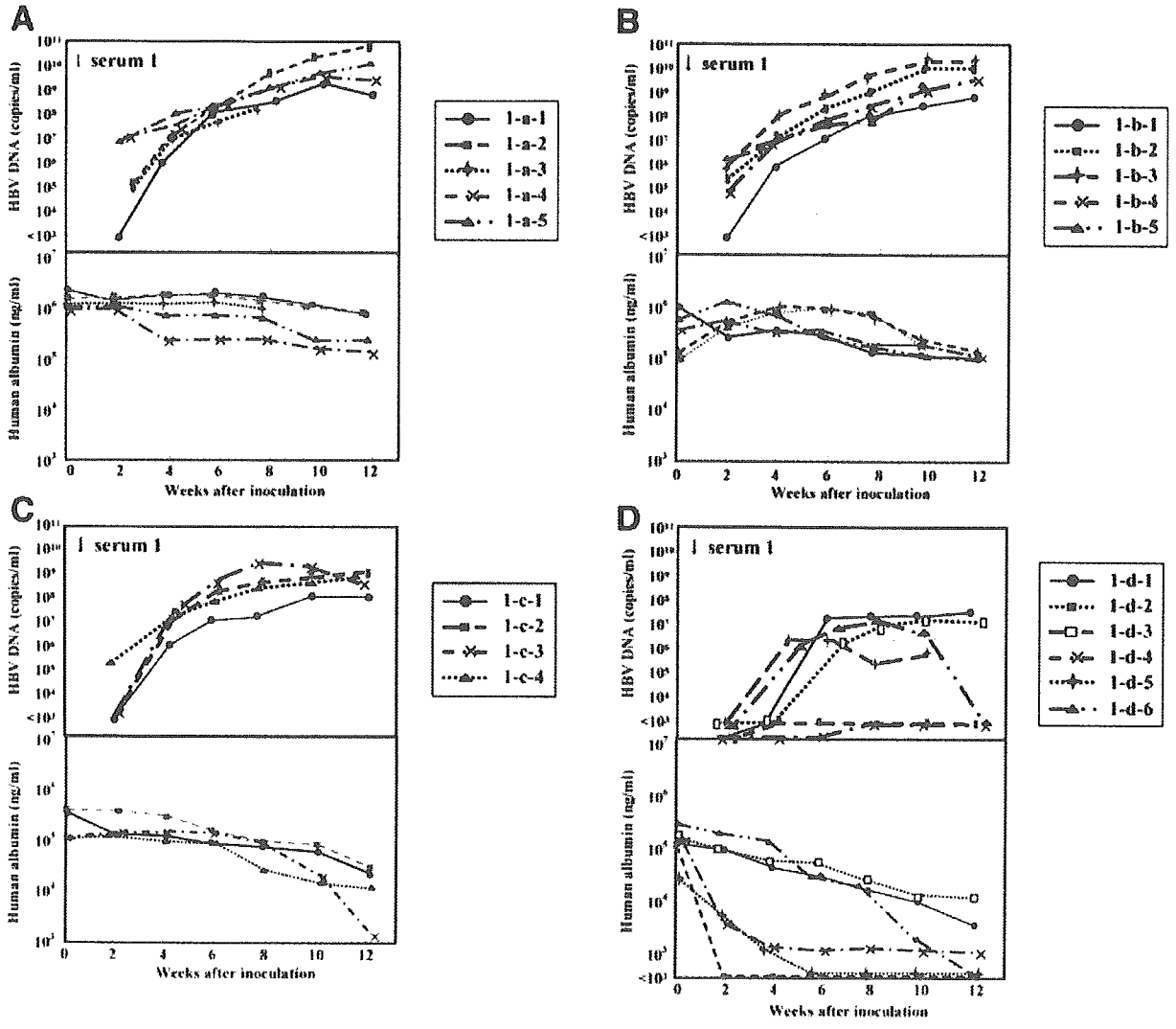

Fig. 1. Time course studies in 20 mice inoculated with human serum samples positive for hepatitis B virus (HBV). Fifty microliters serum samples (serum 1, Table 1) was intravenously injected into each mouse. The upper half of each panel shows HBV DNA, and the lower panel shows concentrations of human serum albumin (HSA). Mice were divided according to the levels of HSA; (A) initial HSA > 1,000,000 ng/mL with only slight decline (n = 5); (B) initial HSA > 500,000 ng/mL, with slight decline (n = 5); (C) initial HSA > 200,000 ng/mL, but declined to less than 100,000 ng/mL during observation (n = 4); (D) initial HSA =100,000 ng/mL, but diminished to less than 30,000 ng/mL (n = 6).

tionated into 500-μL samples, and the density of each fraction was calculated from the weight and volume. Each fraction was diluted 10-fold and tested for HBV DNA by real-time PCR.

Generation of Human Hepatocyte Chimeric Mice and Analysis of Serum Samples. Generation of the uPA$^{+/+}$/SCID$^{+/+}$ mice and transplantation of human hepatocytes were performed as described previously by our group.[19] Animal protocols were performed in accordance with the guidelines of the local committee for animal experiments. Infection, extraction of serum samples, and sacrifice were performed under ether anesthesia. HSA was measured with a Human Albumin ELISA Quantitation kit (Bethyl Laboratories Inc., Montgomery, TX) according to the instructions provided by the manufacturer. Serum samples obtained from mice were aliquoted and stored in liquid nitrogen until use.

Histochemical Analysis of Mouse Liver. The liver specimens of infected mice were fixed with 10% buffered-paraformaldehyde and embedded in paraffin blocks for histological examination. The liver sections were stained with hematoxylin-eosin or subjected to immunohistochemical staining by using an antibody against hepatitis B core antigen (HBc-Ag) (DAKO Diagnostika, Hamburg, Germany) or HSA (Bethyl Laboratories Inc.). Endogenous peroxidase activity was blocked with 0.3% H_2O_2

and methanol. Immunoreactive materials were visualized by using a streptavidin-biotin staining kit (Histofine SAB-PO kit; Nichirei, Tokyo) and diaminobenzidine.

Results

Human Hepatocyte Chimeric Mice Develop High-Level and Long-Term Viremia After Inoculation of Serum Samples Obtained From Carriers. Twenty chimeric mice were inoculated with 50 μL serum 1 (Table 1). We used mice that had relatively low-level HSA because we had previously found that mice with low-level replacement are susceptible to HBV (Chayama K and Tateno C, unpublished results). The HSA of these mice was 300,000 ng/mL (median, range, 40,000-3,090,000, Fig. 1A). All 20 mice tested positive for HBV DNA by nested PCR 2 to 4 weeks after inoculation. Eighteen of 20 mice developed quantitatively measurable viremia, but two mice showed very low-level viremia that was detectable only by nested PCR. Mice with persistently high-level HSA tended to show high virus titer (Fig. 1A-C). The maximum level of viremia was 9.5×10^{10} copy/mL. The viremia reached a plateau 4 to 6 weeks after infection. In contrast, mice with a rapid decrease in HSA or persistently low-level HSA showed low virus titer (Fig. 1D). We also performed infection experiments using serum 2 (Table 1). Of the five mice inoculated with this serum, all developed quantitatively measurable viremia 2 to 4 weeks after inoculation (Fig. 2). The level of viremia reached 1×10^7 to 1×10^9 copies/mL. The level of viremia also tended to be high in mice with high HSA levels.

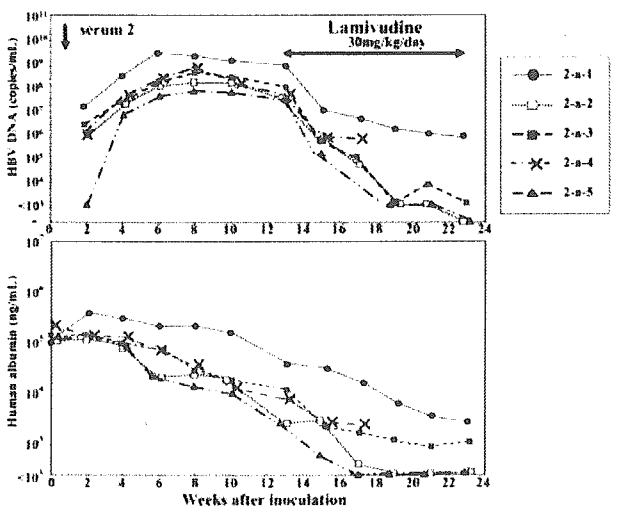

Fig. 2. Time course studies in five mice inoculated with human serum samples positive for hepatitis B virus (HBV). Fifty microliters serum samples (serum 2, Table 1) was intravenously injected into each mouse. The upper panel shows HBV DNA, and the lower panel shows concentrations of human serum albumin. The effects of lamivudine are shown in the upper panels.

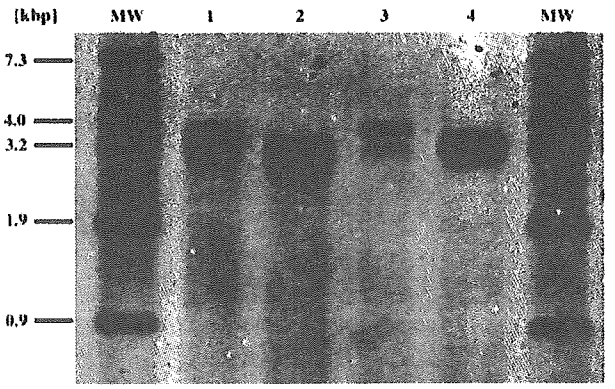

Fig. 3. Formation of fully repaired, relaxed circular hepatitis B virus (HBV) DNA after endogenous polymerase reaction. HBV particles produced into supernatant were immunoprecipitated with a monoclonal antibody against HBs antigen and subjected to Southern blot analysis before (lanes 1 and 2) and after (lanes 3 and 4) endogenous polymerase reaction. The undigested (lanes 1 and 3) and unique *Sma*I-digested DNA was electrophresed in a 1% agarese gel and detected by Southern blot hybridization.

HBV Generated in HepG2 Cell Lines Are Infectious to Human Hepatocyte Chimeric Mice. HBV markers and endogenous polymerase experiments with Southern blot analysis of HBV produced by transiently or stably transfected HepG2 cell lines are shown in Table 1 and Fig. 3. The results indicated that these cell lines produced the expected HBV antigens and HBV DNA into the supernatant. Using virus particles produced by transient transfection of plasmid pTRE-HB-wt, we performed endogenous polymerase chain reaction experiments. Formation of fully double-stranded, relaxed circular DNA was observed after the reaction (Fig. 3). Sucrose density gradient analysis of HBV produced by stably transfected cell line (CA59, Table 1) showed that the produced viruses were sedimented to similar fractions of HBV obtained from the serum of the HBV carrier (Fig. 4), suggesting that HBV particles similar to those in serum are produced in these cell lines.

In the next step, we inoculated each chimeric mouse with 50 μL of the supernatants produced by transiently or stably transfected cell lines (Table 1). Three mice were inoculated with CM3 (Fig. 5A). Four weeks later, one of these three mice developed measurable viremia. It reached a high level (7.3×10^8 copy/mL) at week 14. A serum sample obtained from this mouse at week 6 was stored in liquid nitrogen and used in the subsequent passage experiments. The other two mice developed viremia, but its level was so low that HBV was only detectable by nested PCR. At week 13, these two mice (5-a-2, 5-a-3, Fig. 5A) were inoculated with serum 1, which induced high-level viremia in mice with high HSA levels (Fig. 1). These mice did not develop measurable viremia, suggesting that the

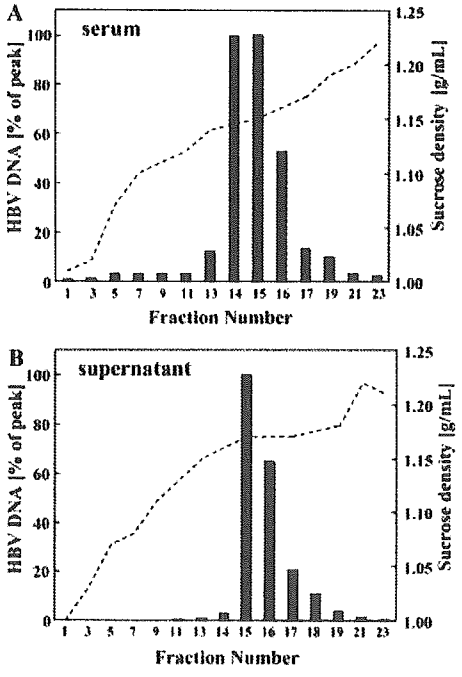

Fig. 4. Sucrose density gradient analysis of hepatitis B virus (HBV) produced into the supernatant of the transfected HepG2 cell line (CA59). Sucrose density is indicated by the dotted line. The amount of HBV DNA in each fraction was measured by real-time PCR.

low-level viremia in the latter two mice was due to low-levels of human hepatocyte replacement. Similarly, six mice were inoculated with supernatant CA59. One of these six mice developed quantitatively measurable viremia (peak, 2.6×10^9 copies/mL) (Fig. 5B). Two of the six mice developed viremia only detectable by nested PCR. For a more efficient reverse genetics infection procedure, we used mice with higher human albumin concentrations and inoculated each with 500 to 1,000 μL freshly prepared high-titer virus particles. This resulted in infection of all 10 mice (Fig. 5C). Thus, we established a highly effective infection procedure of reverse genetics of HBV.

Infection of Genetically Engineered Mutant Viruses. Four mice were inoculated with the supernatant of genetically engineered e-antigen–negative HBV generated in a pTRE-HBV-PC-transfected HepG2 cell line (e-negative, Table 1). Three of these four mice developed quantitatively measurable, but relatively low-level (less than 10^7 copies/mL) viremia, 2 to 6 weeks after inoculation (Fig. 6). Nucleotide sequence analysis of the precore region showed that the sequence obtained from the infected mice was completely in agreement with the transfected plasmid with precore stop codon at 1896.

Passage Experiment of HBV From a Mouse Infected by* In Vitro *Generated HBV to Naïve Chimeric Mice. Each of four naïve mice was injected with 5 μL serum samples obtained from a mouse that developed HBV viremia after inoculation of *in vitro* generated virus (CM3, Table 1). All four mice developed viremia at 2 to 6 weeks after inoculation (Fig. 7). One of the four mice that developed measurable viremia died at week 5 (7-a-3, Fig. 7). Another mouse (7-a-2) was weak and was sacrificed at week 13. The high-level viremia in the third mouse (7-a-1) increased further to 8.5×10^9 copies/mL. The remaining mice developed viremia detectable only by nested PCR (7-a-4).

Histochemical Analysis of the Liver of Mice Infected With HBV. Liver specimens from mice that became positive for HBV DNA after the inoculation of the described passage experiment were subjected to histological and immunohistochemical analyses. Multiple foci of replaced human hepatocytes were noted in hematoxylin-eosin–stained sections (Fig. 8A) that were positive for HSA (Fig. 8B). Such positive human hepatocytes were also positive for the HBV core antigen in serial sections (Fig. 8C).

Effect of Lamivudine Treatment in Mice Infected With HBV. Five mice that became positive for HBV DNA by inoculation with serum 2 (Fig. 2) were fed lamivudine (30 mg/kg/day)-containing food. A rapid reduction of HBV DNA level was observed in all 5 mice. Although two of five mice showed graft failure reflected by a decrease in HSA levels to the lower limits of the assay (2-a-2 and 2-a-5), the reduction of HBV DNA levels appeared before the decrease in HSA in these mice, suggesting that the decrease in HBV DNA was due to both the effect of lamivudine and the loss of virus replicating human hepatocytes (Fig. 2). Similarly, 1 mouse with high-level viremia as described in the above passage experiment (7-a-1, Fig. 7) showed a marked reduction of HBV DNA.

Discussion

The major finding of the current study was the successful establishment of a model of HBV infection with long-term and high-level HBV viremia in the human hepatocyte chimeric mouse. The level of viremia correlated with the degree of human hepatocyte replacement indicated by HSA levels. We also showed that HBV created *in vitro* using HepG2 cell lines are infectious to this mouse model. Thus, a combination of chimeric mouse and molecularly cloned virus enabled us to prepare a practical model for the study of HBV virology. Chimpanzee is also a useful model for the study of HBV virology. Injection of molecularly cloned HBV into the liver of chimpanzee induced HBV infection and hepatitis.[22] However, there might be some difference between hepatocytes of human and chimpanzee that could affect the nature of infection and the replication of this narrow host virus.

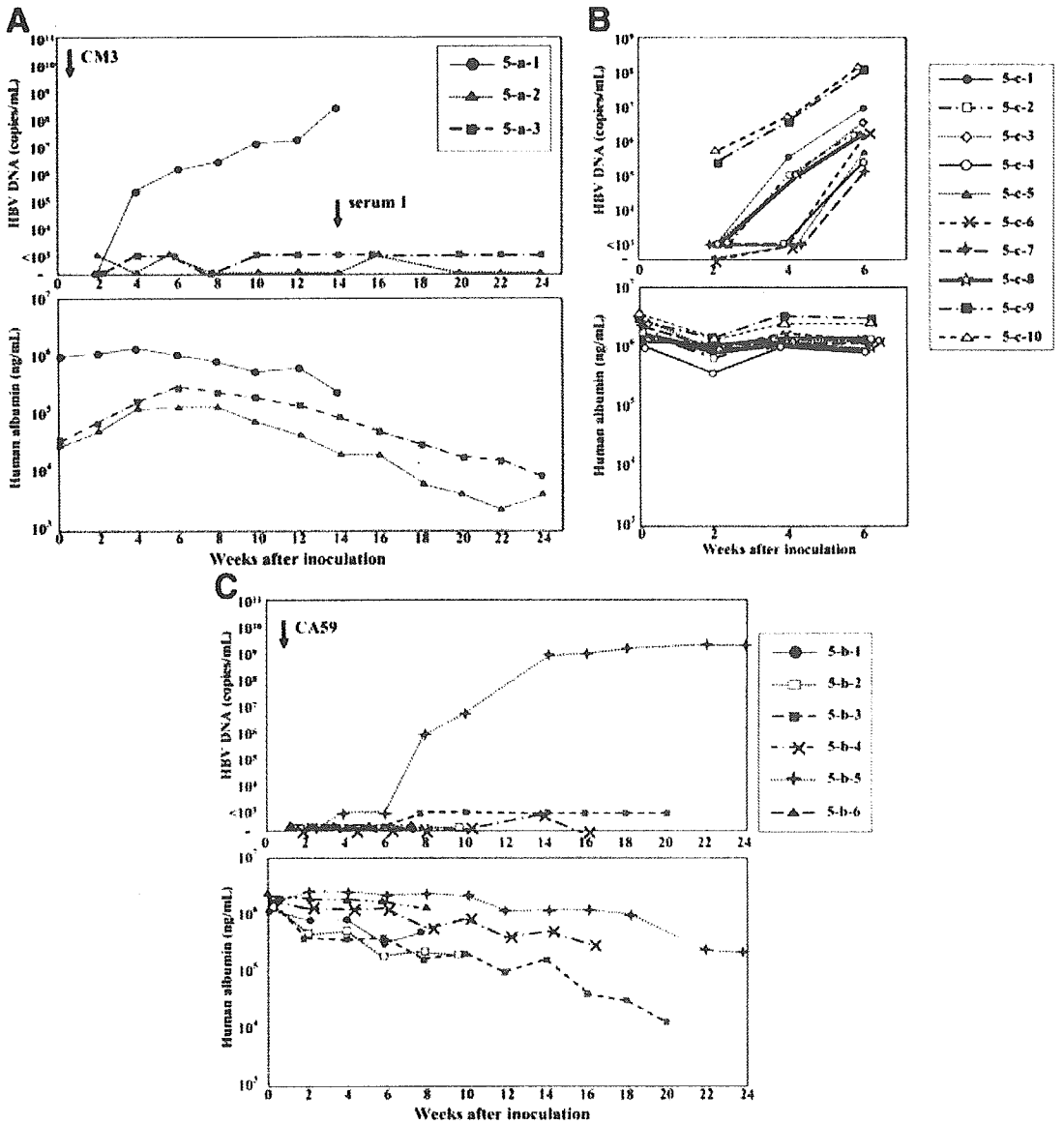

Fig. 5. (A) Time course studies in three mice inoculated with supernatants of HepG2 cell lines transfected with hepatitis B virus (HBV) DNA. Fifty microliters culture supernatant (CM3, Table 1) was inoculated intravenously into each mouse. Upper panel: HBV DNA; lower panel: concentrations of human serum albumin. (B) Time course studies in 6 mice inoculated with supernatants of HepG2 cell lines transfected with HBV DNA. Fifty microliters culture supernatant (CA59, Table 1) was inoculated intravenously into each mouse. (C) Time course studies in 10 mice inoculated with freshly prepared supernatants of HepG2 cell lines transfected with HBV DNA. Five hundred (mice 5-c-1 to 5-c-4) and 1,000 μL culture supernatants (5-c-5 to 5-c-8) and 500 μL concentrated (from 1.1×10^9 copies/mL to 1.3×10^{10} copy/mL by ultrafiltration) supernatant (5-c-9 and 5-c-10) were inoculated intravenously into each mouse.

A critical difference between the chimeric mouse model reported here and chimpanzee is that there is no immune system active for HBV in the mouse model. Although the chimpanzee model is known to cause hepatitis and is suitable for the study of HBV-induced hepatitis,[23] the mouse model is expected to be free from inflammation because these mice are SCID and do not have any human cytotoxic T lymphocytes. Actually, we observed no lymphocyte infiltration or focal necrosis of human hepatocytes in our mouse model. Recently, similar morphological changes in a similar model were reported by Meuleman et al.[24]; they also observed no alteration of liver architecture by HBV and hepatitis C virus infections. Interestingly, however, we observed a poor increase in the viral titer during the early phase of infection in some mice (Figs. 5A, 6). This might represent some innate anti-viral defense mechanism of liver cells themselves against viral infection. Further investigation is necessary to explore this issue.

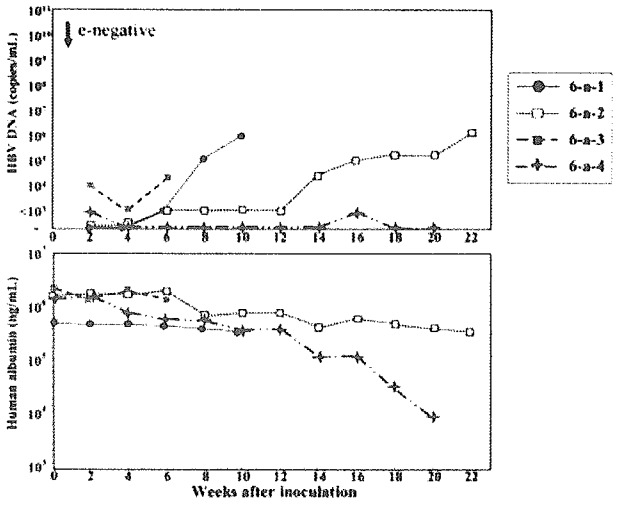

Fig. 6. Time course studies in four mice inoculated with supernatants of HepG2 cell lines transfected with e-antigen-negative (G1896A) hepatitis B virus (HBV) DNA. Fifty microliters culture supernatant (e-negative, Table 1) was inoculated intravenously into each mouse. The upper panel shows HBV DNA, and the lower panel shows the concentrations of human serum albumin.

A mouse model without any inflammation is an advantageous phenotype because it allows the study of HBV replication without any influence of immunological reaction. The model is also beneficial for studying the effects of drugs without any influence of fluctuation of the virus by immunological reaction. The HBV-infected mouse described here opens the way to create a long desired practical small animal model that overcomes economical

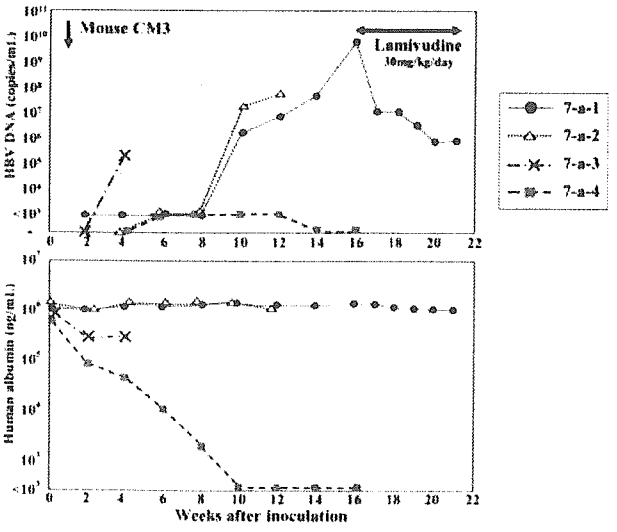

Fig. 7. Passage experiments in four mice. Five microliters mouse serum (mouse CM3, Table 1) was intravenously inoculated into each of four naïve mice. Upper panel: HBV DNA, lower panel: concentrations of human serum albumin.

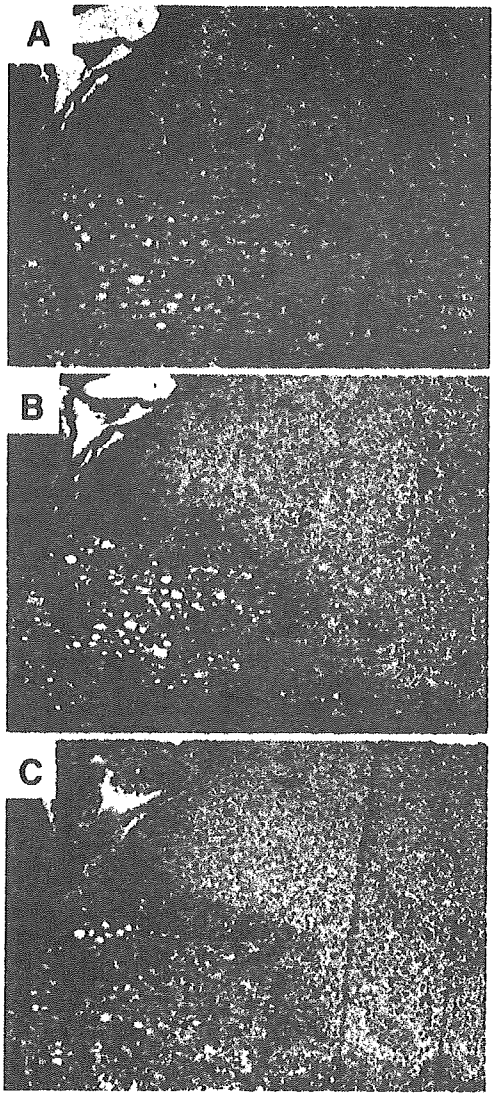

Fig. 8. Histochemical analysis of liver samples obtained from mice infected with hepatitis B virus generated in the 1.4 genome transfected HepG2 cell line. (A) Hematoxylin-eosin staining. Human hepatocytes are indicated by arrows. Immunohistochemical staining with anti-human serum albumin antibody (B) and anti-HBc-Ag antibody (C) (original magnification, ×40.

and ethical problems associated with the chimpanzee model.

We showed in this study that reverse genetics of HBV can be achieved highly efficiently by using mice with high human albumin levels and inoculating mice with large amounts of freshly prepared virus particles (Fig. 5C). We further showed in this study that e-antigen is completely dispensable for infection and replication. The e-antigen–negative HBV-containing serum was previously used in chimpanzee and is known to induce more severe hepatitis.[25] However, it is difficult to exclude the possible presence of a small amount of e-antigen–producing virus that might help infection and

replication of HBe antigen-negative HBV strain. Our results clearly demonstrate that HBV can infect and replicate in the complete absence of e-antigen–producing species. However, the level of viremia was relatively low (less than 1×10^7 copies/mL) in these mice. Whether this is due to lack of e-antigen should be further confirmed in a larger number of mice with high replacement index.

Because the mice treated with lamivudine showed a reduction of viremia (Figs. 2, 7), our infected mouse is suitable for the study of new drugs. Lamivudine is a potent anti-HBV drug that reduces the virus and induces clinical remission and histological improvement.[26-28] Emergence of drug-resistant HBV mutants against this drug as well as other anti-viral drugs is a serious problem in the treatment of HBV,[29-31] as has been seen in the therapy of human immunodeficiency virus infection. Our model is especially useful for the study of the biology and drug susceptibility of such mutants, because almost all such drug resistances are based on only one or two point mutation(s).[29,31]

In conclusion, the mouse model presented in this study is very useful for the study of HBV biology and evaluating of anti-HBV drugs. Furthermore, many applications of this model are expected because we can easily create, manipulate, and modify the model compared with other models.

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

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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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(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

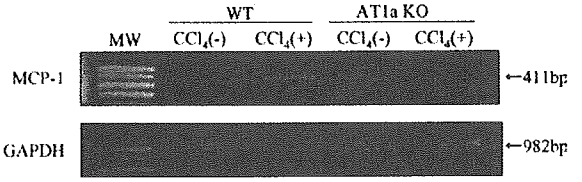

Fig 1. Steady-state hepatic MCP-1 mRNA expression in WT and AT1a KO mice with or without CCl_4 treatment for 4 weeks. An aliquot of each PCR product was loaded onto a 2% agarose gel and stained with ethidium bromide. Amplification of GAPDH was done to confirm the equal amounts of mRNA in each sample.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

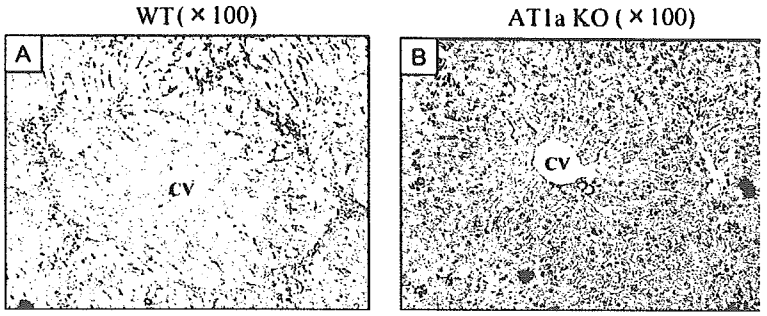

Fig 2. Immunohistochemical staining of MCP-1 in liver tissue from (A) a WT mouse and (B) an AT1A KO mouse after 4 weeks of CCl_4 treatment (1.0 ml/kg). CV, central vein. (Original magnification, × 100.)

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).

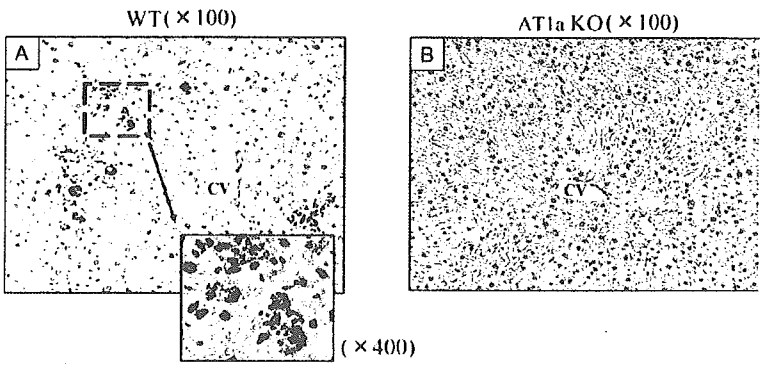

Fig 3. Distribution of CD68-positive cells in the liver. Immunohistocheical staining for CD68-positive mononuclear cells was performed in liver tissue from (A) a WT mouse and (B) an AT1A KO mouse after 4 weeks of CCl_4 treatment (1.0 ml/kg). CV. central vein. (Original magnification. × 100.)

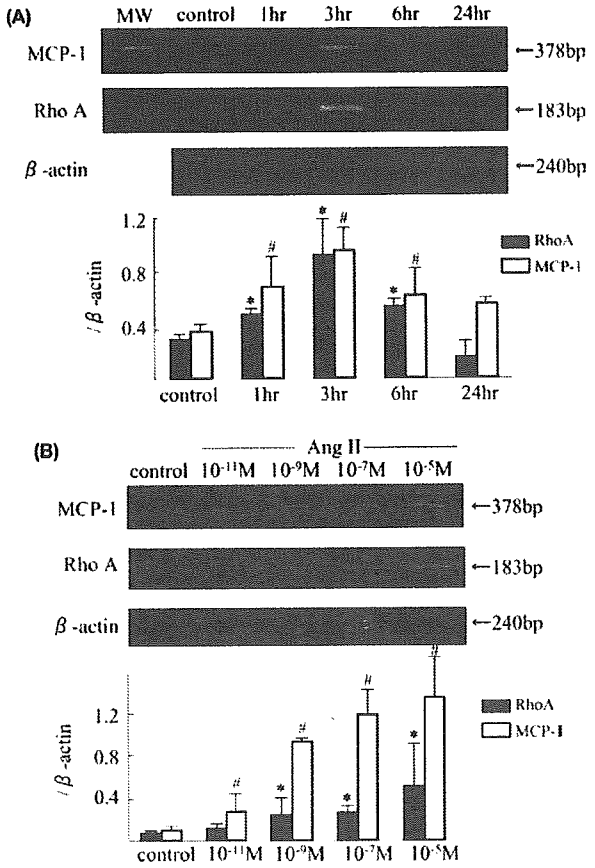

Fig 4. Effect of Ang II on MCP-1 mRNA expression by cultured LI90 cells. (A) Serum-starved LI90 cells were treated with 10^{-7} *M* Ang II at the indicated times, and the changes in Ang II-induced MCP-1 mRNA expression were examined by semiquantitative PCR. (B) Serum-starved LI90 cells were stimulated 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.

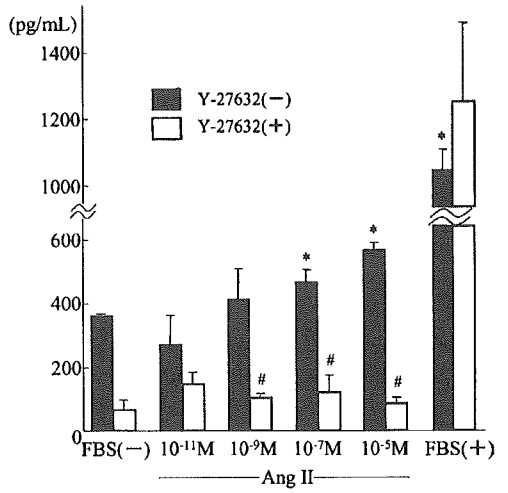

Fig 5. Effect of Ang II on MCP-1 secretion into the culture medium. Serum-starved LI90 cells were stimulated for 48 hr with the indicated concentrations of Ang II in the absence or presence of Y-27632 (10^{-5} *M*). Data from four independent experiments are shown as means ± SD. $^{*}P < 0.05$ vs. serum-free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

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 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

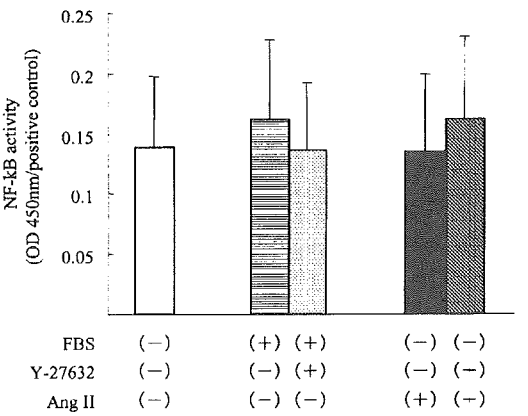

Fig 6. NF-κB activity in nuclear extracts of Ang II-stimulated LI90 cells. The cells were stimulated using Ang II for 1 hr with or without preincubation of Y-27632 (10^{-5} *M*). Then nuclear protein was extracted, and NF-κB activity was estimated. Absorbance values were standardized according to the 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

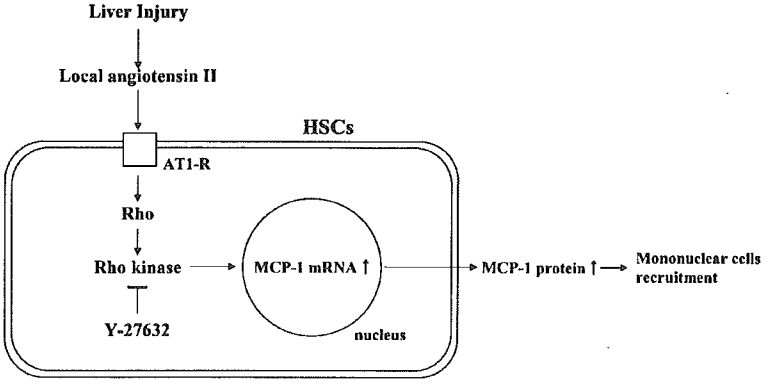

Fig 7. Speculated mechanism of how angiotensin II participates in hepatic inflammation after liver injury. Ang II enhances MCP-1 gene expression and synthesis, partly via the Rho signaling pathway, which modulate the recruitment of inflammatory cells into the liver. AT1-R, angiotensin II type1 receptor;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

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Clinical Studies

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

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

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

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

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

Patients and methods

Patients

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

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

Serological markers for HBV

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

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

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

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

Statistical analysis

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

Results

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

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

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

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

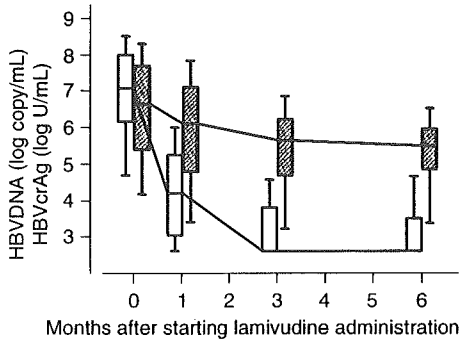

Fig. 1. Changes in the median levels of hepatitis B virus core-related antigen (HBVcrAg) and hepatitis B virus (HBV) DNA during lamivudine administration. The box plots show the 10th, 25th, 50th, 75th and 90th percentiles, with the open boxes indicating HBV DNA and shaded boxes indicating HBVcrAg. The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

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

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

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

Discussion

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

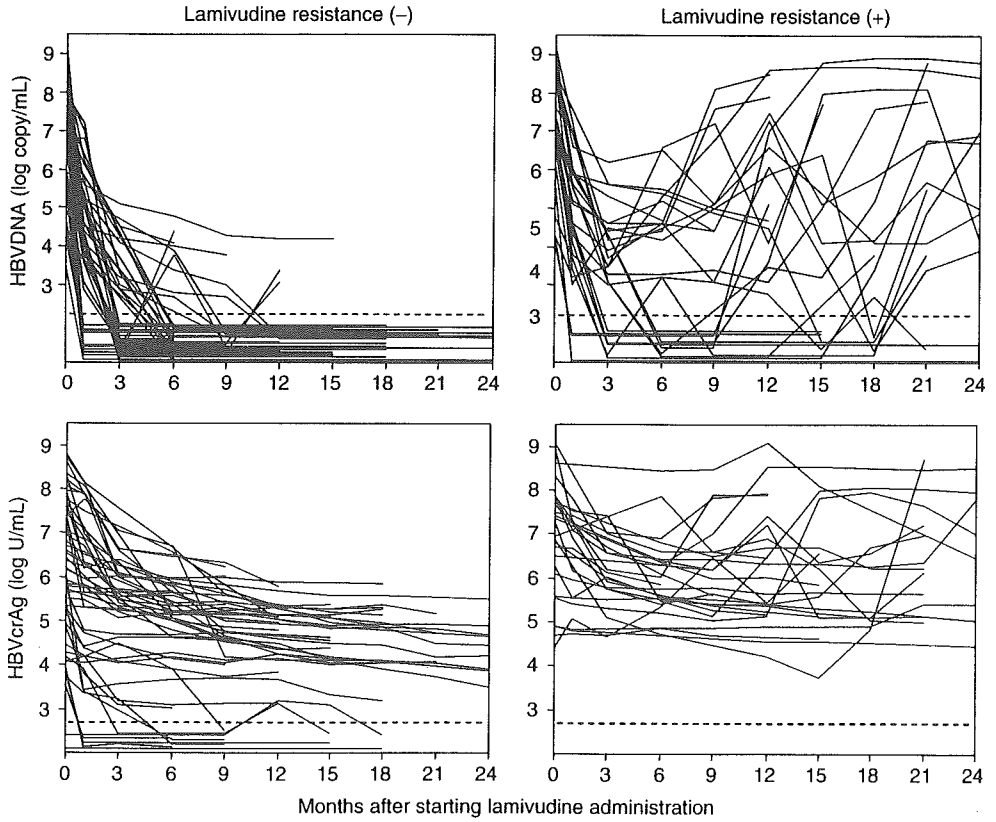

Fig. 2. Comparison of changes in serum hepatitis B virus (HBV) DNA and serum HBV core-related antigen (HBVcrAg) levels between patients who showed lamivudine resistance and those who did not. The broken lines indicate the detection limit of each assay.

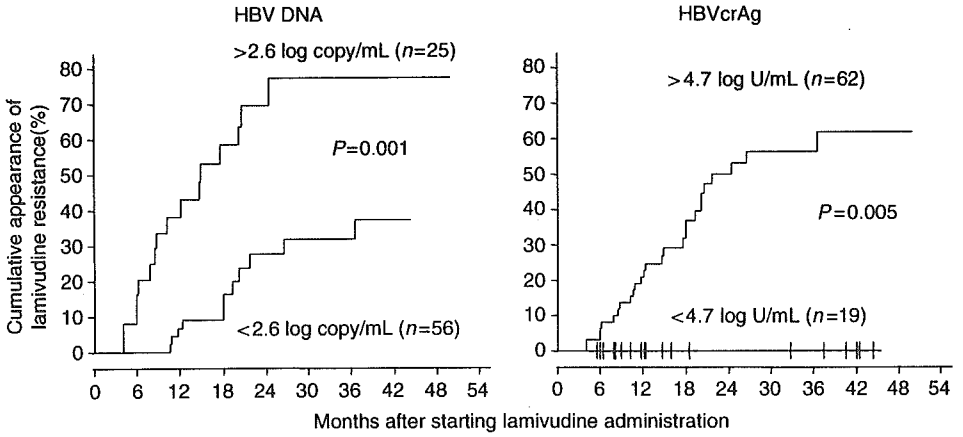

Fig. 3. Comparison of the cumulative occurrence of lamivudine resistance between patients who showed hepatitis B virus (HBV) DNA levels of less than the detection limit (2.6 log copy/ml) at 6 months after starting lamivudine administration and those who did not (left figure), and similarly between patients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

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

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

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

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

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.

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