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Anti-fibrogenic function of angiotensin II type 2 receptor in CCl_4-induced liver fibrosis ☆,☆☆

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Abstract

The renin-angiotensin system (RAS) contributes to fibrogenesis in a variety of organs. We recently showed that a lack of angiotensin (Ang) II type 1 (AT1) receptor activity reduces liver fibrosis. In this study, we investigated whether the Ang II type 2 (AT2) receptor is implicated in the development of liver fibrosis. A comparison was made between AT2-receptor knockout (AT2KO) and wild type (WT) mice after 4 weeks of treatment with carbon tetrachloride (CCl_4). Fibrosis was assessed by Azan–Mallory staining and hepatic hydroxyproline (HP) content. The expression of fibrogenic mRNA was measured by real-time quantitative reverse-transcription polymerase chain reaction (PCR). Liver fibrosis evaluated by regular histological analyses and immunohistochemical α-SMA staining was observed in both groups of mice. The extent of fibrosis was greatest in the AT2KO mice. Fibrosis was associated with increases in hepatic HP content and mRNA expression for TGF-β1 and α-SMA, as well as an increase in hepatic TBARS. These findings suggest that CCl_4 induces oxidative stress which leads to activation of hepatic stellate cells (HSCs). These changes were considerably more pronounced in the AT2KO mice than the WT mice. Taken together, we conclude that AT2 signal has anti-fibrogenic and/or cytoprotective effects on oxidative stress-induced liver fibrosis. We therefore suggest that RAS-associated liver fibrogenesis may be determined by the balance between AT1 and AT2 signals.

Keywords: Renin-angiotensin system (RAS); Angiotensin II type 2 receptor; Liver fibrosis; Oxidative stress

Recent studies have suggested that the renin-angiotensin system (RAS), in addition to having a major role in the regulation of blood pressure and body-fluid homeostasis, is also involved in hepatic fibrogenesis [1–3]. During hepatic fibrogenesis, hepatic stellate cells (HSCs), which are key producers of the extracellular matrix (ECM) during liver injury, proliferate and acquire the characteristics of contractile cells (myofibroblasts) [4]. There is evidence from animal models that blocking angiotensin (Ang) II, the main effector of the RAS, with agents such as angiotensin-converting-enzyme (ACE) inhibitors and Ang-receptor antagonists, induces regression and prevents the development

☆ *Abbreviations:* Ang II, angiotensin II; AT1, Ang II type 1; AT2, Ang II type 2; α-SMA, α smooth-muscle actin; RAS, renin-angiotensin system; CCl_4, carbon tetrachloride; TBARS, thiobarbituric acid-reactive substances; TGF-β1, transforming growth factor β1; HSCs, hepatic stellate cells; HP, hydroxyproline.

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of hepatic fibrosis [5,6]. *In vitro* studies have shown that Ang II type 1 (AT1) receptor is expressed in activated human HSCs and that Ang II may induce the contraction and proliferation of HSCs [7].

Angiotensin II receptors can be differentiated pharmacologically into two distinct types, designated as type 1 (AT1) or type 2 (AT2) receptors. While the majority of the well-known effects of Ang II on the cardiovascular system and renal function are mediated through the AT1 receptor, little is known on the role of the AT2 receptor [8]. However, it is generally accepted that AT2 signals decrease oxidative stress in various tissues [9].

An animal model such as the AT2-receptor knockout (AT2KO) mouse, in which a single receptor is completely eliminated by gene targeting, has provided a new approach to the investigation of receptor regulation and function. Although this mouse model has provided considerable information on the role of these receptors in the cardiovascular system [10,11], it has not been used for the study of hepatic fibrogenesis.

We reported recently that the RAS is involved in hepatic fibrogenesis based on the results of experiments on AT1-receptor knockout (AT1KO) mice. These experiments also showed that Ang II stimulates HSCs to induce monocyte chemoattractant protein (MCP)-1 which is known to amplify hepatic inflammation [12,13]. In the cardiovascular system, AT1 and AT2 receptors are known to have different functions and it has been demonstrated that their functional balance has pathophysiological importance in the development of atherosclerosis [14]. However, little is known about the role of AT2 receptor in liver fibrogenesis. The present study in AT2KO mice therefore had the aim of clarifying whether AT2 receptor has a role in liver fibrogenesis.

Materials and methods

Animals and experimental design. AT2KO mice with a C57BL/6 genetic background were provided by Horiuchi et al. [15], and C57BL/6 mice were obtained from Hiroshima Jikken Doubutsu (Hiroshima, Japan). The experiments were performed on mice at 8–10 weeks of age. During the study, the mice were allowed free access to food and water and were housed at a constant temperature, with a 12-h light/dark cycle. To assess the necrotic and inflammatory changes caused by exposure to carbon tetrachloride (CCl_4) (Wako Pure Chemical Industries, Osaka, Japan), a single intraperitoneal injection was administered at a dose of 1 mL/kg (1:1 in mineral oil). Liver fibrosis was induced by injecting CCl_4 (1 mL/kg) subcutaneously twice weekly for 4 weeks. The mice were sacrificed and blood samples collected 3 days following the last administration for measurement of transaminases and serum thiobarbituric acid-reactive substances (TBARS) [16,17]. Liver samples were harvested and either frozen rapidly in liquid nitrogen for storage at −80 °C or fixed with 4%-paraformaldehyde-periodate-lysine (PLP) solution for histological examination. All animal procedures were carried out in accordance with our institutional guidelines.

Histological examination. The liver samples were fixed by perfusion with 4% PLP solution and then transferred successively into solutions of 10–20% sucrose in 0.1 mol/L phosphate buffer, followed by embedding in paraffin. Sections of 5-μm thickness were then prepared for either hematoxylin and eosin (H and E) or Azan–Mallory stains. Immunohistochemistry for α smooth-muscle actin (α-SMA) was carried out using a monoclonal mouse anti-human smooth-muscle-actin antibody (Dako, Japan) and the Vector M.O.M. Immunodetection Kit (Vector Laboratories, USA) according to the manufacturer's instructions. For the histological examinations, several fields on each slide were selected randomly, with the representative results from three animals being recorded.

Assay of serum transaminases. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using standard methods.

Serum and liver thiobarbituric acid-reactive substances (TBARS). Serum and hepatic TBARS content was measured using the OXI-TEK Assay Kit with some modifications [18,19]. Liver samples (30 mg) were hydrolyzed in NaCl. After centrifugation at 10,000 rpm for 15 min, 40 μL of the supernatant was neutralized. The samples were incubated at 98 °C for 60 min, followed by centrifugation at 3000 rpm for 15 min. The samples were then transferred to a flat-bottomed microtiter plate and the absorbance measured at 560 nm.

Hepatic hydroxyproline content. Hepatic hydroxyproline (HP) content was measured using Kivirikko's method [20], with some modifications. Briefly, liver tissue (50 mg) was hydrolyzed in 6 mol/L HCl at 110 °C for 24 h in a glass test tube. After centrifugation at 3000 rpm for 10 min, 2 mL of the supernatant was neutralized with 8 N KOH. Two grams of KCl and 1 mL of 0.5 mol/L borate buffer were then added to the resultant solution, followed by incubation for 15 min at room temperature and a further incubation for 15 min at 0 °C. Freshly prepared chloramine-T solution was then added and incubated at 0 °C for 1 h, followed by the addition of 2 mL of 3.6 mol/L sodium thiosulfate. The samples were incubated at 120 °C for 30 min and then 3 mL toluene was added with incubation for a further 20 min at room temperature. After centrifugation at 2000 rpm for 5 min, 2 mL of the supernatant was added to 0.8 mL buffer containing Ehrlich's reagent and incubated for 30 min at room temperature. The samples were then transferred to a flat-bottomed microtiter plate and the absorbance was measured at 560 nm. The results were expressed as the magnitude of increase relative to the average HP of liver samples from untreated mice.

Real-time reverse-transcription polymerase chain reaction. Messenger RNA (mRNA) was isolated using a RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Single-stranded complementary DNA (cDNA) was then synthesized by the polymerase chain reaction from 1 μg of mRNA using 0.5 nmol of random primers. Real-time quantitative reverse-transcription polymerase chain reaction (PCR) was performed as described previously [21] using a SYBR green I kit (MJ Research, Inc., Waltham, MA, USA) and Premix Ex TaqTM (Takara Bio Inc., Shiga, Japan). The synthesized cDNA was amplified using the following sequence specific primers. For the AT1 receptor the primers were 5′-AGTCGC ACTCAAGCCTGTCT-3′ (forward) and 5′-ACTGGTCCTTTGGTC GTGAG-3′ (reverse); for the AT2 receptor 5′-CCTGCATGAGTG TCGATAGGT-3′ (forward) and 5′-CCAGCAGACCACTGAGC ATA-3′ (reverse); for β-actin 5′-CCTGTATGCCTCTGGTCGTA-3′ (forward) and 5′-CCATCTCCTGCTCGAAGTCT-3′ (reverse) [22]; for transforming growth factor β1 (TGF-β1), 5′-GCCCTGGATACCAACT ATTGC-3′ (forward) and 5′-GCAGGAGCGCACAATCATGTT-3′ (reverse) [23]; and for α-SMA, 5′-CTGGAGAAGAGCTACGAACTGC-3′ (forward) and 5′-CTGATCCACATCTGCTGGAAGG-3′ (reverse) [24]. The steady-state levels of TGF-β1 and α-SMA mRNA were assessed by real-time PCR, using β-actin as the control.

Statistical analysis. The results were expressed as means ± SD. Statistical analysis was performed using one-way analysis of variance (ANOVA) with p-values <0.05 being considered significant.

Results

AT1 and AT2 receptor mRNA expression in the liver

Steady-state hepatic expression of AT1 receptor mRNA did not change after 4 weeks of CCl_4 administration in either WT or AT2KO mice. The levels of expression were similar in the AT2KO mice compared with the WT control mice (Fig. 1). Expression of AT2 receptor mRNA could

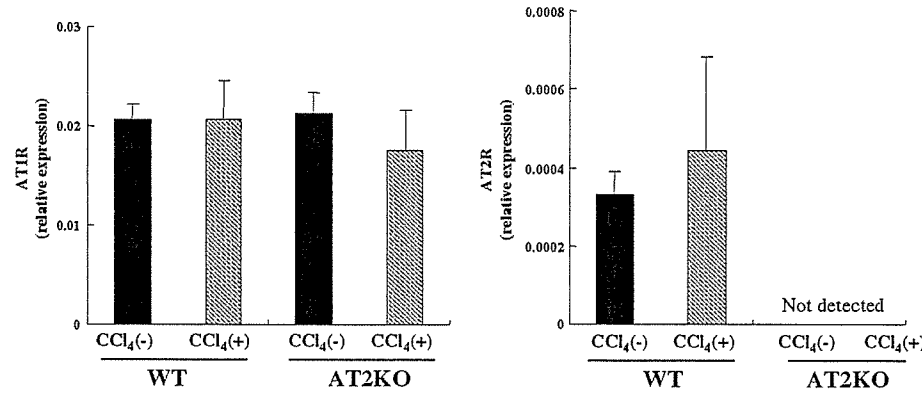

Fig. 1. Steady-state hepatic AT1 and AT2 mRNA levels in WT and AT2KO mice. These results are shown relative to β-actin using real-time PCR. mRNA levels in normal liver and liver after 4 weeks of CCl_4 treatment. After 4 weeks of CCl_4 treatment, AT1 mRNA levels in both WT and AT2KO mice were not changed, AT2 mRNA levels in the WT mice were not increased, and AT2 mRNA was not expressed in the AT2KO mice. Each value represents the mean ± SD of six mice per group.

not be detected in the AT2KO mice, whereas minute levels of expression were observed in the normal livers of WT mice. These low levels did not change after CCl_4 administration. The expression of mRNA expression for hepatic AT1 and AT2 receptors in the AT2KO mice was in a continuously unbalanced state.

Parameters of inflammation

Serum AST and ALT levels were measured in order to determine whether the absence of the AT2 receptor had any influence on inflammation caused by chronic exposure to CCl_4. After administration of CCl_4 for 4 weeks, there were significant differences in the level of serum transaminases between the WT and AT2KO mice, with markedly higher levels being measured in the AT2KO mice (Fig. 2). Following an acute single exposure to CCl_4, the level of serum transaminases in the AT2KO mice was higher than in the WT mice (data not shown).

Histological findings after chronic CCl_4 administration

Inflammatory-cell infiltrates were evident around the portal tracts in the livers of mice treated with CCl_4 for 4 weeks. Mononuclear cells represented the major infiltrating cell type and were more prevalent in the AT2KO mice than in the WT mice (Fig. 3A). In addition to the inflammatory changes, liver fibrosis was found in both the AT2KO and WT mice, with more severe changes, such as the formation of nodules and bridging fibrosis, being observed in the AT2KO mice (Fig. 3B). In agreement with these observations, the expression of α-SMA, an indicator of HSC activation, was detected at high levels in the fibrotic septa of AT2KO mice, whereas it was negligible in the WT mice (Fig. 3C).

Hepatic hydroxyproline content

As the HP content in the liver is known to parallel the extent of fibrosis, this amino acid was measured in order

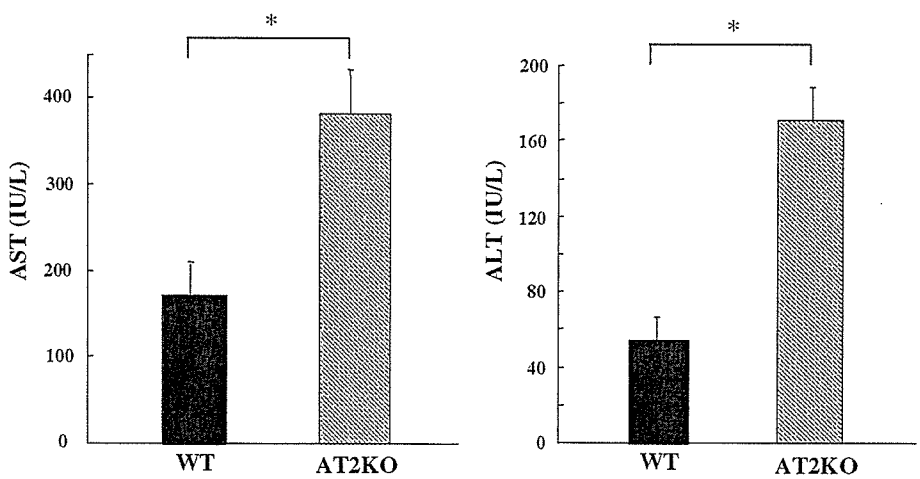

Fig. 2. Effect of 4 weeks of CCl_4 treatment on serum levels of transaminases in WT and AT2KO mice. Chronic serum samples were collected to monitor the effects of the twice-weekly subcutaneous injection of CCl_4 (1 mL/kg) for 4 weeks, and the mice were sacrificed three days after the last injection. Significant differences in serum transaminase levels were found between the two groups ($*p < 0.05$). Serum AST (IU/L) in the AT2KO mice was 2-fold higher, and serum ALT (IU/L) was 3-fold higher than the corresponding values in the WT mice. Each value represents the mean ± SD of six mice per group.

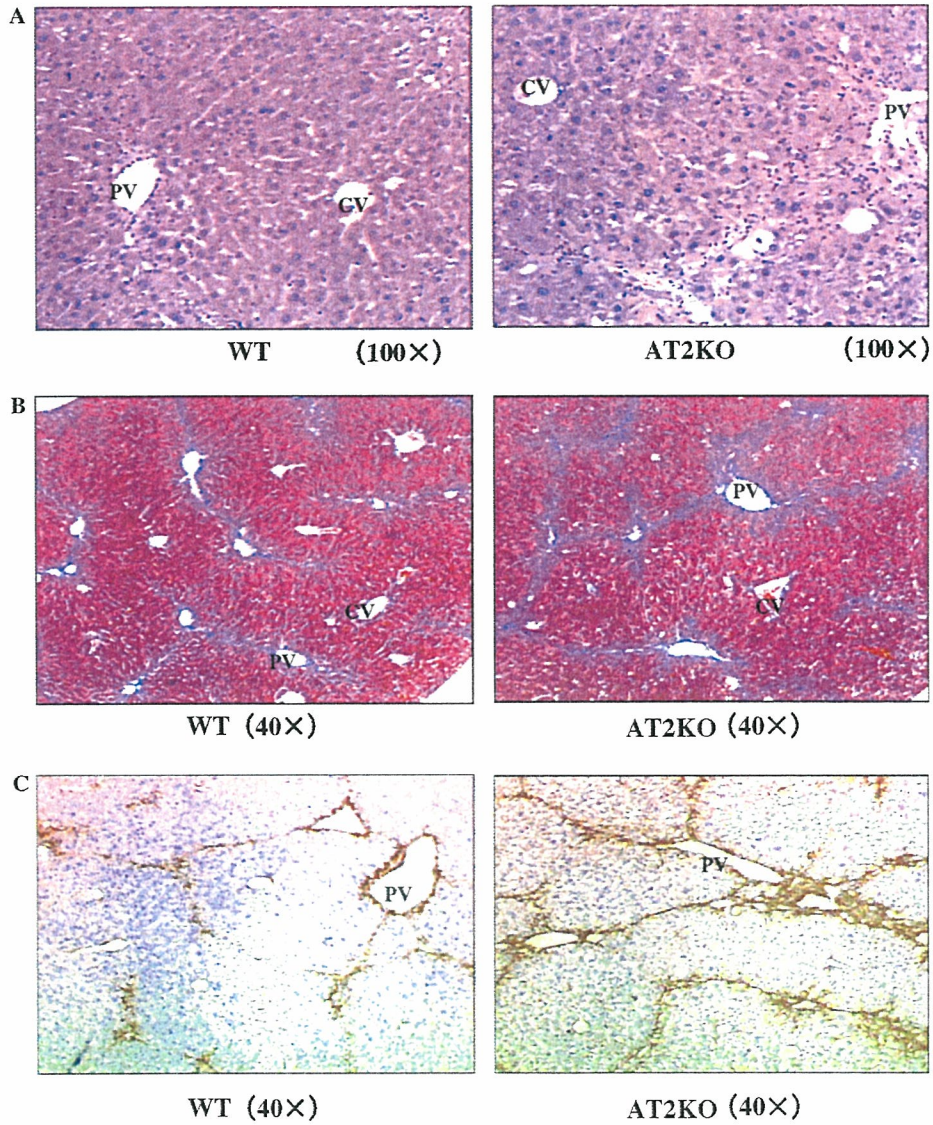

Fig. 3. Representative photomicrographs of the liver after 4 weeks of treatment with CCl_4 [1.0 mL/kg] in WT and AT2KO mice. Paraffin-embedded sections were processed for H and E staining (A; original magnification = 100×), inflammatory cell infiltrates were evident around the portal tracts. Mononuclear cells represented the major infiltrating cell type and were more prevalent in the WT mice than the AT2KO mice. In Azan–Mallory staining (B; original magnification = 40×; PV = portal vein and CV = central vein), the livers of the WT mice showed formation of nodules and bridging fibrosis, and fibrosis was found in the AT2KO mice. Sections were subjected to α-SMA immunostaining (C; original magnification = 100×; PV = portal vein and CV = central vein), the expression of α-SMA, an indicator of HSC activation, was strongly detected in the fibrotic septa of the AT2KO mice, but less so in the WT mice.

to assess the degree of hepatic fibrosis. While the WT mice treated with CCl_4 for 4 weeks had a 5.9-fold increase in HP content compared with the untreated WT mice, the CCl_4-treated AT2KO mice had a 9.0-fold increase compared with the untreated AT2KO mice (Fig. 4). These data indicated that hepatic fibrosis in the AT2KO mice was more severe than in the WT mice.

TGF-β1 and α-SMA mRNA expression

As TGF-β1 and α-SMA are thought to be a central fibrogenic factor and a reliable indicator of HSCs activation, respectively, the transcription levels of these two molecules were analyzed using real-time PCR (Fig. 5). The steady-state levels of TGF-β1 and α-SMA mRNA were significantly higher in the AT2KO mice compared with levels in the WT mice.

Serum and hepatic contents of thiobarbituric acid-reactive substances (TBARS)

TBARS are an established parameter for evaluating the extent of oxidative stress. Serum TBARS levels were increased markedly by chronic CCl_4 administration in both AT2KO mice and WT mice, with significantly higher levels being measured in the CCl_4-treated AT2KO mice

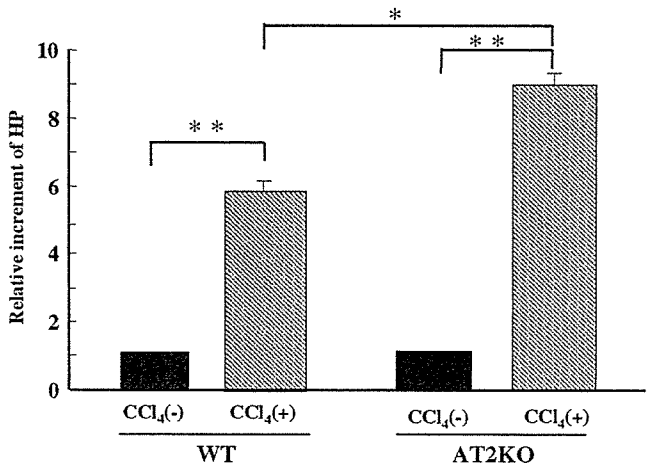

Fig. 4. Hepatic hydroxyproline content after 4 weeks of CCl_4 treatment. The HP content of the liver was significantly increased by CCl_4 treatment in both AT2KO (9.0-fold) and WT (5.9-fold) mice, but more so in the former ($^*p < 0.05$, $^{**}p < 0.01$). These changes were calculated on the basis of the average HP content of untreated livers from the WT and AT2KO mice. Each value represents the mean ± SD of six mice per group.

compared with the treated WT mice (Fig. 6). Interestingly, elevation of hepatic TBARS was evident only in the AT2KO mice.

Discussion

In this study, we provide evidence for the enhancement of CCl_4-induced liver damage in AT2KO mice. This damage was associated with increases in hepatic mRNA expression of TGF-β1 and α-SMA. In addition, hepatic ROS generation was increased in the AT2KO mice, suggesting that the AT2 receptor has a role in reducing oxidative stress and subsequent liver damage. These observations indicate that the AT2 signal has anti-fibrogenesis and/or cytoprotective roles against oxidative stress-induced liver fibrogenesis. Therefore, we presume that RAS-associated liver fibrogenesis may be determined by the balance between the AT1 and AT2 signals.

An important finding in this study was that while mRNA expression for the AT2 receptor was detected in the liver from WT mice, the level of expression was very low (Fig. 1). In agreement with our data, a previous study reported expression of AT2 receptor in human HSCs [25] at the mRNA level but not at the protein level. Presumably, the AT1 receptor may have a predominant role in the liver similar to that observed in other tissues under physiological circumstances. Despite the relatively low expression of the AT2 receptor, it is believed that this receptor has an opposite role to that of the AT1 receptor and that the balance between AT1 and AT2 signaling contributes to the patho-physiology in various tissues including myocardial hypertrophy, fibroblast proliferation, and vascular cell hyperplasia [26]. In this regard, we clarified the pathogenic role of AT2 signal in the liver, using AT2KO mice with CCl_4-induced liver damage. As shown in Fig. 2, we showed that liver damage caused by CCl_4 administration was markedly higher in the AT2KO mice compared with the WT mice. In a previous report we demonstrated that CCl_4-induced liver damage was reduced in AT1KO mice, indicating that these changes were mediated by AT1 signaling [12]. Accordingly, the fact that we observed a marked increase in liver damage in the AT2KO mice treated with CCl_4 indicated that AT2 signaling suppressed CCl_4-induced liver damage mediated by the AT1 signal. Therefore, the balance between AT1 and AT2 signals may play a role in liver patho-physiology similar to that found in other tissues.

As shown in Fig. 3, CCl_4-induced liver damage was associated with liver fibrogenesis in the WT mice. These fibrogenic changes were increased in the livers of the AT2KO

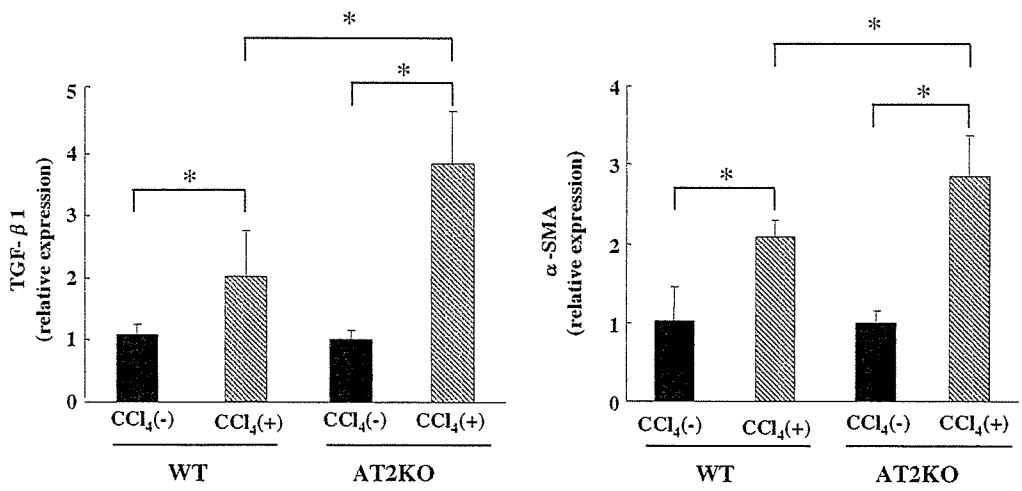

Fig. 5. Steady-state hepatic TGF-β1 and α-SMA mRNA levels in WT and AT2KO mice after 4 weeks of CCl_4 treatment. These results are shown relative to those of the housekeeping gene β-actin by real-time PCR. TGF-β1 and α-SMA mRNA levels are shown. TGF-β1 and α-SMA mRNA expressions of the liver were significantly increased by CCl_4 treatment in both the AT2KO and WT mice, but more so in the former ($^*p < 0.05$). Each value represents the mean ± SD of four mice per group.

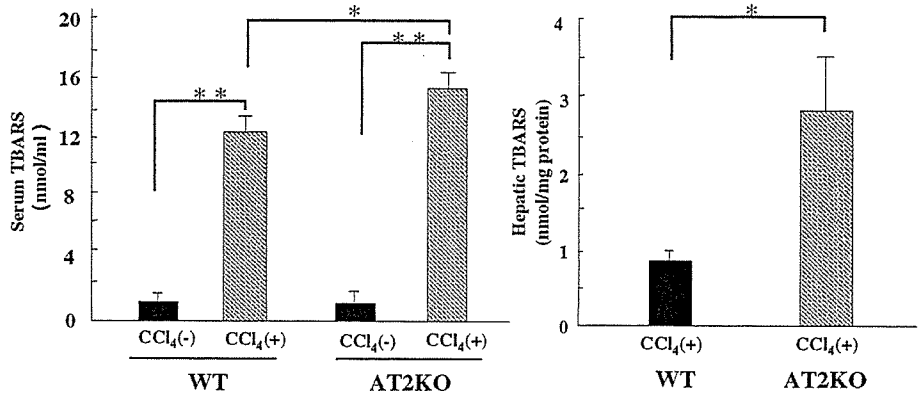

Fig. 6. Serum and hepatic contents of TBARS after 4 weeks of CCl_4 treatment. Serum and hepatic samples were collected to monitor the effects of the twice-weekly subcutaneous injection of CCl_4 (1 mL/kg) over 4 weeks, and the mice were sacrificed three days after the last administration. Serum TBARS levels increased in both the AT2KO (15.0-fold) and WT mice (12.0-fold), with higher levels evident in the former ($^*p < 0.05$, $^{**}p < 0.01$). Hepatic TBARS elevation was evident only in the AT2KO mice (2.8-fold, $p < 0.05$). Each value represents the mean ± SD of six mice per group.

mice. The lack of the AT2 receptor resulted in enhanced liver damage as well as subsequent hepatic fibrosis in the animal model. In addition, we found that HP content was significantly higher in the liver of the AT2KO mice compared with the WT mice (Fig. 4). Similarly, mRNA expression of TGF-β1 and α-SMA was significantly higher in the AT2KO mice compared with the WT mice (Fig. 5). In our previous report, CCl_4-induced liver fibrogenesis was shown to be mediated partly by the AT1 signal as the fibrogenic changes were not as pronounced in the AT1KO mice. Our current data provide further evidence that the AT2 signal suppresses AT1 signaling-mediated liver fibrogenesis. Several other recent studies have also determined the fibrogenic role of Ang II and its receptors in several organs. Ang II was reported to induce cardiac and renal fibrosis, a change that was shown to be prevented by Ang II inhibitors [27–31]. These studies suggested that Ang II and its receptors are involved in the process of cardiac and renal fibrogenesis. Furthermore, we demonstrated recently that the AT2 receptor exerts anti-proliferative effects and pro-apoptotic changes in vascular smooth-muscle cells by counteracting the action of the AT1 receptor in the process of neointimal formation after vascular injury. This finding suggests that the balance between AT1 and AT2 signals has a functional role in the development of atherosclerosis [14]. The data of the current study indicate that the balance between AT1 and AT2 signals also has a role in liver fibrogenesis similar to that found in other tissues.

The underlying mechanism of the fibrogenic action determined by the balance between AT1/AT2 signals is of clinical importance. In this study, enhancement of oxidative stress possibly induced by AT1/AT2 signal imbalance was investigated as a potential pathogenic candidate. As there is evidence that the hepatotoxicity of CCl_4 generates highly reactive trichloromethyl free radicals, leading to lipid peroxidation and membrane damage [32], TBARS levels were used as a marker of oxidative stress and hepatocyte-derived lipid peroxidative products [33] and as shown in Fig. 6 increased with the progression of the CCl_4-induced liver fibrosis. In various other investigations, oxidative stress was demonstrated to activate HSCs [34–36], which in turn induced the progression of liver fibrosis. In fact, we found that elevated levels of serum and liver TBARS were associated with increased α-SMA expression, indicating that general and local oxidative stress was increased in the AT2KO mice. These increases in general and local oxidative stresses found in the AT2KO mice suggest an enhancement of AT1 signal and subsequent progression of liver fibrosis. The production of TGF-β1, a potent fibrocytokine, has been reported to be induced in the livers of mice treated with CCl_4 [37]. In our study, the AT2KO mice had significantly higher expressions of TGF-β1 compared with WT mice, suggesting that the absence of AT2 receptor increased the production of TGF-β1.

In conclusion, the enhancement of liver fibrogenesis found in the AT2KO mice suggests that the AT2 signal acts as an anti-fibrogenic and/or cytoprotective factor against CCl_4-induced liver fibrosis in this mouse model. This anti-fibrogenic action of the AT2 signal is likely to be mediated by the induction of oxidative stress in both the liver and the circulating blood. Taken together with previous data showing that AT1 signal contributes to the progression of hepatic fibrosis, our study indicates that the AT1 and AT2 signals have somewhat reverse functions, and that an imbalance in the activity of the two receptors may lead to liver fibrosis by increasing oxidative stress.

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Serine palmitoyltransferase inhibitor suppresses HCV replication in a mouse model

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Abstract

Serine palmitoyltransferase (SPT) is a first-step enzyme in the sphingolipid biosynthetic pathway. Myriocin is an inhibitor of SPT and suppresses replication of the hepatitis C virus (HCV) replicon. However, it is still unknown whether this SPT inhibitor suppresses HCV replication in vivo. We investigated the anti-HCV effect of myriocin against intact HCV using chimeric mice with humanized liver infected with HCV genotype 1a or 1b. We administered myriocin into HCV infected chimeric mice and succeeded in reducing the HCV RNA levels in serum and liver to 1/10–1/100 of the levels prior to the 8 day treatment. Furthermore, combined treatment with pegylated interferon reduced the HCV RNA levels to less than 1/1000 of the control levels. We strongly suggest that suppression of SPT reduces HCV replication, and therefore that the SPT inhibitor is potentially a novel drug in the treatment of HCV infection.

Keywords: HCV; Myriocin; Serine palmitoyltransferase; Lipid raft; Chimeric mice with humanized liver

Hepatitis C virus (HCV) infection usually causes chronic hepatitis and often leads to cirrhosis of the liver or hepatocellular carcinoma [1,2]. The number of carriers now amounts to approximately 3% (~170 million) of the population worldwide. The most effective treatment against HCV infection is a combination of pegylated interferon (PEG-IFN) and ribavirin [3,4]. However, many people cannot tolerate the serious side effects and thus the number of patients able to receive the therapy is limited. The development of novel drugs to treat HCV with greater safety and better efficacy is therefore urgently required.

HCV is a single-stranded RNA virus that belongs to the Flaviviridae family [5]. The RNA genome produces at least 10 viral proteins, which include structural and non-structural (NS) proteins. The former are involved in the formation of the HCV particle. The latter play a key role in HCV genome replication [6]. It is generally accepted that a complex of NS proteins is associated with the lipid raft on the Golgi and endoplasmic reticulum membranes, where HCV replication occurs [7,8]. Thus, disruption of assembly of the lipid raft may lead to suppression of HCV replication.

Myriocin (ISP-1) is a specific inhibitor of serine palmitoyltransferase (SPT), a first-step enzyme in the sphingolipid biosynthetic pathway (Fig. 1A; [9,10]). Myriocin inhibits SPT activity due to its structural similarity to sphingosine (Fig. 1B), resulting in decreased intercellular sphingomyelin and its intermediates, dihydrosphingosine, sphingosine, ceramide, and sphingosine-1-phosphate (Fig. 1A). Inhibition of SPT by myriocin is thought to eventually lead to disruption of lipid raft assembly, as sphingomyelin is one of the major integral components of its assembly [11].

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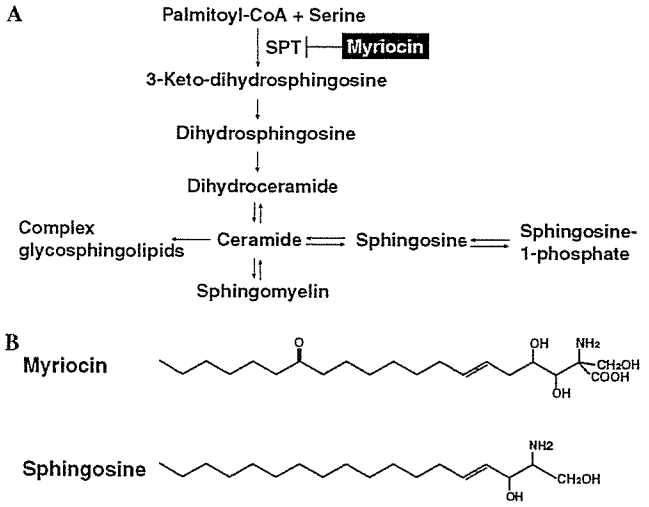

Fig. 1. Sphingolipid biosynthetic pathway. (A) Biosynthesis pathway of sphingolipid. (B) Structure of myriocin and sphingosine.

Previously, we isolated the compound NA255, which suppresses replication of the HCV subgenomic replicon [12]. NA255 is structurally similar to myriocin and inhibits the enzymatic activity of SPT, resulting in suppression of replication without affecting the enzyme activities of HCV NS3 (protease and helicase) or NS5B (RNA-dependent RNA polymerase). Thus, replication of the HCV subgenomic replicon was suppressed by NA255 in response to the decrease in amount of sphingolipid, ceramide, and sphingomyelin. These findings suggest that NA255 disrupts assembly of the lipid raft associated with HCV NS proteins. In the present study, we performed comparative and concomitant trials of one SPT inhibitor, myriocin, and PEG-IFN in chimeric mice with humanized liver (chimeric mice) infected with intact HCV. The results demonstrate for the first time that suppression of SPT inhibits replication of intact HCV in vivo.

Materials and methods

Inhibition assay of replication in HCV replicon cells by myriocin. Myriocin (Sigma, St. Louis, MO, USA) was added in the growth medium of HCV subgenomic replicon cells FLR3-1 (genotype 1b, Con-1; [12]) at a final concentration of 0.2, 1.0, 3.9, 15.6 or 62.5 nM. After 72 h incubation, we performed luciferase assays using the Bright-Glo luciferase assay kit (Promega, Madison, WI, USA).

Measurement of cell viability using the Tetra Color One (WST-8) assay. Myriocin was added to FLR3-1 cells as described above. After 72 h incubation, cell viability was measured using the Tetra Color One kit (Seikagakukougyo, Tokyo, Japan) according to the manufacturer's instructions.

Immunoblotting analysis. Cells were harvested and lysed in lysis buffer (PBS containing 0.5% Triton X-100 and 0.5 mM PMSF), and then 5 μg of protein separated by 12% SDS–PAGE and electro-blotted onto a nitrocellulose membrane (Shleicher & Schuell, Dassel, Germany). A rabbit polyclonal anti-NS3 antibody [12] and anti-actin (20–33) antibody (Sigma, St. Louis, MO, USA) were used as the primary antibodies. The proteins were then detected by an anti-rabbit antibody HRP-linked IgG (Cell Signaling Technology, Beverly, MA, USA).

Immunofluorescent staining of HCV replicon cells. After treatment of 250 nM myriocin for 72 h, FLR3-1 cells were probed with a primary antibody, an anti-NS3 polyclonal antibody, after blocking with TNB blocking buffer (Perkin-Elmer, Wellesley, MA, USA). Next, an anti-rabbit IgG-Alexa-488 conjugate (Invitrogen, Carlsbad, CA, USA) was applied as the secondary antibody.

TLC analysis. Cells were incubated for 2 h with [^{14}C] serine (0.5 μCi/ml) in Opti-MEM (Invitrogen). After the cells were lysed with 0.1% SDS, and total lipids were extracted with chloroform/methanol (1:2 v/v). The extracts were spotted onto Silica Gel 60 thin-layer chromatography (TLC) plates (Merck, Darmstadt, Germany) and chromatographed with methyl acetate/1-propanol/chloroform/methanol/0.25% KCl (25:25:25:10:9, v/v). Radioactive spots were detected by BAS 2000 (Fuji Film, Kanagawa, Japan).

Complementation of sphingolipid intermediates. FLR3-1 cells were incubated with 1 or 2.5 μM of sphingolipid intermediates (dihydrosphingosine, sphingosine, or sphingosine-1-phosphate) and sequentially diluted myriocin then added. After 72 h, the IC_{50} of each combination was measured by the luciferase assay.

Infection of HCV genotype 1a and 1b in chimeric mice. Chimeric mice were purchased from PhenixBio Co., Ltd. (Hiroshima, Japan). The mice were generated by transplanting human primary hepatocytes into SCID mice carrying the urokinase plasminogen activator transgene controlled by an albumin promoter (*Alb-uPA*) [13–15]. Overexpression of this transgene induces a profoundly hypofibrinogenemic state and accelerated hepatocyte death. HCG9 (genotype 1a) and HCR6 (genotype 1b, Accession No: AY045702), originally from patient serum, were intravenously injected at 10^6 copies/mouse at about 40 days after transplantation of human hepatocytes. After 4 weeks, the HCV 1a and 1b RNA levels had reached ~10^8 copies/ml and ~10^7 copies/ml, respectively, in the mice serum.

Administration of myriocin and/or PEG-IFN into chimeric mice infected with HCV 1b. Injections of myriocin or PEG-IFN (Chugai, Tokyo, Japan) or both were administered to HCV genotype 1b (HCR6) infected mice and blood then collected according to the protocol in Table 2.

Quantification of HCV RNA by real-time PCR. Total RNA was purified from 1 μl of serum or 50 μg of liver tissue from chimeric mice using the AGPC method. HCV RNA was quantified by real-time PCR as previously reported [16].

Measurement of human albumin in the serum. Human albumin concentration was measured in 2 μl of serum using the Alb-II kit (Eiken Chemical, Tokyo, Japan) according to the manufacturer's instructions.

Detection of core protein in live tissue. We used chimeric mice with a high RNA levels of HCV genotype 1a (HCG9) in the serum to easily detect HCV RNA and core protein in liver tissue. We administered 2 mg/kg myriocin daily for 6 days to a chimeric 1a-4 mouse and extirpated the liver. For comparison, the livers of non-treated (1a-1), non-infected (1a-2), and PEG-IFN treated (1a-3) mice were also extirpated. The liver tissues were homogenized in RIPA buffer and 100 μg of total protein was used for the detection of core protein using the Ortho HCV core protein ELISA kit (Eiken Chemical).

Immunofluorescent and histological staining of chimeric mouse liver tissue. Liver sections from 1a-1 and 1a-4 mice were probed by biotinylated anti-HCV core protein monoclonal antibody, and human hepatocyte monoclonal antibody (Dako, Glostrup, Denmark) as the primary antibodies, followed by streptavidin-Alexa-488 (Invitrogen) and anti-mouse-IgG-Alexa-546 (Invitrogen). The nuclei were stained using DAPI. Biotinylated normal mouse IgG (Ancell, Bayport, MN, USA) was used as the negative control. For histological analysis, liver sections from 1a-1 and 1a-4 mice were stained by hematoxylin-eosin (H&E staining).

Results

Anti-HCV effect of the SPT inhibitor, myriocin

We examined the anti-HCV effect and cell toxicity of myriocin in the HCV subgenomic replicon cells FLR3-1. Luciferase activity was greatly decreased by myriocin in a dose-dependent manner without affecting cell viability

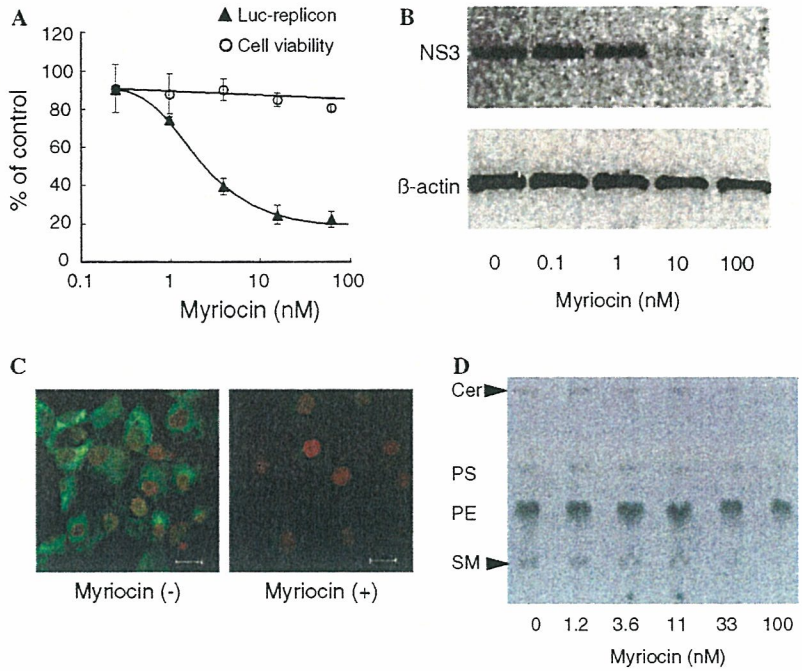

Fig. 2. Anti-HCV effect of myriocin in HCV replicon cells. (A) Luciferase activity and cell viability of FLR3-1 replicon cells in the presence of myriocin. Experiments were conducted independently at least three times. (B) Detection of NS3 protein by immnoblotting analysis. (C) Detection of NS3 protein by immunofluorescent staining in FLR3-1 cells. HCV NS3 protein and nucleus are shown in green and red, respectively. Scale bar: 20 μm. (D) De novo sphingolipid biosynthesis in the presence of myriocin was monitored by TLC. Cer, ceramide; PS, phosphatidylserine; PE, phosphatidylethanolamine; and SM, sphingomyelin.

Table 1
IC_{50} values of myriocin in the presence of sphingolipids

Supplement (μM)		IC_{50} of myriocin (nM)
Absence	0	5.8
Dihydrosphingosine	1.0	77.7
	2.5	>1000
Sphingosine	1.0	22.4
	2.5	>1000
Sphingosine-1-phosphate	1.0	14.7
	2.5	>1000

IC_{50} values of myriocin measured in the presence of 1, and 2.5 μM dihydrosphingosine, sphingosine or sphingosine-1-phosphate, show suppression of HCV replicon replication by myriocin.

(Fig. 2A) or cell growth (data not shown). The maximum inhibition rate was about 80% in the presence of over 62.5 nM myriocin (Fig. 2A), while the 50% inhibitory concentration (IC_{50}) was about 5.8 nM (Table 1). Reduction of NS3 protein, which plays a key role in HCV replication, was also observed by immunoblotting analysis and staining (Figs. 2B and C), suggesting that myriocin has a potent anti-HCV effect.

Relationship between sphingolipid metabolites and HCV replication

To examine the relationship between sphingolipid metabolites and HCV replication, we monitored de novo sphingolipid biosynthesis by FLR3-1 cells in the presence of myriocin. The production of both ceramide and sphingomyelin was inhibited in a dose-dependent manner, whereas production of the phosphatidylethanolamine and phosphatidylserine, metabolites of sphingosine, was unaffected (Fig. 2D). To confirm whether suppression of HCV subgenomic replicon replication was caused by sphingolipid depletion, we examined the anti-HCV effect of myriocin in the presence of three sphingolipids, dihydrosphingosine, sphingosine, and sphingosine-1-phosphate, which are intermediates in the sphingolipid biosynthesis pathway (Fig. 1A). Replication of the HCV replicon was recovered by complementation of the intermediate molecules of sphingolipid biosynthesis (Table 1). These results indicate that suppression of replication by myriocin was due to a reduction in sphingolipid biosynthesis.

Anti-HCV effects of myriocin and PEG-IFN in chimeric mice infected with HCV

The inhibitory ability of myriocin was investigated using chimeric mice infected with HCR6 (genotype 1b). We administered myriocin or PEG-IFN via intraperitoneal or subcutaneous injection, as shown in Table 2. In the myriocin-treated group, the HCV RNA levels were reduced in the serum from 3×10^6–1×10^7 copies/ml to 6×10^5–1×10^4 copies/ml over 8 days (an approximately 1/10–1/100 reduction). The same level of reduction was

Table 2
Administration schedule into chimeric mice infected with HCV genotype 1b

Day	−1	0	1	2	3	4	5	6	7	8
Collection of blood	B		B			B				B
PEG-IFN		I			I				I	
Myriocin		M	M	M	M	M	$M_{1/2}$	$M_{1/2}$	$M_{1/2}$	
Myriocin + PEG-IFN		M/I	M	M	M/I	$M_{1/2}$	$M_{1/2}$		I	

B, I or M indicates that each manipulation was performed as required, and administration of reagents was started from day zero. PEG-IFN was subcutaneously injected at 30 μg/kg. The amount of myriocin intraperitoneally injected was adjusted according to the body weight of the mice. Doses began at 1 mg/kg, and at a 10% reduction in body weight, the dose was reduced to 0.5 mg/kg ($M_{1/2}$). At 20% reduction, administration was discontinued.

observed in the PEG-IFN-treated group, which was injected with a 10-fold larger amount of PEG-IFN than that used in clinical treatment (30 μg/kg body weight). Moreover, combined treatment with myriocin and PEG-IFN reduced the HCV RNA levels to less than 1/1000 of the control levels, and in 2 (1b-7 and 1b-9) of 3 mice HCV RNA was not detected at all on day 8 (Fig. 3A). Concurrently, we monitored the concentration of human albumin (h-Alb) and found slight reductions only in the combined treatment group (Fig. 3B). These results indicate that myriocin suppressed replication of intact HCV without interfering with h-Alb expressed from humanized liver, whereas the combination of myriocin and PEG-IFN synergistically suppressed HCV replication with slight liver damage.

Detection of HCV RNA and core protein in the liver of chimeric mice

To clarify whether HCV was reduced by myriocin from the humanized liver, we examined the livers of the chimeric mice infected with another HCV genotype, 1a (HCG9). The RNA level of HCV 1a in serum reached approximately 1×10^8 copies/ml, which is 10-fold higher than that of HCV 1b (HCR6). Thus, we speculated that HCV core protein in hepatocytes would be easily detected by immunofluorescent staining. The liver of a 1a-4 mouse was extirpated after daily administration of 2 mg/kg of myriocin for 6 days. Following treatment, the HCV RNA level in the serum of the 1a-4 mouse fell to 1×10^5 copies/ml (Fig. 4A). The amount of HCV 1a RNA and core protein

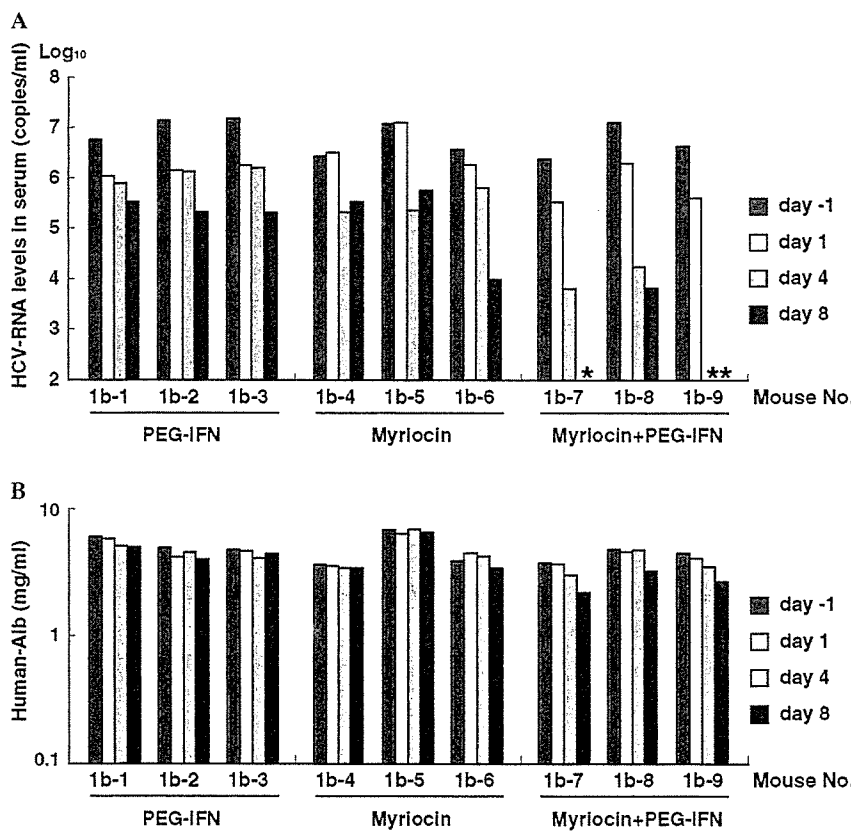

Fig. 3. Anti-HCV effect of myriocin in chimeric mice infected with HCV genotype 1b. (A) HCV RNA levels in the serum of chimeric mice. Asterisks indicate no HCV RNA was detected. (B) Human albumin concentrations in serum of chimeric mice.

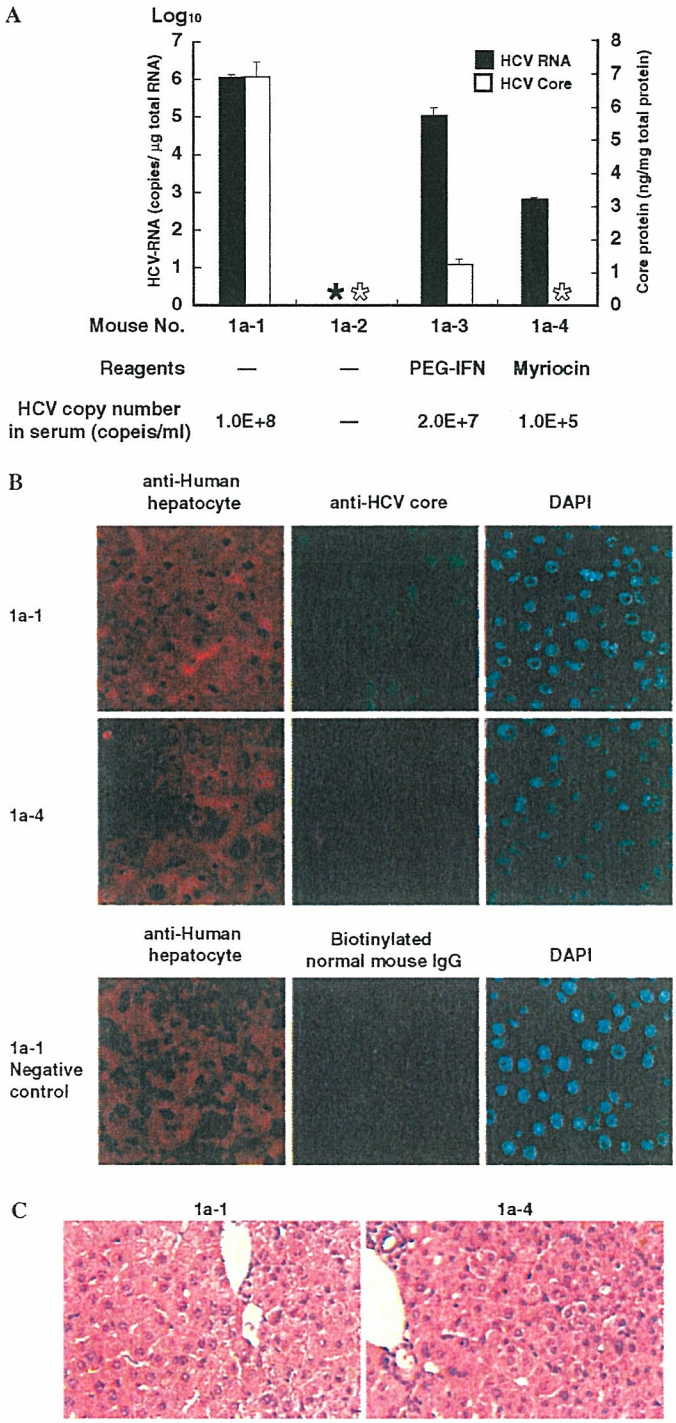

Fig. 4. Analysis of liver tissue from chimeric mice. HCV RNA and core protein were detected in the liver of chimeric mice infected with HCV genotype 1a. 1a-1, non-treated; 1a-2, non-treated and non-infected; 1a-3, PEG-IFN treated; and 1a-4, myriocin treated mouse. (A) Copy number of HCV RNA per 1 μg total RNA, and expression levels of HCV core proteins per 1 mg total protein. Asterisks indicate no HCV RNA or core proteins were detected. (B) Immunofluorescent staining of HCV core protein and human hepatocytes in chimeric mice liver. Human hepatocytes, HCV core protein, and nucleus are shown in red, green, and light blue, respectively. As a negative control of anti-core monoclonal antibody staining, the liver of 1a-1 was also stained with biotinylated normal mouse IgG. (C) H&E staining of liver tissue from chimeric mice 1a-1 and 1a-4. Primary human hepatocytes were observed in these mice and these hepatocytes displayed no significant morphological changes.

in the liver was quantified and had also reduced, as well as that in serum (Fig. 4A). Immunofluorescent staining revealed that the core protein of the non-treated mouse, 1a-1, which had 1×10^8 copies/ml serum (Fig. 4A), expressed a human hepatocyte moiety, whereas the core protein of the 1a-4 mouse disappeared (Fig. 4B). These results indicate that myriocin causes a decrease not only in HCV genotype 1b but also in genotype 1a, and eliminates HCV from the liver.

H&E staining of chimeric mouse liver

We performed histological analysis of the livers from non-treated (1a-1) and myriocin-treated (1a-4) mice (Fig. 4C). No significant morphological differences were observed between the tissues of the 1a-1 and 1a-4 mice. Thus, myriocin did not induce hepatocyte damage in chimeric mice to any biologically significant degree.

Discussion

In the present study, the SPT inhibitor myriocin was shown to inhibit replication of intact HCV in vivo. We initially investigated the fundamental inhibitory effects and mechanisms of myriocin against replication of the HCV replicon and found that inhibition of HCV replicon replication is compatible with a decrease in ceramide and sphingomyelin in the cells. The inhibitory effect of myriocin on replication of the HCV replicon differed slightly from that on de novo biosynthesis of ceramide and sphingomyelin. It has been previously reported that the membranous web formed in HCV replicon cells is the site of viral RNA synthesis and is not observed in naive HuH-7 cells [17]. Furthermore, HCV replication is known to occur on the lipid raft assembly [7]. The lipid raft associated with HCV replicase exists as an aberrant structure that forms a membranous web, and thus the lipid raft formed by HCV differs from the one in healthy cells. Therefore, we speculate that the above difference indicates that the lipid raft associated with HCV replicase is sensitively disrupted by myriocin.

Furthermore, HCV replication inhibition was complemented in the presence of the sphingomyelin biosynthetic intermediates dihydrosphingosine, sphingosine, and sphingosine-1-phosphate. These results indicate that depletion of sphingolipids induces disruption of the lipid raft assembly, resulting in suppression of replication of the HCV replicon. Disruption of the lipid raft assembly would lead to failure of HCV replicase to associate with the raft, and thus interrupt genome replication. This result is consistent with a previous study [12]. In order to demonstrate whether inhibition of SPT can be linked to anti-HCV therapy in vivo, we used a chimeric mouse model.

Recently, Mercer et al. developed a chimeric mouse containing human hepatocytes in which infection and replication of intact HCV occurs [14]. We examined the anti-HCV effect of myriocin in chimeric mice infected with HCV

genotypes 1a and 1b, and succeeded in the effective elimination of both HCV genotypes in their serum. Myriocin did not induce significant liver damage or interfere with the amount of human albumin in the chimeric mice or the ALT levels in Balb/c mice (data not shown), and thus the reduction in HCV RNA levels by myriocin is not due to induction of human hepatocyte damage. In the analysis of liver tissue infected with HCV genotype 1a, both HCV RNA and core protein levels were reduced by myriocin more effectively than by PEG-IFN. Thus, myriocin appears to be able to directly suppress replication of intact HCV in human hepatocytes, regardless of the HCV genotype. This is the first report of an SPT inhibitor suppressing intact HCV replication in vivo. Furthermore, combined treatment was more effective than myriocin or PEG-IFN alone, with HCV RNA levels reduced to less than 1/1000 of the controls, suggesting that myriocin with PEG-IFN cooperatively and synergistically inhibits the replication and proliferation of HCV.

Cholesterol is another major component of the lipid raft assembly, in addition to the sphingolipids [11]. The cholesterol biosynthetic pathway has also been a target for disruption of lipid raft assembly. However, recent studies have shown that the protein associated with geranlygeranylation, rather than cholesterol, is important for HCV replication [7,18,19]. Thus, it appears likely that disruption of lipid raft assembly is effectively caused by inhibition of sphingolipid biosynthesis rather than inhibition of cholesterol biosynthesis.

Myriocin is a known immunosuppressant [9], and mainly inhibits generation of cytotoxic T lymphocytes and T-cell dependent antibody production via inhibition of SPT activity in vivo. In chimeric mice deficient in both T and B cells (SCID), the immunosuppressant effect of myriocin does not cause a reduction in HCV replication but simply causes disruption of sphingolipid biosynthesis. In addition, the inhibitory mechanism in vivo, as in vitro analysis has shown, is likely to be disrupted by myriocin of the lipid raft assembly. Whether the sphingolipid level in liver in vivo is disrupted is currently unknown and the focus of future study. We continually monitored HCV for 14 days after the administration regimen and detected the same level of HCV as before the administration among all groups. Thus, to eliminate HCV completely, it will be necessary to adjust the dosage of myriocin and PEG-IFN and further extend the duration of administration.

In conclusion, we elucidated the mechanism of myriocin inhibition of HCV replication in vitro and determined that myriocin inhibits HCV replication in a chimeric mouse model with humanized liver. Although the toxicity of myriocin renders it unsuitable for use as an anti-HCV drug in human patients, our results suggest that SPT may be an effective target of drugs designed to inhibit HCV replication, and that SPT inhibitors such as myriocin are good candidates on which to base the development of new anti-HCV drugs.

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Clinical Studies

Measurement of hepatitis B virus core-related antigen is valuable for identifying patients who are at low risk of lamivudine resistance

Tanaka E, Matsumoto A, Suzuki F, Kobayashi M, Mizokami M, Tanaka Y, Okanoue T, Minami M, Chayama K, Imamura M, Yatsuhashi H, Nagaoka S, Yotsuyanagi H, Kawata S, Kimura T, Maki N, Iino S, Kiyosawa K, HBV Core-Related Antigen Study Group. Measurement of hepatitis B virus core-related antigen is valuable for identifying patients who are at low risk of lamivudine resistance.
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Abstract: *Objective:* The clinical usefulness of hepatitis B virus core-related antigen (HBVcrAg) assay was compared with that of HBV DNA assay in predicting the occurrence of lamivudine resistance in patients with chronic hepatitis B. *Patients:* Of a total of 81 patients who were treated with lamivudine, 25 (31%) developed lamivudine resistance during a median follow-up period of 19.3 months. *Results:* The pretreatment positive rate of HBe antigen, or pretreatment levels of HBVcrAg or HBV DNA did not differ between patients with and without lamivudine resistance. Levels of both HBVcrAg and HBV DNA decreased after the initiation of lamivudine administration; however, the level of HBVcrAg decreased significantly more slowly than that of HBV DNA. The occurrence of lamivudine resistance was significantly less frequent in the 56 patients whose HBV DNA level was less than 2.6 log copy/ml at 6 months of treatment than in the remaining 25 patients. The cumulative rate of lamivudine resistance was as high as 70% within 2 years in the latter group, while it was only 28% in the former group. Lamivudine resistance did not occur during the follow-up period in the 19 patients whose HBVcrAg level was less than 4.6 log U/ml at 6 months of treatment, while it did occur in 50% of the remaining patients within 2 years. *Conclusion:* These results suggest that measurement of HBV DNA is valuable for identifying patients who are at high risk of developing lamivudine resistance, and that, conversely, measurement of HBVcrAg is valuable for identifying those who are at low risk of lamivudine resistance.

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Lamivudine, a nucleoside analogue that inhibits reverse transcriptases, was first developed as an anti-viral agent against human immunodeficiency virus (HIV). It was later also found to be effective against hepatitis B virus (HBV) because HBV is a member of the Hepadnaviridae family of viruses, which use reverse transcriptases in their replication process (1, 2). Lamivudine was found to inhibit the replication of HBV, reduce hepatitis, and improve histological findings of the liver in long-term treatment (3–5). Furthermore, it has been shown that lamivudine treatment improves the long-term outcome of patients with chronic hepatitis B (6, 7). However, there are a number of problems with lamivudine therapy, such as relapse of hepatitis because of the appearance of YMDD mutant viruses and the reactivation of hepatitis after discontinuation of the treatment (8–11).

The concentration of HBV DNA in serum decreases and usually becomes undetectable during lamivudine administration, but it rapidly increases when HBV becomes resistant to lamivudine. Thus, the measurement of HBV DNA is useful for monitoring the anti-viral effects of lamivudine. However, a negative result of HBV DNA in serum does not necessarily indicate a good outcome of lamivudine therapy, because lamivudine resistance may occur even if HBV DNA levels remain undetectable during therapy (11–13). Recently, a chemiluminescence enzyme immunoassay (CLEIA) was developed in our laboratory for the detection of hepatitis B virus core-related antigen (HBVcrAg) (14, 15). The assay reflects the viral load of HBV in a similar manner to that used in assays, which detect HBV DNA. HBVcrAg consists of HBV core and e antigens; both proteins are transcribed from the precore/core gene and their first 149 amino acids are identical (16–18). The HBVcrAg CLEIA simultaneously measures the serum levels of hepatitis B core (HBc) and e (HBe) antigens, using monoclonal antibodies, which recognize common epitopes of these two denatured antigens. In the present study, we analyzed the clinical significance of the HBVcrAg assay in monitoring the anti-viral effects of lamivudine treatment.

Patients and methods

Patients

A total of 81 patients with chronic hepatitis B, who received lamivudine therapy, were enrolled in the present study. These were 58 men and 23 women with a median age of 49 years (range 24–79 years). The 81 patients were selected retrospectively from six medical institutions in Japan (Shinshu University Hospital, Toranomon Hospital, Nagoya City University Hospital, Kyoto Prefectural University Hospital, Hiroshima University Hospital, National Nagasaki Medical Center). Eight to 25 patients who met the following three criteria were selected consecutively in each institution: the first, a daily dose of 100 mg lamivudine was administered for at least 6 months in a period from 1999 to 2004; the second, histologically confirmed for chronic hepatitis without liver cirrhosis; and the third, serum samples at several time points available for testing. All patients were naive for lamivudine therapy. Chronic hepatitis B was defined as positive hepatitis B surface (HBs) antigen for more than 6 months with elevated levels of serum transaminases. The HBV genotype was A in two patients, B in three and C in 76. Serum HBV DNA was detectable in all patients, and HBe antigen was positive in 51 (63%) of the 81 patients just before lamivudine administration. The median follow-up period was 19 months with a range from 6 to 50 months. Follow-up of patients ended when lamivudine administration was discontinued. Written informed consent was obtained from each patient.

The occurrence of lamivudine resistance was defined as a rapid increase in serum HBV DNA levels with the appearance of the YMDD mutations during lamivudine administration. Using this criteria, resistance appeared in 27 (33%) of the 81 patients. The median period from the start of lamivudine administration to the occurrence of resistance was 12 months with a range from 4 to 37 months.

Serological markers for HBV

HBs antigen, HBe antigen and anti-HBe antibody were tested using commercially available enzyme immunoassay kits (Abbott Japan Co., Ltd., Tokyo, Japan). Six major genotypes (A–F) of HBV can be detected using the method reported by Mizokami et al. (19), in which the surface gene sequence amplified by polymerase chain reaction (PCR) is analyzed by restriction fragment length polymorphism. The YMDD motif, that is, lamivudine resistant mutations in the active site of HBV polymerase, was detected with an enzyme-linked mini-sequence assay kit (HBV YMDD Mutation Detection Kit, Genome Science Laboratories Co., Ltd., Tokyo, Japan) (20).

Serum concentration of HBV DNA was determined using Amplicor HBV monitor kit (Roche, Tokyo, Japan), which had quantitative range from 2.6 to 7.6 log copy/ml. Sera containing

over 7.0 log copy/ml HBV DNA were diluted 10- or 100-fold with normal human serum and re-tested to obtain the end titer.

Serum concentrations of HBVcrAg were measured using the CLEIA method reported previously (10, 11). Briefly, 100 μL serum was mixed with 50 μL pretreatment solution containing 15% sodium dodecylsulfate and 2% Tween 60. After incubation at 70 °C for 30 min, 50 μL pretreated serum was added to a well coated with monoclonal antibodies against denatured HBc and HBe antigens (HB44, HB61 and HB114) and filled with 100 μL assay buffer. The mixture was incubated for 2 h at room temperature and the wells were then washed with buffer. Alkaline phosphatase-labeled monoclonal antibodies against denatured HBc and HBe antigens (HB91 and HB110) were added to the well, and the mixture was incubated for 1 h at room temperature. After washing, CDP-Star with Emerald II (Applied Biosystems, Bedford, MA) was added and the plate was incubated for 20 min at room temperature. The relative chemiluminescence intensity was measured, and the HBVcrAg concentration was determined by comparison with a standard curve generated using recombinant pro-HBe antigen (amino acids, 10–183 of the precore/core gene product). The HBVcrAg concentration was expressed as units/ml (U/ml) and the immunoreactivity of recombinant pro-HBe antigen at 10 fg/ml was defined as 1 U/ml. In the present study, the cutoff value was tentatively set at 3.0 log U/ml. Sera containing over 7.0 log U/ml HBVcrAg were diluted 10- or 100-fold in normal human serum and re-tested to obtain the end titer.

Statistical analysis

The Mann–Whitney *U*-test and Wilcoxon signed-ranks test were utilized to analyze quantitative data, and Fisher's exact test was used for qualitative data. A log-rank test was used to compare the occurrence of lamivudine resistance. Statistical analyses were performed using the SPSS 5.0 statistical software package (SPSS, Inc., Chicago, IL). A *P*-value of less than 0.05 was considered to be statistically significant.

Results

Table 1 shows a comparison of the clinical and virological backgrounds of the 27 patients who showed lamivudine resistance and the 54 patients who did not. Median age, gender distribution and median follow-up period did not differ between the two groups, and the positive rate of HBe antigen was similar. Both HBV DNA and HBVcrAg levels at the beginning of lamivudine administration were similar between the two groups; however, both HBV DNA and HBVcrAg levels at 6 months after the start of lamivudine administration were significantly lower in the lamivudine resistance negative group than in the positive group. ALT level was normal at the beginning in eight (15%) of the 54 patients without lamivudine resistance and in two (7%) of the 27 patients with it ($P > 0.2$).

Table 1. Comparison of the clinical and virological backgrounds of patients who showed lamivudine resistance and those who did not

Characteristics	Appearance of lamivudine resistance: Negative ($n = 54$)	Positive ($n = 27$)	P
Age (years)*	47.0 (24–79)	50.6 (34–67)	0.140†
Gender (male %)	74%	67%	>0.2‡
Follow-up period (months)*	16 (6–50)	21 (9–43)	>0.2†
HBV genotype (A/B/C)	2/2/50	0/1/26	>0.2‡
HBe antigen (positive %)	59%	70%	>0.2‡
ALT (IU/ml)*			
Initial	85 (22–713)	95 (20–1140)	>0.2†
At 6 months	27 (11–115)	30 (15–92)	>0.2†
HBV DNA (log copy/ml)*			
Initial	7.0 (3.5–9.1)	7.3 (4.2–9.2)	>0.2†
At 6 months	<2.6 (<2.6–4.8)	3.3 (<2.6–6.6)	<0.001†
HBVcrAg (log U/ml)*			
Initial	6.2 (<3.0–8.8)	7.3 (4.4–9.1)	0.073†
At 6 months	5.2 (<3.0–6.7)	5.8 (4.7–8.4)	<0.001†

HBe antigen, hepatitis B e antigen; HBV, hepatitis B virus; ALT, alanine aminotransferase; HBVcrAg, HBV core-related antigen. *Data are expressed as median (range). †Mann–Whitney *U* test. ‡χ^2-test.

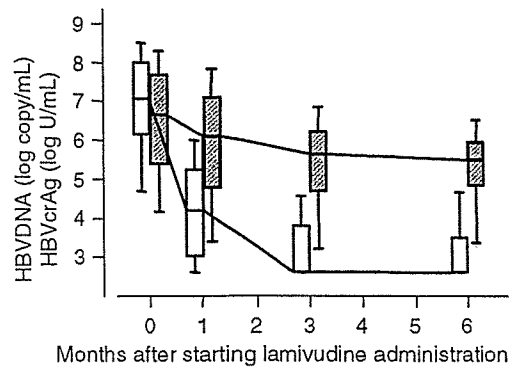

Fig. 1. Changes in the median levels of hepatitis B virus core-related antigen (HBVcrAg) and hepatitis B virus (HBV) DNA during lamivudine administration. The box plots show the 10th, 25th, 50th, 75th and 90th percentiles, with the open boxes indicating HBV DNA and shaded boxes indicating HBVcrAg. The median amount of decrease from the baseline in HBVcrAg levels was significantly smaller (Wilcoxon signed-ranks test) than that in HBV DNA level at 1 (2.80 log copy/ml vs. 0.27 log U/ml, $P < 0.001$), 3 (3.60 log copy/ml vs. 0.83 log U/ml, $P < 0.001$) and 6 months (3.90 log copy/ml vs. 1.15 log U/ml, $P < 0.001$) after the initiation of lamivudine administration.

Figure 1 shows changes in HBV DNA and HBVcrAg levels during lamivudine treatment in all patients. The level of HBV DNA decreased rapidly and became undetectable at 3 months after treatment was initiated. On the other hand, although HBVcrAg levels decreased continuously, the median amount of decrease from the base-line was significantly lower than that in HBV DNA levels at 1, 3 and 6 months after starting lamivudine administration (Wilcoxon signed-ranks test, $P<0.001$ at all analyzed points in time).

Changes in HBV DNA and HBVcrAg levels during lamivudine administration are compared in Fig. 2 between the 27 patients who showed lamivudine resistance and the 54 patients who did not. Serum HBV DNA levels were found to decrease rapidly and become undetectable within 6 months in 45 (83%) of the 54 patients without lamivudine resistance. On the other hand, only 11 (41%) of the 27 patients with lamivudine resistance showed a similar rapid decrease, and the HBV DNA levels of the remaining patients stayed above the detection limit during the follow-up period. HBVcrAg levels decreased but did not reach levels lower than 4.7 log U/ml (5000 U/ml) in the 27 patients with lamivudine resistance. In 19 (35%) of the 54 patients without lamivudine resistance, on the other hand, the levels decreased to levels below 4.7 log U/ml within 6 months after the start of lamivudine administration. The level of HBVcrAg increased rapidly as did the level of HBV DNA when lamivudine resistance occurred.

The occurrence of lamivudine resistance was significantly less frequent in the 56 patients whose HBV DNA level was less than 2.6 log copy/ml at 6 months after the initiation of treatment than in the remaining 25 patients (Fig. 3). The cumulative occurrence of lamivudine resistance was as high as 70% within 2 years in the latter group, while it was only 28% in the former group. There was no occurrence of lamivudine resistance during the follow-up period in the 19 patients whose HBVcrAg levels were less than 4.6 log U/ml at 6 months after the initiation of lamivudine therapy (Fig. 3). On the other hand, lamivudine resistance occurred in 50% of the remaining patients within 2 years.

Discussion

The HBVcrAg assay is a unique assay, which measures the amounts of e and core antigens

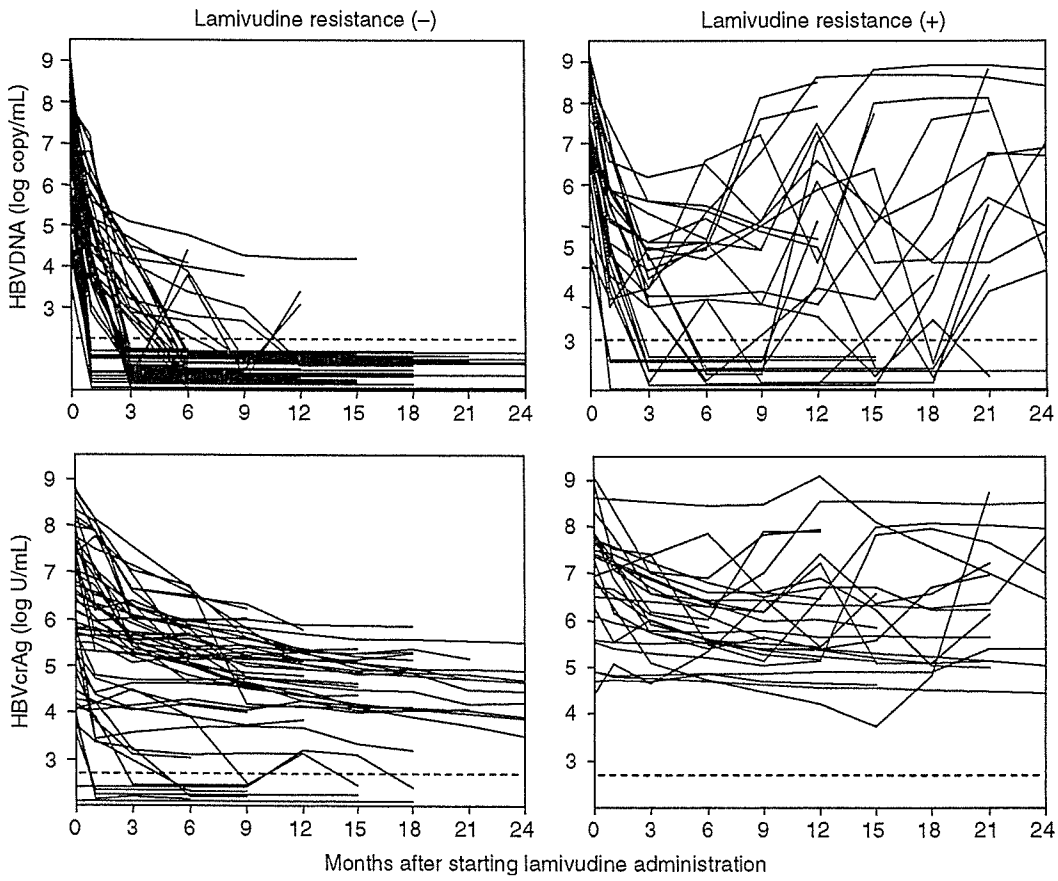

Fig. 2. Comparison of changes in serum hepatitis B virus (HBV) DNA and serum HBV core-related antigen (HBVcrAg) levels between patients who showed lamivudine resistance and those who did not. The broken lines indicate the detection limit of each assay.

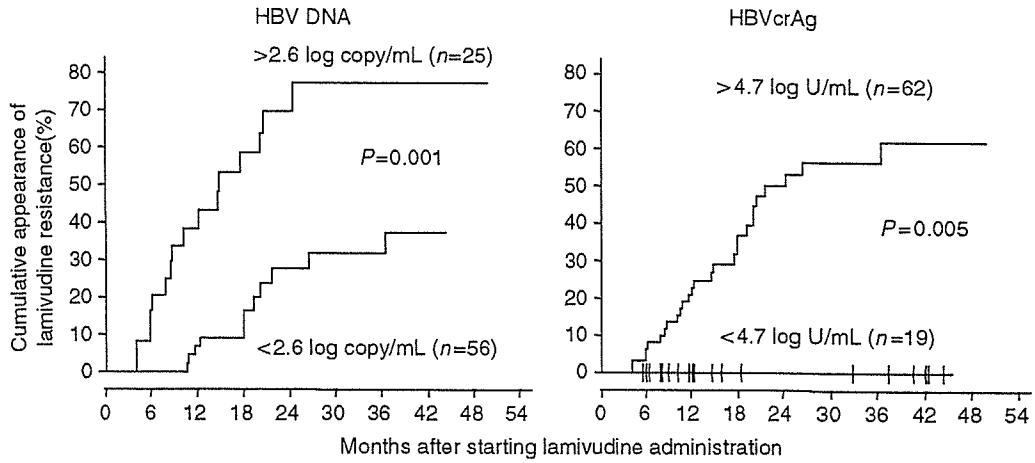

Fig. 3. Comparison of the cumulative occurrence of lamivudine resistance between patients who showed hepatitis B virus (HBV) DNA levels of less than the detection limit (2.6 log copy/ml) at 6 months after starting lamivudine administration and those who did not (left figure), and similarly between patients who showed HBV core-related antigen (HBVcrAg) levels of less than 4.7 log U/ml and those who did not (right figure).

coded by the core gene of the HBV genome with high sensitivity and a wide quantitative range. Serum HBVcrAg levels reflect the viral load in the natural course because these levels correlate linearly with those of HBV DNA (14, 15). On the other hand, the character of HBVcrAg is somewhat different from that of HBV DNA in patients undergoing anti-viral therapies such as lamivudine. That is, HBVcrAg levels decrease significantly more slowly than those of HBV DNA after the initiation of lamivudine administration.

HBV is an enveloped DNA virus containing a relaxed circular DNA genome, which is converted into a covalently closed circular DNA (cccDNA) episome in the nucleus of infected cells (18, 21–23). The cccDNA molecules serve as the transcriptional template for the production of viral RNAs that encode viral structural and non-structural proteins. Reverse transcription of the viral pregenomic RNA and second-strand DNA synthesis occur in the cytoplasm within viral capsids formed by the HBV core protein. Because lamivudine, a nucleoside analogue, inhibits reverse transcription of the pregenomic RNA, it directly suppresses the production of HBV virion. Thus, serum HBV DNA levels decrease rapidly after the initiation of lamivudine administration. On the other hand, the production of viral proteins is not suppressed by lamivudine because the production process does not include reverse transcription. Furthermore, it has been reported that the amount of cccDNA, which serves as a template for mRNA, decreases quite slowly after starting the administration of nucleoside analogues (24–26). Thus, it is reasonable that serum HBVcrAg levels decrease much more slowly than HBV DNA levels after the initiation of lamivudine therapy.

Significant markers that can predict the presence or absence of lamivudine resistance are clinically valuable because the emergence of this resistance and the subsequent recurrence of hepatitis are fundamental problems in lamivudine therapy. Serum markers that reflect the activity of HBV replication have been reported to be associated with the occurrence of lamivudine resistance (11, 12, 27, 28). However, neither the pretreatment existence of HBe antigen nor pretreatment levels of HBV DNA or HBVcrAg were found to be significant markers in the present study. These results may reflect a weak association between the pretreatment activity of HBV replication and the occurrence of lamivudine resistance (13, 29). Changes in HBV DNA and HBVcrAg levels after starting lamivudine administration clearly differed between patients with and without lamivudine resistance. Thus, HBV DNA and HBVcrAg levels at 6 months after starting lamivudine administration were analyzed to determine whether these levels might serve as predictive markers; both were found to be significantly lower in patients without lamivudine resistance at the tested point in time. Furthermore, patients who showed higher levels of HBV DNA and HBVcrAg at 6 months after the initiation of treatment were significantly more likely to develop lamivudine resistance than those who showed lower levels.

We believe that the measurement of HBV DNA levels is useful to identify patients who are at high risk for lamivudine resistance because as many as 70% of patients who were positive for HBV DNA at 6 months after starting lamivudine