negative HCV-RNA PCR result. As normal hepatocytes turn over every 1 year and in chronic inflammation, the duration would be shorter [44, 45].

Although pegylated IFN/ribavirin combination is now available and a most promising therapy, undetectable HCV-RNA at the end of treatment is obtained in about 80% of patients with genotype 1 and high viral load [23–25]. Pegylated IFN/ribavirin combination has the advantage of a low rate of breakthrough or relapse. However, SVR would never been achieved in a residual HCV-RNA-positive patient. Thus to obtain a further high SVR rate, more high prevalence of undetectable HCV-RNA would be necessary. In the present study, a high rate (96%) of HCV-RNA-negative status was obtained by the induction of twice-daily administration of IFN-β followed by ribavirin combination therapy. Although a too small number of patients was enrolled and complicated protocols were included, our data may indicate that twice-daily administration of IFN-β followed by pegylated IFN/ribavirin combination therapy could obtain a further high rate of HCV-RNA-negative status in patients with genotype 1 and high viral load.

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Evolution of Hepatitis C Virus Quasispecies during Ribavirin and Interferon-Alpha-2b Combination Therapy and Interferon-Alpha-2b Monotherapy

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Key Words
Hepatitis C virus quasispecies · Viral resistance · Error catastrophe · Chronic hepatitis C virus infection · Ribavirin

Abstract
Objective: Ribavirin and interferon combination therapy is more effective than interferon monotherapy in patients with chronic hepatitis C virus (HCV) infection. To test the hypothesis that ribavirin induces nucleotide substitutions in the viral genome and reduces viral load by forcing it into error catastrophe in the combination therapy, we investigated the molecular evolution of HCV quasispecies in 3 patients who received combination therapy and 2 patients who received interferon monotherapy. ***Methods:*** The quasispecies were analyzed before and after therapy by sequencing at least 8 clones in five regions of the HCV genome; 5′ untranslated region, E1, E2, NS5A and NS5B. ***Results:*** Marked genetic drift was observed in the NS5A and NS5B regions in patients treated with combination therapy. However, genetic distances between clones obtained after therapy were closer than those obtained before therapy. ***Conclusion:*** Our results suggest that the combination therapy modified HCV quasispecies, but that this did not reflect the induction of error catastrophe by ribavirin. Modification of quasispecies by this therapy requires further investigation in a larger number of patients to elucidate the possible mechanism of viral resistance against the combination therapy.

Introduction

Hepatitis C virus (HCV) infection is a serious health problem worldwide [1–4]. Ribavirin and interferon (IFN) combination therapy induces a significantly higher response rate than IFN monotherapy as shown in recent randomized studies [5–7]. McHutchison et al. [5] and Poynard et al. [6] studied patients with chronic hepatitis C who had not been treated previously, and Davis et al. [7] studied patients with chronic hepatitis C who relapsed after IFN treatment. They reported that the rate of sustained virological response was higher among patients who received combination therapy (31–49%) than among patients who received IFN monotherapy (5–19%).

The mechanism of action of ribavirin is not clearly understood; however, various possible mechanisms have been proposed including: (1) ribavirin inhibits the enzyme inosine monophosphate dehydrogenase (IMPDH)

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Table 1. Clinical and virological characteristics of the patients studied

Patient	Sex	Age years	Histo-pathological staging	Geno-type	Viral load, kIU/ml		
					pretreatment	4 weeks	end of treatment
IFN plus ribavirin therapy							
1	M	60	1[a]	1b	>850[b]	<0.5[b]	<0.5[b]
2	M	56	1[a]	1b	>850[b]	420[b]	450[b]
3	M	35	2[a]	1b	>850[b]	57[b]	190[b]
IFN therapy							
4	M	51	1[a]	1b	>850[b]	64[b]	(+)
5	M	57	1[a]	1b	>850[b]	>850[b]	>850[b]

[a] Staging of chronic hepatitis by Desmet et al. [21].
[b] Viral load was measured by the Amplicor HCV Monitor assay (version 2.0) (Roche, Tokyo, Japan).

and reduces the guanosine triphosphate (GTP) pool in hepatocytes; (2) ribavirin induces a T cell helper (Th)2 to Th1 bias in favor of a host antiviral response via either cytotoxic T lymphocytes (CTLs) or Th1 cytokines; (3) ribavirin inhibits HCV NS5B-encoded RNA-dependent RNA polymerase (RdRp), and (4) ribavirin acts as an RNA mutagen [for review, see 8]. Crotty et al. [9, 10] hypothesized that the antiviral effect of ribavirin is due to induction of nucleotide substitutions in the genome of RNA viruses forcing them into error catastrophe. They used a polio virus system to investigate the effect of ribavirin and demonstrated induction of nucleotide substitutions in the viral genome [9, 10].

The effect of ribavirin on HCV was examined using a replicon system [11, 12]. Contreras et al. [11] assayed mutation frequencies using a replicon system, and reported that ribavirin broadly increased error generation, particularly in otherwise invariant regions (5′ UTR and core). However, to our knowledge, no data are available about the effect of IFN and ribavirin combination therapy on HCV in humans. Sookoian et al. [13] investigated HCV quasispecies by SSCP analysis in hypervariable regions in patients who received ribavirin monotherapy, but they did not analyze nucleotide sequences or quasispecies. In the present study, we determined the HCV quasispecies in patients who received combination therapy of IFN-alpha-2b and ribavirin or IFN-alpha-2b monotherapy. We investigated five conserved and variable regions of the HCV genome including the 5′ untranslated region (UTR), EI, E2 (HVR1), NS5A and NS5B regions. The 5′ UTR was chosen because it plays important roles in key processes in viral infection such as replication of the viral genome and translation of viral protein. The E1 and E2 regions were also selected because they are variable regions as targets of the humoral immune response [14–16]. The NS5A region was studied because of its putative implication in IFN resistance [17, 18]. NS5B is a domain harboring the putative catalytic site (GDD) of the viral polymerase and is a putative target of nucleoside analogs, including ribavirin [19, 20].

Materials and Methods

Patients

Five male Japanese patients chronically infected with HCV genotype 1b who received antiviral therapy at the Department of Gastroenterology, Toranomon Hospital, were enrolled in this study. Three of these 5 patients (patients 1, 2 and 3) received IFN-alpha-2b plus ribavirin (800 mg/day) for 6 months. The remaining 2 patients (patients 4 and 5) were treated with IFN-alpha-2b alone (table 1). Serum samples for sequence analyses were collected just before the start of therapy and at the end of therapy. Informed consent was obtained from each patient and study protocol conformed the ethical guidelines of 1975 Declaration of Helsinki, and institutional approval was obtained.

Amplification of 5 HCV Genomic Regions by Reverse Transcription-Polymerase Chain Reaction

HCV-RNA was isolated from 100-μl serum samples using Sepa Gene RV-R (Sanko Junyaku Co., Japan). HCV-RNA was reverse transcribed with random primer and a reverse transcriptase according to the instructions provided by the manufacturer (ReverTra Ace [Toyobo Co., Osaka, Japan]). HCV cDNA was then amplified using primer sets specific for each region (table 2). For the first and second rounds of nested PCR, 35 cycles of 94°C for 30 s, 55°C for 90 s, and 72°C for 1 min were performed after an initial denaturation step at 94°C for 5 min, followed by a final extension for 7 min at 72°C.

Table 2. Primers used for RT-nested PCR amplification of 5′ UTR, E1, E2, NS5A and NS5B regions

5′ UTR	outer sense primer	5′-CCT GTG AGG AAC TAC TGT C-3′	(32–50)[a]	144 bp[b]
	outer antisense primer	5′-CAA CAC TAC TCG GCT AGC AGT C-3′	(254–233)[a]	
	inner sense primer	5′-TTC ACG CAG AAA GCG TCT AGC-3′	(51–71)[a]	
	inner antisense primer	5′-TTT ATC CAA GAA AGG ACC-3′	(194–176)[a]	
E1	outer sense primer	5′-CAG CCC GGG TAC TAC CCT TGG C-3′	(561–579)[a]	706 bp[b]
	inner sense primer	5′-CTC GAA TTC GGC TTC GCC GAT CTC ATG G-3′	(705–732)[a]	
	antisense primer	5′-CTC GGA TCC CCG CCA GGA CTC CCC AGT G-3′	(1,383–1,410)[a]	
E2	outer sense primer	5′-CAA GAC TGC AAT TGC TCC ATC T-3′	(1,233–1,254)[a]	535 bp[b]
	outer antisense primer	5′-GGT GCC GGA TCC ATC GGT CGT CCC CAC-3′	(1,875–1,901)[a]	
	inner sense primer	5′-CTA CTC CGG ATC CCA CAA GC-3′	(1,383–1,357)[a]	
	inner antisense primer	5′-CAA CAG GGA TCC GAG TGA AGC AAT A-3′	(1,848–1,872)	
NS5A	outer sense primer	5′-TTC CAC TAC GTG ACG GGC ATG AC-3′	(6,624–6,646)[a]	418 bp[b]
	outer antisense primer	5′-CCC GTC CAT GTG TAG GAC AT-3′	(7,590–7,609)[a]	
	inner sense primer	5′-GGG TCA CAG CTC CCA TGT GAG CC-3′	(6,798–6,820)[a]	
	inner antisense primer	5′-GAG GGT TGT AAT CCG GGC GTG C-3′	(7,194–7,215)[a]	
NS5B	outer sense primer	5′-TGG GGT TCT CGT ATG ATA CC-3′	(8,230–8,249)[a]	372 bp[b]
	inner sense primer	5′-CGC TGC TTT GAC TCA ACG GTC AC-3′	(8,250–8,272)[a]	
	antisense primer	5′-CCT GGT CAT AGC CTC CGT GAA-3′	(8,601–8,621)[a]	

[a] Location of nucleotide sequences according to Kato et al. [22].
[b] Size of PCR products in base pairs.

Cloning and Sequencing

PCR products were electrophoresed in 2% agarose gels and purified using Geneclean (Qbiogene Inc., Carlsbad, Calif., USA). Purified DNA was ligated into the plasmid vector pGEM-T Easy Vector (Promega, Madison, Wisc., USA), and transformed into *Escherichia coli*-competent cells according to the instructions provided by the manufacturer. Transformants were grown overnight on LB/ampicillin/IPTG/X-gal plates, and 10 individual clones from each sample were sequenced with an automated DNA sequencer (ABI PRISM 310 Genetic Analyzer, Applied Biosystems Japan, Tokyo).

Phylogenetic Analysis and Evaluation of Genetic Distances

Nucleotide sequences were aligned using the Expansion of CLUSTAL W in DNA Data Bank of Japan (DDBJ). Genetic distances were calculated with the Kimura two-parameter method [23] using these nucleotide alignments. Phylogenetic trees were constructed with the help of MEGA2 software [24] with the neighbor-joining method [25]. Bootstrap resampling (1,000 replicates) was utilized as a pseudo-empirical test of the reliability of the tree topology [26].

Evolution of quasispecies was estimated as described by Pawlotsky et al. [18]. Within-sample genetic distances, before and after treatment, was calculated for the quasispecies in each of 5 patients by comparing the genetic distances of pairs of sequences. Between-sample genetic distances were calculated on the basis of distances between pairs of pre- and post-treatment sequences. These genetic distances were calculated using the Kimura two-parameter method using MEGA program and expressed as mean ± SEM.

Statistical Analysis

Distributions of continuous variables were analyzed by the Mann-Whitney U test. $p < 0.05$ was considered statistically significant. Comparisons of genetic distances were made with the t test.

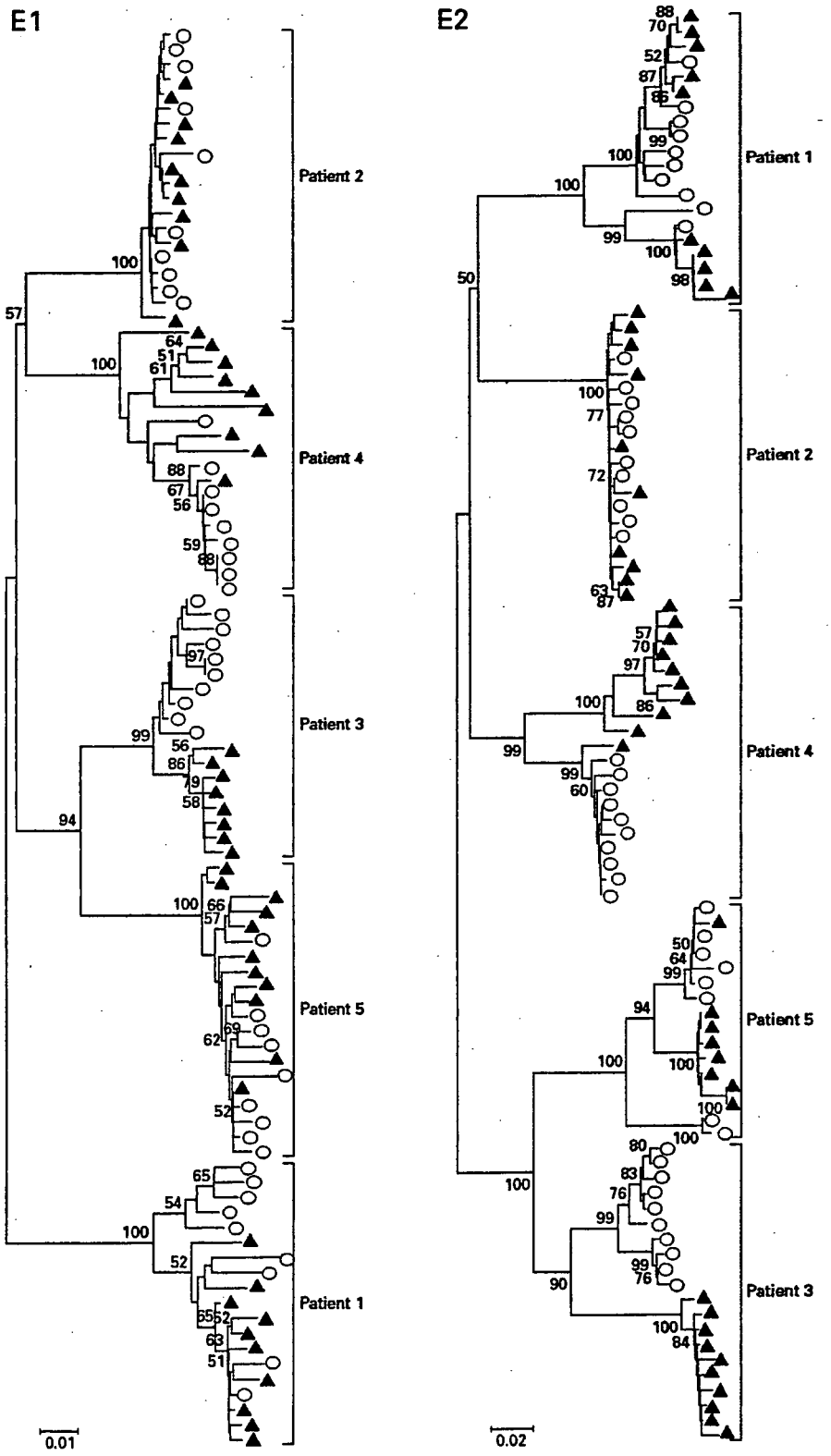
Results

Genetic Drift of HCV Quasispecies before and after Therapy

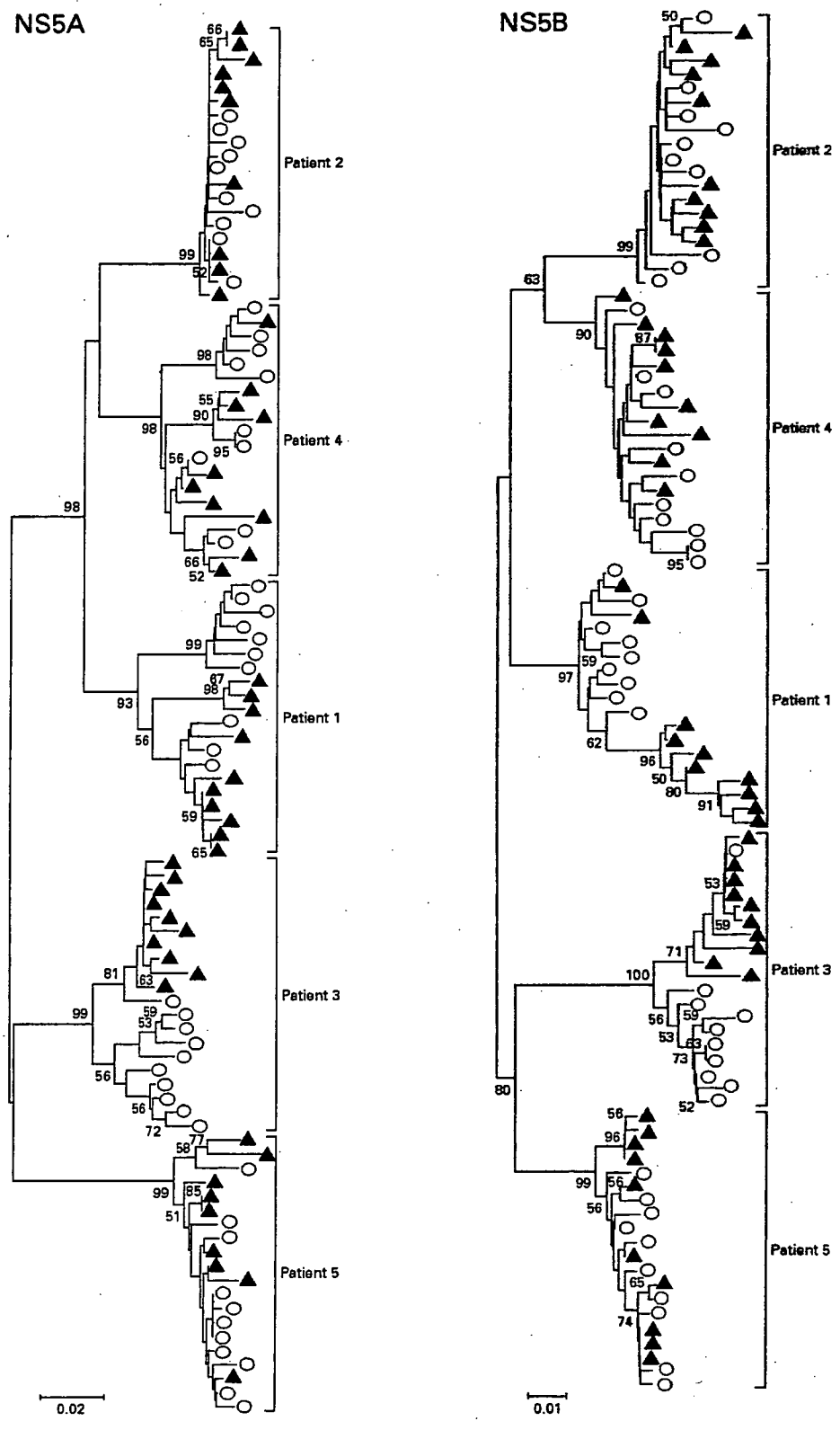
Nucleotide sequences of HCV clones in each region were aligned and phylogenetic trees were constructed (fig. 1). HCV evolution was observed in some patients in certain regions. Typical evolution, for instance, was seen in the phylogenetic tree of the E1 region in patient 3, the E2 region in patient 4, the NS5A region in patients 3 and 5,

(For figure see next pages.)

Fig. 1. Phylogenetic trees based on nucleotide sequences of E1, E2, NS5A and NS5B regions. Open circles represent clones obtained from serum samples extracted before therapy and closed triangles represent clones obtained after therapy. Figures on the branches of the trees represent bootstrap values. Bars represent nucleotide substitutions per site.


E1
E2
Patient 1
Patient 2
Patient 3
Patient 4
Patient 5
0.01
0.02

1


NS5A
Patient 2
Patient 4
Patient 1
Patient 3
Patient 5
0.02
NS5B
Patient 2
Patient 4
Patient 1
Patient 3
Patient 5
0.01

1

and the NS5B region in patients 1 and 3. To evaluate these evolutions, statistical analyses were performed using the MEGA program (fig. 2). To evaluate evolution during therapy, within-pretreatment sample genetic distances were compared with between-treatment sample genetic distances. If the between-treatment sample genetic distances were significantly greater than within-pretreatment genetic distances, the virus exhibited significant evolution. 5′ UTR analyses showed statistically significant evolution in only 1 of the 5 patients. Analyses of the E1 and E2 regions showed significant evolution in patients 3, 4 and 5. Since 2 of these 3 patients (patients 4 and 5) did not receive ribavirin, these evolutions are not related to ribavirin. Significant evolutions were seen in the NS5A and NS5B regions in patients 1 and 3, but not in patients 2, 4 and 5. These evolutions might be the effect of the combination therapy, or evolution of the virus to escape the effect of the therapy and develop resistance to it.

To evaluate whether the combination therapy induced errors in the HCV genome, we compared within-pretreatment sample genetic distances to within-post-treatment sample genetic distances (fig. 3). If the combination therapy induced nucleotide substitutions in the HCV genome, post-treatment sample genetic distances would exceed pre-treatment sample genetic distances. Post-treatment sample genetic distances in the 5′ UTR were significantly greater in 2 of the 3 patients who received combination therapy (patients 2 and 3; fig. 3). However, analyses of the other four regions of the HCV genome did not show such a tendency. The post-treatment genetic distances were smaller in 2 patients in E1. It was therefore difficult to detect error catastrophe from these genetic distance analyses.

Another possible mechanism of HCV evolvement is the acquisition of drug resistance. We compared nucleotide and amino acid sequences of HCV before and after therapy. There was no common amino acid substitution suggestive of resistance to the combination therapy (data not shown).

Discussion

Nucleotide substitutions during viral nucleic acid synthesis are important for viruses to survive under certain pressures of host immune responses and drugs. However, too many substitutions result in so-called error catastrophe. Ribavirin has been shown to induce nucleotide substitutions into RNA virus genomes and to reduce the virus load by inducing error catastrophe [9, 10, 27]. Induction of nucleotide substitutions by ribavirin has been shown in some in vitro systems. Crotty et al. [9, 10] reported that ribavirin induced nucleotide substitutions in the polio virus genome. Airaksinen et al. [27] observed a 10-fold increase in nucleotide substitutions in foot-and-mouth disease virus cultured with ribavirin. Contreras et al. [11] used a HCV full-length replication system and reported that ribavirin induced viral mutations. On the other hand, only limited in vivo data are available for the effect of ribavirin on the HCV viral genome. Querenghi et al. [28] analyzed nucleotide substitutions in the HVR1, NS5A and NS5B regions of HCV in patients treated with ribavirin monotherapy. They observed no significant effect for ribavirin on the amino acid sequence evolution in these regions. Furthermore, Sookoian et al. [13] analyzed HCV quasispecies of the hypervariable region, and concluded that the combination therapy did not affect HCV quasispecies. Since the hypervariable region is known to evolve very rapidly, we considered that analyses of different regions were necessary.

As shown in the phylogenetic tree depicted in figure 1, the apparent evolution of HCV during interferon and ribavirin combination therapy was observed in 2 of the 3 patients, particularly in the NS5A and NS5B regions in patients 3 and 5. These results are consistent with previous observations of Contreras et al. [11] who showed region-specific substitutions induced by ribavirin in vitro. However, investigation of the evolution of the E1 and E2 regions yielded different results. Statistical evaluation showed that not only patients who received combination therapy, but also patients who received interferon monotherapy showed significant evolution (fig. 2; patients 4 and 5). Since these regions encode the envelope protein, these substitutions might be induced by host immune pressure. In contrast, evolution in the NS5A and NS5B regions was seen predominantly in patients who received combination therapy. Such evolution might reflect induction of errors by ribavirin or the development of resistance against the therapy. To clarify this issue, we compared within-pretreatment sample genetic distances to within-post-treatment sample genetic distances. If the ribavirin-interferon combination therapy induced errors in the HCV genome, the post-treatment sample distances should have been greater than the pretreatment sample distances. However, an increase in genetic distance was observed in only limited patients and only in some regions.

We then examined the possibility that the virus developed resistance to the combination therapy. Typical

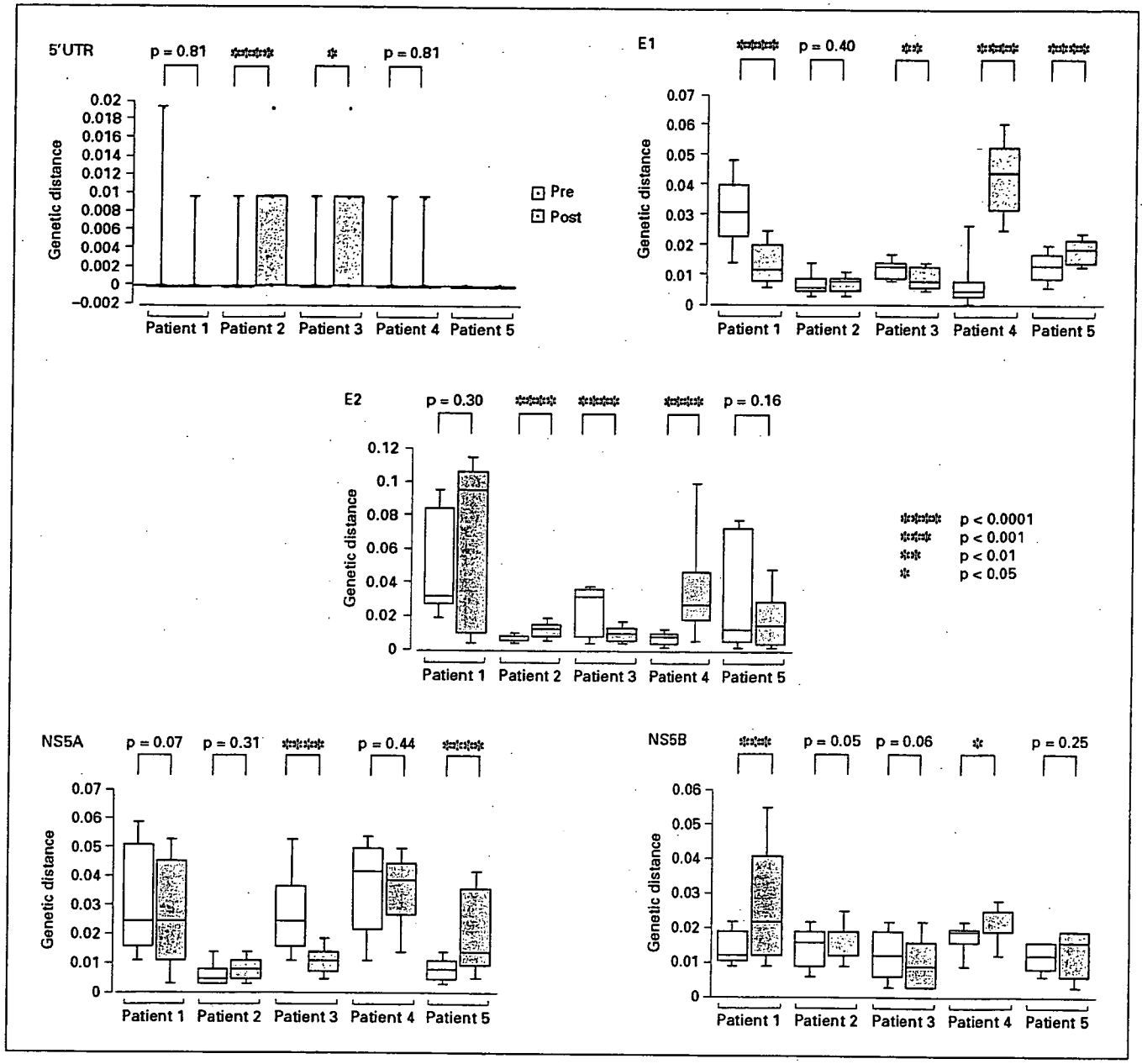


Fig. 2. Comparisons of pretreatment sample genetic distances and between-sample genetic distances. Open bars represent pretreatment sample genetic distances calculated by pairwise comparisons of nucleotide sequences of clones obtained before treatment. Closed bars represent between-sample genetic distances obtained by pairwise comparisons of clones obtained before and after treatment. Median genetic distances are indicated with horizontal bars. The vertical bars indicate the range and the horizontal boundaries of the boxes represent the first and the third quartiles.

nucleotide and amino acid substitutions that are related to resistance of the virus against nucleoside analogs are seen in human immunodeficiency virus and hepatitis B virus reverse transcriptase/polymerase. Amino acid substitutions of the methionine of the YMDD motif to leucine or valine induce strong resistance against lamivudine [29–32]. However, no specific nucleotide or amino acid changes suggestive of resistance to the therapy were

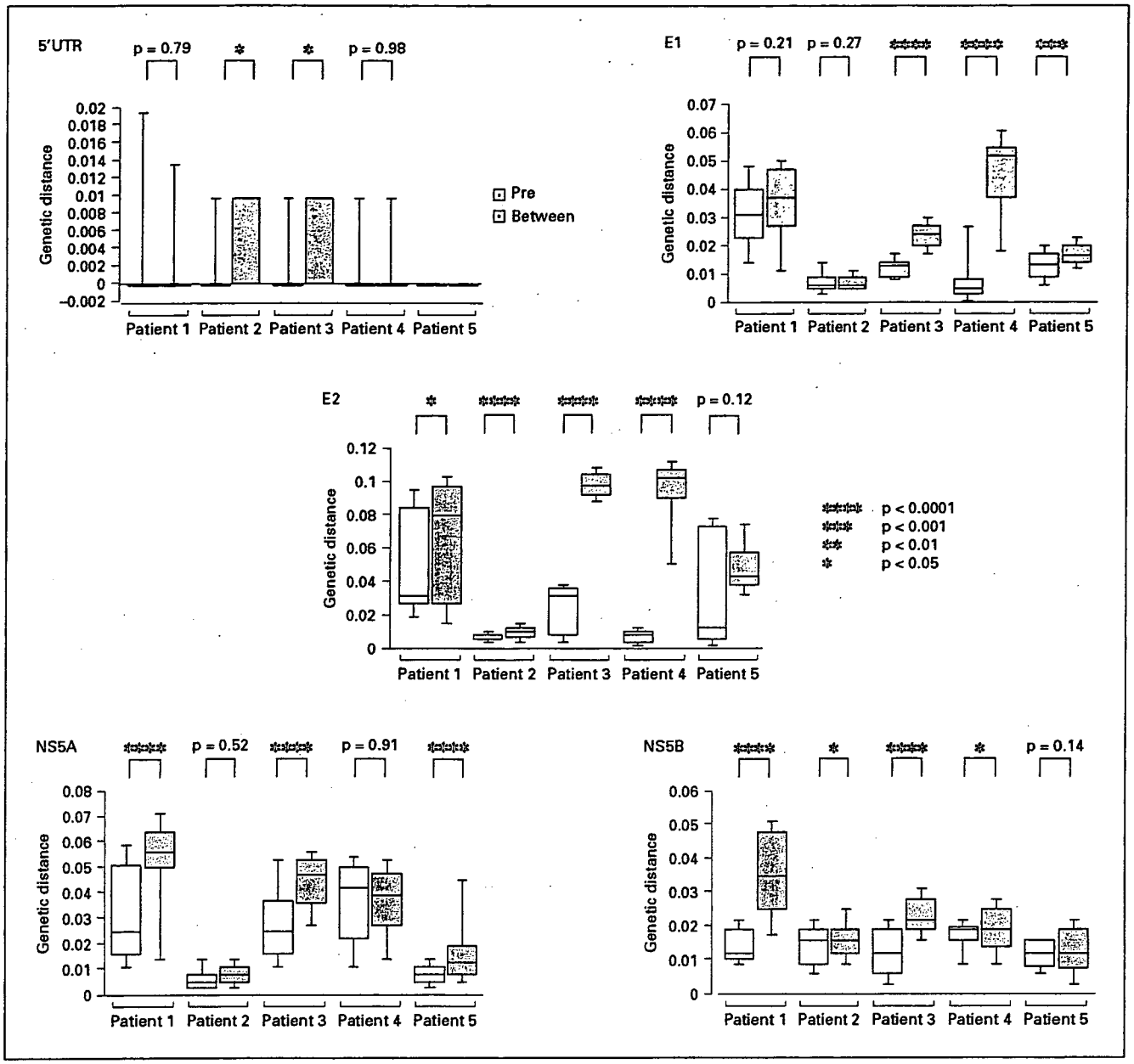


Fig. 3. Comparisons of pretreatment sample genetic distances and post-treatment sample genetic distances. Open bars and closed bars represent distances obtained by comparing nucleotide sequences of clones obtained before and after therapy, respectively. Median genetic distances are indicated with horizontal bars. The vertical bars indicate the range and the horizontal boundaries of the boxes represent the first and the third quartiles.

detected in this study. This finding was consistent with the observations of Lee et al. [33] who analyzed patients who received ribavirin monotherapy and observed no escape mutation of HCV. A possible escape mutation requires analysis in a larger number of patients with comparisons of sequences before and after combination therapy.

Although ribavirin is known to improve liver function without reducing the viral load, the mechanism of the additive effect of ribavirin to interferon therapy is not

yet clear [8]. Some possibilities have been proposed, but there is no definitive evidence to support each hypothesis. Although in vitro findings have suggested the induction of error catastrophe is likely to be the primary mechanism of action of the drug, no in vivo study, including this report, has yielded evidence in support of that hypothesis. One possible explanation for this discrepancy is that we were unable to observe virus with nucleotide substitutions because of the rapid turnover of the virus in vivo.

Clarification of the mechanism of action of these drugs in combination will be useful in developing new treatment strategies against HCV infection. The mechanism of ribavirin in reducing HCV in combination with interferon requires further investigation to enhance eradication of HCV and reduce liver-related deaths from this viral infection.

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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

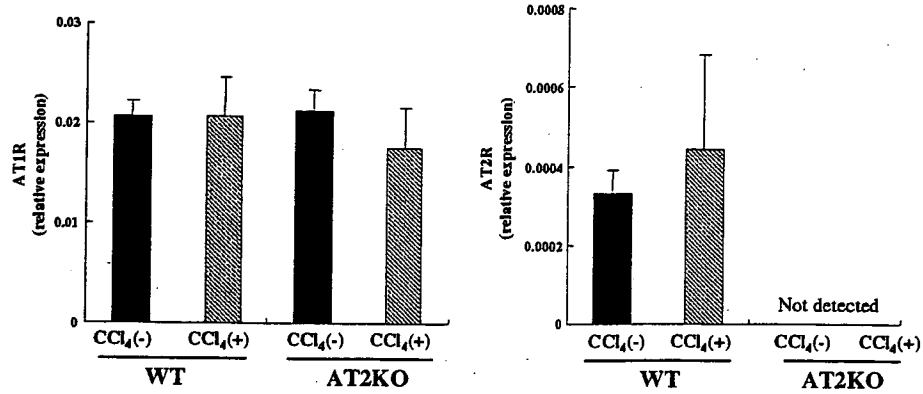

Fig. 1. Steady-state hepatic AT1 and AT2 mRNA levels in WT and AT2KO mice. These results are shown relative to β-actin using real-time PCR. mRNA levels in normal liver and liver after 4 weeks of CCl_4 treatment. After 4 weeks of CCl_4 treatment, AT1 mRNA levels in both WT and AT2KO mice were not changed, 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

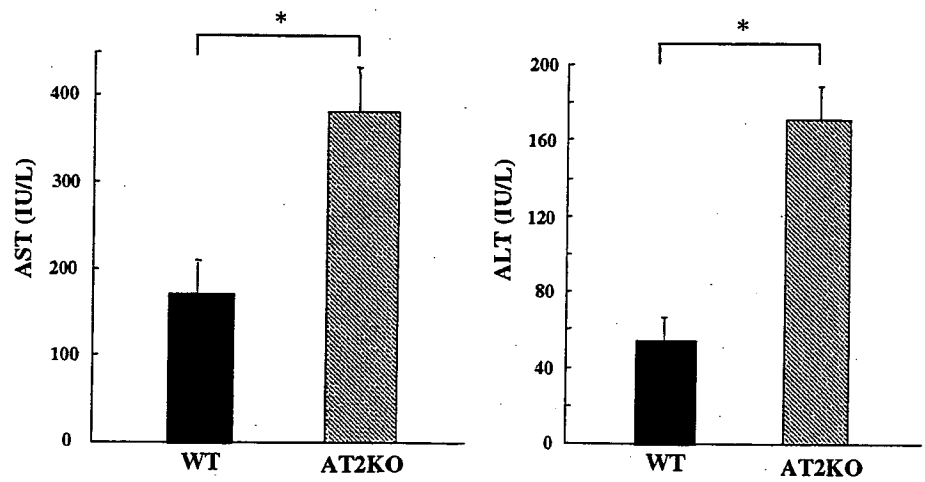

Fig. 2. Effect of 4 weeks of CCl_4 treatment on serum levels of transaminases in WT and AT2KO mice. Chronic serum samples were collected to monitor the effects of the twice-weekly subcutaneous injection of CCl_4 (1 mL/kg) for 4 weeks, and the mice were sacrificed three days after the last injection. 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.

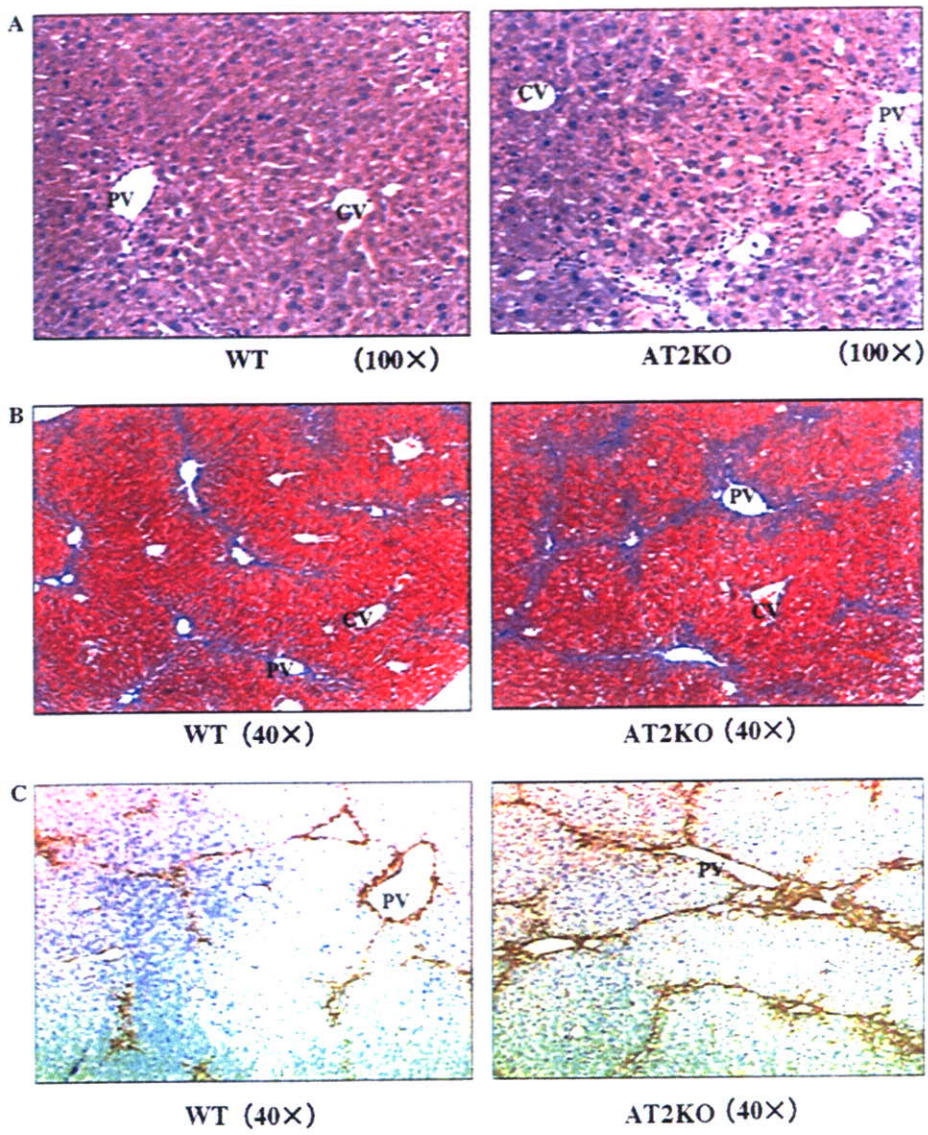

Fig. 3. Representative photomicrographs of the liver after 4 weeks of treatment with CCl_4 [1.0 mL/kg] in WT and AT2KO mice. Paraffin-embedded sections were processed for H and E staining (A; original magnification = 100×), inflammatory cell infiltrates were evident around the portal tracts. Mononuclear 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

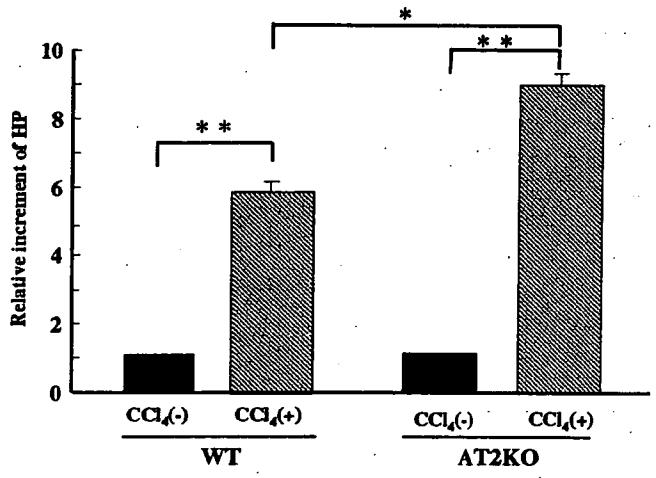

Fig. 4. Hepatic hydroxyproline content after 4 weeks of CCl_4 treatment. The HP content of the liver was significantly increased by CCl_4 treatment in both AT2KO (9.0-fold) and WT (5.9-fold) mice, but more so in the former ($^{*}p < 0.05$, $^{**}p < 0.01$). These changes were calculated on the basis 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

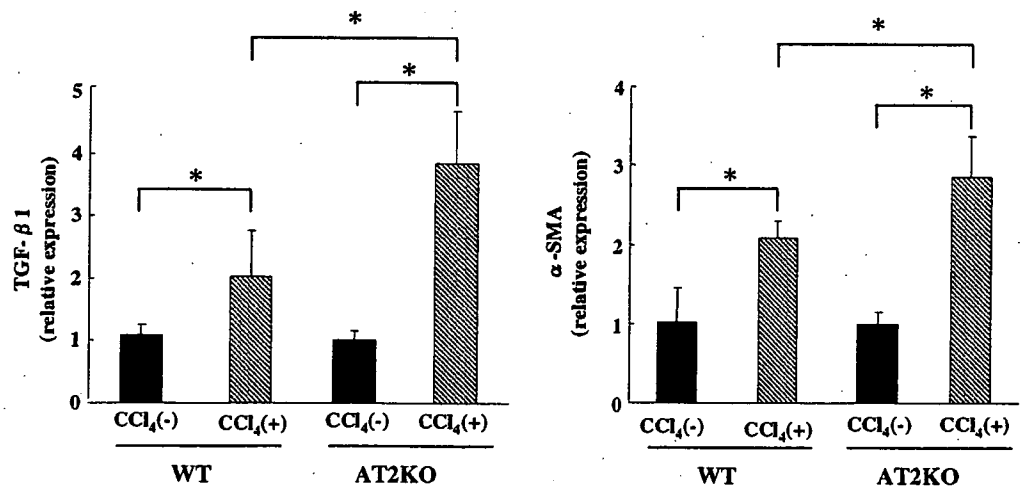

Fig. 5. Steady-state hepatic TGF-β1 and α-SMA mRNA levels in WT and AT2KO mice after 4 weeks of CCl_4 treatment. These results are shown relative to those of the housekeeping gene β-actin by real-time PCR. TGF-β1 and α-SMA mRNA levels are shown. TGF-β1 and α-SMA mRNA expressions of the liver were significantly 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.

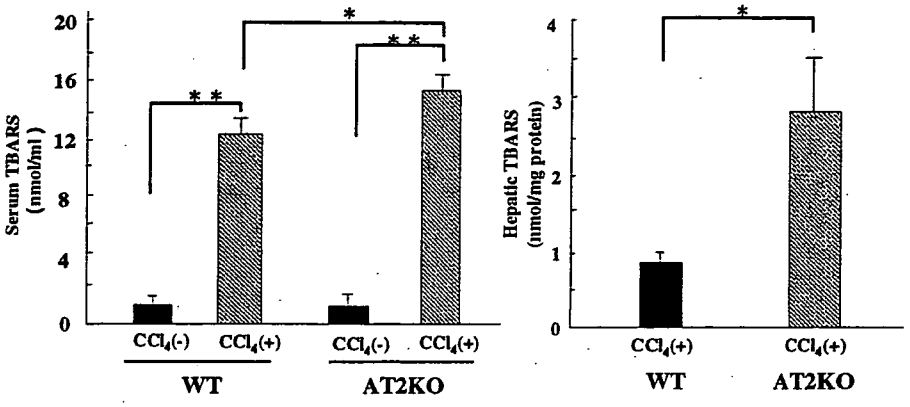

Fig. 6. Serum and hepatic contents of TBARS after 4 weeks of CCl_4 treatment. Serum and hepatic samples were collected to monitor the effects of the twice-weekly subcutaneous injection of CCl_4 (1 mL/kg) over 4 weeks, and the mice were sacrificed three days after the last administration. Serum TBARS 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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