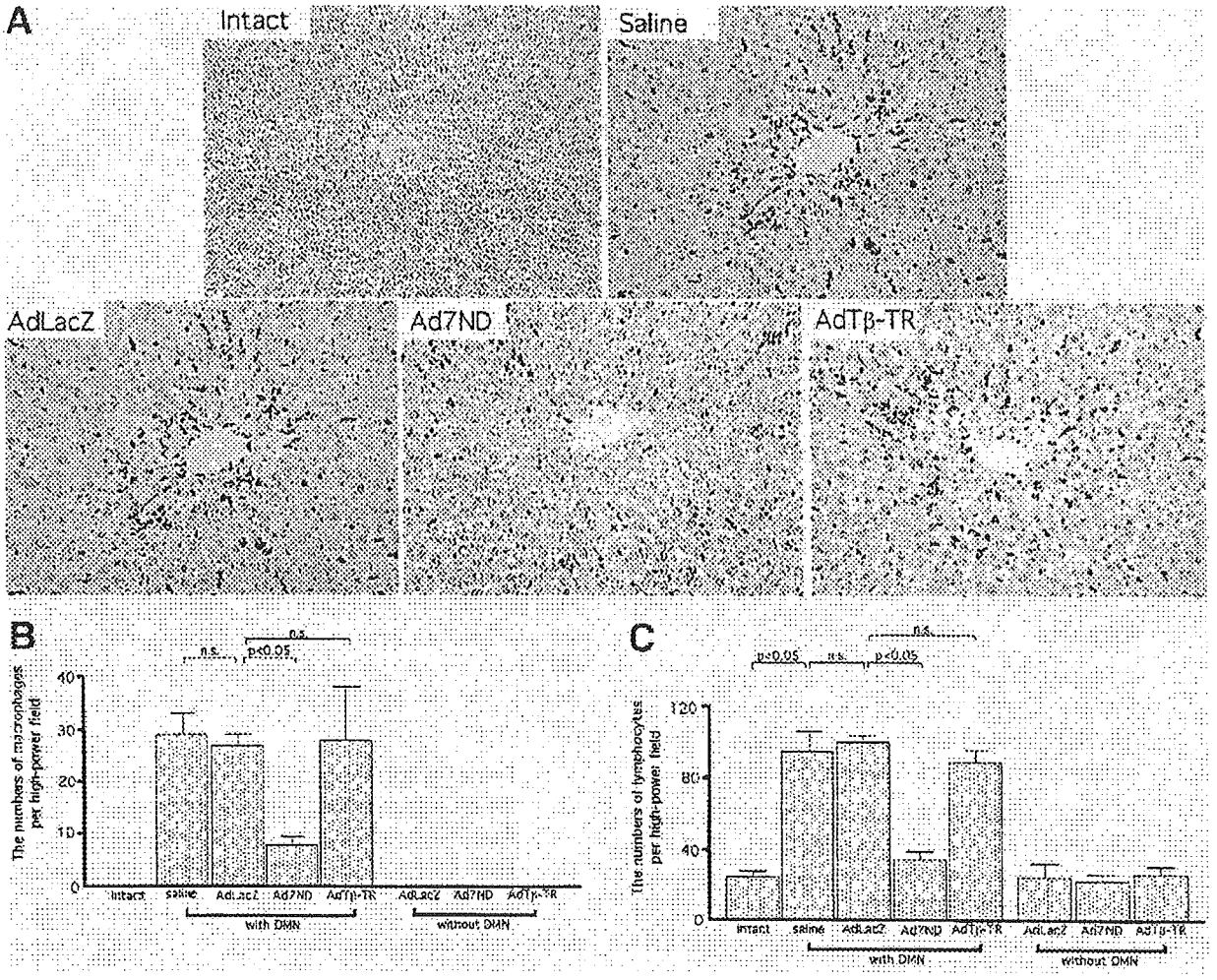

Figure 3. Infiltration of macrophages and lymphocytes into DMN-treated livers. Rats were given a single infusion of saline, AdLacZ, Ad7ND, or AdTβ-TR via the tail vein. Seven days later, some rats were administered DMN once (shown as *with DMN*) and some rats were not administered DMN (shown as *without DMN*; histology not shown). Twenty-four hours after the DMN challenge, they were killed, and liver sections were histologically examined either by immunohistostaining against CD68, to detect macrophages (*A*), or by hematoxylin staining for lymphocytes (not shown; original magnification, 200×). Similar histology was seen in all 4 rats in each group. The numbers of (*B*) macrophages and (*C*) lymphocytes were semiquantitated (see Materials and Methods). Four fields in each of 4 rats (a total of 16 fields in each group) were examined, and the number of cells per high-power field is shown as mean ± SD. n.s., statistically not significant. Rats never treated with adenovirus or DMN were also analyzed (shown as *intact*).

β-galactosidase (AdLacZ), a truncated TGF-β receptor (AdTβ-TR), or a mutated MCP-1 (Ad7ND). Seven days later (when the expression of the introduced molecules had reached a submaximal level), DMN was given. One day after a single injection of DMN, we analyzed liver sections by hematoxylin staining and immunohistostaining against CD68, which is a specific marker for macrophages. Macrophages were detectable in the centrilobular area of the livers of AdLacZ-infected, AdTβ-TR–infected, or saline-injected rats: there were no differences among these 3 groups. However, macrophages were greatly reduced in Ad7ND-treated livers (Figure 3*A*). The numbers of CD68-positive cells (per high-power field) were 29 ± 3.5 in saline-treated livers, 27.5 ± 2.1 in AdLacZ-treated livers, 27.5 ± 11.4 in AdTβ-TR–treated livers, and only 7.1 ± 1.2 in Ad7ND-treated livers (Figure 3*B*). Similarly, the numbers of lymphocytes (histology not shown) were 98 ± 7.5 in saline-treated livers, 101 ± 2.5 in AdLacZ-treated livers, 93 ± 5.5 in AdTβ-TR–treated livers, and only 40 ± 3.5 per high-power field in Ad7ND-treated livers (Figure 3*C*). Without DMN treatment, neither macrophages nor lymphocytes (histology not shown) were increased in the livers of AdLacZ-infected, AdTβ-TR–infected, and

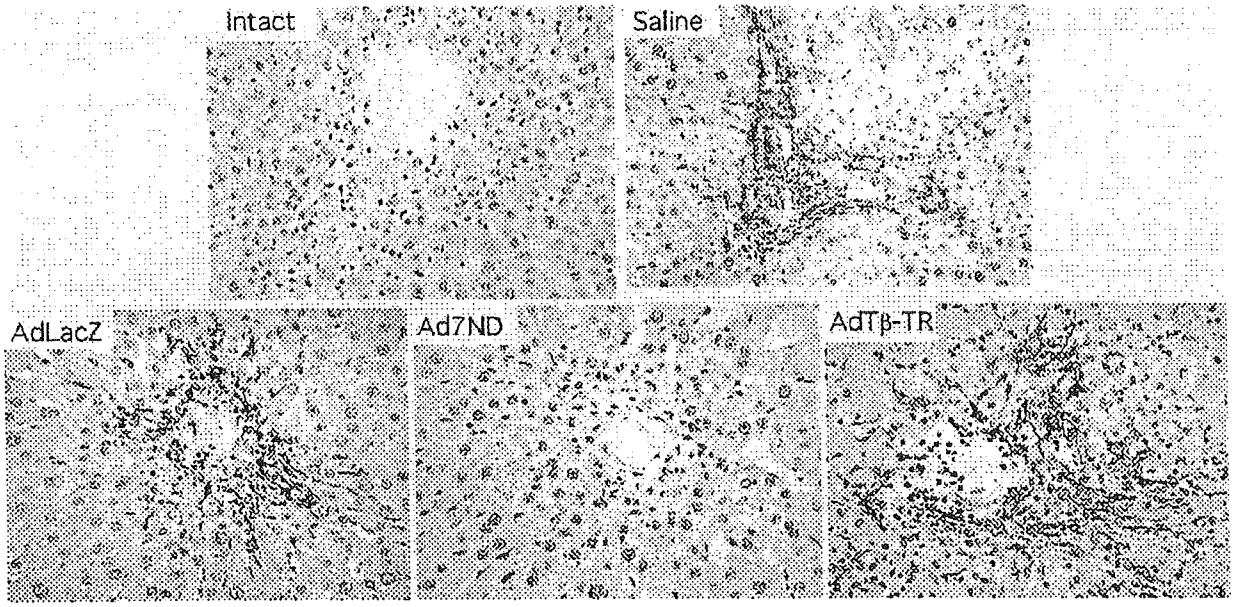

Figure 4. α-Actin–positive cells in DMN-treated livers. Rats were treated with either an adenovirus or saline as described in the legend to Figure 3 and then subjected to DMN for 3 consecutive days. One day after the last DMN injection (the fourth day), livers were examined by immunohistostaining against α-actin (original magnification, 200×). Rats never treated with adenovirus or DMN were also analyzed (shown as *intact*). Similar histology was seen in all 4 rats in each group.

Ad7ND-infected livers compared with intact livers (subjected to no injection of either saline or adenovirus and no DMN treatment; Figure 3*B* and *C*).

Next, after a 3-day DMN treatment, we examined livers for α-actin–positive cells (a marker of activated HSC). They were readily detectable, not only in AdLacZ- or saline-treated, but also in AdTβ-TR–treated livers. In contrast, we could see none in the Ad7ND-treated livers (Figure 4).

Inhibition of Macrophage Infiltration or of Transforming Growth Factor β Signaling Markedly Suppresses Liver Fibrogenesis and Preserves Liver Function

After a 3-week DMN treatment, the hydroxyproline content of livers was measured as a quantitative evaluation of fibrosis (Figure 5). The hydroxyproline contents in the livers of both AdLacZ- and saline-treated rats were approximately 3-fold higher than in intact livers, as previously observed.[10–12] In contrast, in the Ad7ND-treated and AdTβ-TR–treated livers, the hydroxyproline content remained close to the level seen in intact livers.

After the DMN treatment, the serum levels of aspartate aminotransferase, alanine aminotransferase, and total bilirubin were all increased, and both the body and liver weights were decreased, probably because of liver dysfunction. However, these values were preserved or better maintained in the Ad7ND-treated or AdTβ-TR–treated groups (Table 1).

After a 3-week DMN treatment, we analyzed liver histology both by Masson trichrome staining and by immunohistostaining against α-actin. In accordance with the data on hydroxyproline content (Figure 5), both Ad7ND-treated and AdTβ-TR–treated livers showed a fibrotic area that was markedly smaller than that seen in

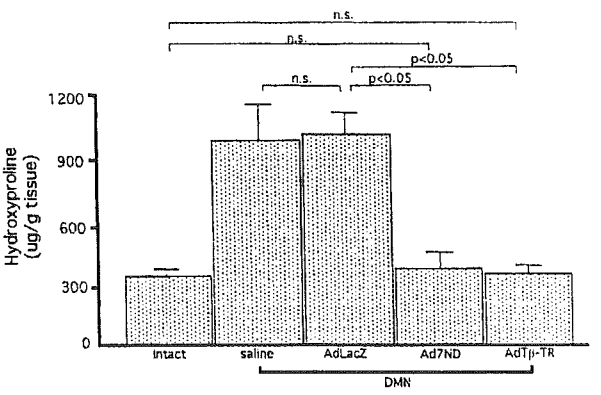

Figure 5. Hydroxyproline content of DMN-treated livers. Rats were treated with either adenovirus or saline as described in the legend to Figure 3 and then subjected to a 3-week DMN treatment. Hydroxyproline content of livers is shown as mean ± SD. Three samples from each of 4 rats were analyzed for each group. n.s., statistically not significant. Rats never treated with adenovirus or DMN were also analyzed (shown as *intact*).

Table 1. Serum Hepatobiliary Parameters and Body and Liver Weights

Variable	Total bilirubin (*mg/mL*)	AST (*IU/mL*)	ALT (*IU/mL*)	Body weight (*g*)	Liver weight (*g*)
Intact	0.2 ± 0.1	68 ± 16	40 ± 9	350 ± 20	3.5 ± 0.1
AdLacZ	0.2 ± 0.1	71 ± 11	39 ± 7	340 ± 20	3.6 ± 0.1
Saline + DMN	0.7 ± 0.6	495 ± 103	245 ± 88	290 ± 20	2.4 ± 0.4
AdLacZ + DMN	0.8 ± 0.7	525 ± 149	232 ± 97	290 ± 30	2.3 ± 0.5
Ad7ND + DMN	0.3 ± 0.1[a]	134 ± 16[a]	69 ± 7[a]	350 ± 10[a]	3.4 ± 0.1[a]
AdTβ − TR + DMN	0.4 ± 0.1[a]	222 ± 84[a]	69 ± 25[a]	350 ± 10[a]	3.5 ± 0.1[a]

NOTE. Rats were given a single infusion of saline, AdLacZ, Ad7ND, or AdTβ-TR via the tail vein. Seven days later, a 3-week DMN treatment was given to some rats (shown as +DMN). After a 3-week DMN treatment, blood was collected, and body and liver weights were measured. Serum total bilirubin, AST, and ALT and body and liver weights are shown as mean ± SE (n = 4). Rats never subjected to adenovirus infection or treated with DMN were also measured (shown as Intact).
AST, aspartate aminotransferase; ALT, alanine aminotransferase.
[a]$P < .05$ vs. AdLacZ + DMN.

the AdLacZ- and saline-injected rats, and α-actin–positive cells were almost undetectable (Figure 6).

In the AdTβ-TR–treated livers, α-actin–positive cells were readily detectable after the initial 3-day DMN treatment (Figure 4). We assumed that activated HSC disappeared through apoptosis under conditions in which the action of TGF-β was suppressed. We therefore performed TUNEL staining on the fourth day after starting DMN treatment. TUNEL-positive cells were increased in the AdTβ-TR–treated livers; however, no such apoptotic cells were observed in the AdLacZ- or saline-injected livers (Figure 7*A*). Immunohistostaining against α-actin confirmed that the TUNEL-positive cells in the AdTβ-TR–treated livers (Figure 7*A*) were indeed α-actin positive (Figure 7*B*).

Discussion

Inflammation induces infiltration by leukocytes and monocytes/macrophages into inflamed tissues.[1] Tissue remodeling or fibrosis then follows the inflammation. MCP-1, one of the CC chemokines, attracts monocytes/macrophages bearing CCR2.[1–3] In this study, the roles of such macrophages in injury-induced liver fibrogenesis were investigated by overexpressing a mutated MCP-1 (7ND), which is reported to suppress the actions of MCP-1.[15,23–25] In the Ad7ND-treated rats, DMN-induced infiltration by macrophages and lymphocytes into injured livers was markedly suppressed (Figure 3), the activation of HSC was eliminated (Figure 4), and liver fibrogenesis was greatly prevented (Figures 5 and 6). The cellular infiltration and activation of HSC observed immediately after infliction of the injury were similar between the AdTβ-TR–treated livers and the controls (saline-infused or AdLacZ-infected rats; Figures 3 and 4). Our study shows that infiltrated macrophages are critical for HSC activation and subsequent fibrogenesis and, importantly, that TGF-β is not an activating factor for HSC. It is suggested that the infiltrated macrophages

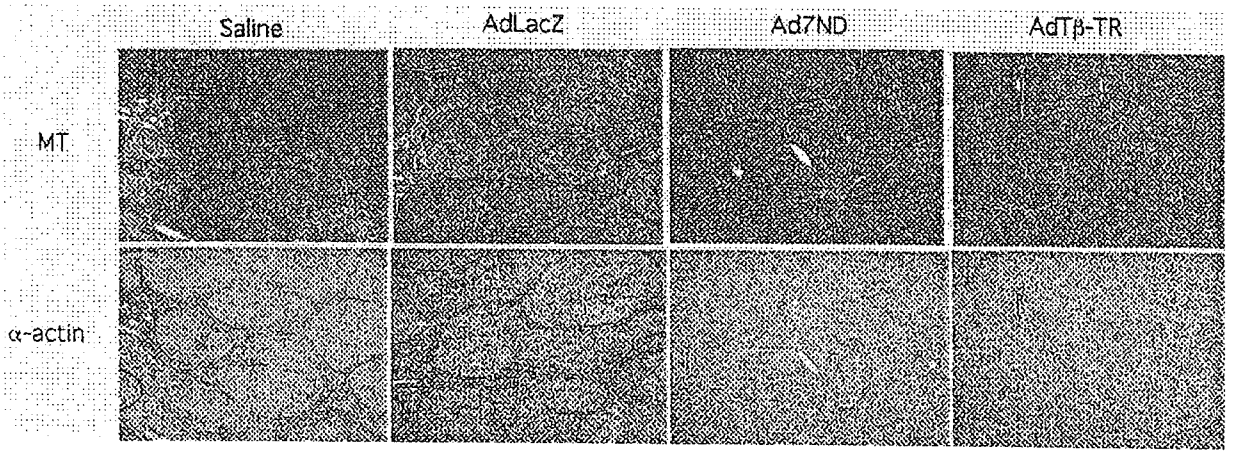

Figure 6. Histology of livers after a 3-week DMN treatment. Rats were treated as described in the legend to Figure 5. Liver sections were histologically examined with the aid of Masson trichrome staining (MT) or by immunohistostaining against α-actin (original magnification, 100×). Similar histology was seen in all 4 rats in each group.

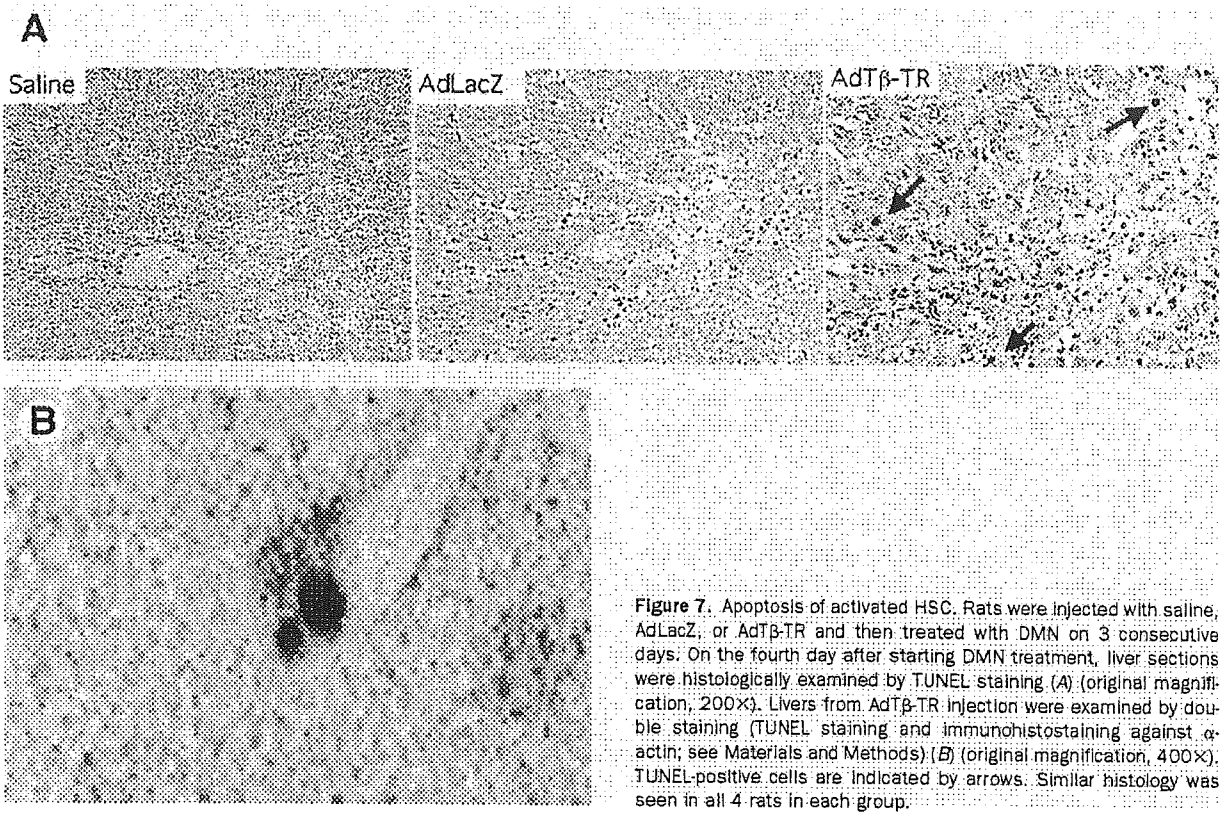

Figure 7. Apoptosis of activated HSC. Rats were injected with saline, AdLacZ, or AdTβ-TR and then treated with DMN on 3 consecutive days. On the fourth day after starting DMN treatment, liver sections were histologically examined by TUNEL staining (*A*) (original magnification, 200×). Livers from AdTβ-TR injection were examined by double staining (TUNEL staining and immunohistostaining against α-actin; see Materials and Methods) (*B*) (original magnification, 400×). TUNEL-positive cells are indicated by arrows. Similar histology was seen in all 4 rats in each group.

may themselves secrete an activating factor or factors for HSC.

We have previously shown that anti–TGF-β intervention inhibits liver fibrogenesis[11,13] and its progression.[12] In this study, we found that suppression of infiltration by macrophages and lymphocytes through overexpression of 7ND led to a powerful suppression of liver fibrogenesis to a similar degree as blockade of TGF-β but that the underlying mechanisms seem to be different. Activation of HSC in the initial stage immediately after injury was already eliminated in the Ad7ND-treated livers (Figure 4). Probably because HSC activation was inhibited, the subsequent progress toward fibrosis was suppressed in the Ad7ND-treated livers, thus supporting the idea that activation of HSC is the initial and critical event that leads to liver fibrosis. It has been considered for a long time that TGF-β is the HSC-activating factor (or at least one of the activating factors).[14] However, our study clearly shows for the first time that TGF-β is not the HSC-activating factor, because a substantial number of activated HSC were present in the AdTβ-TR–treated livers (Figure 4); indeed, the numbers of activated HSC were the same among saline-treated, AdLacZ-treated, and AdTβ-TR–treated livers. We confirmed previously that virtually all liver cells are infected with an adenovirus when one is administered to rats with intact livers,[11,13] so the possibility can be excluded that all of these activated HSC were uninfected with AdTβ-TR. Although substantial numbers of activated HSC were seen after a 3-day DMN treatment, most disappeared during the next 2 weeks of DMN treatment (Figure 6). The activated HSC are probably eliminated through apoptosis under conditions in which TGF-β signaling is inhibited. Indeed, we showed that in the AdTβ-TR–treated livers, but not in the AdLacZ- or saline-injected ones, activated HSC were in apoptosis (Figure 7). Saile et al[26] reported that HSC undergo CD95-mediated spontaneous apoptosis when they are activated, and TGF-β inhibits CD95-agonistic antibody-induced apoptosis of activated HSC in culture.[27] On the basis of these reported findings and our present study, it is likely that TGF-β is required for the activated HSC to survive. Consequently, fibrogenesis was markedly inhibited in the AdTβ-TR–treated livers despite activation of HSC in the initial stage after injury. To judge from our findings, anti–TGF-β intervention ought to be superior to anti–MCP-1 therapy for treating liver cirrhosis patients, most of whom already

have some degree of fibrosis or injury. This issue is now under further investigation in our laboratory.

Marra et al[28] reported that MCP-1 enhances the migration of HSC in culture. Moreover, it has been reported that HSC themselves produce MCP-1[29,30] and that TGF-β induces the secretion of MCP-1.[29] Together with our present study, it is likely that MCP-1 stimulates liver fibrogenesis by 2 mechanisms: (1) MCP-1 induces macrophage infiltration, and macrophages secrete an activating factor(s) for HSC; (2) MCP-1 acts directly on activated HSC to modulate their function. In favor of this notion, it has been reported that in cultured skin fibroblasts, MCP-1 increases the gene expressions of α_1 (I) procollagen and TGF-β[31] and of matrix metalloproteinase 1 and 2 (and of their inhibitor, tissue inhibitor of metalloproteinase 1)[32] and that MCP-1 stimulates the proliferation of cultured vascular smooth muscle cells.[33] Collectively, these observations suggest that MCP-1 secreted from macrophages and from HSC themselves may facilitate the production of TGF-β, as well as of matrix metalloproteinases and their inhibitors, thereby enhancing inflammation and tissue remodeling (fibrogenesis).

We expressed 7ND in livers expecting that it would inhibit MCP-1 as a dominant-negative mutant.[16] It has been reported that a 75:1 molar ratio of 7ND/wild-type MCP-1 is needed for a 50% inhibition of monocyte chemotaxis in vitro.[16] In our setting, the amount of 7ND was substantially higher than that of rat MCP-1 in both sera and livers for at least 2–2.5 weeks under DMN treatment (Figure 2). We have not yet determined how much 7ND is required to inhibit the actions of MCP-1 in vivo, specifically in the case of the DMN-injured rat liver. Thus, it is not certain whether the actions of MCP-1 were indeed inhibited in our experiments or whether the observed inhibition of macrophage infiltration was indeed achieved via a suppression of MCP-1 by 7ND. We would like to add that numerous reports (13, to our knowledge) have been published in which the same 7ND construct as that used in this study was introduced (either by direct injection or by electroporation with an expression plasmid) into various animal models, and suppression of macrophage infiltration and some biological effects were seen in every one of these reports. In 3 of these 13 studies, both 7ND and endogenous MCP-1 proteins in serum were measured, and the values obtained (7ND/MCP-1) were 220/71 pg/mL,[23] 226/85 pg/mL,[24] and 124/92 pg/mL[25] (all in mice). We detected a peak value of 528 ± 182 pg/mL for 7ND and 62 ± 12 pg/mL for endogenous rat MCP-1. Both this peak value for 7ND and the ratio between 7ND and endogenous MCP-1 are the highest among the values reported in the literature so far.

In summary, we have shown that the macrophages that infiltrate into livers immediately after an initial injury are critical both for HSC activation and for the subsequent fibrogenesis, and we also showed that TGF-β, which is required for activated HSC to survive, is not an activating factor for HSC (at least in this situation). Macrophages may themselves secrete an activating factor(s) for HSC.

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Address requests for reprints to: Hikaru Ueno, MD, PhD, Department of Biochemistry and Molecular Pathophysiology, University of Occupational and Environmental Health, School of Medicine, Iseigaoka 1-1, Yahatanishi-ku, Kitakyushu, 807-8555, Japan. e-mail: hueno@med.uoeh-u.ac.jp; fax: (81) 93-692-2777.

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Angiotensin II Participates in Hepatic Inflammation and Fibrosis through MCP-1 Expression

KEISHI KANNO, MD,* SUSUMU TAZUMA, MD,† TOMOJI NISHIOKA, MD,* HIDEYUKI HYOGO, MD,† and KAZUAKI CHAYAMA, MD*

In this study, we assessed the hypothesis that angiotensin (Ang) II could modulate inflammatory cell recruitment into the liver through hepatic expression of monocyte chemoattractant protein (MCP)-1 during liver injury. For in vivo study, Ang II type 1a knockout (AT1a KO) mice and wild-type (WT) mice were treated with CCl_4 for 4 weeks. After CCl_4 treatment, AT1a KO mice showed lower expression of MCP-1 and fewer CD68-positive cells in the liver compared with WT mice. For in vitro study, Ang II was added to LI90 cells. Ang II enhanced MCP-1 mRNA together with RhoA mRNA and also induced secretion of MCP-1 into the culture medium. This change was strongly blocked by Y-27632, a specific Rho-kinase inhibitor. These results suggest that Ang II modulates hepatic inflammation via production of MCP-1 by hepatic stellate cells, and the effect of Ang II on MCP-1 production is, at least partly, mediated by the Rho/Rho-kinase pathway.

KEY WORDS: renin–angiotensin system; monocyte chemoattractant protein-1; hepatic stellate cell; hepatic inflammation; hepatic fibrosis; angiotensin II type 1a knockout mouse; small G protein; Rho/Rho-kinase pathway; carbon tetrachloride.

The renin–anigiotensin system (RAS) not only plays an important role in the regulation of systemic hemodynamics, but also functions as a growth factor in various organs, including the vasculature, kidneys, and liver. Activated hepatic stellate cells (HSCs), which are major producers of extracelluar matrix after liver injury, express the angiotensin (Ang) II receptor (1), and inhibition of Ang II synthesis or blockade of Ang II signaling reduces experimental hepatic fibrosis (2–4). Our previous study showed that mice lacking the Ang II type 1a receptor (AT1a) were resistant to the development of hepatic fibrosis after exposure to carbon tetrachloride (CCl_4) (5). Moreover, local hepatic expression of key components of the RAS was up-regulated in an animal model of bile duct ligation (6), and the major cellular source of Ang II in the fibrotic liver was shown to be HSC (7). Overall, these reports support a contribution of the RAS to hepatic fibrogenesis.

Chemokines are low molecular weight secretory proteins that principally stimulate leukocyte recruitment. There are four defined chemokine subfamilies based on their primary structure, CXC, CC, C, and CX_3C. Monocyte chemoattractant protein (MCP)-1, which belongs to the CC subfamily, regulates the recruitment and activation of inflammatory cells, including monocytes/macrophages and T lymphocytes (8, 9). These inflammatory cells that infiltrate into the liver promote the progression of hepatic fibrosis by releasing various mediators (10). In fact, MCP-1 expression is up-regulated in the livers of patients with active cirrohosis (11), and activated HSCs are predominantly responsible for MCP-1 production (12). MCP-1 is secreted by various types of cultured cells. Among them, rat vascular smooth muscle cells (VSMCs)

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From the *Department of Medicine and Molecular Science, Graduate School of Biomedical Sciences, Hiroshima University, and †Division of General Medicine, Hiroshima University Medical Hospital, Hiroshima, Japan.

Address for reprint requests: Susumu Tazuma, MD, Division of General Medicine, Hiroshima University Medical Hospital, 1-2-3, Kasumi, Minami-ku, Hiroshima 734-8551, Japan; stazuma@hiroshima-u.ac.jp.

(13) and cardiac fibroblasts (14) are stimulated to produce MCP-1 by Ang II.

A small GTPase, Rho, is thought to trigger the intracellular pathways that lead to the activation of several transcription factors and nuclear signaling. Previous studies have detected RhoA in activated HSC, and Rho signaling pathways play a prominent role in the activation of HSCs (15, 16). Administration of Y-27632, a specific Rho-kinase inhibitor, has an inhibitory effect on the progression of experimental liver fibrosis in animal models (17, 18). Furthermore, Rho and Rho-kinase are involved in Ang II-induced expression of MCP-1 by VSMCs (13).

We hypothesized that Ang II may act on HSC to induce MCP-1 during liver injury, thereby modulating inflammatory cell infiltration and subsequent hepatic fibrosis. In addition, we examined the role of Rho/Rho-kinase in Ang II-mediated production of MCP-1 by LI90 cells, an HSC cell line.

MATERIALS AND METHODS

Animals. AT1a knockout (AT1a KO) mice were established and kindly provided by Dr. Sugaya (19). C57BL/6 mice were obtained from Hiroshima Jikken Doubutsu (Hiroshima, Japan). Both strains of mice had the same genetic background and animals 6–8 weeks old were used in this study. The mice were allowed free access to food and water and were housed at a constant temperature with a 12-hr light/dark cycle during the study period. Liver fibrosis was induced by the subcutaneous injection of CCl_4 (Wako Pure Chemical Industries, Osaka, Japan) at a dose of 1.0 ml/kg (1:1 in mineral oil) twice weekly for 4 weeks. Mice were killed and livers were harvested at 3 days after the last injection. All animal procedures were done according to our institutional guidelines.

Immunohistological Examination. Liver tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5-μm-thick sections. Immunohistochemical analysis was routinely performed using either a goat polyclonal antibody for MCP-1 (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) or CD68 (1:50 dilution; Santa Cruz Biotechnology). Several fields per slide were randomly selected for examination, and representative results from three animals are shown.

Cell Culture. LI90 cells (JCRB0160), which were derived from human HSCs (20), were provided by the Japan Health Science Foundation (Tokyo). LI90 cells were grown to confluence in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich, Japan) containing 10% fetal bovine serum (FBS; Gibco, Invitrogen, Japan) in uncoated plastic dishes, and then growth arrest was achieved by culture in DMEM without FBS for 2 days before use in the experiments.

RT-PCR. The steady-state level of each messenger RNA (mRNA) was assessed by a semiquantitative polymerase chain reaction (PCR) using GAPDH or β-actin as the housekeeping gene. RNA was isolated with the RNeasy Mini-kit (Qiagen, Germany) according to the manufacturer's instructions. Then single-stranded complementary DNA (cDNA) was synthesized from 1 μg of RNA using 0.5 nmol of each random primer and subjected to PCR. Subsequently, the synthesized cDNA was amplified using specific sets of primers for mouse MCP-1 (forward, ATGCAGGTCCCTGTCATG; reverse, GCTTGAGGTGGTTGTGGA) (21), mouse GAPDH (forward, TGAAGGTCGGTGTGAACGGATTTGGC; reverse, CATGTAGGCCATGAGGTCCACC AC) (21), human MCP-1 (forward, GACCACCTGGACAAGCAAAC; reverse, CTCAAAACATCCCAGGGGTA) (22), human RhoA (forward, CTGGTGATTGTTGGTGATGG; reverse, GCGATCATAATCTTCCTGCC) (23), and human β-actin (forward, GAGCGGGAAATCGTGCGTGACATT; reverse, GATGGAGTTGAAGGTAGTTTCGTG) (22). The PCR procedure used has been described previously (21–23). An aliquot (10 μl) of each PCR product was loaded onto a 2% agarose gel and stained with ethidium bromide. Then the band intensities were analyzed by densitometry.

Quantification of MCP-1 Protein by ELISA. Culture medium of nonstimulated LI90 cells or LI90 cells stimulated with Ang II for 2 days was collected and centrifuged at 12,000 rpm for 1 min. The supernatant was stored at −80°C until assay. MCP-1 was measured using a commercial enzyme-linked immunosorbent assay kit (Chemicon International, USA) according to the manufacturer's instructions.

NF-κB Activity Assay. LI90 cells were stimulated with 10^{-7} *M* Ang II for 1 hr with or without pretreatment using Y-27632 (Calbiochem–Novabiochem, USA) at a concentration of 10^{-5} *M* for 30 min. Nuclear extracts were prepared with a Nuclear Extraction kit (Active Motif, Japan), and the protein content was standardized. Then NF-κB activity was measured in the nuclear extracts using an NF-κB P65 Transcription Factor Assay kit (Chemicon International) according to the manufacturer's instruction.

Statistical Analysis. Results are expressed as the mean ± SD. Statistical analysis was performed using one-way analysis of variance (ANOVA) and $P < 0.05$ was considered to indicate significance.

RESULTS

Hepatic MCP-1 Expression in CCl_4-Treated Mice. RT-PCR revealed the up-regulation of hepatic MCP-1 mRNA expression in CCl_4-treated WT mice, whereas it was negligible in CCl_4-treated and untreated AT1a KO mice (Figure 1). Immunohistochemical analysis also confirmed the enhanced hepatic expression of MCP-1 protein in CCl_4-treated WT mice. After CCl_4 treatment

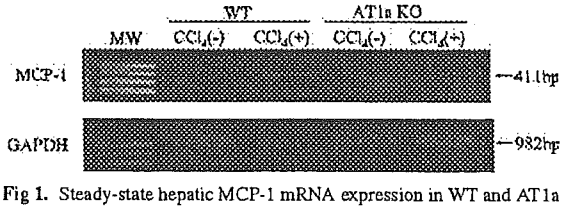

Fig 1. Steady-state hepatic MCP-1 mRNA expression in WT and AT1a KO mice with or without CCl_4 treatment for 4 weeks. An aliquot of each PCR product was loaded onto a 2% agarose gel and stained with ethidium bromide. Amplification of GAPDH was done to confirm the equal amounts of mRNA in each sample. The result shown here is representative of three independent experiments.

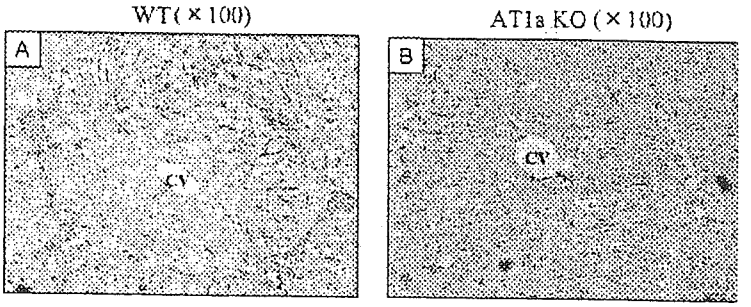

Fig 2. Immunohistochemical staining of MCP-1 in liver tissue from (A) a WT mouse and (B) an AT1A KO mouse after 4 weeks of CCl_4 treatment (1.0 ml/kg). CV, central vein. (Original magnification, ×100.)

for 4 weeks, MCP-1 staining was prominent in the portal tracts and fibrous septa of WT mice (Figure 2A). In contrast, the livers of CCl_4-treated AT1a KO mice showed almost no MCP-1 staining (Figure 2B) and were similar to the livers of the untreated groups (data not shown).

Hepatic CD68-Positive Cells in CCl_4-Treated Mice. As chemokines are considered to affect the recruitment of inflammatory cells, immunohistochemistry for the activated monocyte/macrophage marker CD68 (the main targets of MCP-1) was performed. In CCl_4-treated WT mice, the number of CD68-expressing cells was markedly increased in the portal tracts (Figure 3A). On the other hand, CCl_4 treatment had little influence on the number of CD68-expessing cells in the livers of AT1a KO mice (Figure 3B). These observations demonstrated that the number of CD68-expessing cells in the liver was associated with the expression of MCP-1.

Effect of Ang II on MCP-1 mRNA Expression. LI90 cells were stimulated with Ang II at a concentration of 10^{-7} *M*, and MCP-1 mRNA expression was examined at the indicated times by semiquantitative PCR. The expression of MCP-1 mRNA was enhanced, reaching a peak at 3 hr and returning to the basal level after 24 hr. The time course of MCP-1 mRNA expression was paralleled by the changes in RhoA mRNA (Figure 4A). Then LI90 cells were incubated with various concentrations of Ang II (10^{-11} to 10^{-5} *M*) for 3 hr. The expression of MCP-1 mRNA increased dose dependently and showed a pattern similar to that of RhoA mRNA (Figure 4B).

MCP-1 Protein Level in Culture Medium. To assess MCP-1 protein secretion into the culture medium, LI90 cells were stimulated with various concentrations of Ang II with or without Y-27632 pretreatment at a concentration of 10^{-5} *M*. Ang II dose dependently increased MCP-1 production after 48 hr of stimulation, and a significant difference was seen at a concentration of 10^{-7} or 10^{-5} *M*. Y-27632 markedly inhibited the secretion of MCP-1 protein induced by Ang II, whereas it had no suppressive effect on FBS-induced MCP-1 secretion (Figure 5).

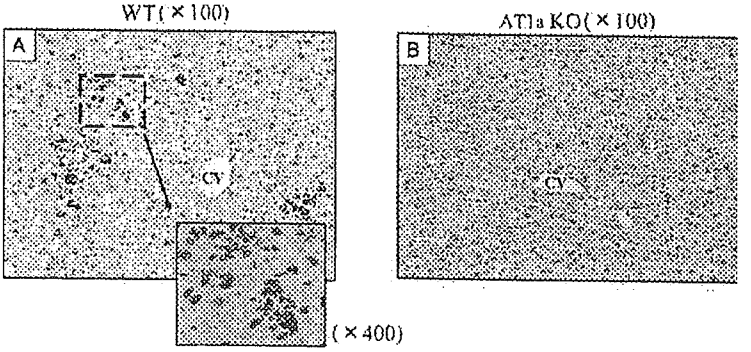

Fig 3. Distribution of CD68-positive cells in the liver. Immunohistocheical staining for CD68-positive mononuclear cells was performed in liver tissue from (A) a WT mouse and (B) an AT1A KO mouse after 4 weeks of CCl_4 treatment (1.0 ml/kg). CV, central vein. (Original magnification, ×100.)

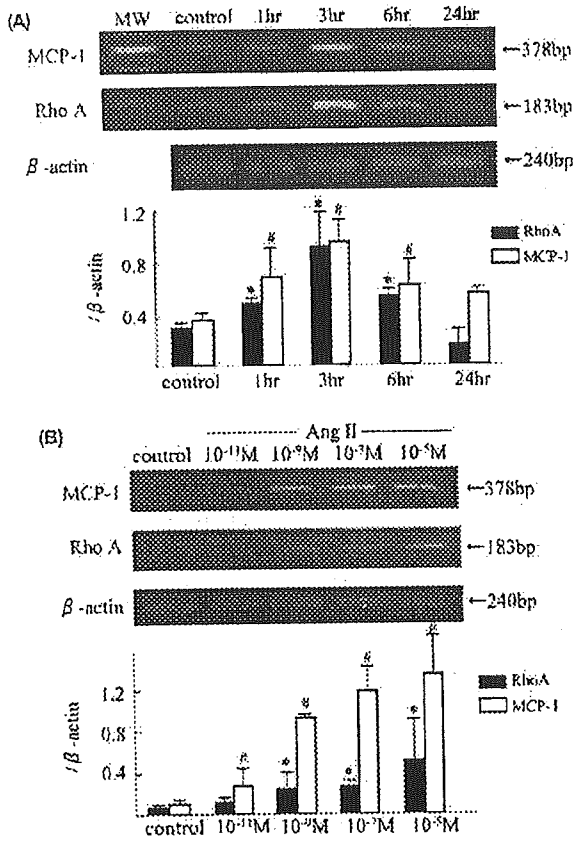

Fig 4. Effect of Ang II on MCP-1 mRNA expression by cultured LI90 cells. (A) Serum-starved LI90 cells were treated with 10^{-7} *M* Ang II at the indicated times, and the changes in Ang II-induced MCP-1 mRNA expression were examined by semiquantitative PCR. (B) Serum-starved LI90 cells were stimulated with Ang II at the indicated concentrations (10^{-11} to 10^{-5} *M*) for 3 hr, and MCP-1 mRNA expression was examined by semiquantitative PCR. Gels were scanned with a digital image analysis system, the products were quantified, and results are shown relative to the level of the housekeeping gene β-actin. Data from four independent experiments are shown as means ± SD. $^{*}P < 0.05$ vs. Rho A/β-actin of serum-free control; $^{\#}P < 0.05$ vs. MCP-1/β-actin of serum-free control.

Effect of Ang II on NF-κB Activity in LI90 Cells. NF-κB is the important factor involved in MCP-1 gene transcription in several cell types. To examine whether NF-κB participated in the induction of MCP-1 in Ang II-stimulated LI90 cells, NF-κB activity was studied. Growth-arrested LI90 cells were incubated with 10^{-7} *M* Ang II for 1 hr. In contrast to the up-regulation of MCP-1, Ang II did not activate NF-κB in LI90 cells (Figure 6).

DISCUSSION

There is accumulating evidence that the RAS is involved in hepatic fibrogenesis. Chronic liver injury up-regulates

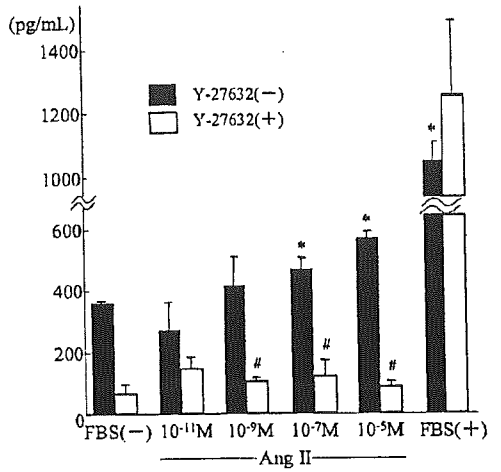

Fig 5. Effect of Ang II on MCP-1 secretion into the culture medium. Serum-starved LI90 cells were stimulated for 48 hr with the indicated concentrations of Ang II in the absence or presence of Y-27632 (10^{-5} *M*). Data from four independent experiments are shown as means ± SD. $^{*}P < 0.05$ vs. serum-free control. $^{\#}P < 0.05$ vs. each group without Y-27632.

key components of the RAS in an animal model of bile duct ligation (6), and RAS blockade ameliorates various types of experimental hepatic fibrosis (2–4). In patients with early chronic hepatitis C, an AT1 receptor antagonist decreased the area of hepatic fibrosis (24). We recently demonstrated that mice lacking the AT1 receptor are protected against CCl_4-induced hepatic fibrosis. Moreover, it was noteworthy that inflammatory infiltrates in the livers

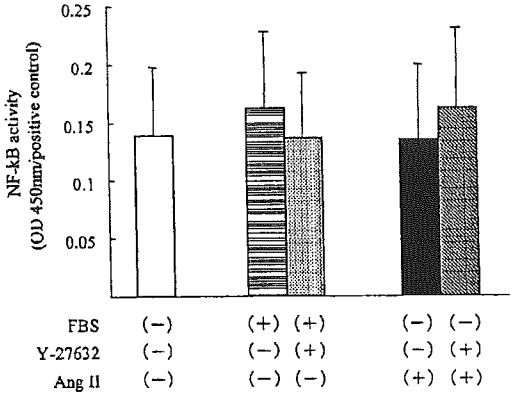

Fig 6. NF-κB activity in nuclear extracts of Ang II-stimulated LI90 cells. The cells were stimulated using Ang II for 1 hr with or without preincubation of Y-27632 (10^{-5} *M*). Then nuclear protein was extracted, and NF-κB activity was estimated. Absorbance values were standardized according to the protein concentration. The absorbance relative to that of the positive control (TNFα-stimulated whole Hela cells) from four independent experiments is shown as the mean ± SD.

of knockout mice were less severe compared with those in WT mice (5). This observation is in agreement with published data showing that prolonged systemic infusion of Ang II in normal rats induces hepatic inflammation (25). Therefore, there appears to be a close link between Ang II signaling and hepatic tissue inflammation.

MCP-1 is one of the potent chemokines that contributes to the accumulation of inflammatory cells. Since the recruitment of inflammatory cells depends on the expression of chemokines and adhesion molecules, hepatic MCP-1 expression is considered to play an important role in the pathogenesis of chronic hepatitis. MCP-1 levels have been reported to be elevated in the liver by treatment with CCl_4, endotoxin, or alcohol in experimental animals as well as in patients with chronic hepatitis (11, 12, 26, 27). In the present study, we demonstrated that mice lacking the AT1a receptor showed lower expression of MCP-1 and fewer CD68-positive cells in the liver after chronic CCl_4 treatment. These results do not mean the total elimination of hepatic inflammation but confirm that Ang II signaling via AT1a is critical for hepatic expression of MCP-1 and for the recruitment of mononuclear cells into the liver. According to the previous report (28), Kupffer cells play a critical role in the pathogenesis of hepatic inflammation and fibrosis through the release of biologically active mediators. In this regard, Ang II modulates hepatic inflammation via control of MCP-1 expression and subsequent mononuclear cells recruitment.

HSC can amplify inflammation through the release of chemokines such as MCP-1, and the up-regulation of such chemokines further amplifies inflammation during the process of liver injury (29, 30). In this study, we examined whether Ang II induces MCP-1 in cultured LI90 cells. Ang II enhanced the expression of MCP-1 mRNA together with RhoA mRNA in LI90 cells and, also, stimulated secretion of MCP-1 protein into the culture medium in a dose-dependent manner. Pharmacological blockade of Rho signaling with Y-27632, a specific inhibitor of Rho-kinase, strongly suppressed the Ang II-induced increase in MCP-1 production. The small G protein Rho is a member of the Rho family of small GTPases that also includes Rac and Cdc42. It is understood that Rho has a role in various cell functions, such as the control of cell morphology, proliferation, apoptosis, and regulation of various transcriptional factors. Recently, considerable attention has been paid to the role of Rho in the pathogenesis of hepatic fibrosis. Rho is reported to regulate the activation and proliferation of cultured HSC (15, 16), while administration of Y-27632 inhibits the development of hepatic fibrosis induced by dimethylnitrosamine (17) and CCl_4 (18) in animals. Furthermore, there are several lines of evidence for a close link between Ang II and the Rho signaling pathway. Ang II activates Rho in cultured VSMCs (13) and rat aortic endothelial cells (31), with induction of MCP-1 occurring in the former cell type. Interestingly, we found that Y-27632 did not suppress MCP-1 production when HSC were stimulated with FBS, which contains various growth factors. This suggests that the repressive effect of Y-27632 on MCP-1 production depends on the type of stimulation applied to HSC.

MCP-1 is produced by various types of cells, including HSC, monocytes, fibroblasts (14), and VSMCs (13), in response to a number of stimuli. Since the effects of different stimuli on MCP-1 expression are quite diverse among cell types, transcriptional activation generally seems to depend on an intricate series of regulatory mechanisms. Previous studies have indicated that NF-κB is the main factor involved in regulating the transcription

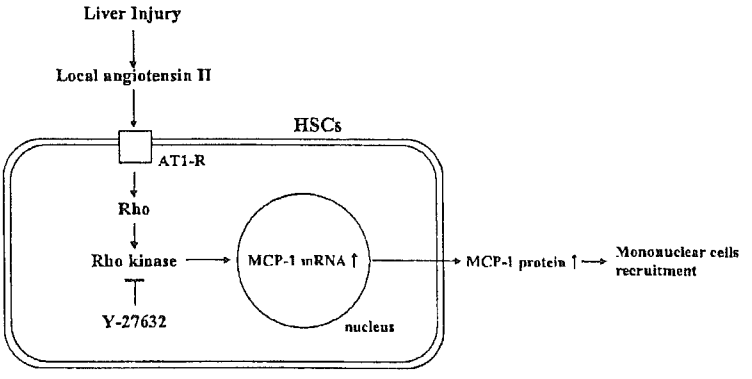

Fig 7. Speculated mechanism of how angiotensin II participates in hepatic inflammation after liver injury. Ang II enhances MCP-1 gene expression and synthesis, partly via the Rho signaling pathway, which modulate the recruitment of inflammatory cells into the liver. AT1-R, angiotensin II type1 receptor; HSCs, hepatic stellate cells.

of MCP-1 induced by LPS, IL-1β, TNF-α, and phorbal esters (32, 33). It was also reported that Ang II promotes MCP-1 expression via activation of NF-κB in cultured glomerular mesangial cells (34), as well as macrophages and VSMCs (13). Moreover, systemic Ang II infusion increases the DNA-binding activity of NF-κB in animals (25). Contrary to our expectation, the present study demonstrated that the level of NF-κB activity in LI90 cells was not altered by Ang II. However, this result is in agreement with the findings of a recent study using primary cultured human HSC (35), so further investigation is needed.

In conclusion, the present study demonstrated that the lack of Ang II signaling reduces the hepatic expression of MCP-1 and recruitment of activated Kupffer cells in a CCl_4-induced hepatic fibrosis model. In cultured LI90 cells, it was also shown that Ang II enhances MCP-1 gene expression and synthesis, partly via the Rho signaling pathway (Figure 7). These findings explain the mechanism by which inhibition of Ang II synthesis or blockade of AT1 signaling can reduce hepatic inflammation and subsequent fibrosis.

ACKNOWLEDGMENTS

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MAJOR ARTICLE

T1653 Mutation in the Box α Increases the Risk of Hepatocellular Carcinoma in Patients with Chronic Hepatitis B Virus Genotype C Infection

Kiyoaki Ito,[1,2] Yasuhito Tanaka,[1] Etsuro Orito,[2] Masaya Sugiyama,[1] Kei Fujiwara,[2] Fuminaka Sugauchi,[2] Takanobu Kato,[1] Hajime Tokita,[3] Namiki Izumi,[4] Michio Kato,[5] Man-Fung Yuen,[6] Ching-Lung Lai,[6] Robert G. Gish,[7] Ryuzo Ueda,[2] and Masashi Mizokami[1]

Departments of [1]Clinical Molecular Informative Medicine and [2]Internal Medicine and Molecular Science, Nagoya City University Graduate School of Medical Sciences, Nagoya, [3]Department of Gastroenterology, National Tokyo Hospital, and [4]Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Tokyo, and [5]National Hospital Organization Osaka National Hospital, Osaka, Japan; [6]Department of Medicine, University of Hong Kong, Queen Mary Hospital, Hong Kong; and [7]Division of Hepatology and Complex Gastroenterology, California Pacific Medical Center, San Francisco

Background. Most patients with chronic hepatitis B virus infection become carriers of inactive virus after hepatitis B e antigen seroconversion; however, a subgroup of patients have persistent abnormal transaminase levels and develop hepatocellular carcinoma after seroconversion.

Methods. In an age-matched case-control study, 40 carriers of inactive virus (mean age ± standard deviation [SD], 50.9 ± 11.1 years), 40 patients with chronic hepatitis (mean age ± SD, 50.2 ± 8.9 years), and 40 patients with hepatocellular carcinoma (mean age ± SD, 50.7 ± 9.4 years) who were infected with hepatitis B virus genotype C and had test results positive for antibody to hepatitis B e antigen were analyzed.

Results. The prevalence of T1653 in the box α was significantly higher among patients with hepatocellular carcinoma than among carriers of inactive virus who did not have hepatocellular carcinoma (70% vs. 25%; $P < .0001$) or chronic hepatitis (70% vs. 35%; $P = .003$). Mutations in the basic core promoter region (T1762/A1764) were frequently found in all groups, regardless of clinical status (in 77.5% of carriers of inactive virus, 77.5% of patients with chronic hepatitis, and 90% of patients with hepatocellular carcinoma). In the multivariate analysis, the presence of T1653, an alanine aminotransferase level of ≥37 U/L, and a platelet count of $<18 \times 10^4$ platelets/mm^3 were independent predictive values for hepatocellular carcinoma (odds ratio [95% confidence interval], 5.05 [1.56–16.35], 12.56 [3.05–51.77], and 11.5 [3.47–38.21], respectively). High α-fetoprotein level was the only independent predictive value for T1653 in patients with hepatocellular carcinoma (odds ratio, 12.67; 95% confidence interval, 1.19–134.17]). Among patients with test results positive for antibody to hepatitis B e antigen who had hepatocellular carcinoma and were infected with different genotypes of hepatitis B virus, the prevalence of T1653 was 40%, 15%, 25%, 25%, 67%, and 23% in patients infected with hepatitis B virus genotypes Aa, Ae, Ba, Bj, C, and D, respectively ($P < .05$ for genotype C vs. genotypes Ae, Ba, Bj, or D).

Conclusions. Our data indicate that the addition of T1653 mutation in the box α to the basic core promoter mutation increases the risk of hepatocellular carcinoma in patients with hepatitis B virus genotype C.

Hepatocellular carcinoma (HCC) is the fifth most frequent cancer and the third leading cause of cancer-related death in the world, with an estimated prevalence of >500,000 cases worldwide per year [1]. It is now accepted that hepatitis B virus (HBV) has a carcinogenic potential in humans. Several mutations in the HBV genome have been reported to occur during the course of persistent viral infection, and there has been increasing evidence of an association between these molecular alterations and the development of HCC in patients with HBV infection.

During persistent HBV infection, carriers frequently undergo seroconversion from hepatitis B e antigen (HBeAg) to the corresponding antibody (anti-HBe). Most patients who acquire chronic HBV infection with HBV genotype C (which is a common genotype in East

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Reprints or correspondence: Dr. Masashi Mizokami, Dept. of Clinical Molecular Informative Medicine, Nagoya City University Graduate School of Medical Science, Kawasumi, Mizuho, Nagoya 467-8601, Japan (mizokami@med.nagoya-cu.ac.jp)

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Asian countries) by perinatal transmission become carriers of inactive virus after seroconversion. A subgroup of patients have persistent abnormal serum transaminase levels and develop HCC in the anti-HBe–positive phase. Many of these patients have active viral replication and are infected with several mutant viruses. The association between different clinical events after seroconversion and specific HBV genomic mutations has not been clearly defined.

Mutations in the basic core promoter (BCP) region at nucleotides (nt) 1762/1764 (T1762/A1764) and mutation in the precore (preC) region at nt 1896 (A1896) are associated with seroconversion and persistent viral replication. It is noteworthy that both BCP and preC mutations are often found in patients with advanced liver disease, (e.g., HCC) [2–8]. The T1762/A1764 mutation alters HBeAg production at the transcription level, and the A1896 in the preC region terminates translation of the precursor protein, abrogates HBeAg production, and results in seroconversion. A1896 was also reported previously to be associated with severe forms of chronic liver disease [7,8].

HBV has been classified into 8 major genotypes with use of the complete nucleotide sequence of the viral genome [10]. HBV genotypes not only have distinct geographical distributions [7, 11, 12] but also have different clinical manifestations and responses to therapy (e.g., IFN therapy). Furthermore, HBeAg positivity and levels of HBV DNA, which are controlled by specific mutations, differ between HBV genotypes (e.g., the BCP double mutation is more prevalent among strains of HBV genotype C, followed by HBV genotype A, and the A1896 mutation is frequently found in HBV genotypes B and D) [13–16].

There have been many studies involving viral mutations associated with clinical features, but most previous studies have ignored age, sex, HBeAg status, and HBV genotypes. In Japan, most patients with HCC experience seroconversion (i.e., they are anti-HBe positive) and have HBV genotype C; therefore, we performed an age-matched case-control study among anti-HBe–positive patients infected with HBV genotype C (including carriers of inactive virus, patients with chronic hepatitis, and patients with HCC) to determine the specific HBV genome mutations associated with disease progression.

PATIENTS AND METHODS

Serum samples. Serum samples were obtained from 211 patients from different regional areas worldwide. A total of 120 patients from Japan who were infected with HBV genotype C (40 carriers of inactive virus, 40 patients with chronic hepatitis, and 40 patients with HCC) were matched with control subjects according to age and HBe status. Control serum samples were obtained from patients with HCC who were positive for anti-HBe and who were infected with HBV genotype Aa (10 subjects), Ae (13), Ba (20), Bj (20), C (15), and D (13). Control subjects were from Hong Kong (19 subjects), Japan (36), and the United States (36). The majority of patients infected with HBV genotypes Aa, Ba, Bj, and C were Asian, and the majority of patients infected with HBV genotypes Ae and D were white and black. None of the subjects had serological test results positive for markers of infection with hepatitis C virus or HIV-1.

The study protocol was approved by ethics committees of the participating institutions in accordance with the 1975 Helsinki declaration. Informed consent was obtained from each patient.

Serological assays for HBV markers. HBeAg and anti-HBe were detected by chemiluminescent EIA (Lumipulse *f*, Fujirebio). HBV genotypes were determined by the restriction fragment–length polymorphism method on the S gene sequence amplified by PCR [29] and ELISA with monoclonal antibodies directed to distinct epitopes on the preS2 region products [18], with use of commercial kits (HBV genotype EIA; Institute of Immunology). The genotypes were also confirmed with use of a phylogenetic tree analysis. α-Fetoprotein and serum protein induced by the absence of vitamin K (antagonist II) were examined with use of chemiluminescent EIA.

Amplification and sequencing of the core promoter and the precore region plus core gene. HBV DNA sequences bearing the core promoter and preC or core regions were amplified by PCR with heminested primers by the method described elsewhere [19]. Thereafter, PCR products were sequenced directly with Prism Big Dye (Applied Biosystems) in the ABI 3100 DNA automated sequencer (Applied Biosystems). Accession numbers for all strains are AB236515–AB236634.

Case-control study. A carrier of inactive virus was defined as an HBsAg-positive individual with normal alanine aminotransferase (ALT) levels for a 2-year period (with at least 4 evaluations at 3-month intervals) and without the presence of portal hypertension. Chronic hepatitis was defined as persistent elevation of ALT levels ($> 1.5 \times$ upper limit of normal [35 U/L]) during a 6-month period (with at least 3 evaluations at 2-month intervals) without a decrease in platelet count or albumin level, and hypersplenism (splenomegaly on ultrasonographic examination). Twenty-one patients were confirmed to have chronic hepatitis by means of a fine-needle biopsy of the liver. Staging and grading (expressed as mean value ± SD [95% CI]) were 1.24 ± 0.64 (0.99–1.58) and 1.36 ± 0.58 (1.07–1.59), respectively, as previously described [30]. None had received antiviral treatment during the follow-up period. Of 40 patients with HCC, 23 patients received a diagnosis of HCC on the basis of a pathologic examination, and 17 patients received a diagnosis of HCC on the basis of results of abdominal ultrasonography, angiography, CT, or MRI, as well as an elevated serum α-fetoprotein level (≥400 ng/mL).

Statistical evaluation. Data were expressed as mean ±

SD. Statistical analyses were performed using χ^2 test and Fisher's exact test for categorical variables. Mann-Whitney U test or 1-way analysis of variance were used for continuous variables, as appropriate. Mantel-Haenszel χ^2 test was used to analyze the trend of frequencies of viral mutations. Multivariate analyses with logistic regression were used to determine the independent factors associated with HCC and T1653. Differences were considered to be significant for P values <.05. The statistical analysis software used was Stata software, version 8.0 (StataCorp).

RESULTS

Table 1 compares ALT level, platelet count, and HBV DNA level, as well as mutations in the box α (enhancer II), core promoter, and preC region, among 40 carriers of inactive virus, 40 patients with chronic hepatitis, and 40 patients with HCC who were infected with HBV genotype C in an age-matched case-control study. ALT and HBV DNA levels were significantly lower among carriers of inactive virus than among patients with chronic hepatitis or patients with HCC ($P<.0001$ and $P = .001$, respectively). Platelet count was lower among patients with HCC than among carriers of inactive virus or patients with chronic hepatitis ($P<.0001$).

The frequency of the T1653 mutation in the box α was significantly higher among patients with HCC (70%) than among carriers of inactive virus (25%) or patients with chronic hepatitis (35%; $P<.0001$) (table 1). Of interest, the T1653 mutation had an opposite correlation with the M1753 mutation. The prevalence of T1762/A1764 was high in all clinical status groups, with no statistically significant difference between groups (table 1). The trend of the frequency of T1653, increasing from carriers of inactive virus to patients with chronic hepatitis to patients with HCC, was analyzed by Mantel-Haenszel χ^2 test (OR, 2.48; 95% CI, 1.59–3.85; $P = .0001$) (figure 1). The trend of the frequency of T1762/A1764 was not statistically significant ($P = .1502$) (figure 1).

The attributable risk of multiple factors, including sex, HBV DNA level, ALT level, platelet count, and the presence of the T1653, M1753, T1762/A1764, and A1896 mutations for HCC in the HBV carriers was determined by multiple logistic regression analysis (table 2). There was a statistically significant association between development of HCC and ALT level >37 U/L (OR, 12.56; 95% CI, 0.55–6.21; $P<.0001$) and platelet count $<18 \times 10^4$ platelets/mm^3 (OR, 11.5; 95% CI, 3.47–38.21; $P<.0001$). The T1653 mutation was still significantly associated with the development of HCC (OR, 5.05; 95% CI, 1.56–16.35; $P = .007$).

The attributable risk of multiple factors, including HBV DNA level, ALT level, platelet count, α-fetoprotein level, protein in-

Table 1. Demographic, clinical, and virologic characteristics of patients infected with hepatitis B virus (HBV) genotype C who were matched for age and hepatitis B e antigen (HBeAg) status.

Variable	Clinical status			
	Carriage of inactive virus (n = 40)	Chronic hepatitis (n = 40)	Hepatocellular carcinoma (n = 40)	P
Male sex	31 (77.5)	37 (92.5)	36 (90)	.171
Age, years	50.9 ± 11.1	50.2 ± 8.9	50.7 ± 9.4	Matched
HBeAg positive	0 (0)	0 (0)	0 (0)	Matched
Anti-HBeAg positive	40 (100)	40 (100)	40 (100)	Matched
HBV genotype C	40 (100)	40 (100)	40 (100)	Matched
Alanine transaminase level, U/L[a]	20.8 ± 7.6	102 ± 108.7	83.2 ± 84.8	.0001
Platelet count, $\times 10^4$ platelets/mm^3[b]	20.7 ± 3.1	17.4 ± 4.1	12.8 ± 5.7	.0001
HBV DNA level, LGE/mL[c]	4.3 ± 0.8	5.9 ± 1.5	5.4 ± 1.5	<.0001
Mutation in the box α: T1653[d]	10 (25)	14 (35)	28 (70)	<.0001
Mutation in the core promoter				
M1753	10 (25)	6 (15)	9 (22.5)	.609
T1762/A1764	31 (77.5)	31 (77.5)	36 (90)	.289
Mutation in the precore region: A1896	25 (62.5)	26 (65)	25 (62.5)	1.0

NOTE. Data are no. (%) of patients or mean value ± SD. Anti-HBeAg, antibody to HBeAg; LGE, log genome equivalents.

[a] $P<.0001$ for carriers of inactive virus vs. patients with chronic hepatitis; $P = .002$ for carriers of inactive virus vs. patients with hepatocellular carcinoma.

[b] $P<.0001$ for patients with hepatocellular carcinoma vs. carriers of inactive virus or patients with chronic hepatitis; $P = .002$ for carriers of inactive virus vs. patients with chronic hepatitis.

[c] $P<.0001$ for carriers of inactive virus vs. patients with chronic hepatitis; $P = .001$ for carriers of inactive virus vs. patients with hepatocellular carcinoma.

[d] $P<.0001$ for carriers of inactive virus vs. patients with chronic hepatitis; $P = .001$ for carriers of inactive virus vs. patients with hepatocellular carcinoma.

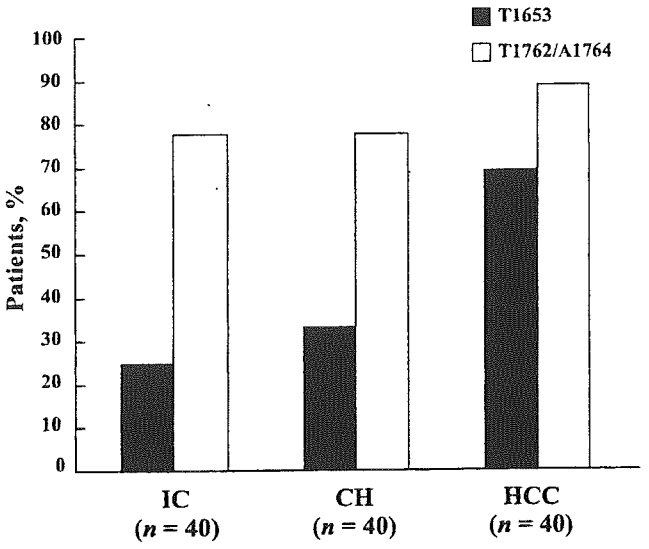

Figure 1. Prevalence of T1653 box α and T1762/A1764 basic core promoter mutations among patients with chronic hepatitis B virus infection, stratified by clinical status. The trend of the frequency of the T1653 mutation was analyzed by Mantel-Haenszel χ^2 test. The OR estimate is an approximation of the OR for carriers of inactive virus (IC), patients with chronic hepatitis (CH), and patients with hepatocellular carcinoma (HCC) having a strain with the mutation (OR, 2.48; 95% CI, 1.59–3.85; $P = .0001$). The trend of the frequency of the T1762/A1764 mutation was not statistically significant according to the Mantel-Haenszel χ^2 test ($P = .1502$).

duced by the absence of vitamin K (antagonist II) level, for T1653 in patients with HCC with HBV genotype C infection was determined by multiple logistic regression analysis (table 3). An α-fetoprotein level >300 ng/mL was the only independent predictive value for the presence of the T1653 mutation in patients with HCC with HBV genotype C infection (OR, 12.67; 95% CI, 1.19–134.17; $P = .035$).

Table 4 compares sex, age, and mutations in the box α, core promoter, and preC region among patients infected with HBV genotypes Aa (10 patients), Ae (13), Ba (20), Bj (20), C (15), and D (13) with the same variables among patients with HCC. Mean age was significantly higher among patients with HBV genotype Bj infection, compared with patients with HBV genotype Ba, genotype C, and genotype D infection ($P < .05$). The prevalence of T1653 among patients with HBV genotype C infection (66.7%) was significantly higher than it was among patients infected with other genotypes (15%–25%; $P < .05$), excluding patients infected with HBV genotype Aa. The prevalence of T1762/A1764 among patients with HBV genotype Ba infection (85%) and HBV genotype C infection (86.7%) was also significantly higher than it was among patients infected with other genotypes (20%–50%; $P < .05$). The prevalence of A1896 among patients with HBV genotype Aa infection and HBV genotype Ae infection was significantly lower than it was among patients infected with other genotypes ($P < .05$).

DISCUSSION

Many previous studies have reported that the clinical course of chronic HBV infection may be modified by several specific viral mutations [5, 20, 21], although the significance of such specific mutations in patients with chronic hepatitis B remains controversial. Because most studies have not controlled for different variables, such as age, HBV genotype, and HBe status, it is unknown whether the mutations were associated with disease progression, greater age of the patient, the specific HBV genotype or subtype, or HBe status. In this study, to exclude any biases, we performed an age-matched case-control study involving only anti-HBe–positive patients infected with HBV genotype C.

In the present case-control study, the prevalence of T1653 was found to be significantly higher among patients with HCC, compared with carriers of inactive virus and patients with chronic hepatitis with HBV genotype C infection; however, the prevalence of T1762/A1764 was high in all clinical status groups. During the anti-HBe–positive phase of infection, T1653 was more reliable than T1762/A1764 as a predicting factor for

Table 2. Multivariate analysis of variables with independent predictive value for development of hepatocellular carcinoma among a group of 120 patients with hepatitis B virus infection.

Variable	OR (95% CI)	P
Sex		
Female	1	
Male	5.06 (0.85–30.15)	.075
HBV DNA level		
<4.8 LGE/mL	1	
≥4.8 LGE/mL	0.34 (0.09–1.21)	.096
Alanine transaminase level		
<37 U/L	1	
≥37 U/L	12.56 (3.05–51.77)	.0001[a]
Platelet count		
≥18 × 10^4 platelets/mm^3	1	
<18 × 10^4 platelets/mm^3	11.51 (3.47–38.21)	.0001[a]
T1653 mutation		
No	1	
Yes	5.05 (1.56–16.35)	.007[a]
M1753 mutation		
No	1	
Yes	1.23 (0.31–5.04)	.770
T1762/A1764 mutation		
No	1	
Yes	2.67 (0.57–12.54)	.214
A1896 mutation		
No	1	
Yes	0.96 (0.29–3.11)	.943

NOTE. Each OR was adjusted for age and other variables in the analysis. LGE, log genome equivalents.

[a] Statistically significant.

Table 3. Multivariate analysis of variables with independent predictive value for the presence of the T1653 mutation among 40 patients with hepatocellular carcinoma.

Variable	OR (95% CI)	*P*
HBV DNA level		
<4.9 LGE/mL	1	
≥4.9 LGE/mL	0.89 (0.16–4.79)	.899
ALT level		
<53 U/L	1	
≥53 U/L	1.72 (0.29–9.96)	.541
Platelet count		
≥12 × 10^4 platelets/mm^3	1	
<12 × 10^4 platelets/mm^3	1.39 (0.28–7.02)	.683
α-Fetoprotein level		
<300 ng/mL	1	
≥300 ng/mL	12.67 (1.19–134.17)	.035[a]
PIVKA-2 level		
<50 mAU/mL	1	
≥50 mAU/mL	0.25 (0.05–1.43)	.120

NOTE. Each OR was adjusted for age and other variables in the table. PIVKA-2, protein induced by the absence of vitamin K (antagonist II).

[a] Statistically significant.

the development of HCC. In fact, in the multivariate analysis, the presence of T1762/A1764 was not an independent predictor of HCC, but ALT level >37 U/L, platelet count <18 × 10^4 platelets/mm^3, and the presence of T1653 were independent predictors of HCC. The T1653 mutation had also been reported by Takahashi et al. [17]; they reported that this specific mutation was prevalent among Japanese patients with HCC, although their study was not a case-control study. These results do not deny that T1762/A1764 is associated with hepatocarcinogenesis, because poor prognosis associated with HBV genotype C infection, compared to that associated with HBV genotype B (Ba and Bj) infection, correlated with a high prevalence of T1762/A1764 [2, 9, 16], indicating that the BCP double mutation is associated with a high potential for hepatocarcinogenesis. The appearance of the T1653 mutation after the occurrence of the T1762/A1764 mutation (the T1762/A1764 mutation usually occurs earlier than the T1653 mutation) could indicate that the virulence of HBV is increasing, which could result in the development of HCC. In the multivariate analysis, however, HBV DNA level was no longer a predicting factor for HCC. One of the reasons for this is that the HBV DNA data used in this study were obtained at the time of diagnosis of HCC. A recent prospective study from Taiwan has indicated that high HBV DNA levels at baseline and infection with HBC genotype C were independent predictors for HCC, but the mean viral load at the time of diagnosis of HCC was significantly lower than at baseline [27]. Although our data could not indicate an association between HBV DNA level and hepatocarcinogenesis, if we could measure the HBV DNA level before diagnosis of HCC, it might found to be be a predicting factor for HCC. Furthermore, an examination of the characteristics of patients with HCC who had the T1653 mutation showed that an elevated α-fetoprotein level (≥300 ng/mL) was the only predictor for the development of HCC in patients with the T1653 mutation. It has been reported that α-fetoprotein level is useful not only for diagnosis but also as a prognostic indicator for patients with HCC [22, 23], suggesting that the T1653 mutation might be associated with poor prognosis for patients with HCC.

The prevalence of several mutations among patients with HCC differed from that among patients with different HBV genotypes (Aa, Ae, Ba, Bj, C, and D) (table 4). The prevalence

Table 4. Demographic and virological characteristics of patients with hepatocellular carcinoma who were positive for antibody to hepatitis B e antigen (anti-HBe), by hepatitis B virus (HBV) genotype.

	HBV genotype						
Variable	Aa (*n* = 10)	Ae (*n* = 13)	Ba (*n* = 20)	Bj (*n* = 20)	C (*n* = 15)	D (*n* = 13)	*P*
Male	10 (100)	12 (92.3)	18 (90)	15 (75)	15 (100)	13 (100)	.10
Age, years[a]	54.4 ± 7.7	55.3 ± 4.4	54.4 ± 14.8	64.9 ± 9.6	47.9 ± 7.6	53.5 ± 8.3	.0002
HBeAg positive	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	Matched
Anti-HBe positive	10 (100)	13 (100)	20 (100)	20 (100)	15 (100)	13 (100)	Matched
Mutation in the box α: T1653[b]	4 (40)	2 (15.4)	5 (25)	5 (25)	10 (66.7)	3 (23.1)	.039
Mutations in the core promoter region							
M1753	3 (30)	3 (23.1)	5 (25)	4 (20)	2 (13.3)	1 (7.7)	.759
T1762/A1764[c]	5 (50)	6 (46.2)	17 (85)	4 (20)	13 (86.7)	5 (38.5)	<.0001
Mutation in the precore region: A1896[d]	0 (0)	0/13 (0)	9/20 (45)	15/20 (75)	9/15 (60)	8/13 (61.5)	<.0001

NOTE. Data are no. (%) of patients or mean value ± SD. HBeAg, hepatitis B e antigen.

[a] $P < .05$ for Bj vs. Ba or D; $P < .0001$ for Bj vs. C.

[b] $P < .05$ for C vs. Ba or Bj or D; $P < .01$ for Ae vs. C.

[c] $P < .05$ for Ae vs. Ba or C; $P < .01$ for D vs. Ba or C; $P < .0001$ for Bj vs Ba or C.

[d] $P < .05$ for Ba vs. Aa or Ae; $P < .005$ for Aa vs. C or D and for Ae vs. Ba or C or D; $P < .0001$ for Bj vs Aa or Ae.

of T1653 was the highest among patients with HBV genotype C infection, followed by those with HBV genotype Aa infection, although the number of patient with HBV genotype Aa infection was too small for any conclusions to be drawn. The prevalence of T1762/A1764 was higher among patients with HBV genotype Ba and HBV genotype C infection than among patients infected with other genotypes. HBV genotype Ba has a sequence that closely resembles that of HBV genotype C in the core promoter region, because it is recombinant HBV between HBV genotype Bj and HBV genotype C from nucleotides 1740 to 2485. Although A1896 was not found in HBV genotype Aa and HBV genotype Ae, as has been reported elsewhere [15], HBV genotype Aa had some specific mutations upstream of the preC initiation codon and encapsidation signal site. Therefore, several HBV genotype–specific mutations would be associated with different mechanisms on seroconversion or HBV replication for each genotype or subtype.

Buckwold et al. [24] reported that T1762/A1764 can no longer bind liver-enriched transcription factors and that the transcription of precore RNA and the expression of HBeAg were reduced. Thereafter, Li et al. [25] reported that this mutation not only removed the nuclear receptor–binding site but also created a hepatic nuclear factor 1 transcription factor–binding site. As for a factor correlated with BCP, the core upstream regulatory sequence, which has a strong stimulation effect on the BCP, was reported. In an earlier article by Yu et al. [28], the box α elements (nucleotides 1646–1668) individually stimulated promoter activity >100-fold. The T1653 mutation converts the box α binding site for CCAAT/enhancer-binding protein and related factors into the perfect palindromic sequence 1648-TCTTATATAAGA, which might enhance binding affinity and core promoter/enhancer II activity. Therefore, it is possible that the mutation in the box α influenced the HBe production and viral replication through the BCP activity. In addition, the T1653 mutation corresponds to an amino acid change from histidine to tyrosine at aa 94 of the X protein, so this alteration of X protein might be hepatocarcionogenesis. Gunther et al. [26] analyzed T1653, T1762, and A1764 mutations in the context of an in vitro study involving wild-type HBV (genotype D, AF043594), and they reported that the preC mRNA and HBeAg secretion was reduced, but the amount of progeny virus DNA in the cells and in the culture medium increased only marginally (if at all), as determined by Southern blot analysis. However, because the genotype was different from that in our study (genotype D vs. genotype C) and the mutant type included not only T1653, T1762, and A1764 mutations but also other mutations in the core promoter, it is possible that some other mutation influenced the results in the earlier study.

In conclusion, the addition of the T1653 mutation in the box α to the BCP mutation increases the risk of HCC in patients with HBV genotype C infection, suggesting that HBV with both the T1653 mutation and the BCP double mutation in patients with chronic hepatitis B should be eradicated by antiviral therapy. Functional analyses of HBV strains with the T1653 mutation are needed in vitro and in vivo.

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