT). NS, not significant. (D) Sequential changes in serum ALT levels after AxEFCre administration. Serum ALT levels were measured at the indicated time-points in CN2-29 transgenic mice (HCV-Tg, closed square) or wild-type mice (WT, opened square) that were injected with 1.0×10^9 PFU AxEFCre. ALT levels are shown as means \pm S.D. of more than three individual specimens. Statistical analysis was performed using an unpaired Student's *t*-test between CN2-29 transgenic mice (HCV-Tg) and wild-type mice (WT). **p* < 0.05; ***p* < 0.01. (E) HCV core protein expression 21 days after AdV administration in transgenic mouse livers. CN2-29 transgenic mice were injected with 1.0×10^9 PFU for each AdV. After 21 days, the livers were harvested and homogenized. The concentrations of HCV core proteins in liver lysates were determined by EIA. The values shown are means \pm S.D. of three individual experiments. Statistical analysis was performed using an ANOVA, followed by the SNK test. **p* < 0.05.

by persistently expressed HCV proteins, we evaluated core proteins by EIA in transgenic mouse livers 21 days post-injection of the AdVs (Fig. 4E). HCV core protein expression was scarcely detectable in the transgenic mice injected with AxCANCre, while the AxEFCre-injected transgenic mice showed significantly higher levels of core protein expression (Fig. 4E). Although, the AxCANCre injection was scarcely observed at day 21 in the transgenic mice (Fig. 4E), HCV

core protein expression induced by AxEFCre injection was observed until at least day 56.

4. Discussion

In the present study, we demonstrated that Cre-expressing AdVs bearing the EF1 α promoter induce HCV gene expression and

HCV protein production without induction of severe liver injury in inducible-HCV transgenic mice. We further observed that increases in serum ALT levels and liver inflammation were related to HCV protein expression mediated by AxEFCre injection. Moreover, AxEFCre injection enabled the transgenic mice to persistently express HCV proteins.

In previous studies, HCV transgenic mice constitutively expressing HCV proteins exhibited symptoms of steatosis and/or hepatocellular carcinoma, but did not show inflammatory or immunopathologic changes (Lerat et al., 2002; Moriya et al., 1997, 1998; Sun et al., 2001). Inducible-HCV transgenic mouse lineages, in which HCV protein expression is regulated, have enabled investigation of the immunopathogenesis of HCV protein expression. HCV transgenic mice regulated by the Cre/loxP system (Sun et al., 2005; Tumurbaatar et al., 2007; Wakita et al., 1998) or the tetracycline regulatory system (Ernst et al., 2007) exhibit inducible and liver-specific expression of HCV proteins. Inducible-HCV transgenic mice using the Cre/loxP system with an AdV that expresses Cre under the control of the CAG promoter (AxCANCre) exhibit HCV-specific immune responses (Wakita et al., 1998, 2000). The inducible-HCV CN2-29 transgenic mice, which express the core, E1, E2, and NS2 proteins, have HCV-specific cytotoxic T lymphocytes (Takaku et al., 2003; Wakita et al., 1998, 2000).

However, they show severe inflammatory responses to AxCANCre itself and thus, HCV protein expression is only transient (Wakita et al., 2000). These significant obstacles have limited the utility of inducible-HCV transgenic mice. Therefore, to deliver the Cre gene into the liver, non-adenoviral induction methods have been developed (Ho et al., 2008; Sun et al., 2005; Zhu et al., 2006). Meanwhile, adenoviral genes that cause cellular immune responses have been identified and modified AdVs that do not trigger host immune responses have been developed (Palmer and Ng, 2005). A recent study demonstrated that immune responses to AdVs bearing the CAG promoter were associated with co-expression of Ad-pIX, whereas immune responses were minimal when transgene expression was controlled by the EF1 α promoter (Nakai et al., 2007). Therefore, we postulated that severe inflammation of mouse livers after administration of Cre-expressing AdVs bearing the CAG promoter (AxCANCre) might be caused by expression of Ad-pIX. In the present study, we generated Cre-expressing AdVs bearing the EF1 α promoter (AxEFCre) and infected HCV transgenic mice. AxEFCre-injected mice expressed much less Ad-pIX mRNA and did not show the increased levels of ALT or severe liver inflammation as did Cre-expressing AdVs under the control of the CAG promoter (Fig. 3). In contrast, AxCANCre administration caused severe liver injury in both HCV transgenic mice and wild-type mice (Fig. 4D; Wakita et al., 2000). AxEFCre administration caused liver injury in the HCV transgenic mice, but not in the wild-type mice (Fig. 4A–D). These results suggest that AxEFCre alone induces only minimal host immune responses compared to AxCANCre; therefore, the liver inflammatory responses exhibited by AxEFCre-injected transgenic mice were clearly due to expression of HCV proteins. Because AxCANCre injection alone causes severe liver injuries, most of the hepatocytes infected with AxCANCre are eliminated and HCV protein expression in the livers of transgenic mice is only transient (Wakita et al., 2000). On the other hand, AxEFCre injection did not induce such severe liver injuries. The AxEFCre-injected HCV transgenic mice showed milder liver inflammation in response to expression of HCV proteins and persistently expressed HCV proteins without elimination of hepatocytes infected with AxEFCre.

In conclusion, HCV gene expression mediated by the Cre/loxP system and a Cre-expressing AdV that bears the EF1 α promoter, AxEFCre, enables Cre-mediated recombination of transgenes in mice without inducing severe liver injury due to the AdV itself. Moreover, this inducible-HCV transgenic mouse model should be

useful for investigation of liver injury due to HCV and the pathogenesis of HCV.

Acknowledgments

The authors wish to thank Mitsugu Takahashi for breeding the transgenic mice. This study was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan; the Program for Promotion of Fundamental Studies in Health Sciences of the Pharmaceuticals and Medical Devices Agency of Japan; and the Ministry of Health, Labor and Welfare of Japan.

References

- Akagi, K., Sandig, V., Vooijs, M., Van der Valk, M., Giovannini, M., Strauss, M., Berns, A., 1997. Cre-mediated somatic site-specific recombination in mice. *Nucleic Acids Res.* 25 (9), 1766–1773.
- Baba, Y., Nakano, M., Yamada, Y., Saito, I., Kanegae, Y., 2005. Practical range of effective dose for Cre recombinase-expressing recombinant adenovirus without cell toxicity in mammalian cells. *Microbiol. Immunol.* 49 (6), 559–570.
- Bangari, D.S., Mittal, S.K., 2006. Current strategies and future directions for eluding adenoviral vector immunity. *Curr. Gene Ther.* 6 (2), 215–226.
- Ernst, E., Schonig, K., Bugert, J.J., Blaker, H., Pfaff, E., Stremmel, W., Encke, J., 2007. Generation of inducible hepatitis C virus transgenic mouse lines. *J. Med. Virol.* 79 (8), 1103–1112.
- Goodman, Z.D., Ishak, K.G., 1995. Histopathology of hepatitis C virus infection. *Semin. Liver Dis.* 15 (1), 70–81.
- Ho, K.J., Bass, C.E., Kroemer, A.H., Ma, C., Terwilliger, E., Karp, S.J., 2008. Optimized adeno-associated virus 8 produces hepatocyte-specific Cre-mediated recombination without toxicity or affecting liver regeneration. *Am. J. Physiol. Gastrointest. Liver Physiol.* 295 (2), G412–G419.
- Kafri, T., Morgan, D., Krahl, T., Sarvetnick, N., Sherman, L., Verma, I., 1998. Cellular immune response to adenoviral vector infected cells does not require de novo viral gene expression: implications for gene therapy. *Proc. Natl. Acad. Sci. U S A* 95 (19), 11377–11382.
- Kanegae, Y., Lee, G., Sato, Y., Tanaka, M., Nakai, M., Sakaki, T., Sugano, S., Saito, I., 1995. Efficient gene activation in mammalian cells by using recombinant adenovirus expressing site-specific Cre recombinase. *Nucleic Acids Res.* 23 (19), 3816–3821.
- Kanegae, Y., Makimura, M., Saito, I., 1994. A simple and efficient method for purification of infectious recombinant adenovirus. *Jpn. J. Med. Sci. Biol.* 47 (3), 157–166.
- Knodell, R.G., Ishak, K.G., Black, W.C., Chen, T.S., Craig, R., Kaplowitz, N., Kiernan, T.W., Wollman, J., 1981. Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. *Hepatology* 1 (5), 431–435.
- Kobayashi, N., Fujiwara, T., Westerman, K.A., Inoue, Y., Sakaguchi, M., Noguchi, H., Miyazaki, M., Cai, J., Tanaka, N., Fox, I.J., Le Boulch, P., 2000. Prevention of acute liver failure in rats with reversibly immortalized human hepatocytes. *Science* 287 (5456), 1258–1262.
- Kremsdorff, D., Brezillon, N., 2007. New animal models for hepatitis C viral infection and pathogenesis studies. *World J. Gastroenterol.* 13 (17), 2427–2435.
- Lerat, H., Honda, M., Beard, M.R., Loesch, K., Sun, J., Yang, Y., Okuda, M., Gosert, R., Xiao, S.Y., Weimann, S.A., Lemon, S.M., 2002. Steatosis and liver cancer in transgenic mice expressing the structural and nonstructural proteins of hepatitis C virus. *Gastroenterology* 122 (2), 352–365.
- Moriya, K., Fujie, H., Shintani, Y., Yotsuyanagi, H., Tsutsumi, T., Ishibashi, K., Matsuura, Y., Kimura, S., Miyamura, T., Koike, K., 1998. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. *Nat. Med.* 4 (9), 1065–1067.
- Moriya, K., Yotsuyanagi, H., Shintani, Y., Fujie, H., Ishibashi, K., Matsuura, Y., Miyamura, T., Koike, K., 1997. Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. *J. Gen. Virol.* 78 (7), 1527–1531.
- Nakai, M., Komiya, K., Murata, M., Kimura, T., Kanaoka, M., Kanegae, Y., Saito, I., 2007. Expression of pIX gene induced by transgene promoter: possible cause of host immune response in first-generation adenoviral vectors. *Hum. Gene Ther.* 18 (10), 925–936.
- Palmer, D.J., Ng, P., 2005. Helper-dependent adenoviral vectors for gene therapy. *Hum. Gene Ther.* 16 (1), 1–16.
- Shepard, C.W., Finelli, L., Alter, M.J., 2005. Global epidemiology of hepatitis C virus infection. *Lancet Infect. Dis.* 5 (9), 558–567.
- Shintani, Y., Yotsuyanagi, H., Mori, K., Fujie, H., Tsutsumi, T., Kanegae, Y., Kimura, S., Saito, I., Koike, K., 1999. Induction of apoptosis after switch-on of the hepatitis B virus X gene mediated by the Cre/loxP recombination system. *J. Gen. Virol.* 80 (12), 3257–3265.
- Sun, J., Bodola, F., Fan, X., Irshad, H., Soong, L., Lemon, S.M., Chan, T.S., 2001. Hepatitis C virus core and envelope proteins do not suppress the host's ability to clear a hepatic viral infection. *J. Virol.* 75 (24), 11992–11998.
- Sun, J., Tumurbaatar, B., Jia, J., Diao, H., Bodola, F., Lemon, S.M., Tang, W., Bowen, D.G., McCaughan, G.W., Bertolino, P., Chan, T.S., 2005. Parenchymal expression of CD86/B7.2 contributes to hepatitis C virus-related liver injury. *J. Virol.* 79 (16), 10730–10739.
- Takaku, S., Nakagawa, Y., Shimizu, M., Norose, Y., Maruyama, I., Wakita, T., Takano, T., Kohara, M., Takahashi, H., 2003. Induction of hepatic injury by hepatitis C-virus-

- specific CD8 \pm murine cytotoxic T lymphocytes in transgenic mice expressing the viral structural genes. *Biochem. Biophys. Res. Commun.* 301, 330–337.
- Takeuchi, T., Katsume, A., Tanaka, T., Abe, A., Inoue, K., Tsukiyama-Kohara, K., Kawaguchi, R., Tanaka, S., Kohara, M., 1999. Real-time detection system for quantification of hepatitis C virus genome. *Gastroenterology* 116 (3), 636–642.
- Tumurbaatar, B., Sun, Y., Chan, T., Sun, J., 2007. Cre-estrogen receptor-mediated hepatitis C virus structural protein expression in mice. *J. Virol. Methods* 146 (1–2), 5–13.
- Wakita, T., Katsume, A., Kato, J., Taya, C., Yonekawa, H., Kanegae, Y., Saito, I., Hayashi, Y., Koike, M., Miyamoto, M., Hiasa, Y., Kohara, M., 2000. Possible role of cytotoxic T cells in acute liver injury in hepatitis C virus cDNA transgenic mice mediated by Cre/loxP system. *J. Med. Virol.* 62 (3), 308–317.
- Wakita, T., Taya, C., Katsume, A., Kato, J., Yonekawa, H., Kanegae, Y., Saito, I., Hayashi, Y., Koike, M., Kohara, M., 1998. Efficient conditional transgene expression in hepatitis C virus cDNA transgenic mice mediated by the Cre/loxP system. *J. Biol. Chem.* 273 (15), 9001–9006.
- Yang, Y., Ertl, H.C., Wilson, J.M., 1994. MHC class I-restricted cytotoxic T lymphocytes to viral antigens destroy hepatocytes in mice infected with E1-deleted recombinant adenoviruses. *Immunity* 1 (5), 433–442.
- Zhu, H.Z., Wang, W., Feng, D.M., Sai, Y., Xue, J.L., 2006. Conditional gene modification in mouse liver using hydrodynamic delivery of plasmid DNA encoding Cre recombinase. *FEBS Lett.* 580 (18), 4346–4352.

Monoclonal Antibody 2-152a Suppresses Hepatitis C Virus Infection Through Betaine/GABA Transporter-1

Masaaki Satoh,¹ Makoto Saito,¹ Takashi Takano,^{1,2} Yuri Kasama,¹ Tomohiro Nishimura,^{1,3} Yasumasa Nishito,⁴ Yuichi Hirata,² Masaaki Arai,² Masayuki Sudoh,⁵ Chieko Kai,⁶ Michinori Kohara,² and Kyoko Tsukiyama-Kohara¹

¹Department of Experimental Phylaxiology, Faculty of Life Sciences, Kumamoto University, Honjo Kumamoto City; ²Department of Microbiology and Cell Biology, Tokyo Metropolitan Institute of Medical Science, Kamikitazawa, Setagaya-ku; ³KAKETSUKEN, Kyokushi, Kikuchi, Kumamoto; ⁴Center for Microarray Analysis, Tokyo Metropolitan Institute of Medical Science; ⁵Kamakura Research Laboratories, Chugai Pharmaceutical Co., Ltd., Kajiwarra, Kamakura-City, Kanagawa; and ⁶Laboratory of Animal Research Center, Institute of Medical Science, University of Tokyo, Shirokane-dai Minato-Ku, Japan

Background. We recently established a monoclonal antibody (2-152a MAb) that binds to 3 β -hydroxysterol- Δ 24-reductase (DHCR24) by immunizing mice with cells (RzM6-LC) persistently expressing hepatitis C virus (HCV). Here, we aimed to analyze the activity of 2-152a MAb against HCV replication and explore the molecular mechanism underlying the antiviral activity.

Methods. We characterized the effects of 2-152a MAb on HCV replication and performed a microarray analysis of antibody-treated HCV replicon cells. The molecules showing a significant change after the antibody treatment were screened to examine their relationship with HCV replication.

Results. The antibody had antiviral activity both in vitro and in vivo (chimeric mice). In the microarray analysis, 2-152a MAb significantly suppressed the expression of betaine/GABA transporter-1 (BGT-1) in 2 HCV replicon cell lines but not in HCV-cured cells. Silencing of BGT-1 expression by small interfering RNA (siRNA) revealed significant suppression of HCV replication and infection without cytotoxicity. Further, BGT-1 expression was significantly increased in the presence of HCV ($P < .05$).

Conclusions. Our results suggest that 2-152a MAb suppresses HCV replication and infection through BGT-1. These findings highlight important roles of BGT-1 in HCV replication and reveal a possible target for anti-HCV therapy.

Hepatitis C virus (HCV) causes chronic hepatitis and hepatocellular carcinoma (HCC) [1–3]. Chronic HCV infection is a major global public health concern because it affects at least 170 million people worldwide [2]. The most effective treatment against HCV currently comprises a combination therapy of PEGylated α -interferon (IFN- α) and ribavirin [4, 5]. However, considering that

sustained virological responses develop in only approximately half of the patients infected with HCV genotype 1, the clinical efficacy of this therapy is limited [6, 7]. Efforts to develop therapies against HCV are further hindered by the high level of viral variation and capacity of the virus to cause chronic infection. Therefore, there is an urgent need to develop effective treatments against chronic HCV infection.

In a previous study, we established a cell line expressing HCV (RzM6-LC) to investigate the effects of persistent HCV expression on cell growth [8]. We also established a monoclonal antibody (2-152a MAb) against the RzM6-LC cell line to produce clones that recognize both cell surface and intracellular molecules. Using this method, we identified 3 β -hydroxysterol-D24-reductase (DHCR24) as the recognition molecule of this antibody.

Received 30 November 2010; accepted 10 May 2011.

Correspondence: Kyoko Tsukiyama-Kohara, PhD, DVM, Department of Experimental Phylaxiology, Faculty of Life Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto-shi, Kumamoto 860-8556, Japan (kkohara@kumamoto-u.ac.jp).

The Journal of Infectious Diseases 2011;204:1172–80

© The Author 2011. Published by Oxford University Press on behalf of the Infectious Diseases Society of America. All rights reserved. For Permissions, please e-mail: journals.permissions@oup.com
0022-1899 (print)/1537-6613 (online)/2011/2048-0005\$14.00
DOI: 10.1093/infdis/jir501

DHCR24 (also termed seladin-1) is an enzyme that catalyzes the conversion of desmosterol to cholesterol in the postsqualene cholesterol biosynthetic pathway [9, 10]. DHCR24 also acts as a hydrogen peroxide scavenger [11]. Therefore, DHCR24 may play a crucial role in maintaining cell physiology through cholesterol synthesis and oxidative stress. We previously demonstrated that HCV infection upregulates DHCR24 expression, and overexpression of DHCR24 inhibits apoptosis and inactivates the tumor suppressor gene p53 [12]. Moreover, silencing of DHCR24 suppressed HCV replication [13]. However, the precise mechanisms through which DHCR24 affects the HCV life cycle are unclear. In this study, we aimed to analyze the activity of 2-152a MAb against HCV replication and explore the molecular mechanism underlying the antiviral activity.

Materials And Methods

Cell Lines and Reagents

Human hepatoma cell line HuH-7 cell-based HCV replicon-harboring cell lines [14] R6FLR-N (genotype 1b) [15], FLR3-1 (genotype 1b) [16], and JFH-1 (genotype 2a) [17] were maintained in Dulbecco's modified Eagle's medium (DMEM) GlutaMAX (Invitrogen) containing 10% fetal calf serum (FCS; Sigma-Aldrich) in the presence of G418 (500 mg/mL for R6FLR-N and FLR3-1, 300 mg/mL for JFH-1; Invitrogen). Cured/HuH-7 histone H3 lysine 4 (K4) cells cured off HCV by interferon treatment [18] were maintained in DMEM GlutaMAX containing 10% FCS without G418. The JFH/K4 cell line persistently infected with the HCV JFH-1 strain and HuH-7 cell lines were maintained in DMEM containing 10% FCS [19]. The human hepatoblastoma HepG2 cell line was also maintained in DMEM containing 10% FCS.

Generation of 2-152a MAb

BALB/c strain of mice was immunized with 7–8 intraperitoneal injections of RzM6-LC cells (5×10^6) in RIBI adjuvant (trehalose dimycolate + monophosphoryl lipid A emulsion; RIBI ImmunoChem Research). After completion of the immunization regimen, their spleens were excised and splenocytes were fused with mouse myeloma plasminogen activator inhibitor (PAI) cells by using PEG1500 (Roche). Hybridoma cells were then selected with hypoxanthine, aminopterin, and thymidine (Invitrogen), and culture supernatants were collected for screening by whole-cell enzyme-linked immunosorbent assay (ELISA).

HCV Infection in Humanized Chimeric Mouse Liver and HCV mRNA Quantification by Real-time Detection Polymerase Chain Reaction

We purchased (from PhoenixBio Co.) chimeric mice that were established by transplanting human primary hepatocytes into severely combined immunodeficient (SCID) mice carrying

a urokinase plasminogen activator (uPA) transgene controlled by an albumin promoter [20]. These mice were then infected with plasma isolated before 2003 from an HCV-positive patient (HCR6) [8, 21], in accordance with the Declaration of Helsinki. The protocols for the animal experiments were preapproved by the local ethics committee, and the animals were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. HCV genotype 1b RNA levels were established at $0.96\text{--}1.84 \times 10^7$ copies/mL in mouse serum samples before the antibody treatment. The antibody (2-152a MAb) and normal immunoglobulin G (IgG, 400 mg/20 g body weight) were intraperitoneally injected into the mice ($n = 4$) at 2-day intervals over a period of 14 days. IFN- α (30 mg/kg) was administered subcutaneously at 2-day intervals over a period of 2 weeks. Human serum albumin in the blood of chimeric mice was measured by using an Alb-II kit according to the manufacturer's instructions (Eiken Chemical). HCV RNA levels in serum and JFH/K4 cells were measured by real-time detection polymerase chain reaction (real-time detection [RTD]-PCR) as described previously [22]. HCV RNA in the cell cultures and supernatants was extracted by using Isogene and Isogene LS (Nippon Gene), respectively.

Replication Assay Using HCV Replicon Cells

We used 3 HCV subgenomic replicon cell lines: R6FLR-N, FLR3-1, and JFH-1. They were seeded at a density of 5×10^3 cells/well in 96-well tissue culture plates in DMEM GlutaMAX (Invitrogen) containing 5% fetal bovine serum (Thermo Scientific). Following incubation for 24 hours at 37°C (in 5% CO₂), the medium was removed and serial dilutions of antibody were added. Luciferase activity was determined by using a Bright-Glo luciferase assay kit (Promega) after 72 hours according to the manufacturer's instructions. The results were calculated as the average percentage relative to the reactivity in untreated cells, which was set at 100%. The viability of the replicon cells was measured by using a WST-8 cell counting kit (Dojindo) according to the manufacturer's instructions.

Immunostaining and Antibodies

Cells were cultured on glass coverslips (1.0 cm diameter) and fixed with 1% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature for 10 minutes in 24-well plates. To permeabilize the cell membranes, the cells were treated with 1% Triton X-100 in PBS at room temperature for 10 minutes. After washing with 0.05% Tween-20 in PBS, the cells were incubated with 2-152a MAb, antiprotein disulfate isomerase (PDI) rabbit polyclonal antibody (Stressgen Bioreagents) or normal mouse IgG for 1 hour and washed with 0.05% Tween-20 in PBS. Alexa Fluor 488-labeled goat antimouse IgG was used as the secondary antibody.

Anti-NS5A antibody was provided by Dr Yoshiharu Matsuura (Osaka University). Anti-myc mouse monoclonal antibody

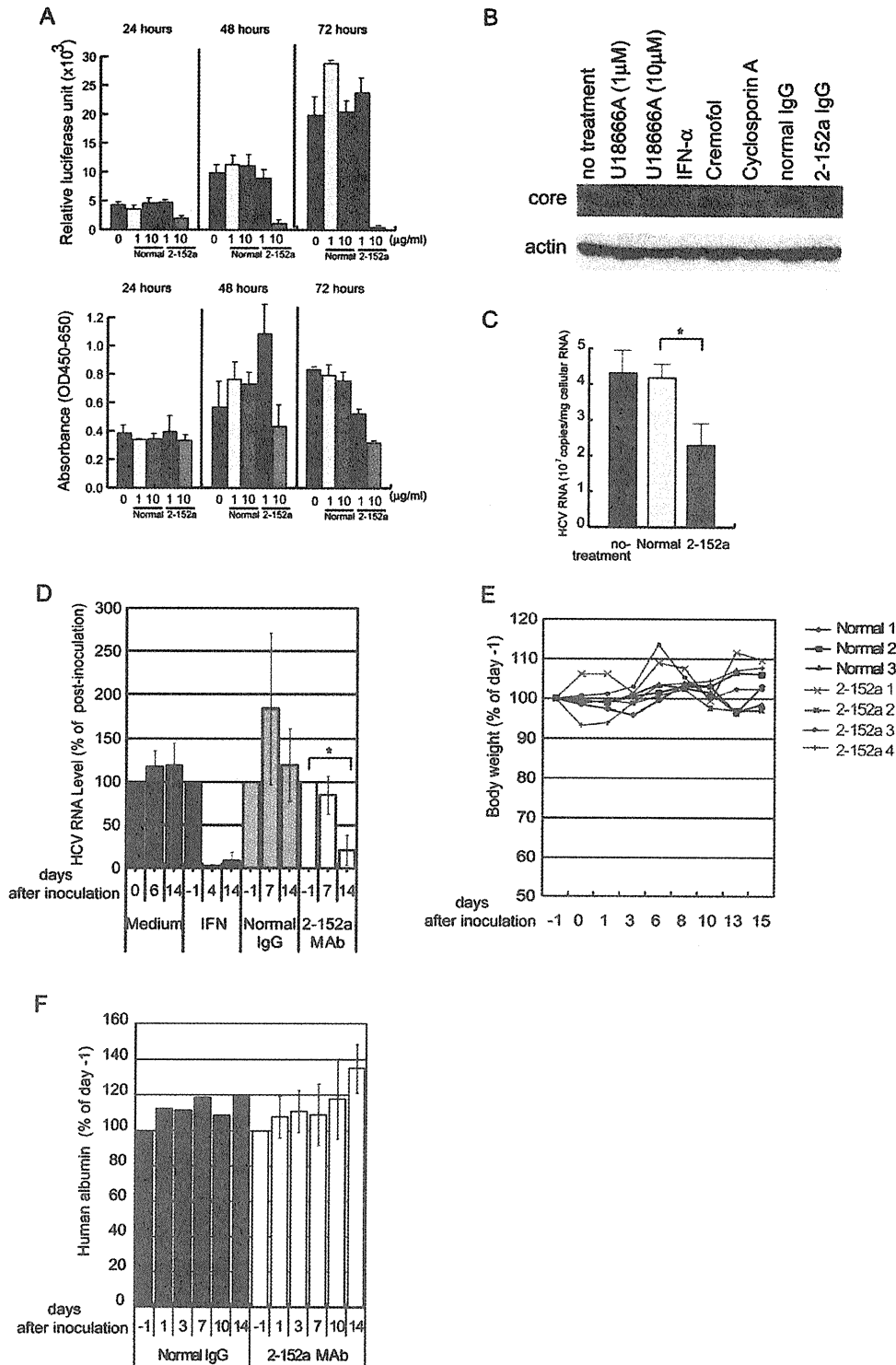


Figure 1. Anti-DHCR24 monoclonal antibody (2-152a MAb) suppresses HCV replication in vitro and in vivo. *A*, The effects of 2-152a MAb on HCV replication were measured by the luminescence activity and cell viability in FLR3-1 cells. The replicon cell line was incubated with IgG from normal mice or 2-152a MAb at 1 or 10 µg/mL for 24, 48, and 72 hours. The mean values from triplicate wells are indicated, and the vertical bars represent the standard deviation. The medium control (2% FCS-DMEM) without IgG is indicated as 0. *B*, The JFH/K4 cells were treated with cholesterol synthesis inhibitor U18666A (1 mM, 10 mM), IFN-α (250 IU/mL), Cyclosporin A (25 µM) and its solvent Cremophor, normal mouse IgG (10 µg/mL), and 2-152a IgG (10 µg/mL). HCV core and actin proteins were detected. *C*, HCV RNA copies were measured in JFH/K4 cells after treatment with normal or 2-152a IgG

(9E10; Cell Signaling Technology) and antiactin mouse monoclonal antibodies (Sigma-Aldrich) were utilized for detecting myc-fusion protein and normalization of the results, respectively.

cDNA Synthesis and Quantitative Reverse Transcriptase PCR

cDNA was synthesized from 0.5 or 1 mg of total RNA with a Superscript II kit (Invitrogen). TaqMan gene expression assays were custom designed and manufactured by Applied Biosystems. The expression was quantified with the ABI 7500 real-time PCR system (Applied Biosystems).

Microarray Analysis

For microarray analysis, total RNAs were extracted using RNAeasy kit (Qiagen), and RNA integrity was assessed using a Bioanalyzer (Agilent Technologies). cRNA targets were synthesized and hybridized with Whole Human Genome Oligo Microarray (G4112F; Agilent) according to the manufacturer's instructions.

RNA Interference, Expression Vector Construction, Transfection, and Rescue Experiments

Small interference RNA (siRNA) targeting betaine/GABA transporter-1 (BGT-1; nucleotides 120–144) was designed by using a program (<https://rnaidesigner.invitrogen.com/>) based on registered sequences in GenBank (5'-CAACAAGATGGAGT TTGTGCTGTCA-3'). Alternative siRNA (BGT-1-siRNA-362; nucleotides 362–386) was similarly designed. The HCV-siRNA (R7) sequence was 5'-GUCUCGUAGACCGUGACCA dTdT-3'.

The coding region of the BGT-1 gene was obtained from RNA of R6FLR-N cells by reverse transcription–polymerase chain reaction (RT-PCR). The PCR products were inserted in *EcoRV*–*XhoI* sites of pcDNA6-myc His, version A (Invitrogen) after digestion of *EcoRV*–*XhoI*. To generate mutant plasmids that contained nucleotide substitutions in the siRNA-targeted site, we introduced point mutations into pcDNA-BGT-1 by using site-directed mutagenesis with a QuickChange multisite-directed mutagenesis kit (Stratagene), according to the manufacturer's instructions, and the following oligonucleotide primer: BGT-1-mut, 5'-CCAATGGACCAA-CAAGATGGAATTCGTCTATTCGGTGGCCGGGGAGCTC ATTGGG-3' (the mutations introduced by mutagenesis are underlined).

Transfection of siRNAs was carried out by reverse transfection using Lipofectamine RNAiMAX according to the manufacturer's protocol (Invitrogen). Transfection of the expression vector was undertaken by using Lipofectamine LTX with Plus reagent (Invitrogen).

The rescue experiment was performed after reverse transfection of BGT-1 siRNA (1.5 nM) into R6FLR-N cells by using RNAiMAX reagent. After 48 hours, wild-type (wt) and mutant (mut) BGT-1 expression vectors (10 ng) were transfected by using Lipofectamine LTX, and the luciferase activity and cell viability were assessed by WST-8 assay (Dojindo) after 24 hours.

Analysis of HCV Infection and BGT-1 Expression

For infection assays, Cured/HuH-7 K4 cells were incubated with JFH/K4 cell-derived HCV (2.0×10^6 copies/mL). At 72 hours after incubation, HCV infection and BGT-1 expression were analyzed by real-time detection (RTD)-PCR and TaqMan expression assay, respectively, as described earlier.

Statistical Analysis

The Student *t* test was used to test the statistical significance of the results. *P* values < .05 were considered statistically significant.

Results

Inhibitory Effect of 2-152a MAb on HCV Replication In Vitro

We examined the effects of 2-152a MAb on HCV replication and the viability in HCV replicon cell lines. The treatment with 2-152a MAb significantly decreased HCV replication after 48 hours and cell viability after 72 hours (Figure 1A). To determine the recognition site of 2-152a MAb, we performed epitope mapping by using serial overlapping deletion mutants of the DHCR24 fusion protein (Supplementary Figure 1A). The recognition site was identified within amino acid residues 259–314 (Supplementary Figure 1B) and the predicted “Diminuto-like protein” homologous region [23] indicated in Supplementary Figure 1A.

Suppression of HCV Infection by 2-152a MAb

To determine the effects of 2-152a MAb on HCV infection, we inoculated the antibody into a persistently HCV-infected cell line (JFH/K4; Figure 1B and C) or uPA-SCID chimeric mice previously transplanted with human hepatocytes [20] and

Figure 1 continued. (10 µg/mL). The error bars indicate the standard deviation, and the asterisk indicates *P* < .005. *D*, Relative amounts of HCV RNA (% copies/mg total RNA on days –1 or 0) in the livers of chimeric mice inoculated with the control medium, PEGylated IFN-α, normal IgG, or 2-152a IgG were estimated by RTD-PCR. For normalization, the HCV RNA level 1 day before the inoculation (day –1) or on the day of inoculation (day 0) was defined as 100%. The graph shows the relative amounts of HCV RNA at –1 day (or day 0), 7 days (or 4 days), and 14 days. The error bars indicate the standard deviation, and the asterisk indicates *P* < .005. *E*, Ratio of body weight of mice inoculated with either normal IgG or 2-152a MAb IgG to that on day –1. *F*, Ratio of albumin concentration in serum samples of mice inoculated with 2-152a MAb IgG or normal IgG to that on day –1. The vertical bars indicate the standard deviation.

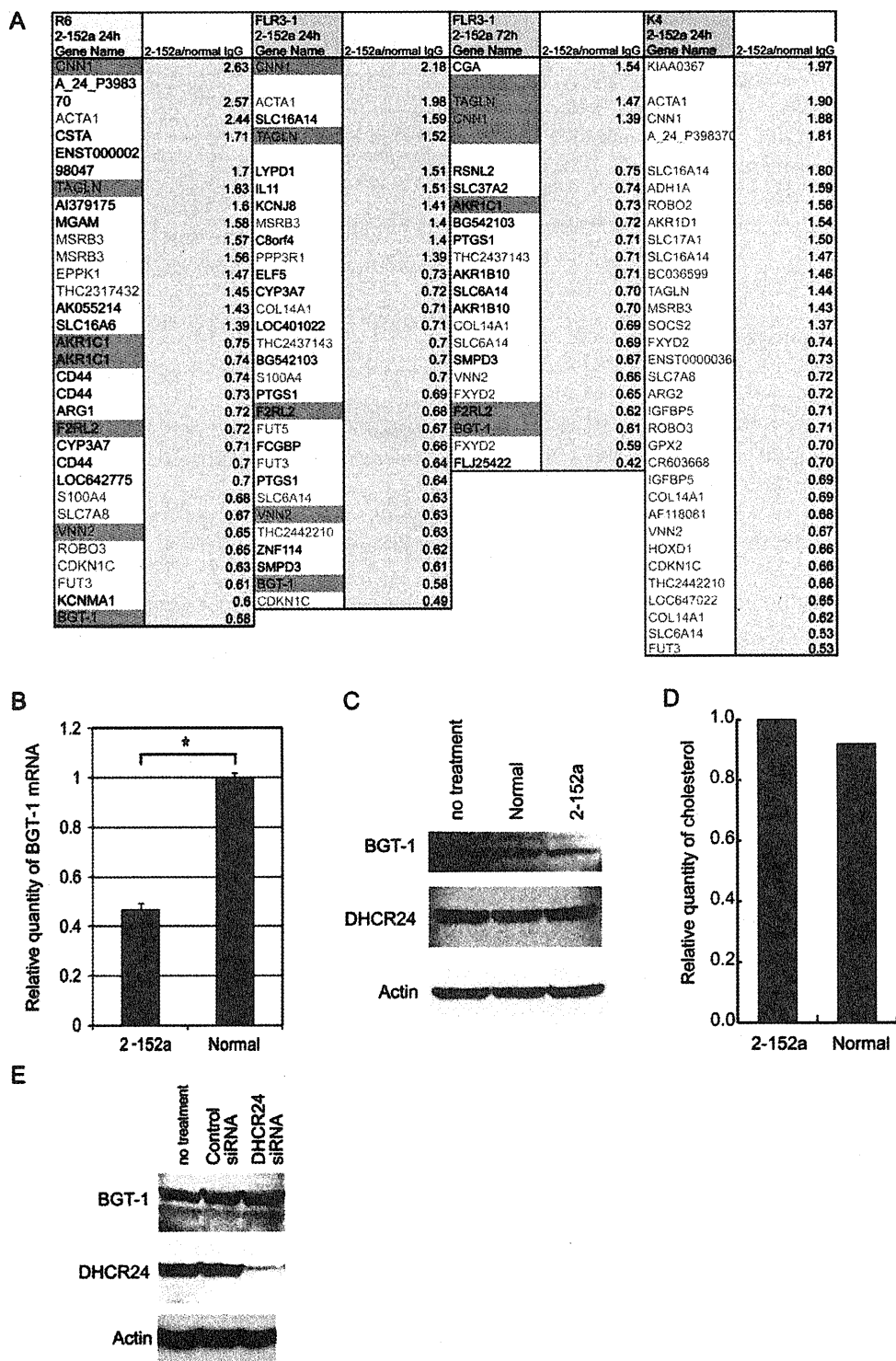


Figure 2. A, Genes that showed significant changes in expression after the 2-152a MAb treatment. HCV replicon cells (FLR3-1 and R6FLR-N) and K4 cells were treated with 2-152a MAb. The symbols shaded in gray indicate the genes that showed significantly changed expression commonly in R6FLR-N and FLR3-1 cells, and those shaded in orange indicate the genes that showed significantly changed expression in K4 cells. The amount of labeled probe for microarray analysis was 7-fold higher than that in the first experiment (Supplementary Table 1). Each value indicates the number of ratios of signal 2-152a MAb/normal IgG treatment. B, TaqMan expression assay of BGT-1 in samples of R6FLR-N cells treated with 2-152a MAb or normal IgG. BGT-1 mRNA (0.5 μ g) samples treated with 2-152a MAb or normal IgG were transcribed by reverse transcriptase, and synthesized cDNAs were used for TaqMan

infected with HCV (Figure 1D and F). We detected viral protein (core) (Figure 1B) or viral RNA in cells (Figure 1C) and mouse blood by using RTD-PCR (Figure 1D). There was a significant reduction in the viral titers with 2-152a MAb treatment compared with that in normal IgG treatment (control) ($P < .005$, Figure 1C and D). No significant effects on body weight were observed by the inoculation of 2-152a MAb (Figure 1E). Further, no significant differences were found among the levels of human albumin in the sera of the normal IgG- and 2-152a MAb-inoculated mice (Figure 1F).

Expression of DHCR24 in Carcinoma Cells and on the Surface of HuH-7-Derived Cells

We observed abundant intracellular expression of DHCR24 in hepatoma cell lines in the previous study [12]; therefore, we characterized its expression on the surface of various carcinoma cell lines by flow cytometric analysis to clarify the mechanism of 2-152a MAb antiviral effects. In this analysis, DHCR24 expression was localized to the surface of the HuH-7 and HuH-7-based cell lines, HCV replicon cell lines (R6FLR-N, FLR3-1, and JFH-1), HCV persistently infected cell line (JFH/K4), and K4 cells; on the other hand, DHCR24 was not significantly expressed on the surface of the HepG2, Hep3B, RzM6-0d, RzM6-LC, WRL68, and PLC/PRF/5 cell lines (Supplementary Figure 1C). To confirm the expression of DHCR24 on the cell surface, we performed immunofluorescence staining (Supplementary Figure 1D). DHCR24 expression was detected in the HuH-7 cells without permeabilization.

Suppression of BGT-1 mRNA Expression in HCV Replicon Cell Lines After Treatment With 2-152a MAb

To determine the molecular mechanism underlying the effects of 2-152a MAb, we performed microarray analysis twice with different amounts of probes and evaluated the changes in gene expression associated with the 2-152a MAb treatment, which were specific to the HCV replicon cells rather than to the HCV-cured K4 cells. Using this methodology, we identified approximately 3–14 genes as upregulated and about 17–20 genes as downregulated following the treatment with 2-152a MAb, compared with the expressions in normal IgG-treated R6FLR-N, FLR3-1, and K4 cells (Figure 2A). Among these genes, the expression level of SLC6A12 (BGT-1; GenBank accession number NM_003044) showed significant downregulation in both the R6FLR-N and the FLR3-1 cell lines but not in the K4 cells (Figure 2A; Table 1). To validate this result, we tested BGT-1 mRNA expression in R6FLR-N cells treated with 2-152a MAb and normal IgG by using TaqMan expression assay. This assay

Table 1. Screened Genes in HCV Replicon Cell Lines After Treatment of IgG

	Gene name	R6FLR-N 24 hours	FLR3-1 24 hours	FLR3-1 72 hours	HuH-7/K4 24 hours
Screened specifically in replicon cells ^a					
1st screening	AKR1C1	0.67	0.62	0.65	NS
	BGT-1	0.53	0.63	0.53	NS
2nd screening (7-fold) ^a	AKR1C1	0.74	NS	0.73	NS
		or 0.75			
	F2RL2	0.72	0.68	0.62	NS
	BGT-1	0.58	0.58	0.61	NS
Screened in replicon and cured K4 cells ^b					
1st screening	CNN1	2.75	0.6	1.62	1.9
2nd screening	CNN1	2.63	2.18	1.39	1.88
(7-fold) ^c	TAGLN	1.63	1.52	1.47	1.44
	VNN2	0.65	0.63	0.66	0.67

Abbreviations: HCV, hepatitis C virus; IgG, immunoglobulin G; NS, not screened.

^a Screened genes were significantly changed in HCV replicon cells but not in HuH-7/K4 cells; each value indicates ratio of signal 2-152a MAb IgG/normal IgG treatment.

^b Screened genes were significantly changed in all cell lines, including replicon cells and HuH-7/K4 cells.

^c Comparing to 1st screening, 7-fold amount of labeled probe was used for microarray.

demonstrated that the relative expression of BGT-1 was significantly suppressed by the treatment with 2-152a MAb ($P < .001$, Figure 2B). Significant downregulation of BGT-1 was also observed by treatment with 2-152a MAb in HCV-JFH-1-infected cells (Figure 2C).

We further addressed the mechanism of action of 2-152a MAb. Treatment with 2-152a MAb did not decrease the level of cholesterol (Figure 2D), and silencing of DHCR24 did not influence BGT-1 significantly (Figure 2E).

Inhibition of HCV Replication and Infection by siRNA Directed Against BGT-1

Because BGT-1 expression was suppressed by the treatment with 2-152a MAb, which had antiviral activity, we attempted BGT-1 silencing in HCV replicon cell lines by using designed siRNAs to examine the potential role of BGT-1 in HCV replication. BGT-1 silencing was confirmed by RT-PCR (Figure 3A). The effect of the siRNAs on HCV replication was examined by Western blotting with anti-NS5A antibody (Figure 3B) and measured by the luminescence level (Figure 3C, left panel) and cell viability (Figure 3C, right panel) in FLR3-1 cells. We also examined the effect of these siRNAs in R6FLR-N and JFH-1 cells (Supplementary Figure 2A) and observed similar inhibitory effects as

Figure 2 continued. gene expression assay. Each value was compensated with values of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA as the internal control and normal IgG. The asterisk indicates $P < .001$, and the vertical bars indicate the standard deviation. C, Level of BGT-1 and DHCR24 proteins detected in JFH/K4 cells after treatment with 2-152a or normal IgG (10 μ g/mL). D, The relative cholesterol amount was measured in R6FLR-N cells treated with 2-152a or normal IgG (10 μ g/mL). E, BGT-1 and DHCR24 proteins were detected in normal IgG- or 2-152a IgG-treated R6FLR-N cells.

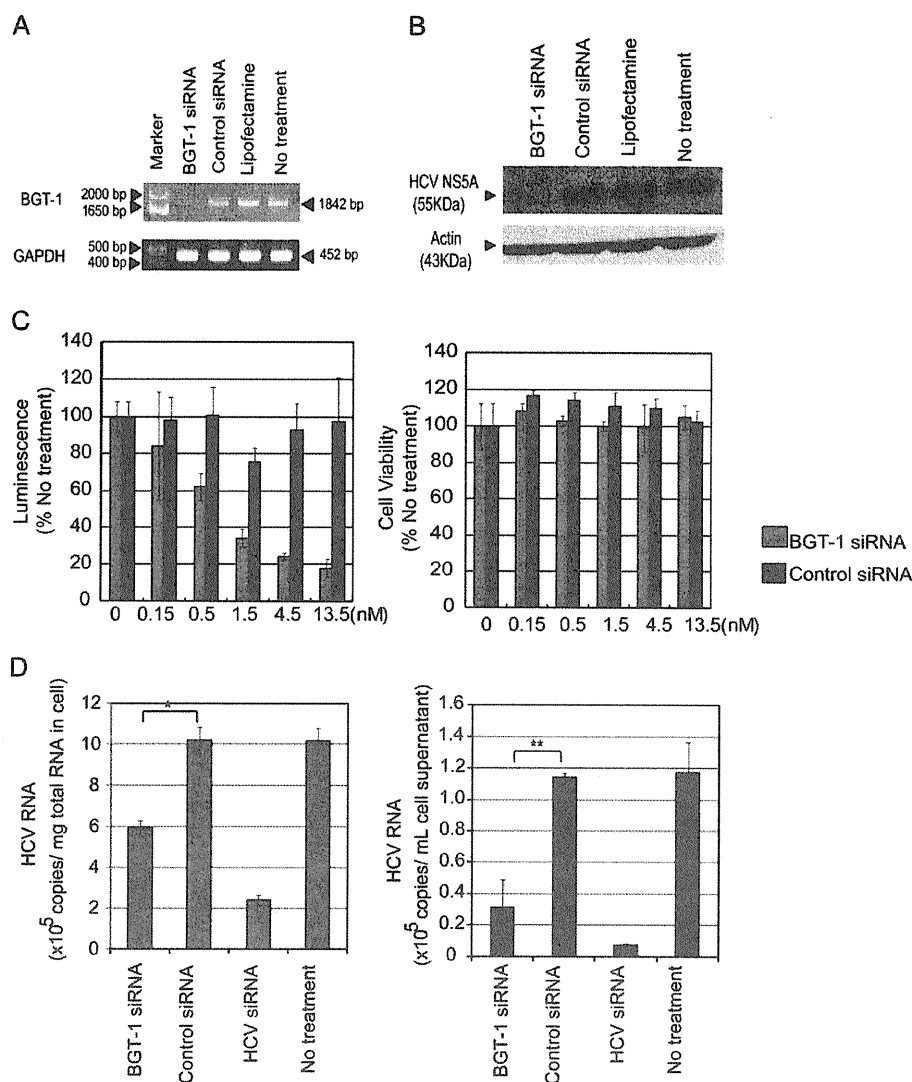


Figure 3. BGT-1 silencing by siRNA inhibits HCV replication in subgenomic HCV replicon cell lines and the persistently infected cell line. *A*, The siRNA targeting BGT-1 suppressed the expression of the corresponding mRNA. The mRNA of each sample was extracted 72 hours after siRNA (10 nM) transfection. Total RNA was transcribed and amplified by RT-PCR using primers specific to the open reading frame (ORF) of the BGT-1 (1842 bp) gene. The experiments were performed in triplicate, and the representative data are presented. *B*, The effects of BGT-1 siRNA (10 nM) on HCV were confirmed by Western blot analysis using an antibody against the HCV NSSA protein (55 kDa). The blots were striped and reprobed with an antibody directed against actin to examine protein loading in each lane. *C*, Levels of HCV replication (left panel) and cell viability (right panel) are presented according to serial concentrations of siRNA targeting BGT-1 and control siRNA in FLR3-1 cells (72 hours after transfection). The inhibition of replication or cell viability following siRNA targeting BGT-1 is defined relative to those of the cells that received no treatment (100%). The error bars represent the standard error of triplicate experiments. *D*, Quantification of HCV RNA by RTD-PCR in HCV persistently infected cells (JFH/K4) after treatment with BGT-1 siRNA. The cells were treated with siRNAs (10 nM) against BGT-1, control, and HCV (HCV R7) and harvested at 72 hours after transfection. TaqMan quantitative RT-PCR was performed for quantitation of HCV RNA in extracted RNA from cells (left panel) and their supernatants (right panel). The single asterisk (*) and double asterisk (**) indicate $P < .005$ and $P < .05$ against the control, respectively. The mean values from triplicate wells are indicated, and the vertical bars indicate the standard deviation.

those in FLR3-1 cells. The median inhibitory concentration (IC_{50}) values of BGT-1 siRNAs in various HCV replicon cell lines were as follows: FLR3-1 cells, 0.93 nM; R6FLR-N cells, 1.37 nM; JFH-1 cells, 5.95 nM. The cell viability was not significantly influenced by the siRNA treatment (Figure 3C, right panel; Supplementary Figure 2A, right panel).

Further, we monitored the levels of HCV RNA in JFH/K4 cells and their supernatants after BGT-1 silencing. Using RTD-PCR, we detected significant suppression in the HCV RNA levels by BGT-1 silencing in these cells ($P < .005$; Figure 3D, left panel) and their supernatants ($P < .05$; Figure 3D, right panel). These results were consistent with the strong inhibitory effects of

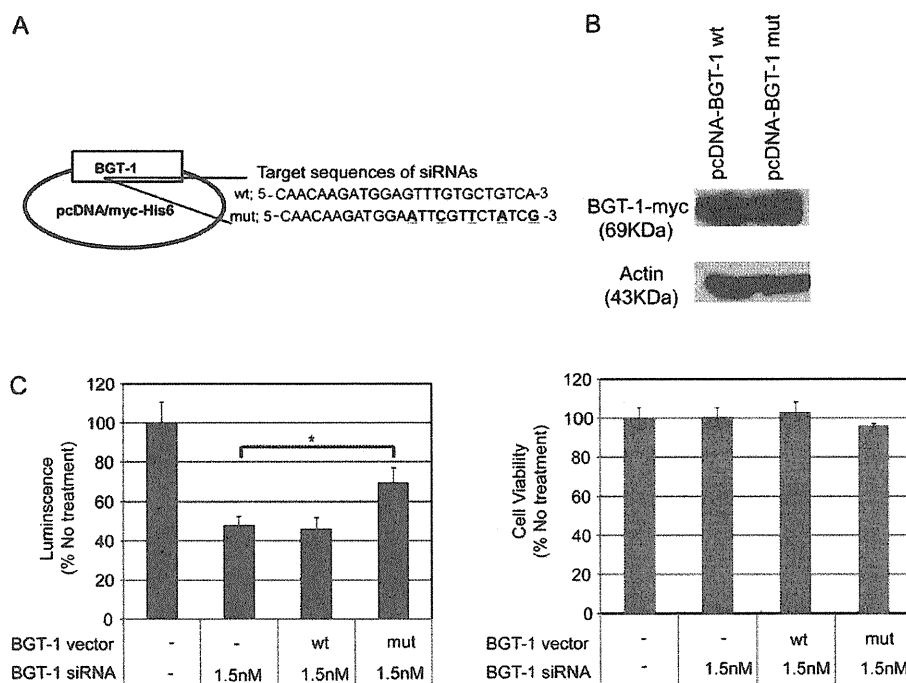


Figure 4. Validation of the inhibitory effects of BGT-1 siRNA on HCV replication in subgenomic HCV replicon cell lines. *A*, Schematic representation of the pcDNA-BGT-1 wild-type (wt) and mutant (mut) plasmids. The siRNA-targeted sites are indicated, and the underlined bold letters indicate the sequences induced by mutagenesis PCR. *B*, The wild type and mutant of the BGT-1-myc fusion protein were detected by using an anti-myc monoclonal antibody (9E10) in transfected R6FLR-N cells (upper panel). The blots were stripped and reprobed with an antibody against actin to determine protein loading for each lane (lower panel). *C*, R6FLR-N cells were transfected with BGT-1 siRNA, and wild-type or mutant expression vectors were transfected after siRNA transfection. After 24 hours of vector transfection, the level of HCV replication (left panel) was measured by luminescence, and cell viability (right panel) was measured by WST-8 assay. The asterisk indicates $P < .05$ compared with transfection of siRNA alone. The mean values from triplicate wells are indicated, and the vertical bars represent the standard deviation.

BGT-1 siRNA on HCV replication, as shown in Figure 3C. We designed alternative siRNA targeting BGT-1 (BGT-1-siRNA-362) and observed its significant inhibitory effect on HCV replication without significant cytotoxicity (Supplementary Figure 2B).

Validation of the Anti-HCV Effects of siRNA Against BGT-1 by Rescue With Expression Vectors

To assess the specificity of BGT-1 silencing, we attempted to rescue HCV replication against the ectopic effects by this silencing. To examine the effect of the rescue, we constructed expression vectors of wild-type and mutant BGT-1 (Figure 4A) and confirmed the expression of each BGT-1-myc-fused protein (Figure 4B). The mutant BGT-1 vector contained 5 base mismatches within the site targeted by the BGT-1 siRNA without a change in the amino acid sequence of the protein (underlined in Figure 4A). We also transfected the pcDNA-BGT-1 plasmid after the siRNA treatment and observed significant recovery of HCV replication with mutant pcDNA-BGT-1 ($P < .05$; Figure 4C, left panel) without significant cytotoxicity (Figure 4C, right panel). BGT-1 expression was increased significantly in K4 cells in the presence of HCV ($P < .05$, Supplementary Figure 2C) at 72 hours after infection compared with the absence of

HCV, and in RzM6-LC cells, which persistently express HCV [8], compared with RzM6-0d cells, which lack HCV expression ($P < .05$, Supplementary Figure 2D).

DISCUSSION

In this study, we determined that 2-152a MAb, which binds to but does not affect the activity of DHCR24, suppresses HCV replication and that BGT-1 is highly downregulated in HCV replicon cell lines treated with this antibody. Further, the efficient rescue of viral replication with a mutant expression vector indicates the specific inhibitory effect of BGT-1 silencing on HCV replication. Therefore, we hypothesize that BGT-1 plays an important role in HCV replication through a pathway that is likely independent of DHCR24, which in its own right can regulate the HCV life cycle [13].

BGT-1 is involved in sodium- and chloride-coupled betaine uptake, which helps in maintaining normal cellular conditions. Previous reports have described that the transcription of BGT-1 mRNA is regulated by a tonicity sensitive element (TonE) in response to hypertonic stress, a result that was first identified in the Madin-Darby canine kidney (MDCK) cell line [24]. BGT-1

is also thought to be responsible for the hyperosmotic stress response and in maintaining cell hydration. Denkert et al [25] reported that BGT-1 gene expression is induced by hyperosmolarity and inhibited by p38 mitogen-activated protein kinase (p38^{MAPK}) inhibitor SB20358. Further, several reports have evidenced that cell hydration affects viral replication and that viral replication increases during cell shrinkage due to hyperosmolarity, a result that was accompanied by increased BGT-1 mRNA expression [26]. Considering the reduction in HCV replication by the BGT-1 siRNA treatment, this treatment may prevent HCV replication by affecting hypoosmotic conditions in HCV-infected cells. Further studies are required to examine in detail the function of BGT-1 in HCV replication.

In summary, we demonstrated that the 2-152a monoclonal antibody inhibits HCV replication in HCV replicon cells and HCV infection in human hepatocytes transplanted into chimeric mice. The inhibitory effect of the monoclonal antibody on viral replication may be mediated by the suppression of BGT-1 expression. We propose BGT-1 as a key target for anti-HCV therapies.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (Supplementary Data).

Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Acknowledgments. The authors thank I. Maruyama, K. Tanaka, T. Seki, and R. Takehara for their excellent technical support, and Y. Tokunaga for the insightful comments and helpful discussion.

Financial Support. This work was supported by grants from the Ministry of Health and Welfare of Japan; Ministry of Education, Culture, Sports, Science and Technology of Japan; Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation; and Cooperative Research Project on Clinical and Epidemiological Studies of Emerging and Reemerging Infectious Diseases.

Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

- Di Bisceglie AM, Carithers RL Jr, Gores GJ. Hepatocellular carcinoma. *Hepatology* 1998; 28:1161–5.
- Hayashi J, Aoki H, Arakawa Y, Hino O. Hepatitis C virus and hepatocarcinogenesis. *Intervirology* 1999; 42:205–10.
- Michielsen PP, Francque SM, van Dongen JL. Viral hepatitis and hepatocellular carcinoma. *World J Surg Oncol* 2005; 3:27.
- Mazzella G, Accogli E, Sottili S, et al. Alpha interferon treatment may prevent hepatocellular carcinoma in HCV-related liver cirrhosis. *J Hepatol* 1996; 24:141–7.
- Bruchfeld A, Stahle L, Andersson J, Schvarcz R. Ribavirin treatment in dialysis patients with chronic hepatitis C virus infection—a pilot study. *J Viral Hepat* 2001; 8:287–92.
- Kohara M, Tanaka T, Tsukiyama-Kohara K, et al. Hepatitis C virus genotypes 1 and 2 respond to interferon-alpha with different virologic kinetics. *J Infect Dis* 1995; 172:934–8.
- Nakamura H, Ogawa H, Kuroda T, et al. Interferon treatment for patients with chronic hepatitis C infected with high viral load of genotype 2 virus. *Hepatogastroenterology* 2002; 49:1373–6.
- Tsukiyama-Kohara K, Toné S, Maruyama I, et al. Activation of the CKI-CDK-Rb-E2F pathway in full genome hepatitis C virus-expressing cells. *J Biol Chem* 2004; 279:14531–41.
- Cramer A, Biondi E, Kuehnle K, et al. The role of seladin-1/DHCR24 in cholesterol biosynthesis, APP processing and A β generation in vivo. *EMBO J* 2006; 25:432–43.
- Waterham HR, Koster J, Romeijn GJ, et al. Mutations in the 3 β -hydroxysterol Δ^{24} -reductase gene cause desmosterolosis, an autosomal recessive disorder of cholesterol biosynthesis. *Am J Hum Genet* 2001; 69:685–94.
- Lu X, Kambe F, Cao X, et al. 3 β -Hydroxysteroid- Δ^{24} reductase is a hydrogen peroxide scavenger, protecting cells from oxidative stress-induced apoptosis. *Endocrinology* 2008; 149:3267–73.
- Nishimura T, Kohara M, Izumi K, et al. Hepatitis C virus impairs p53 via persistent overexpression of 3 β -hydroxysterol Δ^{24} -reductase. *J Biol Chem* 2009; 284:36442–52.
- Takano T, Tsukiyama-Kohara K, Hayashi M, et al. Augmentation of DHCR24 expression by hepatitis C virus infection facilitates viral replication in hepatocytes. *J Hepatol* 2011; 55:512–21.
- Lohmann V, Körner F, Koch J, Herian U, Theilmann L, Bartenschlager R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* 1999; 285:110–3.
- Watanabe T, Sudoh M, Miyagishi M, et al. Intracellular-diced dsRNA has enhanced efficacy for silencing HCV RNA and overcomes variation in the viral genotype. *Gene Ther* 2006; 13:883–92.
- Sakamoto H, Okamoto K, Aoki M, et al. Host sphingolipid biosynthesis as a target for hepatitis C virus therapy. *Nat Chem Biol* 2005; 1:333–7.
- Kato T, Date T, Miyamoto M, et al. Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. *Gastroenterology* 2003; 125:1808–17.
- Blight KJ, McKeating JA, Rice CM. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. *J Virol* 2002; 76:13001–14.
- Wakita T, Pietschmann T, Kato T, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* 2005; 11:791–6.
- Mercer DF, Schiller DE, Elliott JF, et al. Hepatitis C virus replication in mice with chimeric human livers. *Nat Med* 2001; 7:927–33.
- Inoue K, Umehara T, Ruegg UT, et al. Evaluation of a cyclophilin inhibitor in hepatitis C virus-infected chimeric mice in vivo. *Hepatology* 2007; 45:921–8.
- Takeuchi T, Katsume A, Tanaka T, et al. Real-time detection system for quantification of hepatitis C virus genome. *Gastroenterology* 1999; 116:636–42.
- Sarkar D, Imai T, Kambe F, et al. The human homolog of *Diminuto/Dwarf1* gene (*hDiminuto*): a novel ACTH-responsive gene overexpressed in benign cortisol-producing adrenocortical adenomas. *J Clin Endocrinol Metab* 2001; 86:5130–7.
- Takenaka M, Preston AS, Kwon HM, Handler JS. The tonicity-sensitive element that mediates increased transcription of the betaine transporter gene in response to hypertonic stress. *J Biol Chem* 1994; 269:29379–81.
- Denkert C, Warskulat U, Hensel F, Häussinger D. Osmolyte strategy in human monocytes and macrophages: involvement of p38MAPK in hyperosmotic induction of betaine and myoinositol transporters. *Arch Biochem Biophys* 1998; 354:172–80.
- Häussinger D. The role of cellular hydration in the regulation of cell function. *Biochem J* 1996; 313:697–710.

Review Article

Hepatitis C Virus-Related Lymphomagenesis in a Mouse Model

Kyoko Tsukiyama-Kohara,¹ Satoshi Sekiguchi,² Yuri Kasama,¹
Nagla Elwy Salem,^{1,3,4} Keigo Machida,⁵ and Michinori Kohara²

¹Department of Experimental Phylaxiology, Faculty of Life Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto-shi, Kumamoto 860-8556, Japan

²Department of Microbiology and Cell Biology, The Tokyo Metropolitan Institute of Medical Science, 2-1-6 Kamikitazawa, Setagaya-ku, Tokyo 156-8506, Japan

³Department of Medical Virology, Faculty of Life Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto-shi, Kumamoto 860-8556, Japan

⁴Clinical Pathology Department, Faculty of Medicine, Suez Canal University, Round Road Kilo 4.5, Ismailia, Egypt

⁵Department of Molecular Microbiology and Immunology, University of Southern California, Keck School of Medicine, Los Angeles, CA 90033, USA

Correspondence should be addressed to Kyoko Tsukiyama-Kohara, kkohara@kumamoto-u.ac.jp

Received 5 May 2011; Accepted 2 June 2011

Academic Editors: D. Efremov and L. Visser

Copyright © 2011 Kyoko Tsukiyama-Kohara et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

B cell non-Hodgkin lymphoma is a typical extrahepatic manifestation frequently associated with hepatitis C virus (HCV) infection. The mechanism by which HCV infection leads to lymphoproliferative disorder remains unclear. Our group established HCV transgenic mice that expressed the full HCV genome in B cells (RzCD19Cre mice). We observed a 25.0% incidence of diffuse large B cell non-Hodgkin lymphomas (22.2% in male and 29.6% in female mice) within 600 days of birth. Interestingly, RzCD19Cre mice with substantially elevated serum-soluble interleukin-2 receptor α -subunit (sIL-2R α) levels (>1000 pg/mL) developed B cell lymphomas. Another mouse model of lymphoproliferative disorder was established by persistent expression of HCV structural proteins through disruption of interferon regulatory factor-1 (*irf-1*^{-/-}/CN2 mice). *Irf-1*^{-/-}/CN2 mice showed extremely high incidences of lymphomas and lymphoproliferative disorders. Moreover, these mice showed increased levels of interleukin (IL)-2, IL-10, and Bcl-2 as well as increased Bcl-2 expression, which promoted oncogenic transformation of lymphocytes.

1. Introduction

The incidence of non-Hodgkin lymphoma (NHL) is rising worldwide and is higher in developed countries than in Africa and Asia [1]. B cell non-Hodgkin lymphoma is a typical extrahepatic manifestation frequently associated with hepatitis C virus (HCV) infection [2]. The prevalence of HCV infection in patients with B cell non-Hodgkin lymphoma is approximately 15% [3]. The HCV envelope protein E2 binds human CD81 [4], a tetraspanin expressed on various types of cells, including lymphocytes, and activates B cell proliferation [5]. Infection and replication of HCV were observed in B cells [6, 7] although the direct effects, particularly *in vivo*, have not been clarified. To determine the direct effect of HCV infection on B cells *in vivo*, we crossed

transgenic mice with an integrated full-length HCV genome (Rz) under the conditional Cre/*loxP* expression system, with mice expressing the Cre enzyme [8] under transcriptional control of the B lineage-restricted gene *CD19* [9].

To investigate the mechanism of development of lymphoproliferation or B cell non-Hodgkin lymphoma in HCV patients, we also developed a transgenic mouse model that conditionally expressed HCV cDNA (nucleotides 294–3435), including the viral genes that encode the core, E1, E2, and NS2 proteins, by using the Cre/*loxP* system (in core-NS2 [CN2] mice) [10, 11]. Conditional transgene activation of the HCV cDNA (core, E1, E2, and NS2) protects mice from Fas-mediated lethal acute liver failure, by inhibiting cytochrome *c* release from mitochondria [11]. Persistent HCV protein expression is established by targeted disruption

of interferon regulatory factor-1 (*irf-1*), and high incidences of lymphoproliferative disorders are noted in *irf-1*^{-/-} CN2 mice [12].

Previously, transgenic mice that expressed the HCV core protein were established using a promoter derived from hepatitis B virus [13], whereas mice that expressed structural or complete viral proteins were established using promoters derived from the albumin gene [14]. These mice were immunotolerant to the transgene and did not develop hepatic inflammation. However, they developed age-related hepatic steatosis and hepatocellular carcinomas. In contrast, the CN2 mice used in the present study were not immunotolerant to the *HCV* gene and developed hepatitis after the onset of HCV gene expression. However, the expression of *HCV* in these mice was usually lost after 21 days. Therefore, an animal model of persistent HCV protein expression is required for examining the effects of chronic HCV infection *in vivo*.

IFN signaling mediates tumor-suppressor effects and antiviral responses and is regulated by key transcription factors of the interferon-regulatory factor (IRF) protein family, including IRF1, IRF2, IRF3, IRF7, and IRF9. Targeted disruption of *irf-1* results in aberrant lymphocyte development and a marked reduction in the number of CD8⁺ T cells in the peripheral blood, spleen, and lymph nodes [15]. In addition, natural killer cell development is impaired in *irf-1*^{-/-} mice [16]. The mechanisms by which HCV infection induces IFN resistance and influences the development of lymphomas are poorly understood. Therefore, we established an *irf-1*^{-/-} CN2 mouse model of persistent HCV expression, which allowed us to investigate the effects of HCV on lymphatic tissue tumor development.

2. Spontaneous Development of B Cell Lymphomas in the RzCD19Cre Mouse

The full-genome HCV expression was induced by the *Cre/loxP* system with CD19Cre (Figure 1(A)). Expression of HCV was mainly induced in B cells (Figure 1(B)). The incidence of B cell lymphomas in RzCD19Cre mice was 25.0% (22.2% in male and 29.6% in female mice) and was significantly higher than the incidence in the HCV-negative groups. Lymphomas were diagnosed as typical diffuse B cell non-Hodgkin lymphomas. Most were CD45R positive and located in the mesenteric lymph nodes (Figure 1(C)). Some were identified as intrahepatic lymphomas (incidence, 4.2%). *HCV* expression was detected in all B cell lymphomas of RzCD19Cre mice. Indeed, the expression of the *HCV* or HCV proteins induces the spontaneous development of B cell lymphomas, irrespective of the integrated site in the mouse genome.

Serum concentrations of IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-17, eotaxin, G-CSF, GM-CSF, IFN- γ , KC, MCP-1, MIP-1 α , MIP-1 β , RANTES, TNF- α , IL-15, FGF-basic, LIF, M-CSF, MIG, MIP-2, PDGF β , VEGF, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were not significantly different in the presence or absence of B cell lymphomas. Interestingly, the average sIL-2R α level was significantly

higher in the sera from RzCD19Cre mice with B cell lymphomas (830.3 \pm 533.0 pg/mL) than in that from tumor-free control groups, including the RzCD19Cre, Rz, CD19Cre, and wild-type (WT) mice (499.9 \pm 110.2 pg/mL; $P < 0.0057$) (Figure 1(D)). The difference in the average sIL-2R α levels between the sera from groups with tumors other than B cell lymphomas (430.46 \pm 141.15 pg/mL) and that from tumor-free control groups was insignificant ($P > 0.05$). Moreover, all RzCD19Cre mice with a relatively high level of sIL-2R α (>1000 pg/mL) presented with B cell lymphomas. A significant increase in sIL-2R α was also observed in MxCre/CN2-29 mice that expressed the HCV CN2 gene [8] and had B cell lymphomas, compared with tumor-free control (CN2-29) mice.

To examine whether sIL-2R α was derived from lymphoma tissues, we quantified IL-2R α concentrations in splenocytes, peripheral blood lymphocytes (PBLs), and B cell lymphoma tissues. The concentration of IL-2R α was significantly higher in splenocytes from RzCD19Cre mice than in splenocytes from CD19Cre mice. Moreover, the concentration of IL-2R α in B cell lymphoma tissues from RzCD19Cre mice was higher than that in splenocytes [8]. These results strongly suggest that B cell lymphomas directly contribute to the elevated serum concentrations of sIL-2R α in RzCD19Cre mice. They also strongly support the possibility that persistent expression of HCV could directly induce transformation of B cells.

3. Persistent HCV Expression and Lymphoproliferative Disorder

RzCD19Cre mice are immunotolerant to HCV, because *HCV* is expressed in B cells before birth. In contrast, CN2 mice express *HCV* after they are administered the recombinant adenovirus that expresses the Cre enzyme (Figures 2(a) and 2(b)). The expression of HCV in these mice is usually lost after 21 days (Figure 2(b)) through removal of HCV-expressing hepatocytes by the immune response. To establish persistent HCV expression, we disrupted *irf-1* by crossing of RzCD19Cre mice with *irf-1*^{-/-} mice. IRF-1 plays a significant role in the Th1-type immune response and its absence is expected to decrease the elimination of HCV-expressing cells. As expected, HCV expression in *irf-1*^{-/-} CN2 mice persisted for more than 500 days (Figure 2(c)).

A significant percentage of the mice that expressed the HCV core protein (*irf-1*^{-/-} CN2 mice) showed polyclonal lymphoid growth disturbances, including splenomegaly, expanded lymph nodes, adenocarcinoma in the abdomen or leg, and lymphoma of the liver or Peyer's patches (Figures 2(d) and 2(e)). In contrast, hepatocytes with abundant expression of HCV proteins rarely developed into hepatocellular carcinomas. Hematoxylin and eosin (H&E) staining of splenomegalic tissue showed extensive hyperplasia of the white pulp zones, in which the cortical zones contained lymphoid follicles and scattered germinal centers although mitotic figures were rarely observed. These results indicate that persistent expression of HCV proteins frequently induces lymphoproliferative disorders in addition to liver

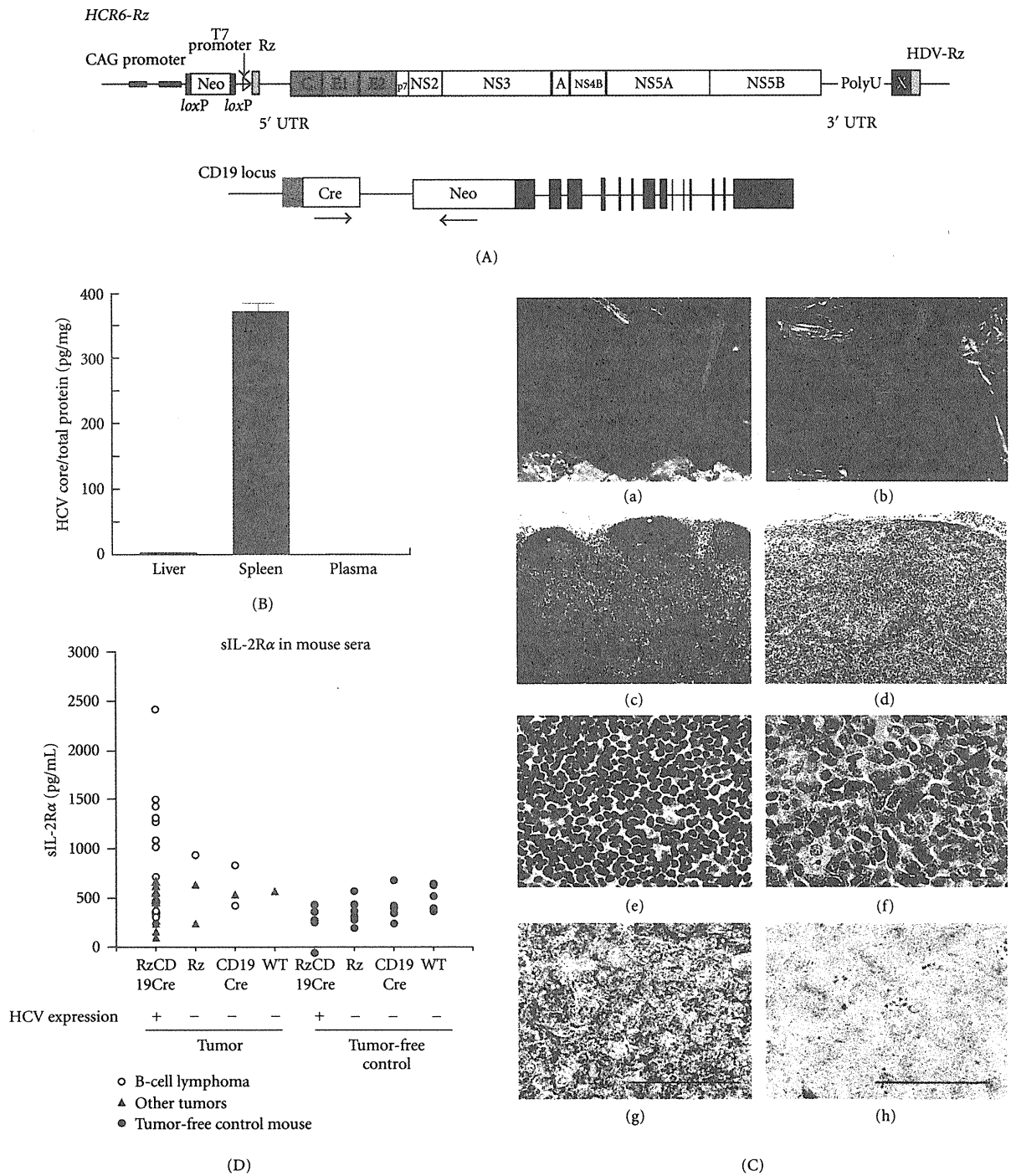


FIGURE 1: (A) The structure of the hepatitis C virus (HCV) transgene (HCR6-Rz); *HCV* gene expression was regulated by the *Cre/loxP* expression cassette (upper). The *Cre* transgene was located in the *CD19* locus (bottom). (B) Expression of HCV core protein in the liver, spleen, and plasma of RzCD19Cre mice was quantified by core ELISA. Data represent the mean (SD) ($n = 3$). (C) Histological analysis of tissues from a normal mouse (a, c, e; lymph node from CD19Cre mouse; b, d, f) and B cell lymphoma (b, d, f; RzCD19Cre mouse). Paraformaldehyde-fixed and paraffin-embedded tumor tissues were stained with hematoxylin and eosin (H&E) (c-f); immunostaining of lymphoma with anti-CD45R (g) and anti-CD3 (h) is indicated. Scale bars, 100 μ m (c, d) and 20 μ m (g, h); arrow heads indicate mitotic cells. (D) Concentration of sIL-2R α in serum samples from tumor-free control mice, and RzCD19Cre and wild-type (WT) mice with or without B-cell lymphomas or other tumors.

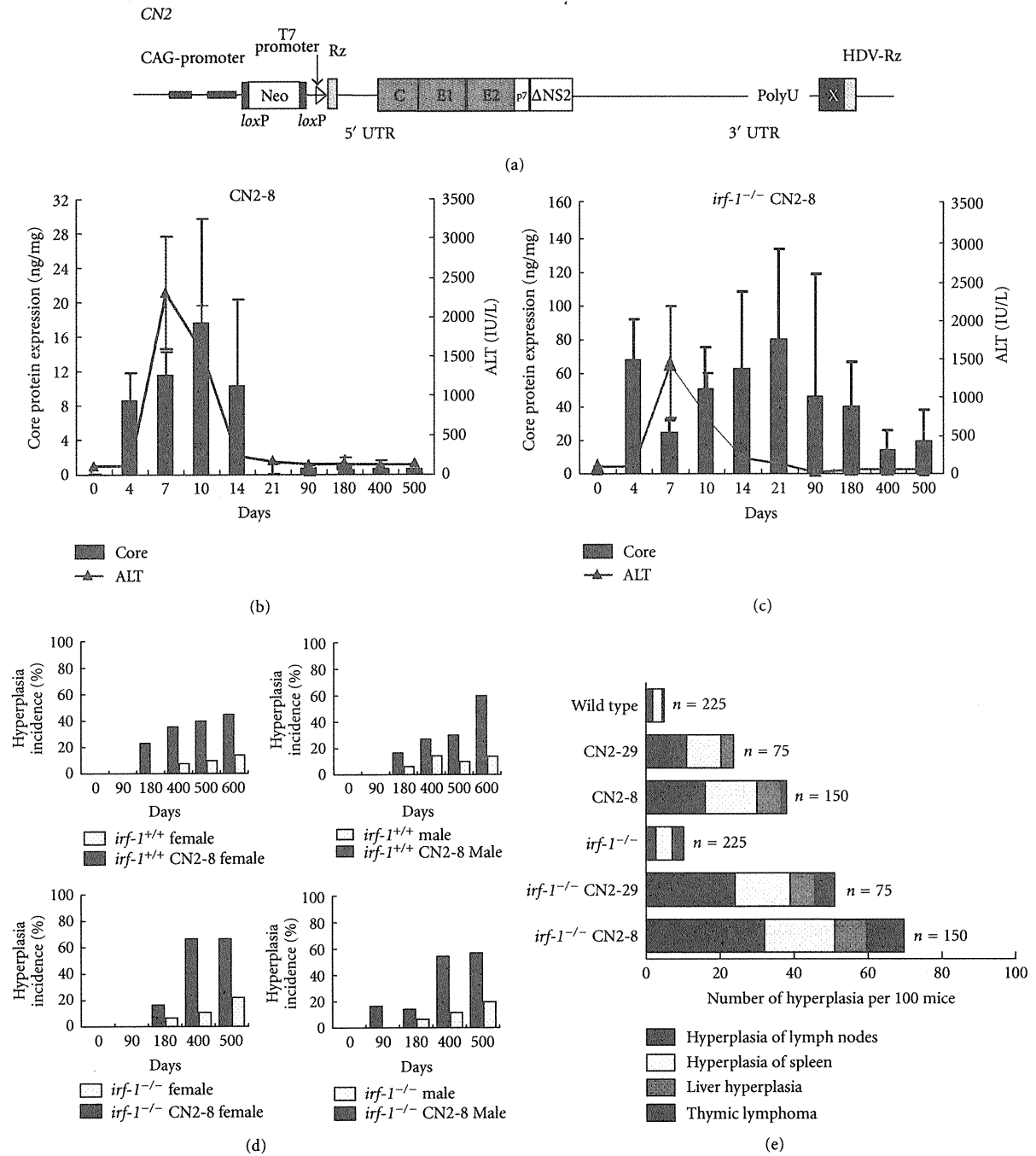


FIGURE 2: (a) The structure of the HCV transgene (core-NS2); gene expression was regulated by the Cre/loxP expression cassette. (b) and (c) Serum alanine aminotransferase (ALT) levels and core protein expression ELISA system in hepatocytes from CN2-8 (b) and *irf-1^{-/-}* CN2-8 (c) mice after administration of AxCANCre ($n = 225$ for *irf-1^{-/-}*, $n = 75$ for *irf-1^{-/-}* CN2-29, $n = 150$ for *irf-1^{-/-}* CN2-8, $n = 225$ for wild type, $n = 75$ for CN2-29, and $n = 150$ for CN2-8; total $n = 900$). (d) HCV protein expression enhanced hyperplasia in male and female CN2 and *irf-1^{-/-}* CN2 mice. The occurrence of hyperplasia was monitored every 7 days for 600 days after administration of AxCANCre. (e) Histological analysis of spontaneous proliferative disturbances in CN2 transgenic mice. Of the 900 mice injected with AxCANCre, 25 of 75 (33%) CN2-29, 47 of 150 (31%) CN2-8, 29 of 75 (39%) *irf-1^{-/-}* CN2-29, and 62 of 150 (41%) *irf-1^{-/-}* CN2-8 mice developed proliferative disturbances.

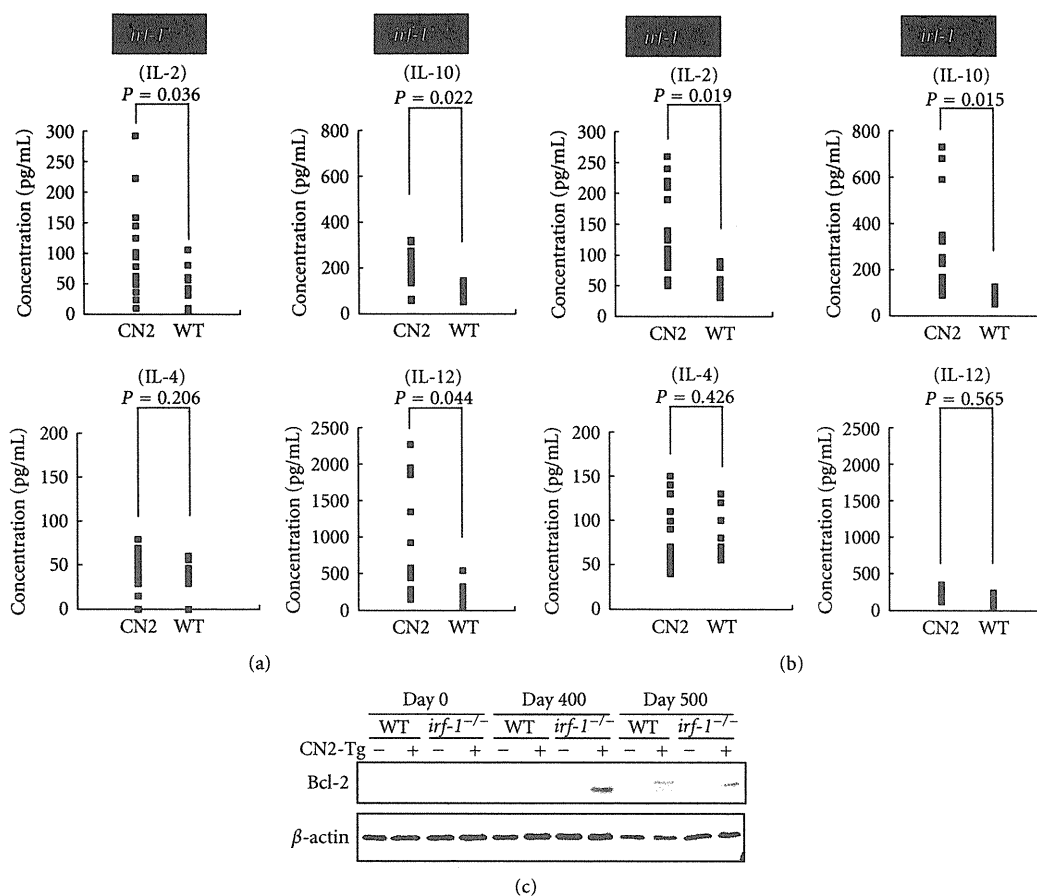


FIGURE 3: (a) Serum IL-2, IL-4, IL-10, and IL-12 levels in *irf-1*^{+/+} CN2 (Tg+) and *irf-1*^{+/+} WT mice measured by ELISA. P values <0.05 were considered significant. (b) Serum IL-2, IL-4, IL-10, and IL-12 levels in *irf-1*^{-/-} CN2 (Tg+) and *irf-1*^{-/-} WT mice measured by ELISA. P values are based on the mean cytokine concentrations. (c) Bcl-2 protein levels in the lymph nodes of *irf-1*^{+/+} WT and *irf-1*^{-/-} WT or transgenic (CN2-29) mice on day 0, day 400, and day 500 after administration of AxCANCre. Bcl-2 migrated at 26 kD.

hyperplasia, which is consistent with the phenotype of patients with hepatocellular carcinoma.

The average ratio of T cells to B cells in the lymph nodes and spleens of CN2 mice was significantly higher than that in WT mice. The majority of CD3⁺ lymphocytes and a few CD8⁺ lymphocytes expressed CD4 on their surfaces. The proliferating cells were mainly CD4⁺ T cells although some were CD45R⁺B cells. The *irf-1*^{-/-} CN2 mice also developed B cell lymphomas (data not shown). These results confirm that HCV protein expression induces lymphoproliferative disorders that involve excessive expansion of both T cells and B cells. The cell population that showed negative results for T cell receptor (α , β , γ , and δ isoforms) staining was smaller in *irf-1*^{-/-} CN2 mice than that in the other mice.

The disruption of *irf-1* inhibited Fas-induced apoptosis, presumably by decreasing the levels of caspase-6 and caspase-7 messenger RNA. These results suggest that the reduced expression of effector caspases delays Fas-mediated apoptosis in *irf-1*^{-/-} mice and prevents the elimination of HCV-expressing cells *in vivo*.

The CN2 mice showed significantly increased levels of serum IL-2, IL-10, and IL-12 (Figure 3(a)). Notably, the CN2 mice with proliferative disturbances in the lymph nodes and spleen had dramatically elevated levels of these cytokines, suggesting that altered cytokine production is involved in aberrant lymphocyte proliferation or differentiation. In contrast, the *irf-1*^{-/-} CN2 mice did not show elevated levels of serum IL-12, but had significantly higher levels of serum IL-2 and IL-10 than did *irf-1*^{-/-} mice (Figure 3(b)). Thus, the disruption of *irf-1* negated the increase in the IL-12 level, but augmented the increases in the levels of IL-2 and IL-10 in CN2 mice. These results indicate that IL-2 and IL-10 play key roles in the induction of the lymphoproliferative phenotype in *irf-1*^{-/-} CN2 mice. A significantly positive correlation was found between the cytokine levels and spleen weights of CN2 gene-expressing mice with the *irf-1*^{+/+} background ($R = 0.43$, $P < 0.05$, and $R = 0.53$, $P < 0.05$, resp.). These results indicate that IL-2 and IL-10 are involved in lymphoproliferation in viral protein-expressing mice.

Bcl-2 is an integral inner mitochondrial membrane protein, overexpression of which blocks the apoptotic death