

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 CCl₄ for 4 weeks. After CCl₄ 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–angiotensin 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 extracellular 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 (CCl₄) (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 CX₃C. 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 cirrhosis (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)

Manuscript received July 27, 2004; accepted September 28, 2004.

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 CCl₄ (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- μ m-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 μ g 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, TGAAGGTCGGTGTGAACGGATTGGC; reverse, CATGTAGGCCATGAGGTCCACC AC) (21), human MCP-1 (forward, GACCACCTGGACAAGCAAAC; reverse, CTCAAAACATCCCAGGGGTA) (22), human RhoA (forward, CTGGTGATTGTTGGTGTATGG; reverse, GCGATCATAATCTTCCTGCC) (23), and human β -actin (forward, GAGCGGGAATCGTGCCTGACATT; reverse, GATGAGTTGAAGGTAGTTTCGTG) (22). The PCR procedure used has been described previously (21–23). An aliquot (10 μ l) 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- κ B 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- κ B activity was measured in the nuclear extracts using an NF- κ B P65 Transcription Factor Assay kit (Chemicon International) according to the manufacturer's instruction.

Statistical Analysis. Results are expressed as the mean \pm 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 CCl₄-Treated Mice.

RT-PCR revealed the up-regulation of hepatic MCP-1 mRNA expression in CCl₄-treated WT mice, whereas it was negligible in CCl₄-treated and untreated AT1a KO mice (Figure 1). Immunohistochemical analysis also confirmed the enhanced hepatic expression of MCP-1 protein in CCl₄-treated WT mice. After CCl₄ treatment

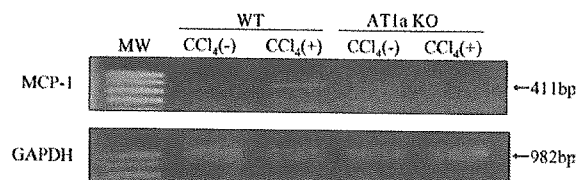


Fig 1. Steady-state hepatic MCP-1 mRNA expression in WT and AT1a KO mice with or without CCl₄ treatment for 4 weeks. An aliquot of each PCR product was loaded onto a 2% agarose gel and stained with ethidium bromide. Amplification of GAPDH was done to confirm the equal amounts of mRNA in each sample. The result shown here is representative of three independent experiments.

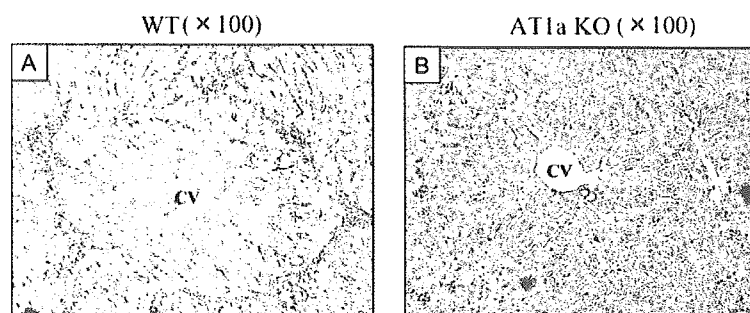


Fig 2. Immunohistochemical staining of MCP-1 in liver tissue from (A) a WT mouse and (B) an AT1A KO mouse after 4 weeks of CCl₄ treatment (1.0 ml/kg). CV, central vein. (Original magnification, $\times 100$.)

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 CCl₄-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 CCl₄-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 CCl₄-treated WT mice, the number of CD68-expressing cells was markedly increased in the portal tracts (Figure 3A). On the other hand, CCl₄ treatment had little influence on the number of CD68-expressing cells in the livers of AT1a KO mice (Figure 3B). These observations demonstrated that the number of CD68-expressing 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 ex-

amined 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).

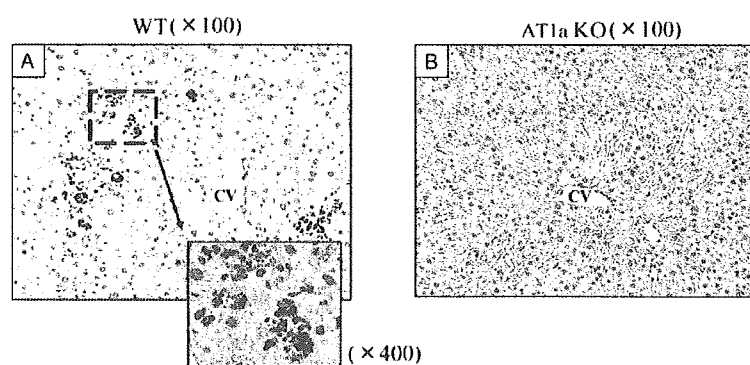


Fig 3. Distribution of CD68-positive cells in the liver. Immunohistochemical staining for CD68-positive mononuclear cells was performed in liver tissue from (A) a WT mouse and (B) an AT1A KO mouse after 4 weeks of CCl₄ treatment (1.0 ml/kg). CV, central vein. (Original magnification, $\times 100$.)

ANGIOTENSIN II AND HEPATIC INFLAMMATION

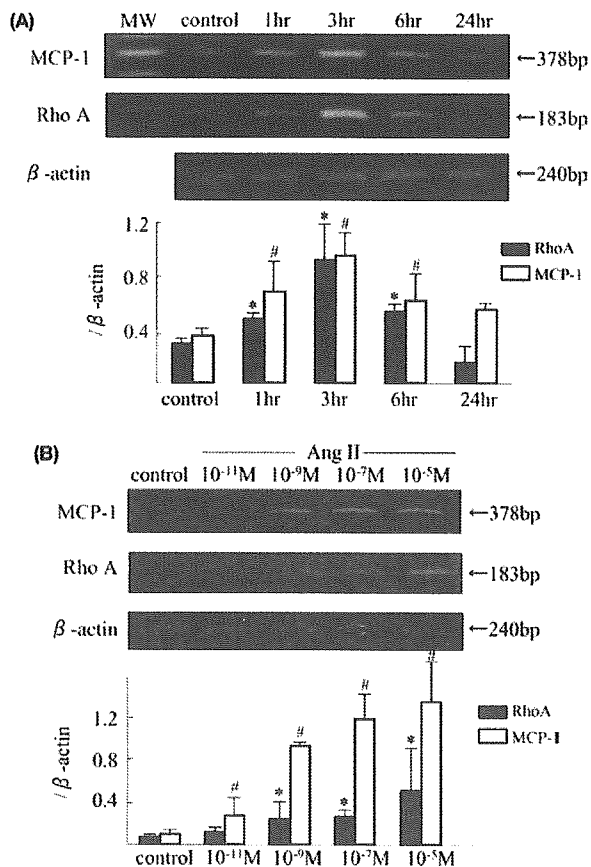


Fig 4. Effect of Ang II on MCP-1 mRNA expression by cultured LI90 cells. (A) Serum-starved LI90 cells were treated with 10^{-7} M Ang II at the indicated times, and the changes in Ang II-induced MCP-1 mRNA expression were examined by semiquantitative PCR. (B) Serum-starved LI90 cells were stimulated 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 \pm 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- κ B Activity in LI90 Cells. NF- κ B is the important factor involved in MCP-1 gene transcription in several cell types. To examine whether NF- κ B participated in the induction of MCP-1 in Ang II-stimulated LI90 cells, NF- κ B 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- κ B in LI90 cells (Figure 6).

DISCUSSION

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

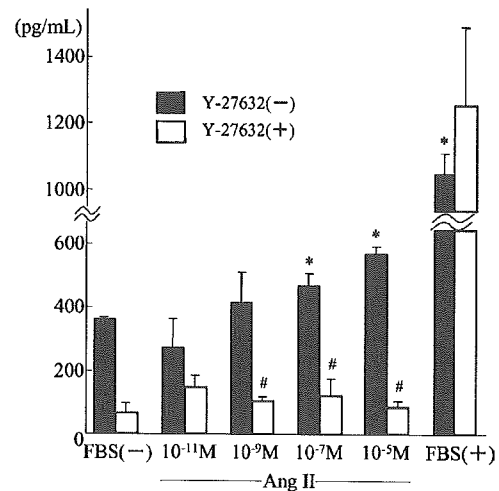


Fig 5. Effect of Ang II on MCP-1 secretion into the culture medium. Serum-starved LI90 cells were stimulated for 48 hr with the indicated concentrations of Ang II in the absence or presence of Y-27632 (10^{-5} M). Data from four independent experiments are shown as means \pm SD. * $P < 0.05$ vs. serum-free 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

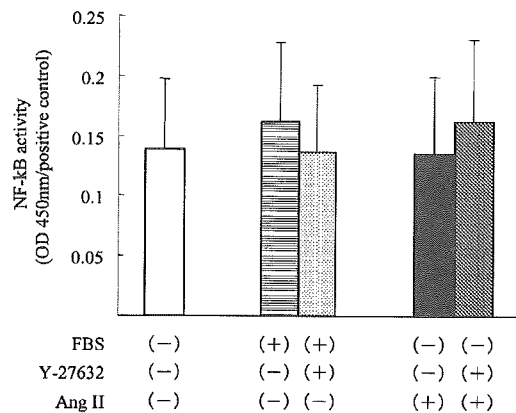


Fig 6. NF- κ B activity in nuclear extracts of Ang II-stimulated LI90 cells. The cells were stimulated using Ang II for 1 hr with or without preincubation of Y-27632 (10^{-5} M). Then nuclear protein was extracted, and NF- κ B activity was estimated. Absorbance values were standardized according to the protein concentration. The absorbance relative to that of the positive control (TNF α -stimulated whole Hela cells) from four independent experiments is shown as the mean \pm 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 CCl₄, 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 CCl₄ 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 CCl₄ (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- κ B is the main factor involved in regulating the transcription

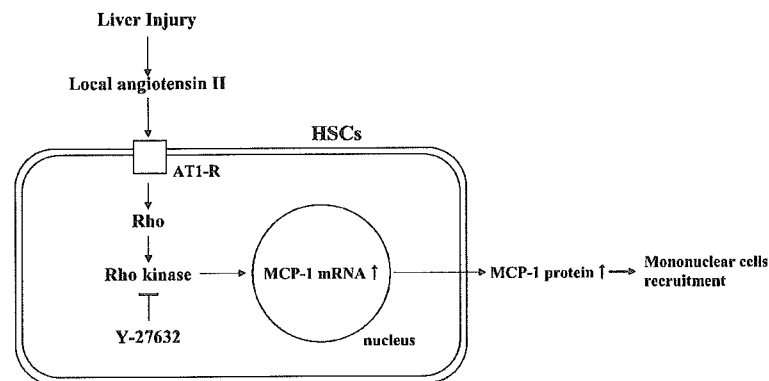


Fig 7. Speculated mechanism of how angiotensin II participates in hepatic inflammation after liver injury. Ang II enhances MCP-1 gene expression and synthesis, partly via the Rho signaling pathway, which modulate the recruitment of inflammatory cells into the liver. AT1-R, angiotensin II type1 receptor;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

of MCP-1 induced by LPS, IL-1 β , TNF- α , and phorbol esters (32, 33). It was also reported that Ang II promotes MCP-1 expression via activation of NF- κ B 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- κ B in animals (25). Contrary to our expectation, the present study demonstrated that the level of NF- κ B 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 CCl₄-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

This study was partly supported by a grant from the Japanese Ministry of Education, Culture, Sports, Science, and Technology to Dr. Tazuma (No. 12670489).

REFERENCES

- Bataller R, Gines P, Nicolas JM, Gorbic MN, Garcia-Ramallo E, Gasull X, Bosch J, Arroyo V, Rodes J: Angiotensin II induces contraction and proliferation of human hepatic stellate cells. *Gastroenterology* 118:1149–1156, 2000
- Wei HS, Li DG, Lu HM, Zhan YT, Wang ZR, Huang X, Zhang J, Cheng JL, Xu QF: Effects of AT1 receptor antagonist, losartan, on rat hepatic fibrosis induced by CCl₄ (4). *World J Gastroenterol* 6:540–545, 2000
- Jonsson JR, Clouston AD, Ando Y, Kelemen LI, Horn MJ, Adamson MD, Purdie DM, Powell EE: Angiotensin-converting enzyme inhibition attenuates the progression of rat hepatic fibrosis. *Gastroenterology* 121:148–155, 2001
- Yoshiji H, Kuriyama S, Yoshii J, Ikenaka Y, Noguchi R, Nakatani T, Tsujinoue H, Fukui H: Angiotensin-II type 1 receptor interaction is a major regulator for liver fibrosis development in rats. *Hepatology* 34:745–750, 2001
- Kanno K, Tazuma S, Chayama K: AT1A-deficient mice show less severe progression of liver fibrosis induced by CCl₄ (4). *Biochem Biophys Res Commun* 308:177–183, 2003
- Paizis G, Cooper ME, Schembri JM, Tikellis C, Burrell LM, Angus PW: Up-regulation of components of the renin-angiotensin system in the bile duct-ligated rat liver. *Gastroenterology* 123:1667–1676, 2002
- Bataller R, Sancho-Bru P, Gines P, Lora JM, Al-Garawi A, Sole M, Colmenero J, Nicolas JM, Jimenez W, Weich N, Gutierrez-Ramos JC, Arroyo V, Rodes J: Activated human hepatic stellate cells express the renin-angiotensin system and synthesize angiotensin II. *Gastroenterology* 125:117–125, 2003
- Loetscher P, Seitz M, Clark-Lewis I, Baggiolini M, Moser B: Monocyte chemotactic proteins MCP-1, MCP-2, and MCP-3 are major attractants for human CD4+ and CD8+ T lymphocytes. *FASEB J* 8:1055–1060, 1994
- Gressner AM: Cytokines and cellular crosstalk involved in the activation of fat-storing cells. *J Hepatol* 22:28–36, 1995
- Bernuau D, Rogier E, Feldmann G: In situ ultrastructural detection and quantitation of liver mononuclear phagocytes in contact with hepatocytes in chronic type B hepatitis. *Lab Invest* 51:667–674, 1984
- Narumi S, Tominaga Y, Tamaru M, Shimai S, Okumura H, Nishioji K, Itoh Y, Okanoue T: Expression of IFN-inducible protein-10 in chronic hepatitis. *J Immunol* 158:5536–5544, 1997
- Marra F, DeFranco R, Grappone C, Milani S, Pastacaldi S, Pinzani M, Romanelli RG, Laffi G, Gentilini P: Increased expression of monocyte chemotactic protein-1 during active hepatic fibrogenesis: correlation with monocyte infiltration. *Am J Pathol* 152:423–430, 1998
- Funakoshi Y, Ichiki T, Shimokawa H, Egashira K, Takeda K, Kaibuchi K, Takeya M, Yoshimura T, Takeshita A: Rho-kinase mediates angiotensin II-induced monocyte chemoattractant protein-1 expression in rat vascular smooth muscle cells. *Hypertension* 38:100–104, 2001
- Omura T, Yoshiyama M, Kim S, Matsumoto R, Nakamura Y, Izumi Y, Ichijo H, Sudo T, Akioka K, Iwao H, Takeuchi K, Yoshikawa J: Involvement of apoptosis signal-regulating kinase-1 on angiotensin II-induced monocyte chemoattractant protein-1 expression. *Arterioscler Thromb Vasc Biol* 24:270–275, 2004
- Yee HF Jr: Rho directs activation-associated changes in rat hepatic stellate cell morphology via regulation of the actin cytoskeleton. *Hepatology* 28:843–850, 1998
- Kato M, Iwamoto H, Higashi N, Sugimoto R, Uchimura K, Tada S, Sakai H, Nakamura M, Nawata H: Role of Rho small GTP binding protein in the regulation of actin cytoskeleton in hepatic stellate cells. *J Hepatol* 31:91–99, 1999
- Tada S, Iwamoto H, Nakamura M, Sugimoto R, Enjoji M, Nakashima Y, Nawata H: A selective ROCK inhibitor, Y27632, prevents dimethylnitrosamine-induced hepatic fibrosis in rats. *J Hepatol* 34:529–536, 2001
- Murata T, Arii S, Nakamura T, Mori A, Kaido T, Furuyama H, Furumoto K, Nakao T, Isobe N, Imamura M: Inhibitory effect of Y-27632, a ROCK inhibitor, on progression of rat liver fibrosis in association with inactivation of hepatic stellate cells. *J Hepatol* 35:474–481, 2001
- Sugaya T, Nishimatsu S, Tanimoto K, Takimoto E, Yamagishi T, Imamura K, Goto S, Imaizumi K, Hisada Y, Otsuka A, Uchida H, Sugiura K, Fukuta K, Fukamizu A, Murakami K: Angiotensin II type 1a receptor-deficient mice with hypotension and hyperreninemia. *J Biol Chem* 270:18719–18722, 1995
- Murakami K, Abe T, Miyazawa M, Yamaguchi M, Masuda T, Matsuura T, Nagamori S, Takeuchi K, Abe K, Kyogoku M: Establishment of a new human cell line, LI90, exhibiting characteristics of hepatic Ito (fat-storing) cells. *Lab Invest* 72:731–739, 1995
- Ousman SS, David S: MIP-1 alpha, MCP-1, GM-CSF, and TNF-alpha control the immune cell response that mediates rapid phagocytosis of myelin from the adult mouse spinal cord. *J Neurosci* 21:4649–4656, 2001

22. Von Bubnoff D, Matz H, Cazenave JP, Hanau D, Bieber T, De La Salle H: Kinetics of gene induction after Fc epsilon RI ligation of atopic monocytes identified by suppression subtractive hybridization. *J Immunol* 169:6170–6177, 2002
23. Kamai T, Arai K, Tsujii T, Honda M, Yoshida K: Overexpression of RhoA mRNA is associated with advanced stage in testicular germ cell tumour. *BJU Int* 87:227–231, 2001
24. Terui Y, Saito T, Watanabe H, Togashi H, Kawata S, Kamada Y, Sakuta S: Effect of angiotensin receptor antagonist on liver fibrosis in early stages of chronic hepatitis C. *Hepatology* 36:1022, 2002
25. Bataller R, Gabele E, Schoonhoven R, Morris T, Lehnert M, Yang L, Brenner DA, Rippe RA: Prolonged infusion of angiotensin II into normal rats induces stellate cell activation and proinflammatory events in liver. *Am J Physiol Gastrointest Liver Physiol* 285:G642–G651, 2003
26. Czaja MJ, Geerts A, Xu J, Schmiedeberg P, Ju Y: Monocyte chemoattractant protein 1 (MCP-1) expression occurs in toxic rat liver injury and human liver disease. *J Leukoc Biol* 55:120–126, 1994
27. Afford SC, Fisher NC, Neil DA, Fear J, Brun P, Hubscher SG, Adams DH: Distinct patterns of chemokine expression are associated with leukocyte recruitment in alcoholic hepatitis and alcoholic cirrhosis. *J Pathol* 186:82–89, 1998
28. Luckey SW, Petersen DR: Activation of Kupffer cells during the course of carbon tetrachloride-induced liver injury and fibrosis in rats. *Exp Mol Pathol* 71:226–240, 2001
29. Phillips MI, Kagiya S: Angiotensin II as a pro-inflammatory mediator. *Curr Opin Investig Drugs* 3:569–577, 2002
30. Pinzani M, Marra F: Cytokine receptors and signaling in hepatic stellate cells. *Semin Liver Dis* 21:397–416, 2001
31. Kunieda Y, Nakagawa K, Nishimura H, Kato H, Ukimura N, Yano S, Kawano H, Kimura S, Nakagawa M, Tsuji H: HMG CoA reductase inhibitor suppresses the expression of tissue factor and plasminogen activator inhibitor-1 induced by angiotensin II in cultured rat aortic endothelial cells. *Thromb Res* 110:227–234, 2003
32. Ueda A, Okuda K, Ohno S, Shirai A, Igarashi T, Matsunaga K, Fukushima J, Kawamoto S, Ishigatsubo Y, Okubo T: NF-kappa B and Sp1 regulate transcription of the human monocyte chemoattractant protein-1 gene. *J Immunol* 153:2052–2063, 1994
33. Hernandez-Presa M, Bustos C, Ortego M, Tunon J, Renedo G, Ruiz-Ortega M, Egido J: Angiotensin-converting enzyme inhibition prevents arterial nuclear factor-kappa B activation, monocyte chemoattractant protein-1 expression, and macrophage infiltration in a rabbit model of early accelerated atherosclerosis. *Circulation* 95:1532–1541, 1997
34. Ruiz-Ortega M, Bustos C, Hernandez-Presa MA, Lorenzo O, Plaza JJ, Egido J: Angiotensin II participates in mononuclear cell recruitment in experimental immune complex nephritis through nuclear factor-kappa B activation and monocyte chemoattractant protein-1 synthesis. *J Immunol* 161:430–439, 1998
35. Bataller R, Schwabe RF, Choi YH, Yang L, Paik YH, Lindquist J, Qian T, Schoonhoven R, Hagedorn CH, Lemasters JJ, Brenner DA: NADPH oxidase signal transduces angiotensin II in hepatic stellate cells and is critical in hepatic fibrosis. *J Clin Invest* 112:1383–1394, 2003

Clinical Studies

Liver International

DOI: 10.1111/j.1478-3231.2005.01200.x

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

Tanaka E, Matsumoto A, Suzuki F, Kobayashi M, Mizokami M, Tanaka Y, Okanoue T, Minami M, Chayama K, Imamura M, Yatsuhashi H, Nagaoka S, Yotsuyanagi H, Kawata S, Kimura T, Maki N, Iino S, Kiyosawa K, HBV Core-Related Antigen Study Group. Measurement of hepatitis B virus core-related antigen is valuable for identifying patients who are at low risk of lamivudine resistance.

Liver International 2006; 26: 90–96. © Blackwell Munksgaard 2005

Abstract: *Objective:* The clinical usefulness of hepatitis B virus core-related antigen (HBVcrAg) assay was compared with that of HBV DNA assay in predicting the occurrence of lamivudine resistance in patients with chronic hepatitis B. *Patients:* Of a total of 81 patients who were treated with lamivudine, 25 (31%) developed lamivudine resistance during a median follow-up period of 19.3 months. *Results:* The pretreatment positive rate of HBe antigen, or pretreatment levels of HBVcrAg or HBV DNA did not differ between patients with and without lamivudine resistance. Levels of both HBVcrAg and HBV DNA decreased after the initiation of lamivudine administration; however, the level of HBVcrAg decreased significantly more slowly than that of HBV DNA. The occurrence of lamivudine resistance was significantly less frequent in the 56 patients whose HBV DNA level was less than 2.6 log copy/ml at 6 months of treatment than in the remaining 25 patients. The cumulative rate of lamivudine resistance was as high as 70% within 2 years in the latter group, while it was only 28% in the former group. Lamivudine resistance did not occur during the follow-up period in the 19 patients whose HBVcrAg level was less than 4.6 log U/ml at 6 months of treatment, while it did occur in 50% of the remaining patients within 2 years. *Conclusion:* These results suggest that measurement of HBV DNA is valuable for identifying patients who are at high risk of developing lamivudine resistance, and that, conversely, measurement of HBVcrAg is valuable for identifying those who are at low risk of lamivudine resistance.

Eiji Tanaka,¹ Akihiro Matsumoto,¹ Fumitaka Suzuki,² Mariko Kobayashi,² Masashi Mizokami,³ Yasuhito Tanaka,³ Takeshi Okanoue,⁴ Masahito Minami,⁴ Kazuaki Chayama,⁵ Michio Imamura,⁵ Hiroshi Yatsuhashi,⁶ Shinya Nagaoka,⁶ Hiroshi Yotsuyanagi,⁷ Sumio Kawata,⁸ Tatsuji Kimura,⁹ Noboru Maki,⁹ Shiro Iino¹⁰, Kendo Kiyosawa¹, and HBV Core-Related Antigen Study Group

¹Department of Medicine, Shinshu University School of Medicine, Matsumoto, Japan, ²Department of Research Institute for Hepatology, Toranomon Hospital, Minato-ku, Tokyo, Japan, ³Department of Clinical Molecular Informative Medicine, Nagoya City University Graduate School of Medical Science, Nagoya, Japan, ⁴Department of Gastroenterology and Hepatology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto, Japan, ⁵Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan, ⁶Clinical Research Center, National Nagasaki Medical Center, Omura, Japan, ⁷Department of Internal Medicine, Division of Gastroenterology and Hepatology, St. Marianna University, Kawasaki, Japan, ⁸Department of Gastroenterology, School of Medicine, Yamagata University, Yamagata, Japan, ⁹Advanced Life Science Institute, Inc., Wako, Japan, ¹⁰Kiyokawa Hospital, Tokyo, Japan

Kiyomi Yasuda (Kiyokawa Hospital, Tokyo, Japan); Hitoshi Togashi and Takatumi Saito (Department of Gastroenterology, School of Medicine, Yamagata University); Masataka Tsuge (Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan); Rumiko Nakao (Clinical Research Center, National Nagasaki Medical Center, Omura, Japan); Chiaki Okuse and Hideaki Takahashi (Department of Internal Medicine, Division of Gastroenterology and Hepatology, St. Marianna University, Kawasaki, Japan).

Key words: chronic hepatitis B – HBV core-related antigen – HBV DNA – lamivudine resistance

Eiji Tanaka, MD, Department of Medicine, Shinshu University School of Medicine, Asahi 3-1-1, Matsumoto 390-8621, Japan.

Tel: +81-263-37-2634

Fax: +81-263-32-9412

e-mail: etanaka@hsp.md.shinshu-u.ac.jp

Received 19 June 2005,

accepted 8 August 2005

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

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

Patients and methods

Patients

A total of 81 patients with chronic hepatitis B, who received lamivudine therapy, were enrolled in the present study. These were 58 men and 23 women with a median age of 49 years (range 24–79 years). The 81 patients were selected retro-

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

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

Serological markers for HBV

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

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

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

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

Statistical analysis

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

Results

Table 1 shows a comparison of the clinical and virological backgrounds of the 27 patients who showed lamivudine resistance and the 54 patients who did not. Median age, gender distribution and median follow-up period did not differ between the two groups, and the positive rate of HBe

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

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

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

antigen was similar. Both HBV DNA and HBVcrAg levels at the beginning of lamivudine administration were similar between the two groups; however, both HBV DNA and HBVcrAg levels at 6 months after the start of lamivudine administration were significantly lower in the lamivudine resistance negative group than in the positive group. ALT level was normal at the beginning in eight (15%) of the 54 patients without lamivudine resistance and in two (7%) of the 27 patients with it (*P* > 0.2).

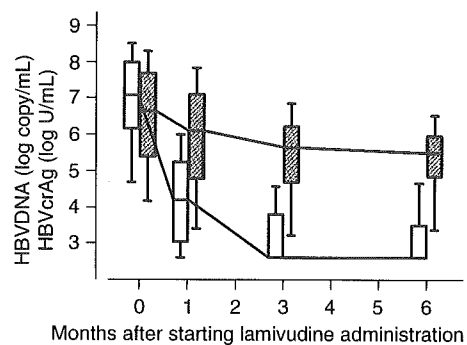


Fig. 1. Changes in the median levels of hepatitis B virus core-related antigen (HBVcrAg) and hepatitis B virus (HBV) DNA during lamivudine administration. The box plots show the 10th, 25th, 50th, 75th and 90th percentiles, with the open boxes indicating HBV DNA and shaded boxes indicating HBVcrAg. The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

Prediction of lamivudine resistance

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

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

resistance. In 19 (35%) of the 54 patients without lamivudine resistance, on the other hand, the levels decreased to levels below 4.7 log U/ml within 6 months after the start of lamivudine administration. The level of HBVcrAg increased rapidly as did the level of HBV DNA when lamivudine resistance occurred.

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

Discussion

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

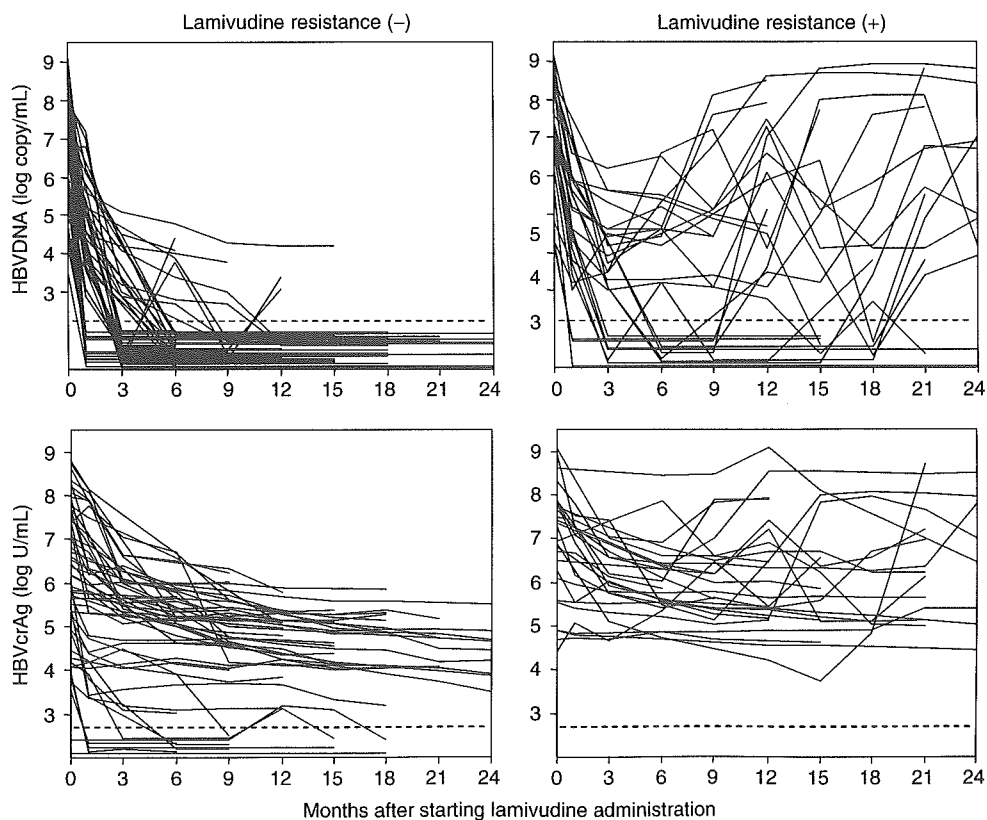


Fig. 2. Comparison of changes in serum hepatitis B virus (HBV) DNA and serum HBV core-related antigen (HBVcrAg) levels between patients who showed lamivudine resistance and those who did not. The broken lines indicate the detection limit of each assay.

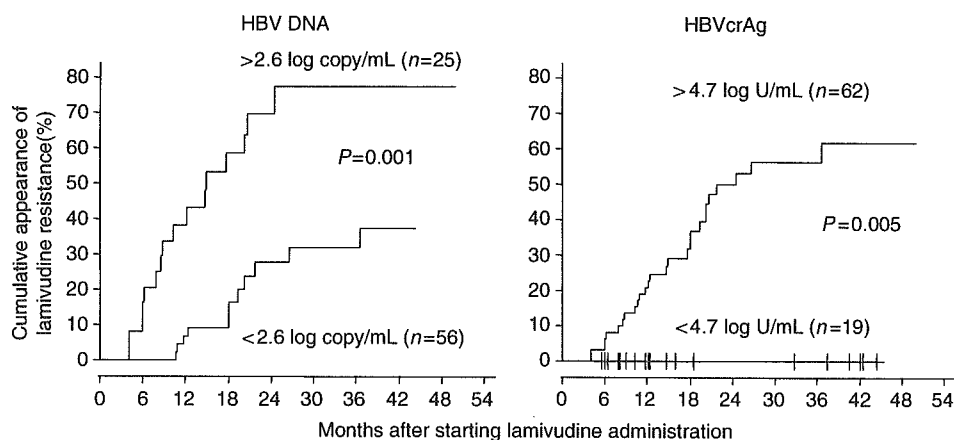


Fig. 3. Comparison of the cumulative occurrence of lamivudine resistance between patients who showed hepatitis B virus (HBV) DNA levels of less than the detection limit (2.6 log copy/ml) at 6 months after starting lamivudine administration and those who did not (left figure), and similarly between patients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

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

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

HBV DNA levels after the initiation of lamivudine therapy.

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

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

administration developed lamivudine resistance within 2 years. However, a negative result of HBV DNA at 6 months does not necessarily guarantee the absence of lamivudine resistance because nearly 30% of such patients developed resistance within 2 years. On the other hand, HBVcrAg levels of less than 4.7 log U/ml at 6 months are a useful indicator of patients who are unlikely to develop lamivudine resistance, because no such patients developed resistance during the follow-up period in the present study. Lower serum HBVcrAg levels may reflect lower levels of cccDNA in hepatocytes because the mRNAs of HBVcrAg are transcribed from the cccDNA (18, 22, 23). This possibility may explain our finding that patients whose HBVcrAg levels decreased sufficiently were unlikely to develop lamivudine resistance, because cccDNA provides the templates for viral and pregenomic messenger RNA (18, 22, 23), which may be a source of lamivudine-resistant strains.

In conclusion, our results suggest that measurement not only of HBV DNA but also of HBVcrAg is useful for predicting the occurrence of lamivudine resistance. HBV DNA measurement is valuable for identifying patients who are at high risk of developing this resistance and HBcrAg measurement is valuable for identifying those who are at low risk.

Acknowledgements

This research was supported in part by a research grant on hepatitis from the Ministry of Health, Labour and Welfare of Japan.

References

- DOONG S L, TSAI C H, SCHINAZI R F, LIOTTA D C, CHENG Y C. Inhibition of the replication of hepatitis B virus in vitro by 2', 3'-dideoxy-3'-thiacytidine and related analogues. *Proc Natl Acad Sci USA* 1991; 88: 8495-9.
- BENHAMOU Y, DOHIN E, LUNEL-FABIANI F, POYNARD T, HURAOX J M, KATLAMA C, et al. Efficacy of lamivudine on replication of hepatitis B virus in HIV-infected patients. *Lancet* 1995; 345: 396-7.
- DIENSTAG J L, GOLDIN R D, HEATHCOTE E J, HANN H W, WOESSNER M, STEPHENSON S L, et al. Histological outcome during long-term lamivudine therapy. *Gastroenterology* 2003; 124: 105-17.
- DIENSTAG J L, PERRILLO R P, SCHIFF E R, BARTHOLOMEW M, VICARY C, RUBIN M. A preliminary trial of lamivudine for chronic hepatitis B infection. *N Engl J Med* 1995; 333: 1657-61.
- LAI C L, CHIEN R N, LEUNG N W, CHANG T T, GUAN R, TAI D I, et al. A one-year trial of lamivudine for chronic hepatitis B. *Asia Hepatitis Lamivudine Study Group. N Engl J Med* 1998; 339: 61-8.
- LIAW Y F, SUNG J J, CHOW W C, et al. Lamivudine for patients with chronic hepatitis B and advanced liver disease. *N Engl J Med* 2004; 351: 1521-31.
- MATSUMOTO A, TANAKA E, ROKUHARA A, et al. Efficacy of lamivudine for preventing hepatocellular carcinoma in chronic hepatitis B: a multicenter retrospective study of 2,795 patients. *Hepatology* 2005; 42: 173-84.
- LING R, MUTIMER D, AHMED M, et al. Selection of mutations in the hepatitis B virus polymerase during therapy of transplant recipients with lamivudine. *Hepatology* 1996; 24: 711-3.
- LOK A S, LAI C L, LEUNG N, YAO G B, CUI Z Y, SCHIFF E R, et al. Long-term safety of lamivudine treatment in patients with chronic hepatitis B. *Gastroenterology* 2003; 125: 1714-22.
- TIPPLES G A, MA M M, FISCHER K P, BAIN V G, KNETEMAN N M, TYRRELL D L. Mutation in HBV RNA-dependent DNA polymerase confers resistance to lamivudine in vivo. *Hepatology* 1996; 24: 714-7.
- LIAW Y F, CHIEN R N, YEH C T, TSAI S L, CHU C M. Acute exacerbation and hepatitis B virus clearance after emergence of YMDD motif mutation during lamivudine therapy. *Hepatology* 1999; 30: 567-72.
- SUZUKI F, TSUBOTA A, ARASE Y, SUZUKI Y S, AKUTA N, HOSAKA T, et al. Efficacy of lamivudine therapy and factors associated with emergence of resistance in chronic hepatitis B virus infection in Japan. *Intervirology* 2003; 46: 182-9.
- ZOLLNER B, SCHAFER P, FEUCHT H H, SCHROTER M, PETERSEN J, LAUFS R. Correlation of hepatitis B virus load with loss of e antigen and emerging drug-resistant variants during lamivudine therapy. *J Med Virol* 2001; 65: 659-63.
- KIMURA T, ROKUHARA A, SAKAMOTO Y, YAGI S, TANAKA E, KLYOSAWA K, et al. Sensitive enzyme immunoassay for hepatitis B virus core-related antigens and their correlation to virus load. *J Clin Microbiol* 2002; 40: 439-45.
- ROKUHARA A, TANAKA E, MATSUMOTO A, KIMURA T, YAMAURA T, ORII K, et al. Clinical evaluation of a new enzyme immunoassay for hepatitis B virus core-related antigen; a marker distinct from viral DNA for monitoring lamivudine treatment. *J Viral Hepatol* 2003; 10: 324-30.
- BRUSS V, GERLICH W H. Formation of transmembraneous hepatitis B e-antigen by cotranslational in vitro processing of the viral precore protein. *Virology* 1988; 163: 268-75.
- GARCIA P D, OU J H, RUTTER W J, WALTER P. Targeting of the hepatitis B virus precore protein to the endoplasmic reticulum membrane: after signal peptide cleavage translocation can be aborted and the product released into the cytoplasm. *J Cell Biol* 1988; 106: 1093-104.
- LEE W M. Hepatitis B virus infection. *N Engl J Med* 1997; 337: 1733-45.
- MIZOKAMI M, NAKANO T, ORITO E, TANAKA Y, SAKUGAWA H, MUKAIDE M, et al. Hepatitis B virus genotype assignment using restriction fragment length polymorphism patterns. *FEBS Lett* 1999; 450: 66-71.
- KOBAYASHI S, SHIMADA K, SUZUKI H, TANIKAWA K, SATA M. Development of a new method for detecting a mutation in the gene encoding hepatitis B virus reverse transcriptase active site (YMDD motif). *Hepatology* 2000; 31: 31-42.
- MASON W S, HALPERN M S, ENGLAND J M, SEAL G, EGAN J, COATES L, et al. Experimental transmission of duck hepatitis B virus. *Virology* 1983; 131: 375-84.
- SUMMERS J, SMITH P M, HORWICH A L. Hepadnavirus envelope proteins regulate covalently closed circular DNA amplification. *J Virol* 1990; 64: 2819-24.
- TUTTLEMAN J S, POURCEL C, SUMMERS J. Formation of the pool of covalently closed circular viral DNA in hepadnavirus-infected cells. *Cell* 1986; 47: 451-60.

Tanaka et al.

24. MORALEDA G, SAPUTELLI J, ALDRICH C E, AVERETT D, CONDREAY L, MASON W S. Lack of effect of antiviral therapy in nondividing hepatocyte cultures on the closed circular DNA of woodchuck hepatitis virus. *J Virol* 1997; 71: 9392-9.
25. WERLE-LAPOSTOLLE B, BOWDEN S, LOCARNINI S, WORS-THORN K, PETERSEN J, LAU G, et al. Persistence of cccDNA during the natural history of chronic hepatitis B and decline during adefovir dipivoxil therapy. *Gastroenterology* 2004; 126: 1750-8.
26. ZHU Y, YAMAMOTO T, CULLEN J, et al. Kinetics of hepadnavirus loss from the liver during inhibition of viral DNA synthesis. *J Virol* 2001; 75: 311-22.
27. LAU D T, KHOKHAR M F, DOO E, GHANY M G, HERION D, PARK Y, et al. Long-term therapy of chronic hepatitis B with lamivudine. *Hepatology* 2000; 32: 828-34.
28. YUEN M F, SABLON E, HUI C K, YUAN H J, DE-CRAEMER H, LAI C L. Factors associated with hepatitis B virus DNA breakthrough in patients receiving prolonged lamivudine therapy. *Hepatology* 2001; 34: 785-91.
29. MIHM U, SARRAZIN C, HERRMANN E, TEUBER G, VON WAGNER M, KRONEA BERGER B, et al. Response predictors and results of a long-term treatment with lamivudine in patients with chronic hepatitis B. *Z Gastroenterol* 2003; 41: 249-54.

G to A Hypermutation of Hepatitis B Virus

Chiemi Noguchi,^{1,2} Hiromi Ishino,¹ Masataka Tsuge,¹ Yoshifumi Fujimoto,³ Michio Imamura,^{1,2} Shoichi Takahashi,^{1,2} and Kazuaki Chayama^{1,2,3}

G to A hypermutation of the human immunodeficiency virus type 1 (HIV-1) is induced by a deaminase APOBEC3G and is related to host antiviral defense. APOBEC3G has also been found to reduce the replication of HIV-1 by an unknown mechanism. This enzyme also reduces the production of hepatitis B virus, although the mechanism for this action has not been clearly elucidated. The hypermutated hepatitis B virus (HBV) is rarely found in usual sequencing analyses. Using peptide nucleic acid mediated by polymerase chain reaction clamping, we detected the hypermutated HBV DNA in 1 of 8 patients with acute HBV infection and 4 of 10 with chronic HBV infection. In the latter group, hypermutated genomes were found only in eAb-positive patients. As much as 72.5% of G residues were mutated in the hypermutated clones. G to A substitutions were predominant in almost all clones sequenced compared with other substitutions. G to A mutated viral genomes also were found in HepG2–derived cell lines that continuously produced HBV into the supernatant. Both alpha and gamma interferon reduced virus production in these cell lines, but they did not alter the frequency of the hypermutation. Transcripts of APOBEC3G, as well as some other deaminases, were found in these cell lines. **In conclusion**, our results show that part of the minus strand DNA of HBV is hypermutated both *in vitro* (HepG2 cell lines) and *in vivo*. The role and mechanism of hypermutation in reducing HBV replication should be further investigated to understand the anti-HBV defense system. (HEPATOLOGY 2005;41:626-633.)

Hepatitis B virus (HBV) is a small enveloped DNA virus that replicates in hepatocytes in a noncytolytic manner. Chronic infection with the virus often leads to chronic hepatitis and liver cirrhosis. Hepatocellular carcinoma arises in chronic carriers at a higher frequency than noninfected individuals.¹⁻⁴

The replication cycle of the HBV includes pregenome RNA synthesis and reverse transcription, resulting in the production of the minus strand DNA, which serves as a template of the plus strand DNA.⁵ The life cycle of this virus resembles that of the human immunodeficiency virus 1 (HIV-1), which also replicates through reverse transcription.⁶

Recent reports showed that a cytosine deaminase APOBEC3G (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G), which is packaged in HIV-1 virions, induces G to A hypermutation to a nascent reverse transcript of HIV-1, which contributes in part to the innate antiviral activity.⁷⁻¹⁰ The antiviral activity of APOBEC3G is species specific^{11,12} and may represent the different actions of the protein.^{13,14} The virion infectivity factor encoded by lentivirus genomes associates with APOBEC3G to prevent the enzyme from being packaged into virions and triggers its proteasomal degradation.¹⁵⁻¹⁸ The negative strand DNA of the HBV might be a target of such antiviral deaminase activity. In fact, naturally occurring HBV genomes bearing the hallmarks of retroviral G to A hypermutation have been reported in clones obtained from 2 HBV carriers.¹⁹ Both of these clones represented subgenomes arising from reverse transcrip-

Abbreviations: HBV, hepatitis B virus; HIV-1, human immunodeficiency virus type 1; APOBEC3G, apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B early antigen; PCR, polymerase chain reaction; PNA, peptide nucleic acid.

From the ¹Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan; ²Hiroshima Liver Research Center, Hiroshima University, Hiroshima, Japan; and ³Laboratory for Liver Diseases, SNP Research Center, Institute of Physical and Chemical Research (RIKEN), Yokohama, Japan.

Received August 27, 2004; accepted December 2, 2004.

Supported in part by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Address reprint requests to: Kazuaki Chayama, M.D., Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan. E-mail: chayama@hiroshima-u.ac.jp; fax: (81) 82-255-6220.

Copyright © 2005 by the American Association for the Study of Liver Diseases.

Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.20580

Conflict of interest: Nothing to report.

tion of packaged spliced mRNA. However, such hypermutated genomes have otherwise never been reported, nor deposited in DNA databases. Moreover, whether such hypermutated sequences are generated in liver cells or in leukocytes is unknown.

Inhibition of HBV replication by APOBEC3G was observed recently in a transient transfection system.²⁰ However, no induction of hypermutations to the HBV genome was observed. Instead, prevention of pre-genome RNA packaging was observed.

The aims of the current study were to determine the frequency of viral genomes with G to A substitutions in HBV carriers and patients with acute HBV infection, and to determine whether the hypermutated sequences are generated in hepatic cell lines. We identified such hypermutated viral genomes in 5 of 18 HBV carriers and patients with acute HBV infection and the expression of known deaminases that are potentially responsible for the hypermutation in cultured hepatoma cell lines.

Materials and Methods

Serum Samples. Serum samples from 18 adult Japanese patients with HBV infection were studied. At the time of the study, 8 of these patients had acute HBV infection and tested positive for immunoglobulin M anti-hepatitis B core antibody. The remaining 10 patients were chronic carriers. All serum samples were stored at -80°C until examined. All patients were negative for serum markers of both hepatitis C virus and HIV-1 infection, and none was on antiviral treatment.

Serological Markers of HBV Infection. Hepatitis B surface antigen (HBsAg) was detected by enzyme immunoassay (Roche Diagnostics, Basel, Switzerland), and hepatitis B early antigen (HBeAg) as well as anti-HBe were detected by radioimmunoassay (Abbott Diagnostics, Abbott Park, IL). HBV DNA was determined by transcription-mediated amplification and hybridization-protection assay (Chugai Diagnostics, Tokyo, Japan), and the results were expressed as log genome equivalents per milliliter. The lower detection limit of this assay is 3.7 log genome equivalents/mL (equivalent to 5,000 copies/mL). The antibody against hepatitis C virus was tested for by the third-generation enzyme immunoassay (Roche Diagnostics).

Analysis of HBV DNA in Cell Lines That Stably Produce HBV. Two cell lines known to produce wild-type HBV and one cell line known to produce lamivudine-resistant HBV (with mutations of L528M and M552V) were created by transfecting 1.4 genome length sequences of HBV to HepG2 cell lines. These cell lines produced HBV that showed a similar sedimentation in

sucrose density gradient centrifugation to HBV extracted from the serum of carriers (M. Tsuge et al., manuscript in preparation) and could infect human hepatocyte chimeric mice (manuscript in preparation). These cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal bovine serum at 37°C and 5% CO_2 . Cells were seeded to semiconfluence in 6-well tissue culture plates and then treated with media containing interferon alpha or gamma. After 3 days of interferon treatment, the cells were harvested and lysed with 250 μL lysis buffer (10 mmol/L Tris-HCl [pH 7.4], 140 mmol/L NaCl, 0.5% [vol/vol] NP-40) followed by centrifugation for 2 minutes at 15,000g. Replicative intermediate of the HBV was immunoprecipitated and subjected to Southern blot analysis and quantitative analysis by light cycler. The effect of lamivudine was analyzed similarly, except that cells were harvested after 5 days of treatment.

Detection of Hypermutated Clones by Polymerase Chain Reaction With PNA Clamping, Cloning, and Sequencing. HBV DNA was extracted from 100 μL serum or culture supernatant by SMITEST (Genome Science Laboratories, Tokyo, Japan) and was dissolved in 20 μL H_2O . The first round of polymerase chain reaction (PCR) was performed with an outer primer set (PLF1 and BR112 [Table 1]) and a second-round PCR with an inner primer set (PLF2 and PLR2 [Table 1]). The peptic nucleic acid (PNA) oligonucleotide, initially designed to detect lamivudine-resistant variant genome,²¹ was an 18-mer (PNA 552 [Table 1]) that exactly matched the 18-nucleotide sequence of the original YMDD sequence of DNA polymerase/reverse transcriptase, which contained GG and TG sequences (AGT TAT ATG GAT GAT GTG). The PCR with PNA clamping was performed in a total volume of 25 μL , consisting of a reaction buffer (100 mmol/L Tris-HCl [pH 8.3], 50 mmol/L KCl and 15 mmol/L MgCl_2), 0.2 mmol/L each of dNTPs, 1 μL of the DNA solution, 12.5 pmol each primer, 150 pmol PNA 552, and 1 unit of Taq DNA polymerase (Gene Taq, Wako Pure Chemicals, Tokyo, Japan) together with 0.2 μg anti-Taq high (Toyobo Co., Osaka, Japan). The amplification conditions included an initial denaturation at 95°C for 4 minutes and 25 cycles of amplification (denaturation at 95°C for 45 seconds, PNA annealing at 73°C for 2 minutes, annealing and extension of primer at 63°C for 50 seconds), followed by a final extension at 63°C for 7 minutes. Part of the X gene was amplified with an outer primer pair (HBV1 and HBV2) and an inner primer (PLF2 and HBV2) (Table 1) for the first- and second-round amplifications, respectively. The amplification for the first-round PCR included initial denaturation at 95°C for 4 minutes and 25 cycles of amplification (denatur-

Table 1. Oligonucleotides and PNAs Used in the Current Study

Primer	Sequence
HBV amplification	
PLF1	5'-GGT ATG TTG CCC GTT TGT CC-3'
BR112	5'-TTC CGT CGA CAT ATC CCA T-3'
PLF2	5'-CCT ATG GGA GTG GGC CTC AG-3'
PLR2	5'-CCA ATT ACA TAT CCC ATG AAG TTA AGG GA-3'
HBV1	5'-CCG GAA AGC TTG AGC TCT TCT TTT TCA CCT CTG CCT AAT CA-3'
HBV2	5'-CCG GAA AGC TTG AGC TCT TCA AAA AGT TGC ATG GTG CTG G-3'
BR109	5'-AAG GGA GTA GCC CCA ACG TT-3'
PNA	
PNA552	H2N-CAC ATC ATC CAT ATA ACT-CON2H
PNA552V	H2N-CAC ATC ATC CAC ATA ACT-CON2H
Amplification of mRNAs of deaminases	
APO1a	5'-CAG AGC ACC ATG ACT TCT-3'
APO1d	5'-ATT GTG GCC AGT GAG CTT CA-3'
APO2a	5'-AGA AGG AAG AGG CTG CTG TG-3'
APO2b	5'-AGA ACG GCT GCC TGC CAA CT-3'
APO2c	5'-GAA GGC TGG TGG CAG GAT GGT GT-3'
APO2d	5'-CAG GTG ACA TTG TAC CGC AG-3'
APO3Aa	5'-TCT TAA CAC CAC GCC TTG AG-3'
APO3Ad	5'-GAA GAT GCG CAG TCT CAC GT-3'
APO3Ba	5'-AGA GCG GGA CAG GGA CAA GC-3'
APO3Bb	5'-GCG TAT CTA AGA GGC TGA AC-3'
APO3Bd	5'-CGA AGG ACC AAA GGG TCA TT-3'
APO3Be	5'-ACA AGT AGG TCT GGC GCC GT-3'
APO3Ca	5'-AGG ACG CTG TAA GCA GGA AG-3'
APO3Cb	5'-CCG ATG AAG TGA AGA GGT GG-3'
APO3Cc	5'-GTC GTC GCA GAA CCA AGA GA-3'
APO3Cd	5'-GAT GTG TAC CAG GTG ACC TG-3'
APO3Da	5'-CTG GGA CAA GCG TAT CTA AG-3'
APO3Dd	5'-AGT CTG AGA TGA AGA GGT GG-3'
APO3Fa	5'-CTT GGG TCC TGC CGC ACA GA-3'
APO3Fd	5'-TCA TCC TTG GCC GGC TAG TC-3'
APO3Ga	5'-GAC TAG CCG GCC AAG GAT GA-3'
APO3Gb	5'-CAC AGT GGA GCG AAT GTA TC-3'
APO3Gc	5'-GTT CGG AAT ACA CCT GGC CT-3'
APO3Gd	5'-ACT CCT GGT CAC GAT GCA GC-3'

ation at 95°C for 45 seconds, PNA annealing at 73°C for 2 minutes, primer annealing at 60°C for 1 minute, and extension of primer at 63°C for 4 minutes), followed by the final extension at 63°C for 7 minutes. The second-round amplification was performed under the same conditions without a primer extension for 3 minutes. The estimated error rate of the Taq DNA polymerase was 1.76×10^{-5} per site in amplifying approximately 10^2 copies of plasmid under the same conditions as described previously and cloning and sequencing.²¹ Products (1 μ L each) of the second-round of PNA PCR were subjected to PCR with primers PLF2 and BR109 for 35 cycles (94°C, 1 minute; 58°C, 1 minute; 72°C, 1.5 minutes) after initial denaturation at 94°C for 4 minutes and followed by the final extension at 72°C for 7 minutes. Amplicons were purified by electrophoresis on 2% (wt/vol) agarose gel and cloned into pGEM-T Easy Vector (Promega, Madison, WI) with the standard method, and then transformed

into *Escherichia coli* JM 109 (Takara Shuzo Co., Otsu, Japan). Sequencing was performed in the ABI PLISMTM 310NT Genetic analyzer (Applied Biosystems, Tokyo, Japan) with Big Dye terminator version 3.0 Cycle Sequencing Ready Reaction kit (Applied Biosystems). Ten independent clones from each serum sample of patients or supernatant of cell cultures were sequenced for analysis and compared for nucleotide sequences obtained by direct sequencing of PCR products. Hypermutation was defined as clones with a statistically significant number of G to A substitutions.

Sequence Analysis. Nucleotide sequences were aligned and parameters of hypermutation were evaluated with Hypermut Program Package²² (<http://www.hiv.lanl.gov/HYPERMUT/hypermut.html>). We used nucleotide sequences obtained by direct sequencing as reference sequences and tentatively labeled clones with a statistically significant ($P < .05$ by Fisher's exact test) number of G to A substitutions as "hypermutated."

Detection of mRNA of Known Deaminases by Reverse Transcription and PCR. Total RNA was extracted from HepG2 cell lines by using cell-to-cDNAII kit (Ambion, Austin, TX). The extracted RNA was reverse transcribed with random primer and M-MLV reverse transcriptase (ReverTra Ace, TOYOBO, Osaka, Japan) at 42°C for 60 minutes according to the instructions provided by the manufacturer. Synthesized cDNAs were used to detect mRNAs of known deaminases using primers listed in Table 1. Each of these primers was carefully designed to amplify only the target member of the APOBEC families. Amplification of specific deaminases was confirmed by amplifying each deaminase cDNA by using cDNAs obtained from organs reported to be positive for the expression of each deaminase. The amplicons were analyzed in 2% agarose gel, and the nucleotide sequences were confirmed by direct sequencing.

Results

Frequent Detection of G to A Substituted HBV Genomes by PCR With PNA Clamping in Patients With Acute or Chronic Hepatitis B Virus Infection.

Using PCR with PNA clamping, clones with multiple G to A substitutions were found (Table 2). In contrast, only small numbers of other substitutions were identified in these clones. A hypermutated genome of HBV was found in 1 of 8 patients with acute HBV infection and 4 of 10 patients with chronic HBV infection (Table 2). We cloned and sequenced more than 20 clones without PNA and found no hypermutated clones. Among patients with chronic HBV infection, hypermutated clones were identified only in eAb-positive patients (Table 2). Figure 1

Table 2. Nucleotide Substitutions of Clones Amplified by PCR With PNA Clamping and Clinical Features of Patients With Acute and Chronic Hepatitis B Virus Infections

Patient	No. of Substitutions*		No. of Clones†	Pre-core‡	CP§	eAg	eAb	HBV DNA	ALT
	G to A	Other							
A-1	27	3	8 (1)	G	A/G	42	0	5.1	2,517
A-2	13	4	8	G	A/G	7.8	88	6.1	3,778
A-3	12	2	5	A/G	A/G	190	0	<3.7	1,417
A-4	11	0	4	G	A/G	58.3	0	4.5	2,550
A-5	11	3	9	G	A/G	170	0	8.3	175
A-6	7	7	9	A/G	Mixed	260	0	7.8	28
A-7	1	2	4	G	Mixed	0.1	99.4	4.1	2,295
A-8	1	1	3	A	T/A	0.7	91	7.1	6,183
C-1	152	2	10 (10)	A	T/A	0.3	100	5.5	394
C-2	44	12	9 (4)	A/G	T/A	18.2	73.4	6.2	340
C-3	30	4	10 (1)	A/G	T/A	0.3	97	7.3	53
C-4	23	1	3	G	A/G	140	0	5.9	2,770
C-5	22	1	8 (1)	A	T/A	0.4	95	6.5	105
C-6	19	9	9	A/G	Mixed	200	0	8.2	113
C-7	18	5	7	G	T/A	170	0	6.6	31
C-8	17	1	7	G	A/G	200	0	7.7	92
C-9	12	4	7	G	T/A	180	0	>8.8	56
C-10	6	4	7	A	A/G	2.5	95	8.3	267

*Total number of nucleotide substitutions in 10 clones compared with sequences obtained by direct sequencing.

†Number of different clones of 10 clones sequenced. Figures in parentheses represent the number of clones with hypermutation (those with a statistically significant number of G to A substitutions).

‡Nucleotide sequence of codon 28 of pre-core protein (nucleotide 1896).

§Nucleotide sequence of basic core promoter (nucleotides 1762 and 1764). Mixed represents mixture of A/G and T/A.

illustrates hypermutations found in an eAb-positive patient with chronic HBV infection (C-1 in Table 2). As much as 72.5% (29 of 40) of G residues were mutated in such hypermutated clones. Hypermutation was found in both the envelope/polymerase region (Fig. 1A) and x region (Fig. 1B) of HBV genome obtained from this patient. Preference of G to A mutation was similar with those reported in HIV-1; that is, G residues in GA sequences were the most frequently hypermutated (Fig. 2).

In contrast, the G residues in CxG context were less frequently substituted (Fig. 2). Numerous G to A nucleotide substitutions were identified in clones lacking a statistically significant number of G to A hypermutations (Table 2). The number of such substitutions was apparently greater than “other substitutions” (Table 2). There was no relationship between the degree of hypermutation and serum alanine aminotransferase concentration or HBV DNA level (Table 2).

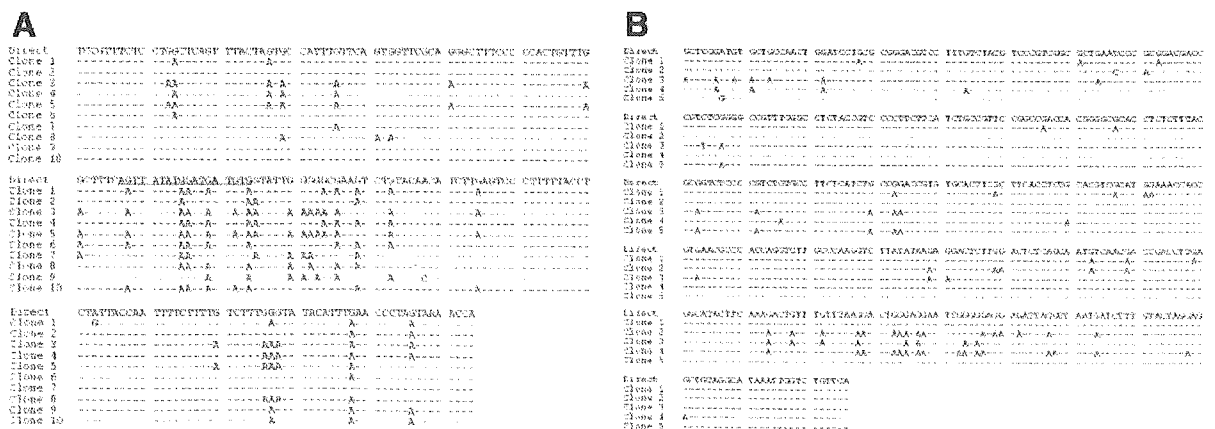


Fig. 1. G to A hypermutations detected in sequences of HBV DNA in sera extracted from an HBe antibody-positive HBV carrier (Patient C-1, Table 2) by PCR with PNA clamping. (A) DNA sequence alignment in the HBs antigen/polymerase region of the HBV. The nucleotide sequences that were obtained by direct sequencing were used as a reference sequence (top line). The target sequence of PNA annealing is underlined. (B) DNA sequence alignment in the x region of the HBV.

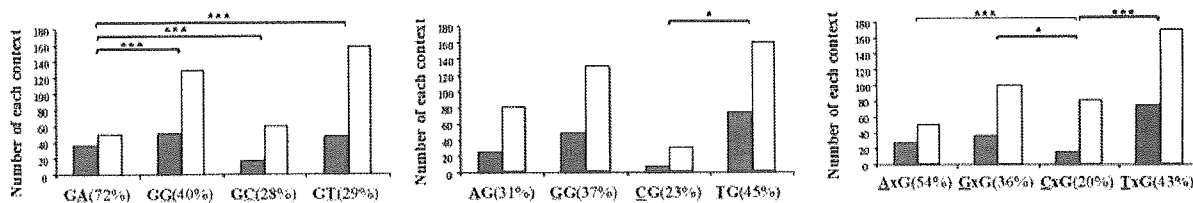


Fig. 2. Preferred nucleotide contexts of G to A hypermutation in 10 clones from patient C-1 (Table 2). The preferred nucleotide letter one letter after (left), one letter before (middle), and two places before (right, x = any) the target G residue. **Open bars:** number of occurrences of each context in the sequence analyzed. **Gray bars:** number of G residues mutated to A. The percentage in parentheses represents the rate of mutated G residues. * $P < .05$, *** $< .001$ (Fisher's exact test or chi-square test).

G to A Hypermutation in HBV-Producing Cell Lines. We established HepG2 cell lines that continuously produced HBV into the medium and examined the frequency of hypermutation. Hypermutated clones were identified in one of these cell lines (Table 3 and Fig. 3). The preference of G to A mutation was similar to that found in serum samples obtained from patients (data not shown). Various levels of HBsAg, HBeAg, and HBV DNA were released into the medium from these cells (Table 3). No relationship was found between the frequency of the hypermutated genome and intracellular intermediates of HBV DNA and HBsAg and HBeAg levels (Table 3). Figure 4 shows replicative intermediates of the HBV produced in these cell lines detected by Southern blot analysis (Fig. 4). No noticeable difference was observed between a cell line with hypermutated genomes and those without hypermutated genomes (lanes 1 and 2 in Fig. 4).

G to A Hypermutation During Antiviral Treatment. We treated the cell lines with alpha and gamma interferon and lamivudine. Both interferons reduced HBV DNA production from these cells in a dose-dependent manner (Fig. 5). The frequency of G to A hypermutation did not increase in those treated cell lines (Fig. 6), suggesting that G to A hypermutation is not responsible

for antiviral defense through these interferons. Treatment of a cell line with lamivudine resulted in marked reductions in the production of HBV in the supernatant as well as intracellular viral intermediates (Fig. 7) and completely abolished identification of G to A substitution (Fig. 6). A similar reduction of detection of hypermutated clones was observed in serum samples obtained from patients who were treated with lamivudine (data not shown).

Expression of Deaminases in HepG2 Cell Lines.

We examined the expression of known deaminases to see whether any such enzymes are active in HepG2 cells. As shown in Fig. 8, mRNA expression of 5 of 8 of these deaminases was detected, although the expression level of some deaminases was very low. mRNA of Apobec3G, a key enzyme for the hypermutation of HIV-1, was expressed in HepG2 cells, but the cDNA of this enzyme was only found by nested PCR. The expression level of the mRNA was similar in HBV-producing cells with various levels of hypermutations of HBV as well as parent HepG2 cells (detected by only nested PCR).

Discussion

In this study, we detected the mutated HBV genome in some patients by using PCR with PNA clamping. PNA is a DNA analog in which the ribose-phosphodiester backbone of DNA has been replaced by *N*-(2-aminoethyl) glycine linkages.²³ The PNA anneals strongly to DNA like a complementary DNA, but with higher affinity.²³ The annealing of the PNA to the target sequence thus prevents amplification of the target DNA in the PCR. In our previous study,²¹ we attempted to block the amplification of lamivudine-sensitive wild-type YMDD motif strain and detected a very small amount (1/10,000) of YMDD motif mutant. Because the target sequence of this system contained many Gs with GA and GG (AGT TAT ATG GAT GAT GTG), we assumed that we could detect very rare hypermutated genomes.

Because we did not detect any hypermutated sequence without PNA, we assumed that the rate of the hypermutated genome is very low. This low frequency of hyper-

Table 3. Nucleotide Substitutions of Clones Amplified by PCR With PNA Clamping in Three Cell Lines That Produce the Hepatitis B Virus

Cell Line	No. of Substitutions*		No. of Clones†	eAg	HBs Ag	HBV DNA
	G to A	Other				
Cell line 1	102	0	10 (7)	17	4.7	5.2
Cell line 2	19	0	7	10	4.9	4.6
Cell line 3	21	1	6	14	2.8	4.6

*Total number of nucleotide substitutions in ten clones compared with sequences of the transfected clone.

†Number of different clones of 10 clones sequenced. The figure in parentheses represents the number of clones with hypermutation (those with a statistically significant number of G to A substitutions). Codon 28 of the pre-core gene of the transfected clone was wild (Trp), and nucleotides 1762/1764 were T/A.

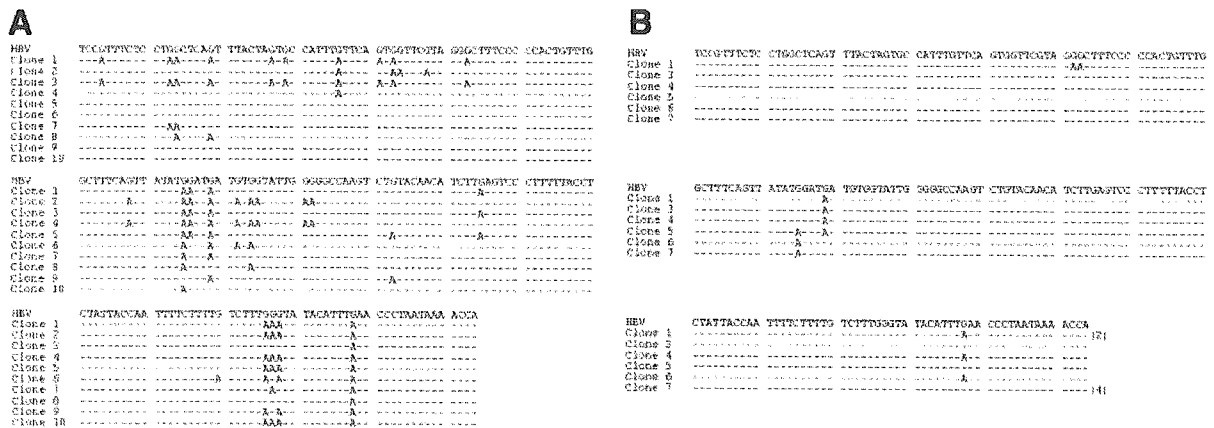


Fig. 3. G to A hypermutations detected in sequences of the HBV DNA (produced by HBV DNA-transfected cell lines to the supernatants). The nucleotide sequences of the transfected clone were used as a reference sequence (top line). DNA sequence alignments in the HBs antigen/polymerase region of cell line 1 (A) and cell line 2 (B) of the HBV. Numbers in parentheses are numbers of clones.

mutated genomes accounts for the lack of reports of such sequences with only one exception until recently,¹⁹ in which the presence of two clones of hypermutated sequences in spliced genomes was reported. One may assume that the rare hypermutated genome might be produced in peripheral blood mononuclear cells because the HBV genome was previously found in such cells.²⁴⁻²⁸ However, we showed that these genomes are found in HBV-transfected cell lines. Our results clearly demonstrate that hypermutation actually occurs in hepatocytes. The reason(s) for such a low frequency of hypermutation

is not clear. The low expression level of deaminases in hepatocytes might account for the low frequency. In fact, we observed a very low expression level of APOBEC3G (transcripts was only detected by nested PCR [Fig. 8]) in HepG2 cell lines.

Recently, Turelli et al.^{20,29} suggested that overexpression of APOBEC3G inhibits the replication of HBV by preventing encapsidation of the virus. However, they did not observe an increase in G to A hypermutation. In contrast, Rosler et al.³⁰ reported that G to A substitutions significantly increased in HepG2 cells when co-transfected with APOBEC3G cDNA. They found only 50 G to A substitutions by cloning 223 clones,³⁰ suggesting that the frequency of G to A substitutions is rare despite overexpression of APOBEC3G. Our preliminary data suggest that overexpression of APOBEC3G does not produce a

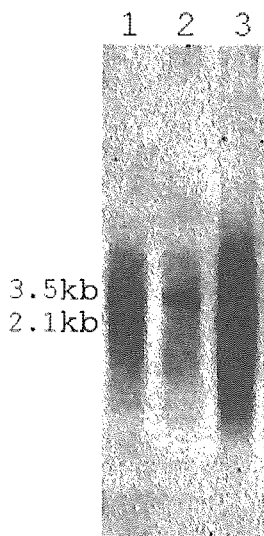


Fig. 4. Southern blot analysis of the HBV DNA extracted from cell lines that stably produce HBV into the supernatant. Two YMDD wild-type virus sequences (lanes 1 and 2) and one YVDD mutant virus sequence (lane 3) were transfected into the HepG2 cell line.

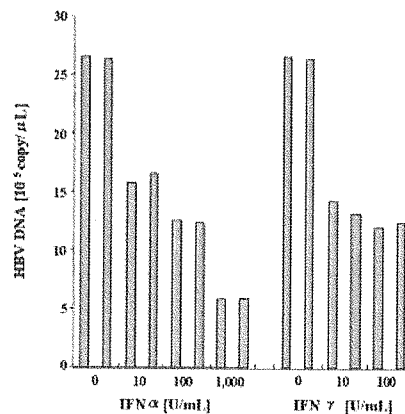


Fig. 5. Effects of interferon alpha and gamma on production of HBV DNA by cell line 1. Experiments were performed in duplicate with increasing amounts of each interferon.