protein (NS) 3/4A protease inhibitors (PIs) boceprevir (approved by the FDA on May 13, 2011) and telaprevir (approved by the FDA on May 23, 2011), used in combination with PEG-IFN-alpha and RBV for HCV genotype 1 infections, have increased cure rates to approximately 70% (Bacon et al., 2011; Jacobson et al., 2011; Poordad et al., 2011). However, these triple-therapy regimens may result in unfavourable side effects and emergence of drug-resistant HCV (Bacon et al., 2011; Jacobson et al., 2011; Poordad et al., 2011), which may reduce virus susceptibility and applicability of current HCV triple therapies (Ozeki et al., 2011). Recently, two more effective compounds have been approved for HCV treatment: the protease inhibitor simeprevir (approved by the FDA in November, 2013) and the nucleotide polymerase inhibitor sofosbuvir (approved by the FDA on December 6, 2013). Among patients infected with HCV, less than 10% are treated and cured, and the major challenge in controlling HCV infections is the identification of those already infected, most of whom are situated in the poorest regions of the world (Thomas, 2013), and to find the most effective, tolerable and affordable direct acting antivirals (DAA) combination that can cure people in the shortest period (Poveda et al., 2014). In the NEUTRINO phase III trial of treatment-naive patients, 12 weeks of triple combination therapy with sofosbuvir (400 mg) once daily resulted in SVR rates of 89% in patients with HCV genotype 1 (92% for subtype 1a and 82% for subtype 1b), and 96% in patients with genotype 4 (Lawitz et al., 2013). Moreover, in the FISSION trial of HCV-2/3 treatment-naive patients receiving sofosbuvir/RBV for 12 weeks, 95% of patients with genotype 2 and 56% of patients with genotype 3 achieved an SVR (Lawitz et al., 2013). In addition, most DAA agents are characterised by a low genetic barrier to the development of resistance, except sofosbuvir, which showed a very high resistance barrier. This is the reason most current DAA-based therapies under evaluation must be co-administered with either PEG-IFN-alpha and ribavirin or different compounds belonging to different DAA classes (Poveda et al., 2014).

Pycnogenol® (PYC; trademark of Horphag Research, Geneva, Switzerland) is a French maritime pine extract produced from the outer bark of *Pinus pinaster* ssp. *atlantica*, and is generally considered safe for human use (American Botanical Council, 2010). The main PYC constituents are procyanidins (68.4%), taxifolin (21.87%), ferulic acid (3.70%), catechin (2.53%), and caffeic acid (3.51%) (Lee et al., 2010). PYC has been reported to have antioxidative and anti-inflammatory effects, and to reduce cardiovascular risk factors associated with type 2 diabetes (Maimoona et al., 2011; Zibadi et al., 2008). A recent report suggests that PYC can inhibit encephalomyocarditis virus replication in the mouse heart by suppressing expression of proinflammatory cytokines, and genes related to cardiac remodelling and mast cells (Matsumori et al., 2007). PYC has also been reported to inhibit binding of human immunodeficiency virus type-1 to host cells, and to cause other significant changes, including increased expression of manganese superoxide dismutase (Feng et al., 2008).

HCV gene expression elevates reactive oxygen species (ROS) levels via calcium signalling. In addition, HCV Core, NS3, and NS5A proteins have all been shown to induce oxidative stress (Choi et al., 2004). The reported link between HCV and oxidative stress makes this pathway a promising anti-HCV therapeutic strategy. To date, however, the effect of PYC on HCV infection has not been investigated. This study evaluated the inhibitory effects of Pycnogenol® on HCV replication *in vitro* and *in vivo*.

2. Materials and methods

2.1. Cell culture and reagents

Genotype 1b HCV subgenomic replicon cell lines, R6FLR-N (R6, genotype 1b, strain N) (Watanabe et al., 2006), FLR3-1 (genotype 1b, Con-1) (Sakamoto et al., 2005) and Rep JFH Luc3-13 genotype 2a (Takano et al., 2011), strain JFH-1 (Wakita et al., 2005) (Supplementary Fig. 1) were cultured at 37 °C (5% CO_2) in Dulbecco's modified Eagle medium-GlutaMAX-I (DMEM-GlutaMAX-I; Invitrogen, Carlsbad, CA, USA) containing 10% foetal bovine serum and 0.5 mg/mL G418 (Invitrogen, Carlsbad, CA, USA) (Sakamoto et al., 2005; Watanabe et al., 2006). The JFH-1/K4 cell line, which comprises HuH-7 cells persistently infected with the HCV JFH-1 strain, was maintained in DMEM with 10% FCS (Takano et al., 2011).

PYC was supplied by Horphag Research Co., Pegylated IFN-alpha-2a was obtained from Chugai Pharmaceutical Co., Japan.

2.2. HCV replicon cell reporter assay

Cells were seeded into 96-well plates (5×10^3/well). After incubation for 24 h at 37 °C (5% CO_2), the medium was removed and replaced with growth medium containing serial dilutions of PYC, IFN-alpha, RBV, telaprevir or simeprevir (Janssen Pharma Co., Tokyo, Japan). After 72 h, luciferase activity was measured using the Bright-Glo luciferase assay kit (Promega, Madison, WI). Measurements were made in triplicate using an AccuFLEX Lumi 400 luminometer (Aloka, Tokyo, Japan), and the results expressed as the average percentage of the control.

2.3. Generation of telaprevir-resistant replicon cell lines and analysis

Telaprevir-resistant R6FLR-N subgenomic replicon cell lines were established as described previously (Katsume et al., 2013). Briefly, wild-type R6FLR-N replicon cells were seeded in 10-cm dishes in the presence of 0.5 mg/mL G418 and treated with telaprevir. The cells were incubated for 51 days with no-compound control or telaprevir (1.8 μM and 2.7 μM serially diluted in media). Fresh media and telaprevir were added every 3 days. Most cells incubated with 2.7 μM telaprevir died; however, after 3 weeks small colonies started to appear and were expanded for 4 weeks. Deep sequencing was performed as described previously (Katsume et al., 2013) and revealed a mutation profile in NS3 (V36A, T54V and A156T) and NS5A (Q181H, P223S and S417P) which confer resistance to telaprevir. Resistant replicon cells were seeded at 5×10^3/well. After incubation for 24 h at 37 °C (5% CO_2), culture medium was removed and replaced with growth medium containing serial dilutions of PYC or telaprevir alone or in combination. After 72 h, luciferase activity was determined using the Bright-Glo luciferase assay kit (Promega, Madison, WI, USA). Measurements were made in duplicate using a GloMax-Multi detection system (Promega, Madison, WI, USA). Cytotoxicity was measured using WST-8 cell counting kit (Dojindo, Kumamoto, Japan). Western blot analysis was performed, as described previously (Nishimura et al., 2009). Briefly, HCV replicon cells (2×10^5) were grown in a 60-mm cell culture dish. After 24 h, cells were treated with PYC for 72 h. Cells were collected and lysed with radioimmunoprecipitation buffer (1% sodium dodecyl sulphate, 0.5% Nonidet P-40, 150 mmol NaCl, 0.5 mmol ethylenediaminetetraacetic acid, 1 mmol dithiothreitol, and 10 mmol Tris, pH 7.4). Total protein (30 μg) was electrophoresed on a 12% sodium dodecyl sulphate–polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Billerica, MA, USA). HCV NS3 and NS5B proteins were detected using rabbit NS3 (R212) polyclonal antibody or anti-NS5B (5B14) monoclonal antibody. Beta-actin was detected using an actin monoclonal antibody (Sigma, St. Louis, MO, USA).

2.4. Quantitative real-time polymerase chain reaction

Quantification of HCV RNA was performed using real-time reverse transcription polymerase chain reaction (qRT-PCR) based

on TaqMan chemistry using the forward primer R6-130-S17 (nucleotides 130–146), 5′-CGGGAGAGCCATAGTGG-3′; the reverse primer R6-290-R19 (nucleotides 290–272), 5′-AGTACCA-CAAGGCCTTTCG-3′; and the Taq-Man probe R6-148-S21FT (nucleotides 148–168), 5′-FAM-CTGCGGAACCGGTGAGTACAC-TAMRA3′, as described previously (Takeuchi et al., 1999). HCV RNA was extracted from PYC-treated, persistently-infected JFH-1/K4 HCV cells, using the ISOGEN RNA extraction kit (Nippon Gene, Japan).

2.5. In vivo effects of PYC

We produced chimeric mice by transplanting human primary hepatocytes into severe combined immunodeficient mice carrying a urokinase plasminogen activator transgene controlled by the albumin promoter (Mercer et al., 2001; Tateno et al., 2004). All animals received humane care according to National Institute of Health criteria outlined in the Guide for Care and Use of Laboratory Animals. The hepatocytes were infected with HCV-G9 (genotype 1a) (Inoue et al., 2007). HCV 1a RNA levels reached 2.9–18.0 $\times$ 10^6 -copies/mL in mice sera after 1–2 months of infection. PYC (40 mg/kg) was administered intraperitoneally once daily. PEG-IFN (30 μg/kg) was administered subcutaneously at 0, 3, 7, and 10 days either alone or in combination with PYC. Each treated group contained at least 3 chimeric mice. HCV RNA was purified from 2 μL chimeric mouse serum using SepaGene RV-R (Sanko Junyaku Co., Ltd., Tokyo, Japan). HCV RNA levels were quantified using qRT-PCR as reported previously (Takeuchi et al., 1999).

2.6. ROS assay

Formation of ROS in the HuH-7 cell-based HCV-replicon-harbouring cell line (R6FLR-N), and in R6FLR-N cured of HCV by interferon treatment (Blight et al., 2002) was measured using the OxiSelect ROS assay kit (Cell Biolabs, San Diego, CA, USA) according to the manufacturer's instructions. Duplicate samples at 1 $\times$ 10^7 -cells/mL from each culture were then incubated with dichlorodihydrofluorescein DiOxyQ (DCFH-DiOxyQ). Under these conditions, ROS species rapidly oxidise DCFH into the highly fluorescent 2′, 7′-dichlorodihydrofluorescein (DCF). Fluorescence intensity, which is proportional to the total ROS levels in the sample, was measured with a fluorescence spectrophotometer reader at 480-nm excitation and 530-nm emission.

2.7. Statistical analysis

Data are presented as means ± standard error of triplicate experiments. Data were analysed using Kruskal–Wallis test and Mann–Whitney *U* tests. A p-value <0.05 was considered statistically significant. To evaluate the synergistic or antagonistic effects of combinations of PYC and IFN-alpha or PYC and telaprevir, we calculated the combination index (CI) using CalcuSyn (Biosoft, Ferguson, MO, USA), where CI < 1 indicates synergism, CI = 1 indicates an additive effect, and CI > 1 indicates antagonism. The weighted average CI was calculated using the formula: $CI = [CI_{50} + 2CI_{75} + 3CI_{90} + 4CI_{95}]/10$, where CI_{50}, CI_{75}, CI_{90}, and CI_{95} are the CI values at 50%, 75%, 90% and 95% inhibition, respectively (Bassit et al., 2008; Chou and Talalay, 1984).

3. Results

3.1. PYC inhibits HCV replication and protein synthesis in vitro

We assessed the effect of PYC on HCV in R6FLR-N and FLR3-1 cell lines after 72 h (Fig. 1). The data are expressed as relative values using the relative light unit count for the 0 μg/mL treatment sample as 100% (Fig. 1A). The results showed that PYC inhibited luciferase activity in R6FLR-N cells (50% inhibitory concentration [IC_{50}] = 5.78 ± 3.75 μg/mL, 50% effective concentration [EC_{50}] = 4.33 μg/mL (2.2–8.5) in a dose-dependent manner. To rule out the possibility that the antiviral activity was caused by cytotoxic effects, cell proliferation was analysed using the WST-8 assay; no significant differences in cell viability (50% cytotoxic concentration [CC_{50}] > 60 μg/mL PYC; Selectivity index [SI] > 14.1) (Fig. 1B). These results indicate that PYC suppresses HCV (genotype 1b) replication.

Consistent with results showing the inhibitory effects of PYC on HCV replication, we observed that HCV NS3 protein levels decreased significantly in PYC and IFN-alpha-treated HCV replicon cell lines (Fig. 1C). HCV NS3 and NS5B proteins levels were progressively suppressed in HCV replicon cell lines at various PYC concentrations (0, 5, 10, and 20 μg/mL) (Fig. 1D). These results suggest that HCV protein synthesis was inhibited by PYC in a concentration-dependent manner.

3.2. Combinations of IFN-alpha, RBV, and PYC inhibit HCV replication

R6FLR-N cells were treated with IFN-alpha and RBV alone or in combination with several concentrations of PYC and incubated for 48 h (Fig. 2A). HCV replication was suppressed by approximately 20% following treatment with 5 μg/mL RBV, and by approximately 40% following treatment with 1 IU/mL IFN-alpha. Treatment with both RBV and IFN-alpha led to approximately 50% suppression. PYC showed a dose-dependent additive effect when administered in combination with RBV and IFN-alpha (Fig. 2A). Treatment with both PYC (5 μg/mL) and IFN-alpha (1 IU/mL) showed a synergistic effect (CI = 0.253) in suppressing HCV replication without cytotoxicity (Fig. 2A and B).

3.3. Effect of PYC on HCV JFH-1 replication

JFH Luc3-13-N cells were inoculated with IFN-alpha (5 IU/mL) or several concentrations of PYC (5–50 μg/mL) and incubated for 72 h (Fig. 2C). HCV (genotype 2a) replication was suppressed by approximately 50% following treatment with 40 μg/mL PYC (Fig. 2C) without significant cytotoxicity (Fig. 2D).

3.4. Anti-HCV activity of PYC in the HCV JFH-1 infection system

PYC, IFN-alpha, and RBV treatments were also evaluated in JFH-1/K4 HCV (genotype 2a) infected cells (Fig. 2E). HCV RNA levels decreased in the presence of PYC (10 or 20 μg/mL) to levels comparable to treatment with 1 IU/mL IFN-alpha in cell culture supernatant after 72 h. Treatment with a combination of IFN-alpha (1 IU/mL), RBV (5 μg/mL), and PYC (10 or 20 μg/mL) had a greater effect than IFN-alpha or PYC alone (Fig. 2E).

3.5. PYC inhibits HCV replication in telaprevir-resistant replicon cells and inhibits additively with telaprevir

We next examined the efficacy of PYC in DAA-resistant HCV. To select telaprevir-resistant replicons, cells with genotype 1b HCV replicons were treated for 14 passages with 1.8 μM and 2.7 μM telaprevir, concentrations 4–6 times the reported IC_{50} (Katsume et al., 2013). These telaprevir-resistant replicon cells showed some cross-resistance to another protease inhibitor, simeprevir (Supplementary Fig. 2). We investigated whether incubation of the wild-type HCV and telaprevir-resistant replicon with PYC alone or with telaprevir would inhibit HCV replication. The susceptibility of the replicon to PYC was measured after treating the cells with increasing concentrations of PYC and telaprevir for 72 h (Fig. 3). Fig. 3A shows that PYC reduced luciferase activity in

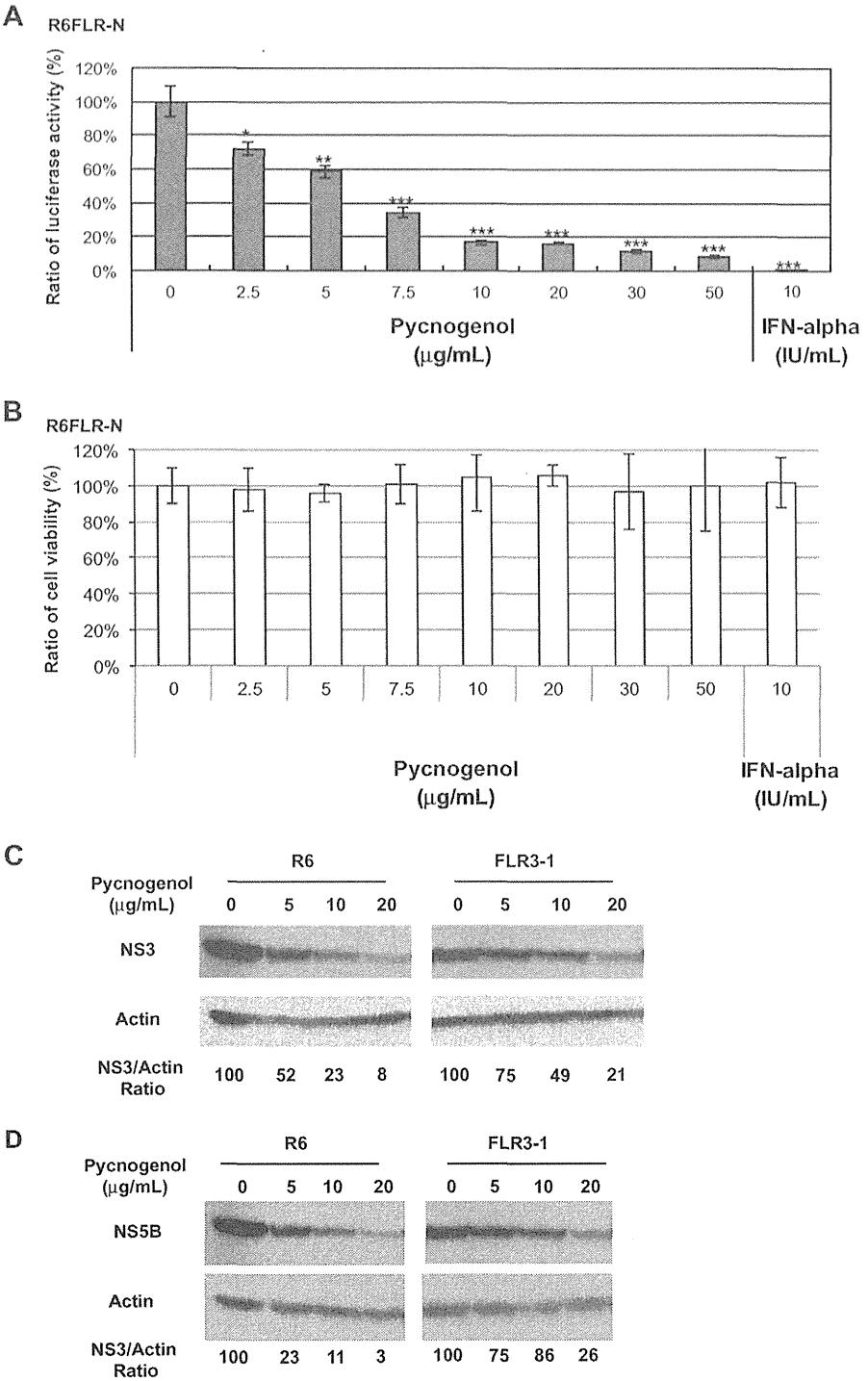

Fig. 1. Effect of PYC on HCV RNA replication in HCV replicon cell lines. (A) Dose-dependent inhibition of HCV by PYC in the R6 replicon cell line. (B) Cytotoxicity in the R6 HCV-replicon cell line treated with PYC. The ratio (%) of viability compared to the no-treatment control value is indicated. (C and D) HCV protein expression level decreased with PYC treatment in the HCV R6 and FLR3-1 replicon cell lines. (C and D) Dose-dependent effects of PYC on HCV protein synthesis (C NS3, D NS5B) in R6 and FLR3-1 replicon cell lines. Error bars = standard deviation. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. The ratio of luciferase was calculated as compared to non-treatment values.

a dose dependant manner in a wild-type HCV replicon and 2 telaprevir-resistant replicon cell lines. In addition, PYC had an additive effect with telaprevir (CI = 1.05) (Fig. 3B). Further, inhibition was greater in telaprevir (1.8 μM) than telaprevir (2.7 μM) and combined PYC (10 μg/mL) and telaprevir (1.8 μM and 2.7 μM) treatment reduced luciferase levels to those reached by PYC alone at 10 μg/mL. Moreover, the resistant mutants remain as sensitive to IFN-alpha as the wild-type replicon (Fig. 3A). After a 72-h incubation with PYC and telaprevir, no significant cytotoxicity, as evaluated in the WST-8 based cell viability assay, was observed in the replicon cells (Fig. 3C).

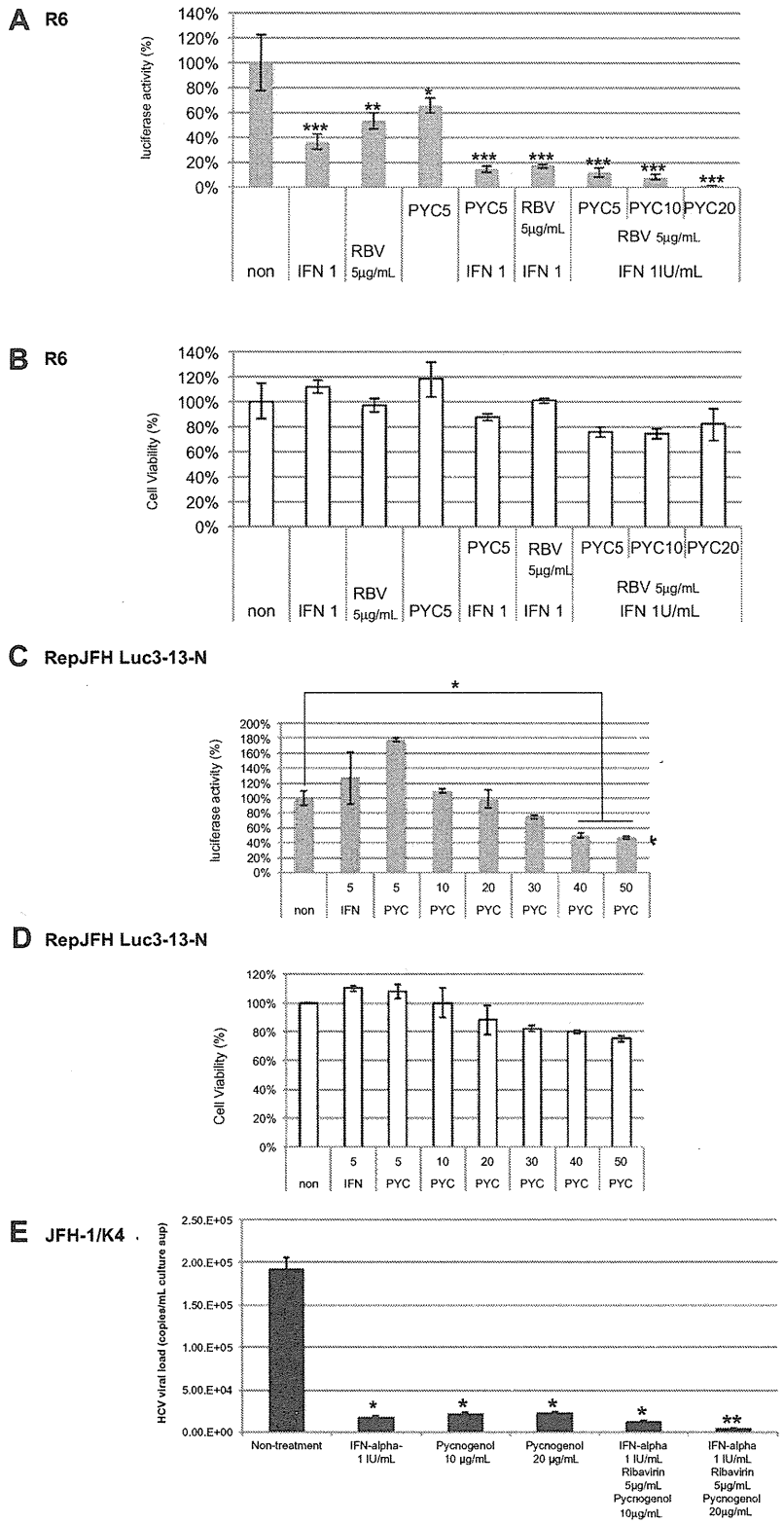

Fig. 2. Effect of PYC and IFN-alpha on HCV replication. (A) Effect of PYC (5 μg/mL), IFN-alpha (1 IU/mL), and RBV (5 μg/mL) on antiviral activity in the R6 HCV replicon cell line. (B) Cytotoxicity in the R6 HCV replicon cell line treated with PYC, IFN-alpha, and RBV. (C) Effect of IFN-alpha (5 IU/mL), and PYC (5–50 μg/mL) on JFH-1 replicon cells (RepJFH Luc3-13-N) (replication; left, viability; right). (D) Cytotoxicity in the RepJFH Luc3-13-N cell treated with PYC. (E) Synergistic effect of PYC, IFN-alpha, and RBV in the culture supernatant of HCV infection system in JFH-1/K4 cell line. Error bars = standard deviation. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

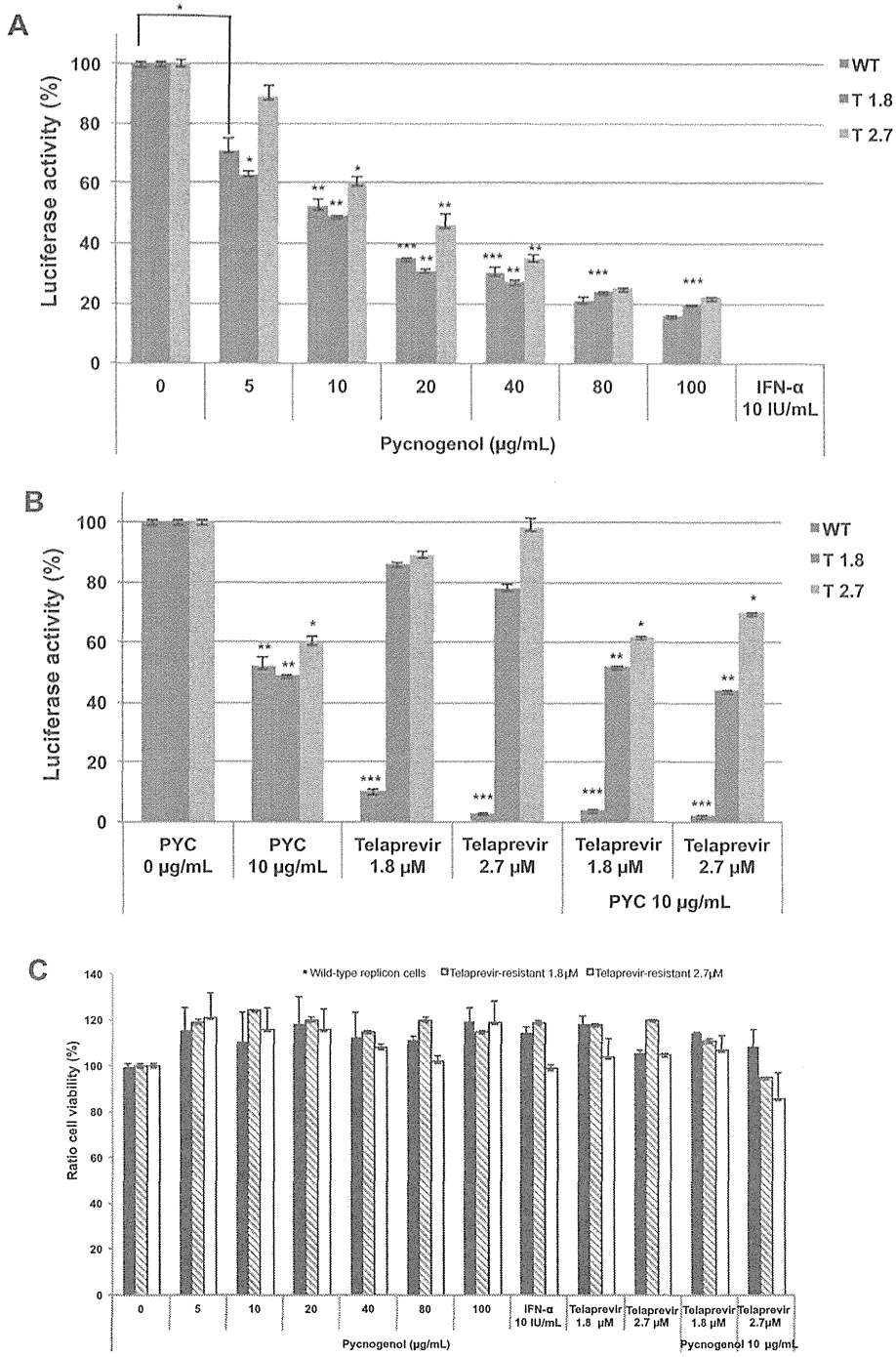

Fig. 3. Effect of PYC on HCV replication in telaprevir-resistant replicon cells. (A) Dose-dependent inhibition of HCV by PYC in wild type (WT) and telaprevir-resistant replicon cell lines (V1.8, V2.7). (B) Effect of PYC and telaprevir in the R6 replicon cell line. (C) PYC cytotoxicity in the R6 HCV replicon cell line treated with PYC, IFN-alpha, and telaprevir. Ratio of viability (%) to cells without PYC is indicated. Error bars = standard deviation. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

3.6. PYC suppresses HCV replication more effectively than procyanidin or taxifolin

Because procyanidin and taxifolin are the main constituents of PYC (Lee et al., 2010), we examined their ability to suppress HCV replication (Supplementary Fig. 3). Procyanidin could not inhibit HCV replication in R6FLR-N cells at concentrations between 15 and 60 μg/mL (Supplementary Fig. 3A). Cytotoxicity was not observed even at this high dose (data not shown). In JFH-1/K4 HCV-infected cell lines, procyanidin suppressed supernatant HCV RNA levels after 72 h and worked synergistically with IFN-alpha (Supplementary Fig. 3B). Moreover, we also examined taxifolin efficacy, but did not observe any effect on HCV replication (Supplementary Fig. 3C) or HCV infection in JFH-1/K4 cells (data not shown).

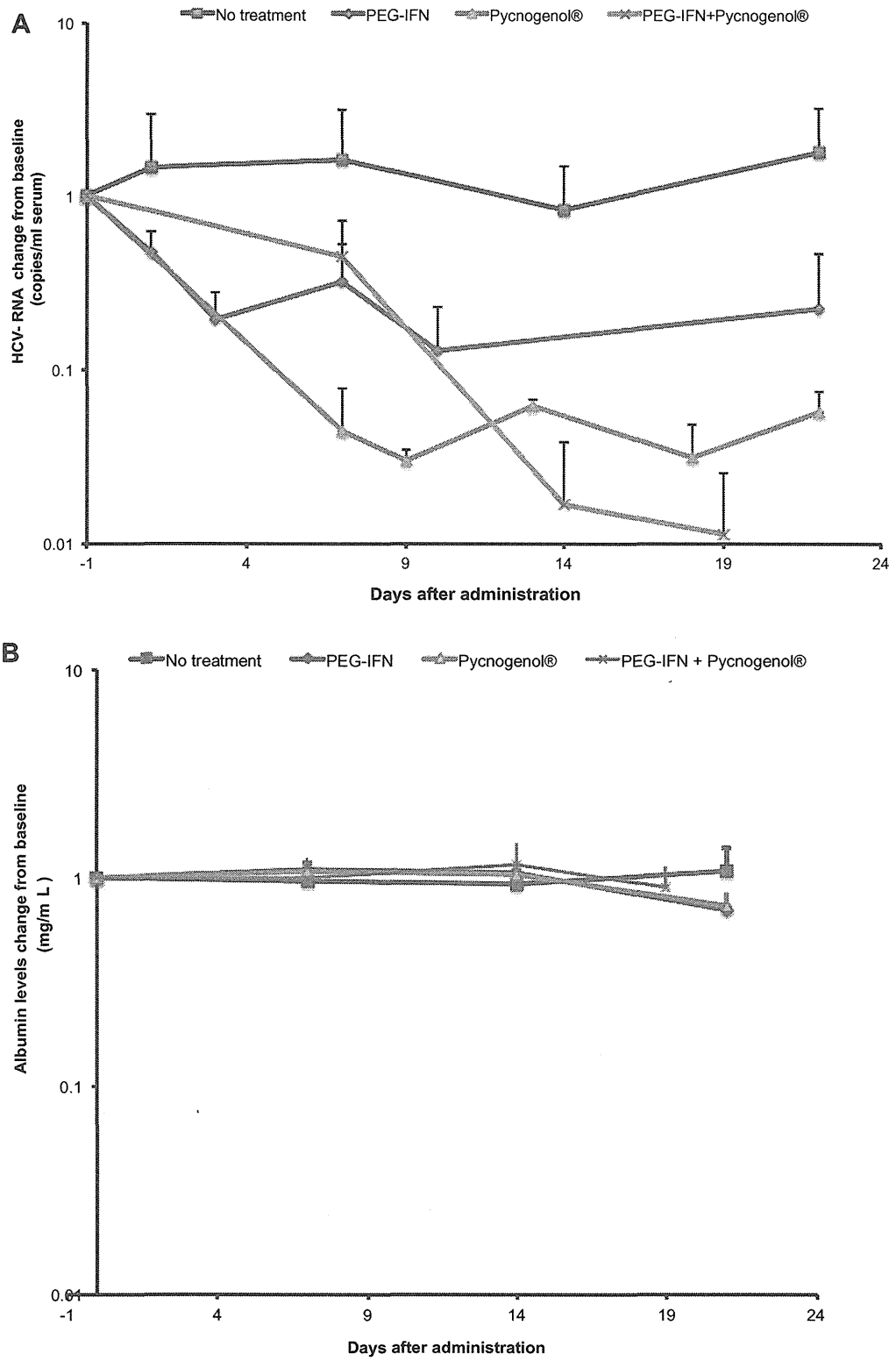

Fig. 4. *In vivo* effects of PYC on HCV replication. (A) Median change from baseline serum relative HCV RNA levels (HCV RNA amount at the beginning; average 4.8×10^7 copies/mL) in chimeric mice treated with PYC (40 mg/kg/day, intraperitoneally) or PEG-IFN (30 μg/kg, subcutaneously) alone, in combination with PEG-IFN, and untreated controls (in each group, $n = 3$). (B) Serum human albumin levels in chimeric mice during treatment.

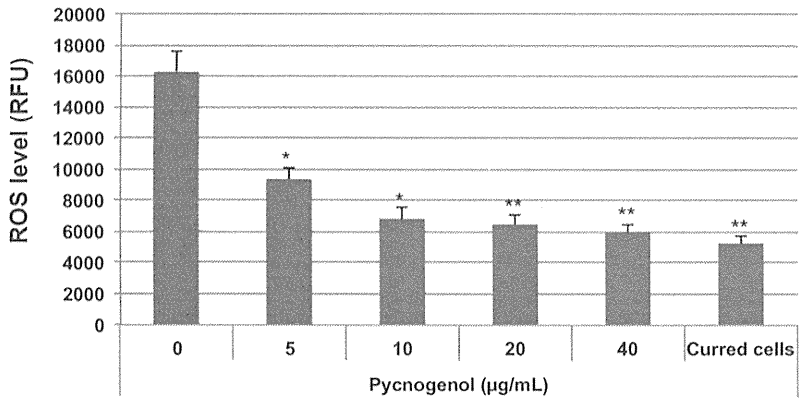

Fig. 5. Effect of PYC on ROS production in HCV replicon cells. HCV replicon and cured cell lines were incubated for 72 h with various concentrations of PYC and without PYC treatment. Data are shown as mean ± standard error of mean. ROS levels in HCV-negative cured cells are also shown. $^{*}P < 0.05$; $^{**}P < 0.01$.

3.7. PYC inhibits HCV replication in chimeric mice

To evaluate the *in vivo* effects of PYC on HCV, we used chimeric mice with a humanized liver infected with HCV G9 (genotype 1a). In the untreated control group ($n = 3$ mice), no decrease in HCV genome RNA levels was observed. In the group treated with PYC (40 mg/kg/day) ($n = 3$ mice), serum HCV RNA levels decreased rapidly, and within 9 days the effect was greater than with PEG-IFN treatment (30 µg/kg) ($n = 3$ mice) (Fig. 4A). Treatment with both PYC (40 µg/kg) and PEG-IFN (30 µg/kg) significantly reduced HCV RNA levels after 14 days compared to either PEG-IFN or PYC monotherapy (Kruskal–Wallis test, $p = 0.0008$). To exclude the possibility that this decline was caused by indirect effects on human hepatocyte viability, we measured human albumin levels in chimeric mice and found no significant variation during treatment ($p = 0.728$) (Fig. 4B), indicating that PYC has an antiviral effect and acts synergistically with PEG-IFN in chimeric mice with humanized livers infected with HCV.

3.8. PYC suppresses intracellular ROS production in R6FLR-N cells

A ROS assay was used to assess the ability of PYC to act as a free radical scavenger. Fluorescence intensity was measured for each sample. Total ROS production was significantly decreased by PYC in the HCV replicon cell line in a dose-dependent manner (Fig. 5). Treatment with PYC at 40 µg/mL reduced ROS to levels comparable to cells cured of the HCV replicon by IFN treatment (Blight et al., 2002), suggesting that PYC may scavenge ROS in HCV replicon cell lines.

4. Discussion

Oxidative stress has been identified as a key mechanism of HCV-induced pathogenesis (de Mochel et al., 2010; Ke and Chen, 2012; Quarato et al., 2013; Tardif et al., 2005). Moreover, several studies have reported a correlation between oxidative stress and IFN treatment response, and have observed that oxidative stress was reduced to normal levels after viral eradication (Levent et al., 2006; Serejo et al., 2003). These data provide a firm theoretical basis for investigation of antioxidants as therapeutics. PYC is a mixture of various chemical groups and exhibits radical-scavenging antioxidant, anti-inflammatory, and antiviral activities (Maimoona et al., 2011). In addition, PYC protects biomolecules such as proteins against oxidative damage (Voss et al., 2006). To our knowledge, this is the first report to demonstrate a direct antiviral effect of PYC against HCV. Our results show that PYC inhibits HCV replication in HCV replicon cell lines and JFH-1 without cytotoxicity. Moreover, this result is in line with a recent report, based on data obtained from 5723 subjects that showed side effect incidence rates of 2.4% and 0.19% in patients and healthy subjects, respectively (American Botanical Council, 2010). The study also found PYC to be nontoxic at doses of 20–100 mg/day for extended periods (months) and 100–300 mg for shorter periods (American Botanical Council, 2010).

Treatments of replicon and JFH-1 cell lines using combinations of PYC with RBV, IFN, and telaprevir showed that co-administration of these compounds increased HCV antiviral activity. In addition, we found that PYC suppressed HCV replication in telaprevir-resistant replicon cells and may improve the response to protease inhibitors. In this report, we found that procyanidins, oligomeric compounds formed from catechin and epicatechin, but not taxifolin, inhibited HCV replication at doses between 15 and 60 µg/mL and had a synergistic effect with IFN treatment without cytotoxicity. Moreover, procyanidin B1 extracted from *Cinnamomum cassia* cortex suppresses hepatitis C virus replication (Li et al., 2010). Other studies have also shown that epicatechin, catechin-derived compounds, and caffeic acid phenethyl ester inhibit HCV replication and attenuate the inflammation induced by the virus (Khachatoorian et al., 2012; Lin et al., 2013; Shen et al., 2013). However, PYC showed greater antiviral effects than procyanidin and taxifolin. PYC efficacy was much stronger than procyanidin or taxifolin; therefore, a combination of components or unknown factor(s) in PYC may contribute to inhibition of viral replication.

Constitutive activation of NF-kappa B and STAT-3 by HCV is implicated in acute and chronic liver disease (Gong et al., 2001; Waris et al., 2003, 2005). Consistent with these data, a previous study showed that PYC inhibits NF-kappa B and activator protein-1, and abolishes the degradation of I-kappa B alpha (Cho et al., 2000). Moreover, a recent study showed that PYC also inhibits its expression and secretion of tumour necrosis factor-alpha and interleukin 6, reducing calcium uptake and suppressing NF-kappa B activation (Choi and Yan, 2009). We observed PYC free radical scavenging activity against ROS in HCV replicon cell lines. These data support our finding that PYC exerts its antioxidant effects directly by scavenging of ROS and indirectly by enhancing cellular antioxidant enzymes (Packer et al., 1999).

5. Conclusion

Our study shows that the natural product PYC inhibits HCV replication both *in vitro* and *in vivo*. Our results indicate that *in vitro* combinations of PYC/IFN-alpha/RBV and PYC/telaprevir lead to a

much stronger antiviral response than with either agent alone and that PYC suppresses replication in telaprevir-resistant replicon cells. Future clinical trials are necessary to assess which patients, for example, naïves, non-responders, or those with severe liver disease, could benefit from co-administration of PYC with PEG-IFN-alpha, RBV, or DAAs. Addition of PYC may be a viable strategy to improve the efficacy of HCV therapies using the recently licensed antiviral molecules.

Conflict of interest

The authors declare that they have nothing to disclose regarding funding or conflicts of interest relating to this manuscript.

Acknowledgements

This research was supported by a grant from the Adaptable and Seamless Technology Transfer Program through Target-driven R&D (Japan Science and Technology Agency), grants from the Ministry of Health, Labor, and Welfare, Japan, and the Ministry of Education, Culture, Sports, Science, and Technology, Japan. Sayeh Ezzikouri is supported by a Japan Society for the Promotion of Science (JSPS) Fellowship for Foreign Researchers. The authors thank Drs Yuko Tokunaga and Makoto Ozawa for their support during experiments, Dr Lin Li for combination index calculation and Horphag Research Co., Geneva, Switzerland, for their generous gift of Pycnogenol® powder.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2014.10.017.

References

American Botanical Council, 2010. Scientific and clinical monograph for Pycnogenol.
Antaki, N., Craxi, A., Kamal, S., Moucari, R., Van der Merwe, S., Haffar, S., Gadano, A., Zein, N., Lai, C.L., Pawlotsky, J.M., Heathcote, E.J., Dusheiko, G., Marcellin, P., 2010. The neglected hepatitis C virus genotypes 4, 5 and 6: an international consensus report. Liver Int. 30, 342–355.
Bacon, B.R., Gordon, S.C., Lawitz, E., Marcellin, P., Vierling, J.M., Zeuzem, S., Poordad, F., Goodman, Z.D., Sings, H.L., Boparai, N., Burroughs, M., Brass, C.A., Albrecht, J.K., Esteban, R., Investigators, H.R., 2011. Boceprevir for previously treated chronic HCV genotype 1 infection. N. Engl. J. Med. 364, 1207–1217.
Bassit, L., Grier, J., Bennett, M., Schinazi, R.F., 2008. Combinations of 2′-C-methylcytidine analogues with interferon-alpha2b and triple combination with ribavirin in the hepatitis C virus replicon system. Antivir. Chem. Chemother. 19, 25–31.
Blight, K.J., McKeating, J.A., Rice, C.M., 2002. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. J. Virol. 76, 13001–13014.
Cho, K.J., Yun, C.H., Yoon, D.Y., Cho, Y.S., Rimbach, G., Packer, L., Chung, A.S., 2000. Effect of bioflavonoids extracted from the bark of *Pinus maritima* on proinflammatory cytokine interleukin-1 production in lipopolysaccharide-stimulated RAW 264.7. Toxicol. Appl. Pharmacol. 168, 64–71.
Choi, Y.H., Yan, G.H., 2009. Pycnogenol inhibits immunoglobulin E-mediated allergic response in mast cells. Phytother. Res. 23, 1691–1695.
Choi, S.H., Kim, S.Y., Park, K.J., Kim, Y.J., Hwang, S.B., 2004. Hepatitis C virus core protein is efficiently released into the culture medium in insect cells. J. Biochem. Mol. Biol. 37, 735–740.
Chou, T.C., Talalay, P., 1984. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv. Enzyme Regul. 22, 27–55.
de Mochel, N.S., Seronello, S., Wang, S.H., Ito, C., Zheng, J.X., Liang, T.J., Lambeth, J.D., Choi, J., 2010. Hepatocyte NAD(P)H oxidases as an endogenous source of reactive oxygen species during hepatitis C virus infection. Hepatology 52, 47–59.
Feng, W.Y., Tanaka, R., Inagaki, Y., Saitoh, Y., Chang, M.O., Amet, T., Yamamoto, N., Yamaoka, S., Yoshinaka, Y., 2008. Pycnogenol, a procyanidin-rich extract from French maritime pine, inhibits intracellular replication of HIV-1 as well as its binding to host cells. Jpn. J. Infect. Dis. 61, 279–285.
Gong, G., Waris, G., Tanveer, R., Siddiqui, A., 2001. Human hepatitis C virus NS5A protein alters intracellular calcium levels, induces oxidative stress, and activates STAT-3 and NF-kappa B. Proc. Natl. Acad. Sci. U.S.A. 98, 9599–9604.
Hoofnagle, J.H., Seeff, L.B., 2006. Peginterferon and ribavirin for chronic hepatitis C. N. Engl. J. Med. 355, 2444–2451.
Inoue, K., Umehara, T., Ruegg, U.T., Yasui, F., Watanabe, T., Yasuda, H., Dumont, J.M., Scalfaro, P., Yoshiba, M., Kohara, M., 2007. Evaluation of a cyclophilin inhibitor in hepatitis C virus-infected chimeric mice in vivo. Hepatology 45, 921–928.
Jacobson, I.M., McHutchison, J.G., Dusheiko, G., Di Bisceglie, A.M., Reddy, K.R., Bzowej, N.H., Marcellin, P., Muir, A.J., Ferenci, P., Flisiak, R., George, J., Rizzetto, M., Shouval, D., Sola, R., Terg, R.A., Yoshida, E.M., Adda, N., Bengtsson, L., Sankoh, A.J., Kieffer, T.L., George, S., Kauffman, R.S., Zeuzem, S., Team, A.S., 2011. Telaprevir for previously untreated chronic hepatitis C virus infection. N. Engl. J. Med. 364, 2405–2416.
Katsume, A., Tokunaga, Y., Hirata, Y., Munakata, T., Saito, M., Hayashi, H., Okamoto, K., Ohmori, Y., Kusanagi, I., Fujiwara, S., Tsukuda, T., Aoki, Y., Klumpp, K., Tsukiyama-Kohara, K., El-Gohary, A., Sudoh, M., Kohara, M., 2013. A serine palmitoyltransferase inhibitor blocks hepatitis C virus replication in human hepatocytes. Gastroenterology 145, 865–873.
Ke, P.Y., Chen, S.S., 2012. Hepatitis C virus and cellular stress response: implications to molecular pathogenesis of liver diseases. Viruses 4, 2251–2290.
Khachatoorian, R., Arumugaswami, V., Raychaudhuri, S., Yeh, G.K., Maloney, E.M., Wang, J., Dasgupta, A., French, S.W., 2012. Divergent antiviral effects of bioflavonoids on the hepatitis C virus life cycle. Virology 433, 346–355.
Lawitz, E., Mangia, A., Wyles, D., Rodriguez-Torres, M., Hassanein, T., Gordon, S.C., Schultz, M., Davis, M.N., Kayali, Z., Reddy, K.R., Jacobson, I.M., Kowdley, K.V., Nyberg, L., Subramanian, G.M., Hyland, R.H., Arterburn, S., Jiang, D., McNally, J., Brainard, D., Symonds, W.T., McHutchison, J.G., Sheikh, A.M., Younossi, Z., Gane, E.J., 2013. Sofosbuvir for previously untreated chronic hepatitis C infection. N. Engl. J. Med. 368, 1878–1887.
Lee, H.H., Kim, K.J., Lee, O.H., Lee, B.Y., 2010. Effect of pycnogenol on glucose transport in mature 3T3-L1 adipocytes. Phytother. Res. 24, 1242–1249.
Levent, G., Ali, A., Ahmet, A., Polat, E.C., Aytac, C., Ayse, E., Ahmet, S., 2006. Oxidative stress and antioxidant defense in patients with chronic hepatitis C patients before and after pegylated interferon alfa-2b plus ribavirin therapy. J. Transl. Med. 4, 25.
Li, S., Kodama, E.N., Inoue, Y., Tani, H., Matsuura, Y., Zhang, J., Tanaka, T., Hattori, T., 2010. Procyanidin B1 purified from Cinnamomi cortex suppresses hepatitis C virus replication. Antivir. Chem. Chemother. 20, 239–248.
Lin, Y.T., Wu, Y.H., Tseng, C.K., Lin, C.K., Chen, W.C., Hsu, Y.C., Lee, J.C., 2013. Green tea phenolic epicatechins inhibit hepatitis C virus replication via cycloxygenase-2 and attenuate virus-induced inflammation. PLoS ONE 8, e54466.
Maimoona, A., Naeem, I., Saddiqe, Z., Jameel, K., 2011. A review on biological, nutraceutical and clinical aspects of French maritime pine bark extract. J. Ethnopharmacol. 133, 261–277.
Matsumori, A., Higuchi, H., Shimada, M., 2007. French maritime pine bark extract inhibits viral replication and prevents development of viral myocarditis. J. Card. Fail. 13, 785–791.
Mercer, D.F., Schiller, D.E., Elliott, J.F., Douglas, D.N., Hao, C., Rinfret, A., Addison, W.R., Fischer, K.P., Churchill, T.A., Lakey, J.R., Tyrrell, D.L., Kneteman, N.M., 2001. Hepatitis C virus replication in mice with chimeric human livers. Nat. Med. 7, 927–933.
Mohd Hanafiah, K., Groeger, J., Flaxman, A.D., Wiersma, S.T., 2013. Global epidemiology of hepatitis C virus infection: new estimates of age-specific antibody to HCV seroprevalence. Hepatology 57, 1333–1342.
Nishimura, T., Kohara, M., Izumi, K., Kasama, Y., Hirata, Y., Huang, Y., Shuda, M., Mukaidani, C., Takano, T., Tokunaga, Y., Nuriya, H., Satoh, M., Saito, M., Kai, C., Tsukiyama-Kohara, K., 2009. Hepatitis C virus impairs p53 via persistent overexpression of 3beta-hydroxysterol Delta24-reductase. J. Biol. Chem. 284, 36442–36452.
Ozeki, I., Akaike, J., Karino, Y., Arakawa, T., Kuwata, Y., Ohmura, T., Sato, T., Kamiya, N., Yamada, I., Chayama, K., Kumada, H., Toyota, J., 2011. Antiviral effects of peginterferon alpha-2b and ribavirin following 24-week monotherapy of telaprevir in Japanese hepatitis C patients. J. Gastroenterol. 46, 929–937.
Packer, L., Rimbach, G., Virgili, F., 1999. Antioxidant activity and biologic properties of a procyanidin-rich extract from pine (*Pinus maritima*) bark, pycnogenol. Free Radic. Biol. Med. 27, 704–724.
Perz, J.F., Alter, M.J., 2006. The coming wave of HCV-related liver disease: dilemmas and challenges. J. Hepatol. 44, 441–443.
Poordad, F., McCone Jr., J., Bacon, B.R., Bruno, S., Manns, M.P., Sulkowski, M.S., Jacobson, I.M., Reddy, K.R., Goodman, Z.D., Boparai, N., DiNubile, M.J., Sniukiene, V., Brass, C.A., Albrecht, J.K., Bronowicki, J.P., Investigators, S., 2011. Boceprevir for untreated chronic HCV genotype 1 infection. N. Engl. J. Med. 364, 1195–1206.
Poveda, E., Wyles, D.L., Mena, A., Pedreira, J.D., Castro-Iglesias, A., Cachay, E., 2014. Update on hepatitis C virus resistance to direct-acting antiviral agents. Antiviral Res. 108, 181–191. http://dx.doi.org/10.1016/j.antiviral.2014.05.015.
Quarato, G., Scrima, R., Agriesti, F., Moradpour, D., Capitanio, N., Piccoli, C., 2013. Targeting mitochondria in the infection strategy of the hepatitis C virus. Int. J. Biochem. Cell Biol. 45, 156–166.
Sakamoto, H., Okamoto, K., Aoki, M., Kato, H., Katsume, A., Ohta, A., Tsukuda, T., Shimma, N., Aoki, Y., Arisawa, M., Kohara, M., Sudoh, M., 2005. Host sphingolipid biosynthesis as a target for hepatitis C virus therapy. Nat. Chem. Biol. 1, 333–337.

Serejo, F., Emerit, I., Filipe, P.M., Fernandes, A.C., Costa, M.A., Freitas, J.P., de Moura, M.C., 2003. Oxidative stress in chronic hepatitis C: the effect of interferon therapy and correlation with pathological features. Can. J. Gastroenterol. 17, 644–650.

Shen, H., Yamashita, A., Nakakoshi, M., Yokoe, H., Sudo, M., Kasai, H., Tanaka, T., Fujimoto, Y., Ikeda, M., Kato, N., Sakamoto, N., Shindo, H., Maekawa, S., Enomoto, N., Tsubuki, M., Moriishi, K., 2013. Inhibitory effects of caffeic acid phenethyl ester derivatives on replication of hepatitis C virus. PLoS ONE 8, e82299.

Takano, T., Tsukiyama-Kohara, K., Hayashi, M., Hirata, Y., Satoh, M., Tokunaga, Y., Tateno, C., Hayashi, Y., Hishima, T., Funata, N., Sudoh, M., Kohara, M., 2011. Augmentation of DHCR24 expression by hepatitis C virus infection facilitates viral replication in hepatocytes. J. Hepatol. 55, 512–521.

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, 636–642.

Tardif, K.D., Waris, G., Siddiqui, A., 2005. Hepatitis C virus, ER stress, and oxidative stress. Trends Microbiol. 13, 159–163.

Tateno, C., Yoshizane, Y., Saito, N., Kataoka, M., Utoh, R., Yamasaki, C., Tachibana, A., Soeno, Y., Asahina, K., Hino, H., Asahara, T., Yokoi, T., Furukawa, T., Yoshizato, K., 2004. Near completely humanized liver in mice shows human-type metabolic responses to drugs. Am. J. Pathol. 165, 901–912.

Thomas, D.L., 2013. Global control of hepatitis C: where challenge meets opportunity. Nat. Med. 19, 850–858.

Voss, P., Horakova, L., Jakstadt, M., Kiekebusch, D., Grune, T., 2006. Ferritin oxidation and proteasomal degradation: protection by antioxidants. Free Radic. Res. 40, 673–683.

Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krausslich, H.G., Mizokami, M., Bartenschlager, R., Liang, T.J., 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. 11, 791–796.

Waris, G., Livolsi, A., Imbert, V., Peyron, J.F., Siddiqui, A., 2003. Hepatitis C virus NS5A and subgenomic replicon activate NF-kappaB via tyrosine phosphorylation of IkappaBalpha and its degradation by calpain protease. J. Biol. Chem. 278, 40778–40787.

Waris, G., Turkson, J., Hassanein, T., Siddiqui, A., 2005. Hepatitis C virus (HCV) constitutively activates STAT-3 via oxidative stress: role of STAT-3 in HCV replication. J. Virol. 79, 1569–1580.

Watanabe, T., Sudoh, M., Miyagishi, M., Akashi, H., Arai, M., Inoue, K., Taira, K., Yoshiba, M., Kohara, M., 2006. Intracellular-diced dsRNA has enhanced efficacy for silencing HCV RNA and overcomes variation in the viral genotype. Gene Ther. 13, 883–892.

Zibadi, S., Rohdewald, P.J., Park, D., Watson, R.R., 2008. Reduction of cardiovascular risk factors in subjects with type 2 diabetes by Pycnogenol supplementation. Nutr. Res. 28, 315–320.

Exp. Anim. 63(4), 367–374, 2014

—Review—

Tupaia Belangeri as an Experimental Animal Model for Viral Infection

Kyoko TSUKIYAMA-KOHARA[1,2], and Michinori KOHARA[3]

[1]*Transboundary Animal Diseases Center, Joint Faculty of Veterinary Medicine, Kagoshima University, 1-2-24 Korimoto, Kagoshima 890-0065, Japan*
[2]*Laboratory of Animal Hygiene, Joint Faculty of Veterinary Medicine, Kagoshima University, 1-2-24 Korimoto, Kagoshima 890-0065, Japan*
[3]*Department of Microbiology and Cell Biology, Tokyo Metropolitan Institute of Medical Science, Tokyo 113-8613, Japan*

Abstract: Tupaias, or tree shrews, are small mammals that are similar in appearance to squirrels. The morphological and behavioral characteristics of the group have been extensively characterized, and despite previously being classified as primates, recent studies have placed the group in its own family, the Tupaiidae. Genomic analysis has revealed that the genus *Tupaia* is closer to humans than it is to rodents. In addition, tupaias are susceptible to hepatitis B virus and hepatitis C virus. The only other experimental animal that has been demonstrated to be sensitive to both of these viruses is the chimpanzee, but restrictions on animal testing have meant that experiments using chimpanzees have become almost impossible. Consequently, the development of the tupaia for use as an animal infection model could become a powerful tool for hepatitis virus research and in preclinical studies on drug development.

Key words: genome, HBV, HCV, *Tupaia*, virus

Taxonomic Classification

Tupaia belangeri belongs to the family Tupaiidae, which consists of four genera and 19 extant species (Table 1) [13, 19]. The members of *Tupaia*, which are colloquially referred to as tree shrews, were first recorded in a sketch by William Ellis on a voyage with Captain Cook in 1780 [7]. With a body weight ranging between 45–350 g (Table 1), members of the genus *Tupaia* are similar in appearance to squirrels (Fig. 1). The natural habitat of *Tupaia* spp. consists of the tropical rainforest in South East Asia where they feed on fruits, insects and small vertebrates [7].

Similarities between *Tupaia* spp. and primates were first reported in the 1920s; for example, Le Gros Clark proposed that tree shrews and primates were closely related based on brain anatomy [20]. However, recent molecular studies have separated tupaias from the primates and placed them in the order Scandentia and within the grandorder Euarchonta, which also contains the Primates and Dermoptera [17].

Handling of Tupaia

Tupaia is active during daytime, and animal rooms are illuminated from 7:00 am to 9:00 pm with a relative humidity of 50–60%, and temperature at 26°C. Their foods are CMS-1M (CREA, Japan) 20 g, apple, banana and boiled egg, everyday. They usually slip into the boxes as soon as somebody enters the room, then we can catch them by net. We can bleed approximately 0.5 ml from the tail or leg vein once in 2 weeks. Tupaias can be

(Received 27 January 2014 / Accepted 18 April 2014 / Published online in J-STAGE 22 July 2014)
Address Corresponding: K. Tsukiyama-Kohara, and M. Kohara, Department of Microbiology and Cell Biology, Tokyo Metropolitan Institute of Medical Science, Tokyo 113-8613, Japan

©2014 Japanese Association for Laboratory Animal Science

Table 1. Composition of family Tupaiidae [1]

Taxa	Morphological characteristics	Reproductive characteristics	Weaning and longevity	Distribution
Family: Tupaiidae				
Genus: *Tupaia*				
*Species: *belangeri*	BW: 50–270 g	GP: 41–55 d	W: ca. 30 d	
Subspecies:	HBL: 12–21 cm	L: 1–5	L: 9–12 yr	
belangeri chinensis	NN: 1–3 pairs	NBW: 6–10 g		
**Species:*				Tropical forests in Southeast Asia
chrysogaster, dorsalis, glis, gracilis, javanica, longipes, minor, moellendorffi, montana, nicobarica, palawanensis, picta, splendidula, tana				
Genus: *Anathana ellioti*	BW: 180 g	UK	UK	
	HBL: 19 cm			
	NN: 3 pairs			
Genus: *Dendrogale melanura, murina*	BW: 60 g	GP: 41–55 d	W: ca. 30 d	
	HBL: 13 cm	L: 1–5	L: 9–12 yr	
	NN: 1 pair	NBW: 6–10 g		
Genus: *Urogale everetti*	BW: 220–359 g	GP: 30 d	W: ca. 30 d	
	HBL: 20 cm	L: 1–4	L: 6 yr	
	NN: 2 pairs	NBW: 10 g		

*BW: body weight; HBL: head-body length; NN: number of nipples; GP: gestation period; L: litter size; NBW: Newborn body weight; W: weaning; L: life span; UK: unknown.

Fig. 1. Adult female tupaia (*Tupaia belangeri*) maintained at the Department of Animal Hygiene, Kagoshima University.

breeding after 6–9 months age and easily to give average 4 babies after approximately 45 days of pregnancy. Tupaia usually possesses few health problems, but sometimes shows diarrhea by *Escherichia coli*, *Klebsiella pneumonia* or protozoa, which can be checked by quarantine. The inbred tupaia has not been established yet.

Genetic Characteristics of Tupaia Spp

Evolutionary characterization of 7S RNA-derived short interspersed elements (SINEs) revealed that 7S RNA is a component of the cytoplasmic signal recognition particle [33] in primates [5], tupaia [25] and rodents [18], i.e. all of the members of the placental mammalian order Supraprimates and the superorder Euarchontoglires. The fossil *Alu* monomer was previously considered to be the oldest common ancestor of all 7S RNA-derived SINEs [27], and was thought to be restricted to primates [17]. *Tupaia* possesses specific, chimeric, Tu-type II SINEs, which may share a common ancestor with rodent B1 SINEs [27]. Phylogenetic analysis of 7S L RNA-derived SINEs has shown that tupaias can be grouped with primates and Dermoptera in the Euarchonta, while the Rodentia and Lagomorpha can be grouped with the Glires [17].

Whole-genome analysis by several groups ([8], Tsukiyama-Kohara *et al.*, *in preparation*) revealed a genetic relationship between tupaias and humans. Similarly, phylogenetic analysis based on whole genome sequences showed that humans are closer to tupaias than they are to mice (Fig. 2). Further, several of the same highly conserved and variable genes have been identified

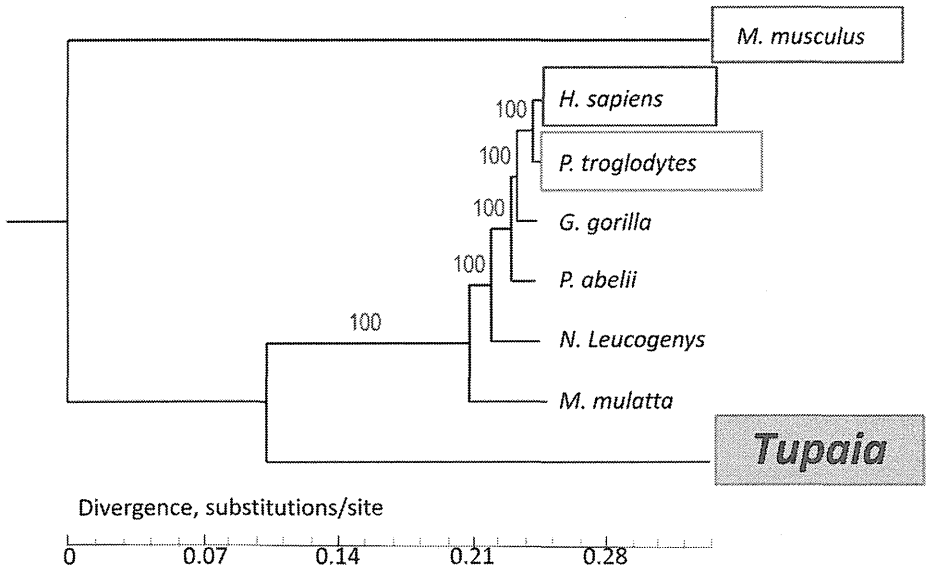

Fig. 2. Dendrogram showing relationships between primates, tree shrews and rodents. Phylogenetic tree constructed using orthologous genes at 4-fold degenerate sites by the maximum likelihood method. Branch lengths represent the neutral divergence rate and blue characters indicate bootstrap values.

in both tupaia and humans. For example, relatively high homology has been observed between human and *Tupaia* hepatitis C virus (HCV) viral receptor CD81 (Fig. 3A), scavenger receptor class B member I (SR-BI), the tight junction proteins claudin I and occludin I [16], as well as the hepatitis B virus (HBV) receptor, sodium-taurocholate cotransporting polypeptide (NTCP) (Fig. 3B) [38], particularly in the receptor and virus envelope surface glycoprotein regions that interact with the transmembrane proteins. It is possible that these highly conserved molecules could be a missing link during the evolution of tupaia, and detailed analysis of this hypothesis is currently underway.

Tupaia as an Experimental Animal Model

The high degree of genetic homology between several neuromodulator receptor proteins in tree shrews and primates has meant that *Tupaia* has been extensively utilized in preclinical research, particularly in the areas of toxicology and virology [10]. Although adult male tupaias exhibit strong territoriality in their natural habitat, the coexistence of two males in visual and olfactory contact in the laboratory leads to the establishment of a stable dominant-subordinate relationship, with subordinates showing distinct stress-induced alterations to behavior, physiology and central nervous activity [9]. These alterations exhibited by the subordinate male tupaias are similar to those observed in depressed human patients, and could be applicable to preclinical research of antidepressant drugs [11]. Various aspects of human behavior, infant development, communication and social structure could also potentially be studied in tupaia [22, 23].

Tupaia as Viral Hepatitis Model

Tupaia have also been employed in studies of viral infection, especially on hepatitis B and C viruses (HBV and HCV) [12]. For these viruses, the only existing natural-infection animal model is the chimpanzee. However, because chimpanzees are long-lived (>50 years), very expensive, and subject to stringent animal welfare regulations, several groups have attempted to develop *Tupaia* for use as an animal infection model. Pathogenesis of HCV was characterized using various transgenic mouse animal models and they can develop chronic hepatitis, liver cirrhosis and hepatocellular carcinoma [30], however natural infection is difficult to be established in these mice. HCV can successfully establish infection in the humanized chimeric mice liver [15, 24], but they do not have immune response, therefore, patho-

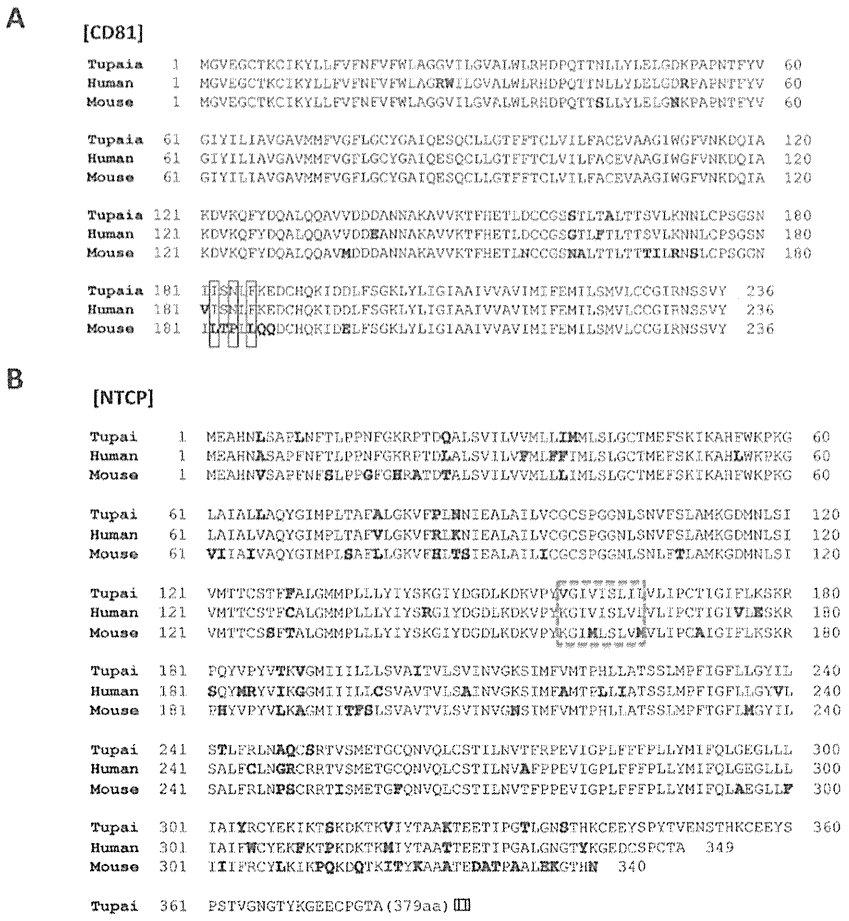

Fig. 3. Alignment of amino acid sequences of viral receptors. (A) Alignment of CD81 amino acid sequences from tupaia, human and mouse. Different amino acids were indicated with red colour. Significant amino acids for binding to HCV E2 protein were surround by square (Ile182, Asn184 and Phe186) [14, 6]. (B) Alignment of NTCP amino acid sequences from tupaia, human and mouse. Different amino acids were indicated with red colour. HBV pre-S1 binding region [37] was surrounded by break line box.

genicity of HCV could not be characterized.

We previously conducted infection experiments using HCV in *Tupaia* and characterized the pathogenesis in this animal [2]. Chronic HCV infection, which manifests as liver cirrhosis and hepatocellular carcinoma, is easily established [1]. Currently, approximately 170 million people around the world may be infected with HCV [35]. The current standard therapy for chronic hepatitis C is a combination of pegylated interferon (IFN) alpha-2a and nucleoside analog ribavirin. Recently, IFN-free combinations of direct-acting antiviral agents have been tested for clinical use and can achieve significant antiviral activity [29]. However, no vaccines against HCV infection have been developed to date, mainly because of the lack of suitable animal experimental systems.

We injected tupaias with serum from a chronic hepatitis C patient (HCR6; 3.7×10^4 50% chimpanzee infectious dose/ml) or reconstituted virus (RCV; genotype 1b). Inoculation with patient serum caused marked fluctuations in the serum alanine aminotransferase (ALT) concentrations − from 2–5 fold in both tupaias − suggesting acute hepatitis (Figs. 4 and 5). Quantitation of viral RNA by reverse transcription PCR revealed HCV viremia in *Tupaia* (Tup. 5 and 6, Fig. 5A). Inoculation with RCV showed sustained viremia for up to 10 weeks (Tup. 4 and 8; Fig. 5B). Histological examination re-

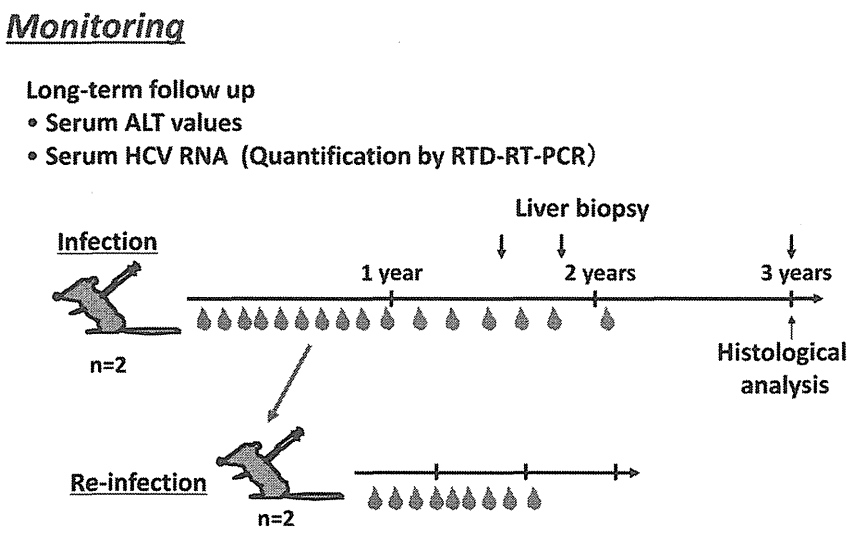

Fig. 4. Experimental design of HCV infection and re-infection of tupaia.

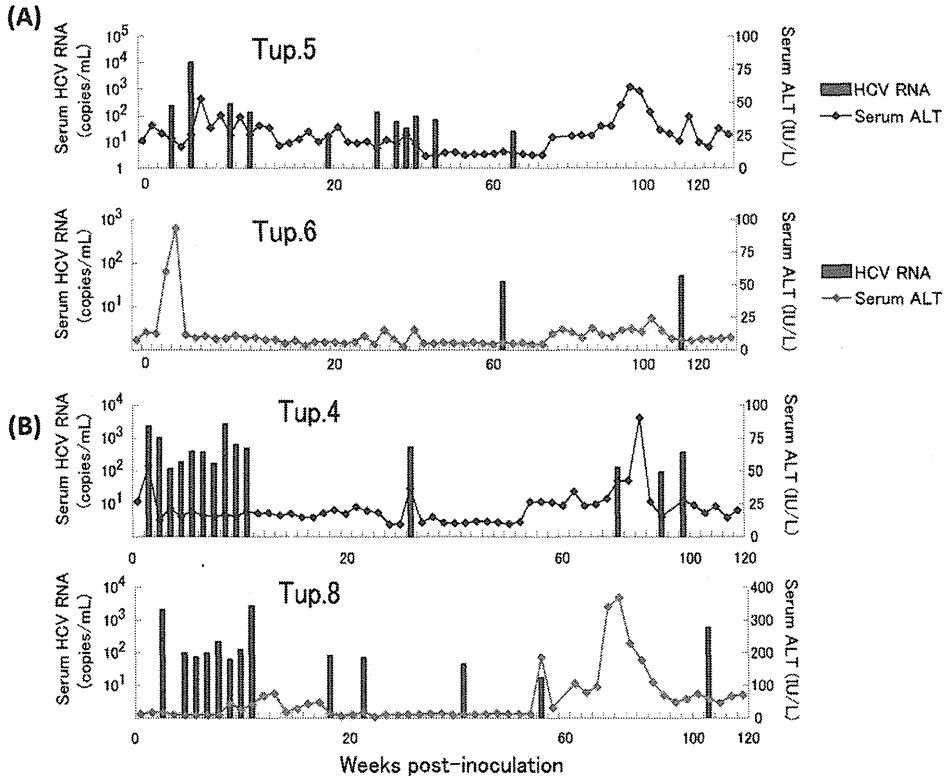

Fig. 5. Course of HCV infection in tupaia. (A) Tupaias No. 5 and 6 were inoculated with patient serum HCR6. Serum ALT (IU/ml) and viral loads, measured as amount of HCV RNA (copies/ml), were measured for over 120 weeks. Set point for serum ALT in untreated tupaias was 22.3 IL/ml (n=23). Negative control animals showed no significant ALT fluctuations for more than 2 years (n=3). No HCV RNA was detected in the negative controls after more than 2 years (n=3). (B) Tupaias No. 4 and 8 were inoculated with RCV as for the HCR6 inoculated animals.

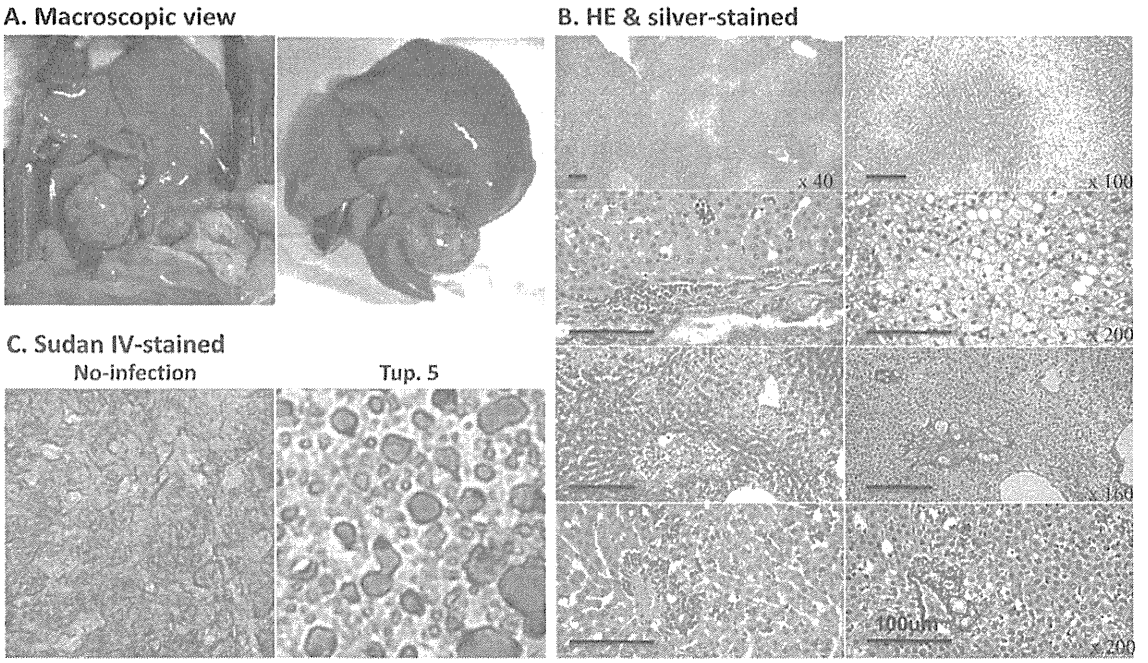

Fig. 6. (A) Macroscopic view of liver inoculated with patient serum HCR6 after 2 years (Tup.5, serum ALT value was 25 IU/l at autopsy). (B) HE staining (×40, ×100, ×200; scale bars indicate **) and silver staining (×160, ×200) of liver tissue (Tup 5) were indicated. Lymphocytic infiltration, steatosis and fibrogenesis were observed. (C) Sudan IV staining of the liver tissue of Tup5 (right) and non-infection (left).

vealed that HCV caused chronic hepatitis, fibrosis and cirrhosis (Fig. 6), with progressive lipid degeneration observed in tupaias over the course of infection. Macroscopic observations also indicated that liver cirrhosis worsened and large surface nodules were observed (Fig. 6). Transmission of viral RNA-positive serum to naïve animals reproduced acute hepatitis and viremia, indicating that HCV infection could reproduce the pathogenesis typically associated with acute and chronic hepatitis in tupaia. However, sustained seroconversion was not observed in tupaia and production of HCV and antibody only occurred at specific time points. To increase the susceptibility of tupaia to HCV infection and to develop a sensitive HCV infection model, these differences between HCV infection in tupaias and humans should be examined in future. HCV infection studies in tupaias have been examined using x-rays [41] and metabolic analysis [31], and the efficacy of natural products for treating HCV-infected tupaia has also been evaluated [39].

Several groups have successfully infected tupaias with HBV, as follows. In culture medium, infection by HBV has been shown to produce HBs antigen (Ag) and HBeAg. HBV infection in newborn and adult tupaias induced the production of HBsAg, HBsAb, HBcAb and HBeAb; all of the adults were successfully infected [34]. Experimental infection of tupaias with HBV was successful in approximately 55% of the animals inoculated [38]. HBV infection and aflatoxin B1 exhibited a synergistic effect in hepatocarcinogenesis [21]. To establish chronic infection by HBV, newborn tree shrews were infected with HBV [36]. Six of 46 newborn babies were found to be susceptible to HBV infection at 48 weeks post inoculation. Histological analysis of liver tissues from infected tupaias revealed chronic hepatitis symptoms, such as hydropic, fatty and eosinophilic degeneration of hepatocytes, lymphocytic infiltration, and hyperplasia of small bile ducts in the portal area [28]. One tupaia infected with HBV for more than 6 years showed multiple necrotic areas [28]. These findings show that although the efficacy of infection needs to be improved in future, tupaias are potentially well suited for use as a model for HBV infection.

Tupaias have also been reported to be infected by

specific viruses, such as tupaia herpes virus, which induces tumorigenicity [4], and potentially with non-pathogenic tupaia paramyxovirus [32]. Tupaias have also been infected with TTV [26], tupaia adenovirus [3], and influenza virus [40].

Conclusion

Tupaia shares considerable genetic homology with both humans and primates, and is considered to be well suited for use as a model for studies on viral infection and preclinical drug development. At present, difficulties associated with maintaining and handling tupaia are major factors limiting the widespread adoption of this animal for use in infection studies. However, optimizing these issues will facilitate the use of tupaias as an experimental animal. In addition, development of genetic methods for modifying the tupaia genome would also increase the potential value of tupaia as a model animal, as this would facilitate detailed studies of virus pathogenesis and drug evaluation.

Acknowledgments

We thank the staff of the Department of Animal Hygiene in the Joint Faculty of Veterinary Medicine at Kagoshima University, and at the Department of Microbiology and Cell Biology of The Tokyo Metropolitan Institute of Medical Science for their assistance with animal care, especially Dr. Yutaka Amako. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan and the Ministry of Health, Labour and Welfare of Japan.

References

1. Alter, M.J., Kruszon-Moran, D., Nainan, O.V., McQuillan, G.M., Gao, F., Moyer, L.A., Kaslow, R.A., and Margolis, H.S. 1999. The prevalence of hepatitis C virus infection in the United States, 1988 through 1994. *N. Engl. J. Med.* 341: 556–562. [Medline] [CrossRef]
2. Amako, Y., Tsukiyama-Kohara, K., Katsume, A., Hirata, Y., Sekiguchi, S., Tobita, Y., Hayashi, Y., Hishima, T., Funata, N., Yonekawa, H., and Kohara, M. 2010. Pathogenesis of hepatitis C virus infection in Tupaia belangeri. *J. Virol.* 84: 303–311. [Medline] [CrossRef]
3. Brinckmann, U., Darai, G., and Flügel, R.M. 1983. Tupaia (tree shrew) adenovirus DNA: sequence of the left-hand fragment corresponding to the transforming early region of human adenoviruses. *EMBO J.* 2: 2185–2188. [Medline]
4. Darai, G., Koch, H.G., Flügel, R.M., and Gelderblom, H. 1982. Tree shrew (Tupaia) herpesviruses. *Dev. Biol. Stand.* 52: 39–51. [Medline]
5. Deininger, P.L., Jolly, D.J., Rubin, C.M., Friedmann, T., and Schmid, C.W. 1981. Base sequence studies of 300 nucleotide renatured repeated human DNA clones. *J. Mol. Biol.* 151: 17–33. [Medline] [CrossRef]
6. Drummer, H.E., Wilson, K.A., and Poumbourios, P. 2002. Identification of the hepatitis C virus E2 glycoprotein binding site on the large extracellular loop of CD81. *J. Virol.* 76: 11143–11147. [Medline] [CrossRef]
7. Eliot, O. 1971. Bibliography of the tree shrews. *Primates* 12: 323–414.
8. Fan, Y., Huang, Z.Y., Cao, C.C., Chen, C.S., Chen, Y.X., Fan, D.D., He, J., Hou, H.L., Hu, L., Hu, X.T., Jiang, X.T., Lai, R., Lang, Y.S., Liang, B., Liao, S.G., Mu, D., Ma, Y.Y., Niu, Y.Y., Sun, X.Q., Xia, J.Q., Xiao, J., Xiong, Z.Q., Xu, L., Yang, L., Zhang, Y., Zhao, W., Zhao, X.D., Zheng, Y.T., Zhou, J.M., Zhu, Y.B., Zhang, G.J., Wang, J., and Yao, Y.G. 2013. Genome of the Chinese tree shrew. *Nat. Commun.* 4: 1426. [Medline] [CrossRef]
9. Fuchs, E., Kramer, M., Hermes, B., Netter, P., and Hiemke, C. 1996. Psychosocial stress in tree shrews: clomipramine counteracts behavioral and endocrine changes. *Pharmacol. Biochem. Behav.* 54: 219–228. [Medline] [CrossRef]
10. Fuchs, E. and Flügge, G. 2002. Social stress in tree shrews: effects on physiology, brain function, and behavior of subordinate individuals. *Pharmacol. Biochem. Behav.* 73: 247–258. [Medline] [CrossRef]
11. Fuchs, E. 2005. Social stress in tree shrews as an animal model of depression: an example of a behavioral model of a CNS disorder. *CNS Spectr.* 10: 182–190. [Medline]
12. Han, J.B., Zhang, G.H., Duan, Y., Ma, J.P., Zhang, X.H., Luo, R.H., Lü, L.B., and Zheng, Y.T. 2011. [Sero-epidemiology of six viruses natural infection in *Tupaia belangeri chinensis*]. *Zool. Res.* 32: 11–16 (in Chinese). [Medline]
13. Helgen, K.M. Order Scandentia, 2005. *Tupaia belangeri.* In: Mammal Species of the World: A Taxonomic and Geographic Reference, 3rd ed. (Wilson, D.E., and Reeder, D.M., eds.), Vol. 1, pp. 104–108. John Hopkins University Press.
14. Higginbottom, A., Quinn, E.R., Kuo, C.C., Flint, M., Wilson, L.H., Bianchi, E., Nicosia, A., Monk, P.N., McKeating, J.A., and Levy, S. 2000. Identification of amino acid residues in CD81 critical for interaction with hepatitis C virus envelope glycoprotein E2. *J. Virol.* 74: 3642–3649. [Medline] [CrossRef]
15. Inoue, K., Umehara, T., Ruegg, U.T., Yasui, F., Watanabe, T., Yasuda, H., Dumont, J.M., Scalfaro, P., Yoshiba, M., and Kohara, M. 2007. Evaluation of a cyclophilin inhibitor in hepatitis C virus-infected chimeric mice in vivo. *Hepatology* 45: 921–928. [Medline] [CrossRef]
16. Jeulin, H., Velay, A., Murray, J., and Schvoerer, E. 2013. Clinical impact of hepatitis B and C virus envelope glycoproteins. *World J. Gastroenterol.* 19: 654–664. [Medline] [CrossRef]
17. Kriegs, J.O., Churakov, G., Jurka, J., Brosius, J., and Schmitz, J. 2007. Evolutionary history of 7SL RNA-derived SINEs in Supraprimates. *Trends Genet.* 23: 158–161. [Medline] [CrossRef]
18. Krayev, A.S., Kramerov, D.A., Skryabin, K.G., Ryskov, A.P.,

Bayev, A.A., and Georgiev, G.P. 1980. The nucleotide sequence of the ubiquitous repetitive DNA sequence B1 complementary to the most abundant class of mouse fold-back RNA. *Nucleic Acids Res.* 8: 1201–1215. [Medline] [CrossRef]
19. Kumar, S. and Hedges, S.B. 1998. A molecular timescale for vertebrate evolution. *Nature* 392: 917–920. [Medline] [CrossRef]
20. Le Gros Clark, W.E.1924. On the brain of the tree shrew (*Tupaia minor*). In: *Proc. Zoolo. Soc., London.* pp. 1053–1074.
21. Li, Y., Su, J.J., Qin, L.L., Yang, C., Ban, K.C., and Yan, R.Q. 1999. Synergistic effect of hepatitis B virus and aflatoxin B1 in hepatocarcinogenesis in tree shrews. *Ann. Acad. Med. Singapore* 28: 67–71. [Medline]
22. Martin, R.D. 1968. Reproduction and ontogeny in tree-shrews (Tupaia belangeri), with reference to the general behaviour and taxonomic relationships. *Z. Tierpsychol.* 25: 409–495. [Medline] [CrossRef]
23. Martin, R.D. 1968. Reproduction and ontogeny in tree-shrews (Tupaia belangeri), with reference to their general behavior and taxonomic relationships. *Z. Tierpsychol.* 25: 505–532.
24. Nakagawa, S., Hirata, Y., Kameyama, T., Tokunaga, Y., Nishito, Y., Hirabayashi, K., Yano, J., Ochiya, T., Tateno, C., Tanaka, Y., Mizokami, M., Tsukiyama-Kohara, K., Inoue, K., Yoshiba, M., Takaoka, A., and Kohara, M. 2013. Targeted induction of interferon-λ in humanized chimeric mouse liver abrogates hepatotropic virus infection. *PLoS ONE* 8: e59611. [Medline] [CrossRef]
25. Nishihara, H., Terai, Y., and Okada, N. 2002. Characterization of novel Alu- and tRNA-related SINEs from the tree shrew and evolutionary implications of their origins. *Mol. Biol. Evol.* 19: 1964–1972. [Medline] [CrossRef]
26. Okamoto, H., Nishizawa, T., Takahashi, M., Tawara, A., Peng, Y., Kishimoto, J., and Wang, Y. 2001. Genomic and evolutionary characterization of TT virus (TTV) in tupaias and comparison with species-specific TTVs in humans and non-human primates. *J. Gen. Virol.* 82: 2041–2050. [Medline]
27. Quentin, Y. 1994. A master sequence related to a free left Alu monomer (FLAM) at the origin of the B1 family in rodent genomes. *Nucleic Acids Res.* 22: 2222–2227. [Medline] [CrossRef]
28. Ruan, P., Yang, C., Su, J., Cao, J., Ou, C., Luo, C., Tang, Y., Wang, Q., Yang, F., Shi, J., Lu, X., Zhu, L., Qin, H., Sun, W., Lao, Y., and Li, Y. 2013. Histopathological changes in the liver of tree shrew (Tupaia belangeri chinensis) persistently infected with hepatitis B virus. *Virol. J.* 10: 333. [Medline] [CrossRef]
29. Schinazi, R., Halfon, P., Marcellin, P., and Asselah, T. 2014. HCV direct-acting antiviral agents: the best interferon-free combinations. *Liver Int.* 34: 69–78. [Medline] [CrossRef]
30. Sekiguchi, S., Kimura, K., Chiyo, T., Ohtsuki, T., Tobita, Y., Tokunaga, Y., Yasui, F., Tsukiyama-Kohara, K., Wakita, T., Tanaka, T., Miyasaka, M., Mizuno, K., Hayashi, Y., Hishima, T., Matsushima, K., and Kohara, M. 2012. Immunization with a recombinant vaccinia virus that encodes nonstructural proteins of the hepatitis C virus suppresses viral protein levels in mouse liver. *PLoS ONE* 7: e51656. [Medline] [CrossRef]
31. Sun, H., Zhang, A., Yan, G., Piao, C., Li, W., Sun, C., Wu, X., Li, X., Chen, Y., and Wang, X. 2013. Metabolomic analysis of key regulatory metabolites in hepatitis C virus-infected tree shrews. *Mol. Cell. Proteomics* 12: 710–719. [Medline] [CrossRef]
32. Tidona, C.A., Kurz, H.W., Gelderblom, H.R., and Darai, G. 1999. Isolation and molecular characterization of a novel cytopathogenic paramyxovirus from tree shrews. *Virology* 258: 425–434. [Medline] [CrossRef]
33. Walter, P. and Blobel, G. 1982. Signal recognition particle contains a 7S RNA essential for protein translocation across the endoplasmic reticulum. *Nature* 299: 691–698. [Medline] [CrossRef]
34. Walter, E., Keist, R., Niederöst, B., Pult, I., and Blum, H.E. 1996. Hepatitis B virus infection of tupaia hepatocytes in vitro and in vivo. *Hepatology* 24: 1–5. [Medline]
35. Wasley, A. and Alter, M.J. 2000. Epidemiology of hepatitis C: geographic differences and temporal trends. *Semin. Liver Dis.* 20: 1–16. [Medline] [CrossRef]
36. Wang, Q., Schwarzenberger, P., Yang, F., Zhang, J., Su, J., Yang, C., Cao, J., Ou, C., Liang, L., Shi, J., Yang, F., Wang, D., Wang, J., Wang, X., Ruan, P., and Li, Y. 2012. Experimental chronic hepatitis B infection of neonatal tree shrews (Tupaia belangeri chinensis): a model to study molecular causes for susceptibility and disease progression to chronic hepatitis in humans. *Virol. J.* 9: 170. [Medline] [CrossRef]
37. Yan, H., Zhong, G., Xu, G., He, W., Jing, Z., Gao, Z., Huang, Y., Qi, Y., Peng, B., Wang, H., Fu, L., Song, M., Chen, P., Gao, W., Ren, B., Sun, Y., Cai, T., Feng, X., Sui, J., and Li, W. 2012. Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. *Elife* 1: e00049. [Medline] [CrossRef]
38. Yan, R.Q., Su, J.J., Huang, D.R., Gan, Y.C., Yang, C., and Huang, G.H. 1996. Human hepatitis B virus and hepatocellular carcinoma. I. Experimental infection of tree shrews with hepatitis B virus. *J. Cancer Res. Clin. Oncol.* 122: 283–288. [Medline] [CrossRef]
39. Yang, M., Li, N., Li, F., Zhu, Q., Liu, X., Han, Q., Wang, Y., Chen, Y., Zeng, X., Lv, Y., Zhang, P., Yang, C., and Liu, Z. 2013. Xanthohumol, a main prenylated chalcone from hops, reduces liver damage and modulates oxidative reaction and apoptosis in hepatitis C virus infected Tupaia belangeri. *Int. Immunopharmacol.* 16: 466–474. [Medline] [CrossRef]
40. Yang, Z.F., Zhao, J., Zhu, Y.T., Wang, Y.T., Liu, R., Zhao, S.S., Li, R.F., Yang, C.G., Li, J.Q., and Zhong, N.S. 2013. The tree shrew provides a useful alternative model for the study of influenza H1N1 virus. *Virol. J.* 10: 111. [Medline] [CrossRef]
41. Xie, Z.C., Riezu-Boj, J.I., Lasarte, J.J., Guillen, J., Su, J.H., Civeira, M.P., and Prieto, J. 1998. Transmission of hepatitis C virus infection to tree shrews. *Virology* 244: 513–520. [Medline] [CrossRef]

VIM-2014-0039-ver9-Zare-Bidaki_1P
Type: review-article

VIRAL IMMUNOLOGY
Volume 27, Number 7, 2014
© Mary Ann Liebert, Inc.
Pp. 1–6
DOI: 10.1089/vim.2014.0039

Toll-like Receptor 4 and Hepatitis B Infection: Molecular Mechanisms and Pathogenesis

Mohammad Zare-Bidaki,[1] Kyoko Tsukiyama-Kohara,[2] and Mohammad Kazemi Arababadi[1]

Abstract

AU1 ▶ Hepatitis B virus (HBV) infection mainly causes liver disease, including inflammation, cirrhosis, and hepatocellular carcinoma (HCC). It has been documented that prolonged hepatitis B–infected patients are unable to clear HBV from hepatocytes completely. Previous investigations have suggested that various genetic and immunologic parameters may be responsible for the induction of prolonged infection forms. Toll-like receptors (TLRs), as members of pathogen recognition receptors (PRRs), play critical roles in the recognition of viruses and the induction of appropriate immune responses. Thus, TLRs may be considered as essential sensors for the recognition of HBV and the induction of immune responses against this virus. It has been documented that TLR4 plays key roles in the detection of several microbial pathogen-associated molecular pattern molecules, including bacterial lipopolysacharide (LPS), as well as endogenous ligands (damage-associated molecular pattern molecules) and subsequently activates pro-inflammatory transcription factors in either MYD88 or TRIF dependent pathways. Previous investigations have proposed that TLR4 might be involved in appropriate immune responses against HBV. Therefore, the aim of this review is to present the recent data regarding the important roles of TLR4 in HBV recognition and regulation of immune responses against this virus, and also its roles in the pathogenesis of cirrhosis and HCC as complications of prolonged hepatitis B infections.

Introduction

In humans, hepatitis B virus (HBV) is the most dangerous and prevalent infectious agent of the liver, which induces hepatitis B (8,9,33). It has been documented that long-term HBV-infected patients with chronic, asymptomatic, and occult HBV infection are unable to eradicate HBV from either hepatocytes or sera completely (6,13). Previous investigations have suggested that prolonged HBV infections are the main causes of liver cirrhosis and hepatocellular carcinoma (HCC) (42,43). Although the main mechanisms responsible for the development of prolonged forms of HBV infection are yet to be clarified, it has been hypothesized that genetic and immunologic differences between subjects who successfully clear HBV infections (clearance group) and patients who suffer from prolonged forms of hepatitis B may be the main reasons for the persistent infection (1,6,8). Toll-like receptors (TLRs) belong to the family of pathogen recognition receptors (PRR). These molecules play crucial roles in pathogen-associated molecular pattern (PAMP) and damage-associated molecular pattern (DAMP) recognition, and subsequently induce immune responses against viral infections, including hepatitis B (34).

TLR/PAMP or DAMP interactions result in various immune cell functions ranging from production of inflammatory cytokines (26), migration to the infected sites (44), and phagocytosis (25), to activation of the NADPH oxidase pathway (10). Accordingly, TLR4 activation leads to upregulation of major histocompatibility complex (MHC), inflammatory cytokines, and homing molecules via the recognition of extracellular viral PAMPs in either MYD88 or TRIF dependent pathways (28). Thus, defected expression of TLR4 may lead to impaired immune responses against HBV infection. Because patients with prolonged forms of HBV are unable to clear HBV from hepatocytes completely (4,7,32), it seems that the immune system of these patients is attenuated in some parameters. Based on the crucial roles played by TLR4 in the viral PAMPs recognition and induction of immune responses, it may be hypothesized that alterations in TLR4 expression levels may lead to HBV survival in the infected liver of long-term HBV-infected patients. Therefore, the aim of this review article is to consider recent information regarding the relationship between TLR4 and HBV infection, as well as its crucial roles in the development of prolonged hepatitis B infections. This review also presents recent information regarding the

[1]Immunology of Infectious Diseases Research Center, Rafsanjan University of Medical Sciences, Rafsanjan, Iran.
[2]Transboundary Animal Diseases Centre, Joint Faculty of Veterinary Medicine, Kagoshima University, Kagoshima, Japan.

VIM-2014-0039-ver9-Zare-Bidaki_1P.3d 06/27/14 10:11pm Page 2

plausible mechanisms leading to alteration in expression of TLR4 and its signaling molecules in these patients.

TLR4 Signaling Pathway

TLR4, which is also known as CD284 and age-related macular degeneration10 (ARMD10), has been determined as a receptor for several microbial PAMPs. The gene of TLR4 is located on 9q33.1 and, like other TLRs, is highly conserved (47,66). The TLR4 molecule contains three domain complexes, including extracellular leucine-rich repeats (LRRs), hydrophobic transmembrane, and cytoplasmic toll/ interleukin-1 receptor (TIR) domains (Fig. 1). Classically, TLR4 recognizes the microbial lipids in homodimer format, and thus activates various intracellular signaling pathways. It has been established that TLR4 interacts with various ligands, which leads to activation of intracellular signaling pathways (65). TLR4 is unique among the TLRs in its ability to combine TRIF and MYD88 adapters, which leads to transcription from pro-inflammatory cytokines such as IL-12, IL-6, TNF-α, and type I interferons (65). In the MYD88-dependent pathway, dimerization of TLR4 with myeloid differentiation 2 (TLR4-MD2) at the cell surface results in recruitment of two adaptor proteins—TIRAP (MAL) and MYD88—which results in activation of NFκB as a pro-inflammatory transcription factor and consequently production of pro-inflammatory cytokines (30). Additionally, TLR4 is internalized into endosomes and recruits

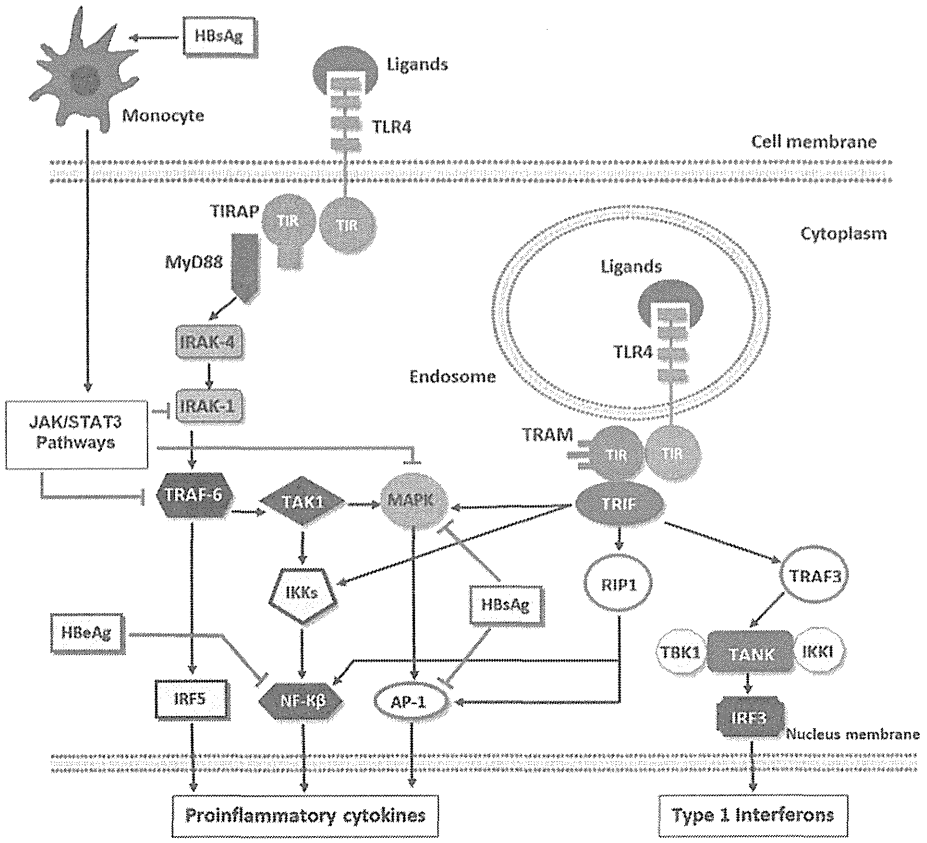

FIG. 1. The intracellular signaling of toll-like receptor 4 (TLR4) and inhibitory effects of HBV. The figure shows how TLR4/ligands interactions in cytoplasmic and endosomes membranes lead to intracellular signaling via either myeloid differentiation primary response (MYD88) or TIR-domain-containing adapter-inducing interferon-β (TRIF), respectively. The figure also shows that HBsAg indirectly suppresses TLR4 signaling via promotion of monocytes to produce IL-10. HBsAg also directly inhibits mitogen-activated protein kinase (MAPK) and activator protein 1 (AP-1) molecules. Hepatitis e antigen (HBeAg) suppresses nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) activation. HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; PRR, pathogen recognition receptors; RIP1, receptor-interacting protein 1; IRAK1, interleukin-1 receptor associated kinase-1; TRAM, TRIF-related adaptor molecule; TIRAP, TIR domain-containing adaptor protein; TRAF6, TNF receptor associated factor; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; TAK1, transforming growth factor b-activated kinase 1; TBK1, TANK-binding kinase 1; IKK, IκB kinase; IKKi, IκB kinase inhibitor.

TRAM and TRIF, which activates another transcription factor, IRF3, and induces transcription from type I interferons (31). Lipopolysacharide (LPS), as the most important TLR4 ligand, is recognized by the TLR4/CD14/MD2 complex. Following LPS secretion, LPS binding protein (LBP) binds LPS to CD14 (a glycosylphosphatidylinositol-anchored protein). CD14 transfers the LPS to MD2. MD2 is a soluble protein that is associated with the extracellular domain of TLR4 in a noncovalent format. LPS/MD-2 binding leads to a conformational change in MD-2 and results in binding of TLR4-MD-2 complex to a second TLR4 receptor. Hence, TLR4 homodimerization is assembled in intracellular signaling, which is discussed in the next section. In addition to LPS, several molecules are also recognized by TLR4 such as high-mobility group box-1, hyaluronan, heat shock protein 60, free fatty acids, allergenic nickel, and the adjuvant monophosphoryl lipid A (MPLA) (2,22). Interestingly, the proposed endogenous TLR4 ligands are not only capable of activating TLR4 directly, but could also bind and transport LPS, which increases sensitivity of cells to LPS. Therefore, it appears that many endogenous TLR4 ligands may be described as PAMP binding/sensitizing molecules (21).

It has been documented that TLR4/ligands interactions lead to the recruitment of TIR-containing adaptor molecules, including TIRAP and MYD88, which participate in stimulation of TLR4-MYD88–dependent signaling pathway, and TRAM and TRIF, which play important roles in the induction of TLR4-TRIF–dependent signaling pathway (12). In MYD88-dependent signaling pathway, TLR4/ligands interaction at the plasma membrane results in binding of TIRAP, which allows AU2 ▶ MyD88 to be recruited as an adaptor protein. Recruited MYD88 leads to phosphorylation of several intracellular signaling molecules, which results in activation of NF-κB, AP-1, and IRF5 transcription in response to several pro-inflammatory cytokines (Fig. 1) (65). Interestingly, upon internalization of TLR4 and interaction with its ligands in the endosomes, TRIF-dependent signaling pathway is activated, resulting in TRAM binding to TIR domain of TLR4 (23). TRIF is an adaptor protein that is recruited to the binding of TRAM and activates (phosphorylates) TRAF3 and RIP1 molecules (23). Phosphorylation of TRAF3 leads to activation of IRF3 and translocation into the nucleus to produce transcripts in response to type I interferons, while phosphorylation of RIP1 leads to activation of other transcription factors, including AP-1 and NF-κB (Fig. 1) (39).

TLR4 and Hepatitis B

According to the fact that expression levels of TLR4 are altered in HBV-infected patients, it appears that TLR4 plays essential roles in hepatitis B infection. For instance, Wei *et al.* revealed that expression levels of TLR4 were increased in the hepatocytes of chronic HBV-infected patients (59). Xing *et al.* demonstrated that TLR4 was upregulated AU3 ▶ on the monocytes of patients with liver cirrhosis and ~~acute on chronic liver failure~~ when compared to healthy controls (64). Another study reported that expression levels of TLR4 were upregulated on monocytes of HBV-infected patients when compared with healthy controls (67). Therefore, it may be concluded that TLR4 activations not only participate in induction of immune responses but also participate in the pathogenesis of hepatitis B–related complications, including cirrhosis. Additionally, previous investigations revealed that patients with prolonged forms of HBV infection are unable to elicit appropriate immune responses against HBV. Hence, the infection persists (5,48). According to the critical roles of TLR4 in the induction of immune responses, it seems that the expression of TLR4 may be impaired in patients infected with prolonged forms of HBV. Chen *et al.* reported that mRNA levels of TLR4 were significantly decreased in PBMCs from chronic HBV-infected patients in a selected Chinese population (16). Another study from China revealed that the expression levels of TLR4 on peripheral monocytes, hepatocytes, and Kupffer cells did not differ between untreated HBeAg positive in comparison to HBeAg negative chronic HBV-infected patients (55). Heiberg *et al.* identified that chronic HBV-infected patients displayed lower IFN-α secretion in comparison to healthy controls, following stimulation with specific ligands for TLR4 (24). Therefore, it appears that TLR4 is downregulated in long-term HBV-infected patients. Some evidence shows that TLR4/ligands interaction may be a main route to activation of immune responses against HBV. For example, it has been identified that TLR4 is expressed on the hepatocytes, sinusoidal endothelial cells, biliary epithelial cells, and hepatic dendritic cells, which is capable of responses to LPS (49,53). Additionally, Wu *et al.* revealed that the supernatants from TLR4-stimulated Kupffer cells from C57/BL6 wild-type mice can suppress HBV replication (62). Zhou *et al.* reported that upregulation of TLR4 in proximal tubular epithelial cells led to inhibition of expression of HBV antigen, as well as HBV-DNA replication (69). Moreover, another study revealed that TLR4 suppresses HBV replication via upregulation of inducible nitric oxide synthase (iNOS) (14,15). Isogawa *et al.* also demonstrated that administration of ligands specific for TLR4 results in inhibition of HBV replication (27). Furthermore, the pivotal roles played by TLR4 in promotion of homing of HBV specific CD8+ T-lymphocyte to the infected liver were reported by John *et al.* (29). It has also been evidenced that LPS level in serum has a positive relation with the severity of clinical presentation in HBV-infected patients (45). Therefore, it appears that TLR4 plays important roles in the induction of appropriate immune responses against HBV and upregulation of endogenous LPS during hepatitis B, confirming this hypothesis. Due to the aforementioned studies, it appears that TLR4 and its signaling pathways can be considered as important mechanisms to fight HBV. Additionally, according to the aforementioned studies, it seems that TLR4 is downregulated in prolonged forms of hepatitis B. Accordingly, it can be hypothesized that three main mechanisms may be responsible for downregulation of TLR4 in prolonged HBV-infected patients. First, HBV and its related antigens may interfere with TLR4 expression directly. A study by Wu *et al.* confirms the direct effects of HBsAg on the expression of TLR4. They show that HBsAg is capable of suppressing NF-κB, as a TLR4 downstream signaling molecule, leading to virus escape from immune surveillance (63). Wang *et al.* revealed that HBsAg could inhibit the JNK-MAPK pathway (57). Lang *et al.* reported that HBeAg suppressed TIR-mediated activation of NF-kB via disruption of homotypic TIR/TIR interactions (35). Wilson *et al.* also reported that HBeAg blocked NF-kB activation in hepatocytes (60).