Hepatitis C Virus Acts as a Tumor Accelerator by Blocking Apoptosis in a Mouse Model of Hepatocarcinogenesis

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We developed hepatitis C virus (HCV) core-E1-E2 and HCV core transgenic mice on a common genetic background to assess the contribution of HCV structural proteins to hepatocarcinogenesis. Eight-week-old core-E1-E2, core, and nontransgenic mice inbred on the FVB×C57Bl/6 background were treated with diethylnitrosamine (DEN) and sacrificed at 32 weeks old. Proliferation and apoptosis were assessed by immunohistochemistry. The effect of viral proteins on apoptosis was evaluated in HepG2 cells in which apoptosis was induced by anti-Fas antibody. HCCs were identified at 32 weeks in the majority of DEN-treated mice from all three groups. The mean size of HCCs was significantly larger in core-E1-E2 transgenic (4.63 ± 1.48 mm), compared with core transgenic (0.78 ± 0.26 mm, $P = .01$), and nontransgenic (1.0 ± 0.19 mm, $P = .002$) mice. While there were no differences in proliferation, the apoptotic index in core-E1-E2 transgenic HCCs was significantly lower than those found in core and non-transgenic HCCs. Core-E1-E2 transfected HepG2 cells demonstrated a significantly lower apoptotic index (0.35 ± 0.11) compared with that of core transfected cells (0.74 ± 0.07, $P = .0103$). Analysis of a Fas-induced apoptosis model in HCV transgenic mice confirmed that core-E1-E2 transgenic liver underwent significantly less apoptosis than transgenic tissue expressing core only. In conclusion, HCV core-E1-E2 transgenic mice develop significantly larger tumors than transgenic mice expressing core alone or nontransgenic mice. The accelerated tumor phenotype is attributable to suppression of apoptosis rather than enhanced proliferation. These data implicate HCV E1 and/or E2 in conjunction with core as antiapoptotic, tumor accelerator proteins. (HEPATOLOGY 2005; 41:660-667.)

Hepatitis C virus (HCV) infects an estimated 170 million people worldwide, and 2.7 million people in the United States harbor active HCV infection.[1] Chronic HCV infection has been independently

established as a leading cause of hepatocellular carcinoma (HCC). Unlike hepatitis B virus (HBV), the RNA genome of HCV does not integrate into the host chromosome. HCV-related hepatocarcinogenesis is, therefore, not likely to involve insertional mutagenesis. Rather, it has been hypothesized that HCV produces HCC through the cumulative effects of chronic infection, injury and repair. Whether HCV proteins are directly oncogenic has not been established. Unfortunately, the lack of an appropriate small animal model of HCV has impeded progress in defining the molecular mechanisms of HCV-induced carcinogenesis.

We originally developed an HCV transgenic mouse model encoding the core, E1, and E2 structural proteins under the control of the albumin promoter on the FVB background; despite high-level protein expression, this model did not develop hepatic pathology.[2] However, more recently, Moriya et al. developed a transgenic mouse model on the C57Bl/6 background that overexpresses

HCV core protein under the control of the HBV en-hancer that is capable of inducing HCC in a subset of animals following the development of steatosis.[3-6] These apparently contradictory findings could be due to inher-ent differences in the transgenes, HCV core dose effect, mouse genetic background, or a combination of these.

To reconcile these findings, we expressed the HCV core and HCV core-E1-E2 transgenes on a common mouse genetic background (FVB×C57Bl/6). We addi-tionally studied whether HCV acts as a cocarcinogen in a diethylnitrosamine (DEN)-induced model of hepatocar-cinogenesis.

Materials and Methods

Transgenic Mice

Alb-core-E1-E2 Transgenic (Core-E1-E2 Tg). The *Pvu*I-*Nsi*I fragment from pAlb-HCV, containing the core, E1, and E2 regions, was purified and microinjected into mouse oocytes from FVB-inbred mice (Taconic, Germantown, NY) as previously described.[2] Transgenic mice were identified by subjecting 1 μg of tail DNA to PCR amplification using the HCV core primers (5'-AT-GAGCACAAATCCTAAACCTC-3' and 5'-CAAG-CGGAATGTACCCCATGAG-3'). The resulting 418-bp fragment was visualized on 1.2% agarose gels. From the original seven lines created, we selected the line with the strongest core protein expression, AC 1-0,[2] as the founder. This line was then mated with wild-type C57Bl/6 mice (Taconic) to produce an HCV core-E1-E2 line on a hybrid background (FVB×C57Bl/6).

HBV enh-core Transgenic (Core Tg). A 1.2-kb *Kpn*I-*Hind*III fragment containing HCV core transgenic construct (pBEP39), previously used in the study associ-ating HCV core with HCC[6] was generously provided by Dr. Kazuhiko Koike (University of Tokyo, Tokyo, Ja-pan).[3-6] Seven transgenic mouse lines were created on the C57Bl/6 background, and HCV core protein expression was confirmed by Western blotting using a mouse anti-hepatitis C core monoclonal antibody (gift from Dr. Michinori Kohara, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan),[7] and one line with the strongest core expression was selected as the founder. Transgenic mice were identified by PCR using HCV core primers (5'-GCCCACAGGACGTTAAGTTC-3' and 5'-TAGTTCACGCCGTCCTCCAG-3') yielding a 438 bp fragment. Again, animals from the founder line were mated with wild type FVB mice (Taconic) to pro-duce an HCV core line on a hybrid background (FVB×C57Bl/6). Mice were housed in a controlled envi-ronment and fed regular sterile mouse chow and water. All procedures involving animals were approved by the Insti-tutional Animal Care and Use Committee.

Table 1. Schedule of Treatments

Age (weeks)	Treatment
0	
7	DEN 75 mg/kg
8	DEN 75 mg/kg
9	DEN 75 mg/kg
10	DEN 100 mg/kg
11	DEN 100 mg/kg
12	DEN 100 mg/kg
16	Sacrifice
24	Sacrifice
32	Sacrifice

NOTE. Animals were injected intraperitoneally with 75 mg/kg body weight of DEN weekly for 3 weeks and then 100 mg/kg of DEN weekly for 3 weeks. Control groups received saline intraperitoneal injections.

Diethylnitrosamine (DEN) Treatment

Eight-week-old male Alb-core-E1-E2 transgenic (core-E1-E2 Tg), HBV enh-core transgenic (core Tg), and non-transgenic (non-Tg) mice on the FVB×C57Bl/6 background were divided into 6 groups: (1) core-E1-E2 Tg with DEN (n = 15); (2) core Tg with DEN (n = 8); (3) non-Tg with DEN (n = 18); (4) Alb-core-E1-E2 Tg without DEN (n = 3); (5) HBV enh-core Tg without DEN (n = 5); and (6) non-Tg without DEN (n = 4) (Table 1). Animals from groups 1, 2, and 3 were injected intraperitoneally with 75 mg/kg body weight of DEN weekly for 3 weeks and then 100 mg/kg of DEN weekly for 3 weeks according to the protocol of Shiota.[8] As con-trols for DEN effects, animals from groups 4, 5, and 6 received saline intraperitoneal injections.

Tumor Incidence

DEN-treated mice were sacrificed at 16, 24, and 32 weeks of age. Control animals, treated with saline only, were sacrificed at 32 weeks to determine the background HCC rate. HCC tissues and matching nonmalignant tis-sue from the animals in groups 1, 2, and 3 were isolated and immediately frozen in OCT cryostat embedding compound (Tissue-Tek, Torrance, CA) or placed in 10% formalin for histological analysis and a portion of each tissue was stored frozen at −80°C for protein analysis. Histological evaluation of the formalin-fixed liver tissues by H&E staining was also carried out by a pathologist (L.Z.) in a blinded manner.

Immunohistochemical Analysis of Tumor Cell Proliferation

Proliferation index was evaluated by immunohisto-chemical analysis of fresh frozen liver tissue sections using anti-Ki67 antibody. Cryostat sections (6μ) of the unfixed frozen samples were cut, air dried for 1 hour, and fixed in cold acetone for 10 minutes. Samples were then incu-

bated with blocking buffer (1% bovine serum albumin) for 30 minutes at room temperature. Endogenous peroxidase activity was blocked by immersing in 0.3% H_2O_2 and methanol for 30 minutes. Mouse anti-Ki67 polyclonal antibody (Novocastra Laboratories Ltd., Newcastle, UK) was applied at a 1:500 dilution for two nights at 4°C. After washing, specimens were incubated with a peroxidase-labeled anti-mouse goat IgG F(ab')$_2$ fragment (DakoCytomation, Carpinteria, CA) at 1:500 dilution for 3 hours at room temperature, followed by 3,3'-diaminobenzidine tetrahydrochloride (DAB; DakoCytomation) as chromogen. Nuclei were counterstained with methylgreen. All positive and negative cells were counted in each of four randomly selected high power fields (40×) for each tumor and nontumor tissue. The difference in cell number between groups was compared using two-tailed unpaired Student t test. P values less than .05 were considered to be significant.

Apoptotic Index

In situ detection of DNA fragmentation was carried out using a terminal deoxyribonucleotidyl transferase mediated dUTP-digoxigenin nick-end labeling (TUNEL) assay (ApopTag In Situ Apoptosis Detection kit; Serological Corp., Norcross, GA). Cryostat sections (6 μm) of the fresh frozen samples were cut, air dried for 1 hour, and fixed in 4% paraformaldehyde at 4°C for 10 minutes. Apoptotic cells were counted in each of four randomly selected high power fields (40×).

The apoptotic index (AI) was calculated as the number of apoptosis-positive hepatocytes divided by the number of all hepatocytes in each high-power field. The difference in AI between groups was compared using the two-tailed unpaired Student t test. P values less than .05 were considered significant.

Western Blots of HCV Core Protein

Nontumor tissues from each group were homogenized in RIPA buffer and protein expression was determined by Western blotting using a mouse anti–hepatitis C core monoclonal antibody from Dr. Kohara.[7] Equal quantities of protein were utilized for HCV core protein determinations.

Apoptotic Index in HCV Core and Core-E1-E2 Transfected Cells

To elucidate the effects of HCV E1-E2 protein on apoptosis, apoptotic index was determined in HCV core-expressing cells. Approximately 5×10^4 HepG2 cells per well of a 24-well plate were seeded 24 hours prior to transfection. Cells were transfected using Lipofectamine (Invitrogen, Carlsbad, CA) with 0.4 μg of plasmids

(pAlb-HCV containing the core-E1-E2 regions and pBEP39 containing the core region) and incubated for 8 hours. To induce apoptosis, transfected cells were then exposed to 0.5 μg/mL of anti-Fas agonistic antibody (EOS9.1 monoclonal Ab, eBioscience, San Diego, CA) for 12 hours.

Cells were trypsinized and fixed with 1% paraformaldehyde in PBS for 10 minutes at room temperature and dried on microscope slides. HCV core expression was stained by mouse anti-hepatitis C core monoclonal antibody from Dr. Kohara[7] followed by FITC-F(ab')$_2$ goat anti-mouse IgG as secondary antibody (Zymed Laboratories Inc., San Francisco, CA). A second stain for detection of DNA fragmentation was carried out using a rhodamine labeled TUNEL assay (ApopTag Red In Situ Apoptosis Detection kit; Serological Corp.). Apoptotic cells were counted in each of ten randomly selected high-power fields (40×). For cell lines, the AI was calculated as the number of apoptosis-positive cells divided by the number of HCV core protein-positive cells in each high-power field. The difference in AI between groups was compared using two-tailed unpaired Student t test. P values less than .05 were considered to be significant.

Apoptotic Index in Fas-Induced Apoptosis in HCV Transgenic Mouse Models

To further confirm the effect of E1 and/or E2 protein in apoptosis, AI was assessed in a Fas-induced apoptosis model using HCV core-E1-E2 transgenic, core transgenic, and non-transgenic mice. Two micrograms of anti-Fas agonistic antibody (monoclonal Ab clone Jo2, BD Biosciences, Palo Alto, CA) in 0.5 mL of normal saline was injected intraperitoneally into mice of 8 months of age in three groups: (1) core-E1-E2 Tg (n = 3), (2) core Tg (n = 3), and (3) non-Tg (n = 3). The animals were sacrificed 4 hours later, and liver samples were fixed in 10% formaldehyde. Apoptosis detection was carried out using a peroxidase labeled TUNEL assay (ApopTag In Situ Apoptosis Detection kit; Serological Corp.). Apoptotic cells were counted in each of four randomly selected high-power fields (40×). The AI was calculated as the number of apoptosis-positive hepatocytes divided by the number of hepatocytes in each high-power field. The difference in AI between groups was compared using two-tailed unpaired Student t test. P values less than .05 were considered to be significant.

Results

Phenotype of Transgenic Mice. We observed no spontaneous hepatocellular carcinomas or adenomas in mice bearing either transgene when they were both ex-

pressed on the FVB×C57Bl/6 background. These mice were observed for as long as 21 months of age. To test the ability of these transgenes to accelerate tumorigenesis after an initial hepatic injury, we then compared the effects of the two transgenes on tumorigenesis after treatment with DEN. Saline-treated HCV-core-E1-E2 and HCV-core transgenic mice were phenotypically normal with no apparent delays in growth or development. In contrast, all three lines of mice (HCV-core-E1-E2, HCV-core, and nontransgenic) treated with DEN experienced growth retardation at 20 weeks of age. There was neither obvious inflammatory cellular infiltrate in the liver nor significant change in serum ALT levels in any of these three lines (data not shown).

Development of Hepatocellular Carcinoma. HCC did not develop prior to 24 weeks of age in any of the mice examined. HCC developed at 32 weeks of age in all DEN-treated mice, but not in saline-treated mice. The mean number of adenomas and HCCs was 1.00 ± 0.38/mouse (core-E1-E2), 0.75 ± 0.25/mouse (core), and 0.92 ± 0.35/mouse (nontransgenic). These differences were not significant (Fig. 1A). The mean size of adenomas and HCCs was 4.18 ± 1.36 mm (core-E1-E2), 0.87 ± 0.23 mm (core), and 1.1 ± 0.16 mm (nontransgenic). The difference between core-E1-E2 and core mice ($P = .008$), and between core-E1-E2 and nontransgenic mice ($P = .003$) was statistically significant (Fig. 1B). The mean number of HCCs was 0.87 ± 0.40/mouse (core-E1-E2), 0.50 ± 0.27/mouse (core), and 0.75 ± 0.37/mouse (nontransgenic). These differences were not statistically significant. The mean size of HCCs was 4.63 ± 1.48 mm (core-E1-E2), 0.78 ± 0.26 mm (core), and 1.00 ± 0.19 mm (nontransgenic). The difference between core-E1-E2 and core mice ($P = .01$) or core-E1-E2 and nontransgenic mice was statistically significant ($P = .002$, Fig. 1C).

The morphology of HCCs was variable. Most HCCs consisted of a well-differentiated hepatic cord with proliferating hepatocytes, while some contained thickened trabeculae or acini of dysplastic hepatocytes (Fig. 2). Mild steatosis was observed in less than 5% of the hepatocytes in all mice studied. No fibrosis or inflammation was observed in any of the mice studied.

No Difference in Proliferation Between HCCs Was Observed. Ki67 proliferation index (PI) in HCC tissue was 68.1 ± 3.7 for core-E1-E2 transgenic, 58.3 ± 4.5 for core transgenic, and 63.2 ± 3.8 for nontransgenic mice. The PI in nontumor tissue was 42.4 ± 5.4 for core-E1-E2 transgenic, 37.5 ± 3.6 for core transgenic, and 35.8 ± 3.9 for nontransgenic mice. There were no significant differences within HCCs or within nontumor tissues according to group; however, PI was significantly different between HCC and nontumor tissues for each group of mice ($P =$
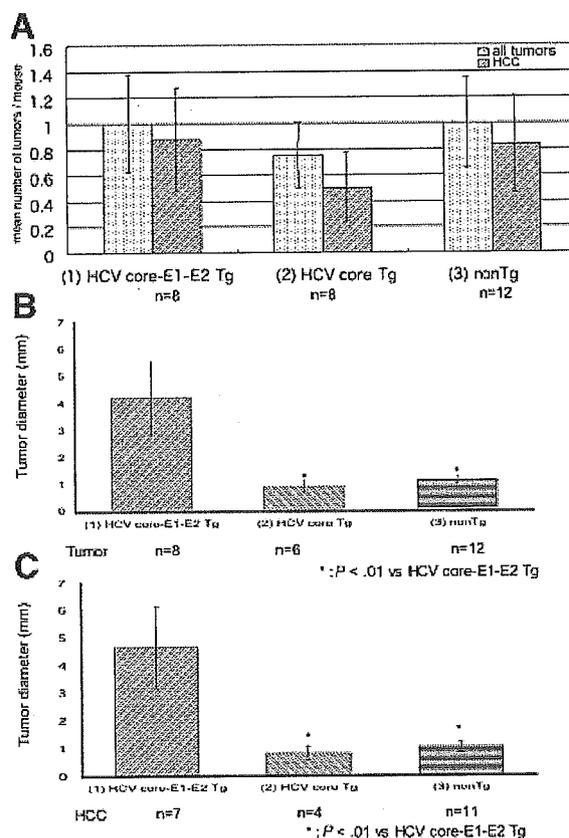

Fig. 1. Tumor formation in DEN-treated mice. (A) Number of tumors in DEN-treated mice at 32 weeks. Liver nodules appeared in groups (1) core-E1-E2 Tg with DEN, (2) core Tg with DEN, and (3) non-Tg with DEN; however, no tumors or steatosis were observed in groups (4) core-E1-E2 without DEN, (5) core Tg without DEN, and (6) non-Tg without DEN. There was no significant difference in number of tumors between any of the three groups. (B) Mean size of tumors (HCCs and adenomas), DEN-treated animals. The mean size of the tumors in group 1 was 4.18 mm, compared with groups 2 (0.87 mm, $P = .008$), and 3 (1.1 mm, $P = .003$). (C) Mean size of HCCs, DEN-treated animals. The mean HCC size in group 1 was 4.63 mm, 0.78 mm in group 2 ($P = .01$ group 1 vs. 2), and 1.00 mm in group 3 ($P = .002$, group 1 vs. 3).

$.0005$, $P = .0003$, $P = .002$ in core-E1-E2, core, nontransgenic, respectively). (Fig. 3).

Apoptosis Is Suppressed in HCV Core-E1-E2 HCCs. The mean apoptotic index (AI) of HCC tissue was 19.9 ± 3.2 in core-E1-E2 transgenic mice, 52.0 ± 7.6 in core transgenic mice, and 47.0 ± 2.3 in nontransgenic mice. The mean AI of nontumor was 6.6 ± 1.0 in core-E1-E2 transgenic mice, 36.0 ± 3.0 in core transgenic mice, and 27.8 ± 1.0 in nontransgenic mice. There was a significant difference in AI of HCC between core-E1-E2 and core ($P < .0001$), and core-E1-E2 and nontransgenic ($P < .0001$) animals. A significant difference in AI was also found in nontumor tissues between core-E1-E2 and core ($P < .0001$), and core-E1-E2 and nontransgenic mice

Fig. 2. Microscopic appearance of HCC. Original magnification ×100, HCV core-E1-E2 Tg HCC. Note the acinar and trabecular arrangement of the hepatoma cells.

($P < .0001$) (Fig. 4A-G). These data indicate that a global suppression of apoptosis occurs in both tumor and non-tumor tissues in HCV core-E1-E2 transgenic mice.

HCV core protein was expressed at equivalent levels in core-E1-E2 transgenic and core transgenic mice, confirming that the observed differences cannot be attributable to an HCV core dose effect (Fig. 5).

Apoptosis Is Suppressed in HCV Core-E1-E2 Transfected Hepatocytes. To confirm that the observed differences in apoptosis were attributable to expression of the respective HCV transgenes, we performed transfection studies in HepG2 hepatocytes using individual HCV gene constructs. We performed double staining for apoptosis and viral protein expression in a model of apoptosis induced by the anti-Fas agonistic antibody. AI was scored among cells expressing HCV core to determine whether core-E1-E2 expression was antiapoptotic (*i.e.*, where apoptosis was suppressed). Representative fields demonstrating the feasibility of the double staining are shown in Fig 6A-B for HCV core-E1-E2- and HCV core–transfected cells, respectively. The mean AI of ten high power fields was significantly lower in cells transfected with core-E1-E2 (0.35 ± 0.11) compared to core alone (0.74 ± 0.07, $P = .01$) (Fig. 6C). The mean AI in nontransfected cells was identical to that of core-transfected cells (data not shown). These data implicate E1 and/or E2 expression in suppression of apoptosis.

Fas-Induced Apoptosis Is Suppressed in HCV Core-E1-E2 Transgenic Liver Tissue. To further confirm the suppressive effect of HCV E1 and/or E2 proteins on apoptosis, we analyzed AI in a Fas-induced model of apoptosis in core-E1-E2 Tg, core Tg, and non-Tg liver tissue. As shown in representative fields (Fig. 7A-F), core-

E1-E2 Tg liver tissue underwent significantly less apoptosis than liver tissue expressing core only or non-Tg tissue. The mean AI of four high-power fields of core-E1-E2 Tg liver (40.3 ± 2.6) was significantly lower than that seen in core Tg (75.2 ± 2.1, $P < .001$) or non-Tg (79.8 ± 2.6, $P < .001$) liver. As a comparison, mesenchymal cells, which do not express HCV proteins by staining (data not shown), underwent diffuse apoptosis in each group analyzed, including the core-E1-E2 group. These data indicate that the suppression of apoptosis occurred only in core-E1-E2 expressing hepatocytes and suggest that E1 and/or E2 have a suppressive effect on Fas-induced apoptosis *in vivo*.

Discussion

HBV and HCV are important risk factors for hepatocellular carcinoma. However, the precise role of each agent in carcinogenesis has not been well defined. In HBV-associated HCC, exposure to aflatoxin B1 (*i.e.*, chemical hepatocarcinogenesis) early in life is believed to play a key role in carcinogenesis, as is integration of HBV into the host genome (*i.e.*, insertional mutagenesis). In HCV-associated HCC, chronic injury and regeneration have been postulated to underlie transformation; however, growing evidence suggests that HCV replication itself may directly lead to carcinogenesis.

HCV contains structural (core, E1, E2) and regulatory proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B). The core nucleocapsid packages the viral genomic RNA; it may also promote apoptosis and cell proliferation through its physical interaction with p53.[1] Additionally, two regions of the envelope protein E2, designated hypervariable regions 1 and 2, have an extremely high rate of mutation, believed to be the result of selective pressure
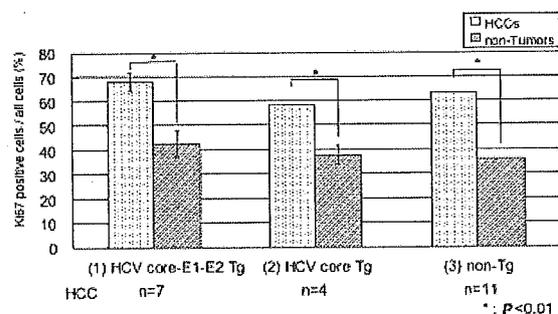

Fig. 3. Immunohistochemical expression of Ki67 in HCCs and non-tumor tissues. Proliferation index in HCCs and adjacent non-tumor tissues, DEN-treated animals. Immunohistochemical evaluation was performed using Ki67 polyclonal antibody. Ki67-positive cells (proliferating cells) were counted. There were significant differences in PI between all HCCs and non-tumor groups. However, there was no significant difference between the three groups.
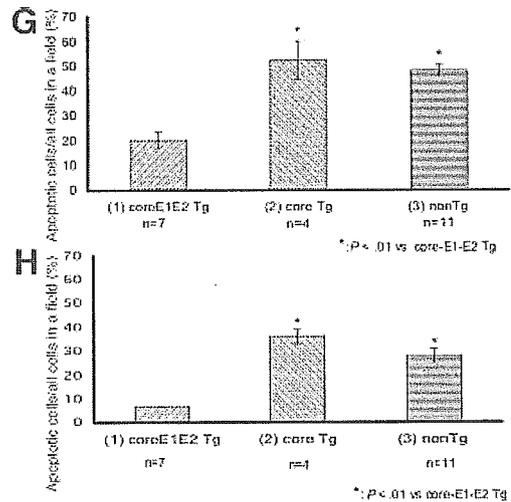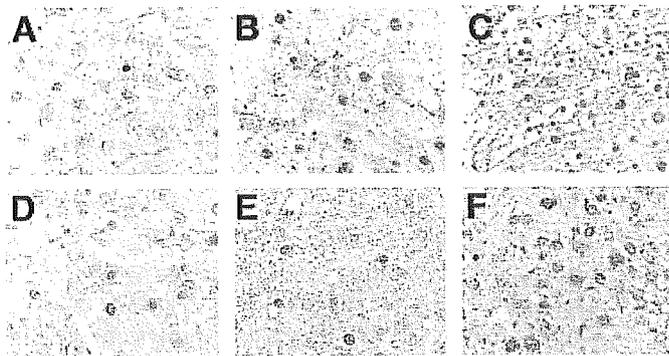
Fig. 4. Immunohistochemical expression of apoptosis in HCCs and non-tumor tissue. (A) core-E1-E2 Tg, HCC, (B) core Tg, HCC, (C) non-Tg, HCC, (D) core-E1-E2 Tg, non-tumor, (E) core Tg, non-tumor, (F) non-Tg, non-tumor. (apoptosis positive: brown nuclei, apoptosis negative: blue-green nuclei), 40×, G-H; Statistical analysis of apoptotic index. (G) Apoptotic index in tumor tissues. (H) Apoptotic index in nontumor tissues. In tumor tissues, AIs were significantly higher than those in nontumor tissues in all three groups (core-E1-E2 Tgs, core Tgs, non Tgs), and AI was significantly lower in core-E1-E2 Tg than core Tgs and non Tgs.

imposed by the humoral immune system. To date, a role of the structural proteins in hepatocarcinogenesis has not been suggested.

To better define the respective role(s) of the HCV core, E1, E2, and nonstructural proteins in hepatocarcinogenesis, various mouse transgenic models utilizing core, E1 + E2, E2, core + E1 + E2, as well as the entire HCV genome have been developed by several groups. These transgenic mice have exhibited two phenotypes: those with and those without hepatic tumors. The tumor phenotype has been reported to occur in transgenic mice for core,[6] the entire region,[9] or core + E1 + E2.[9] However, in different investigations mice transgenic for core,[10] E2,[10] E1 + E2,[3] and core + E1 + E2,[2] did not exhibit the tumor phenotype.

Among HCV transgenic mice without tumor phenotype, two models expressing core have been reported by Pasquinelli et al.[10] and our group.[2] Neither model showed histologically recognizable steatosis, adenoma, or HCC.

Fig. 5. HCV core expression by Western blotting. Both core-E1-E2 and core transgenic mouse liver showed demonstrable core protein expression (core > core-E1-E2).

Although it remains unclear why these two models did not develop HCC, possible explanations include differences in genetic background of the mice, lengths of observation, outcome measures, transgene constructs, and
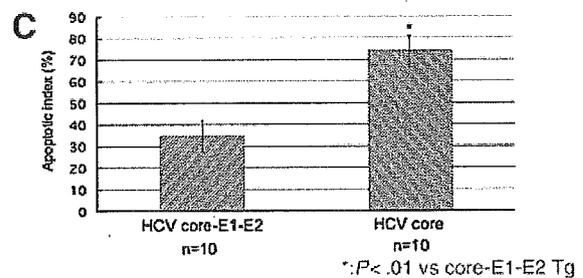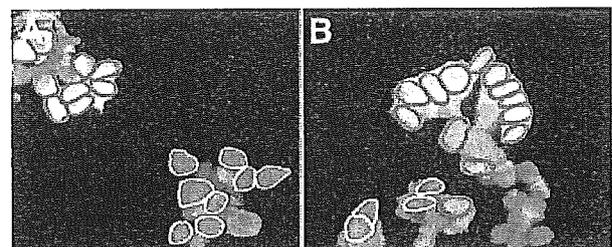

Fig. 6. Double staining for apoptosis and HCV core expression in transfected HepG2 cells. (A) Representative example of HCV core-E1-E2 transfected HepG2 cells; cells staining positive for core (cell color green, circled in white), cells staining positive for apoptosis (cell color red), double positive cells (cell color yellow, circled in red). (B) Representative example of corresponding stains for HCV core-transfected HepG2 cells. (C) Apoptotic index in core-E1-E2 vs. core-transfected HepG2 cells among those cells staining (+) for core. Index represents mean of 10 high-powered fields.
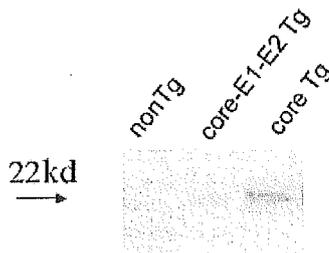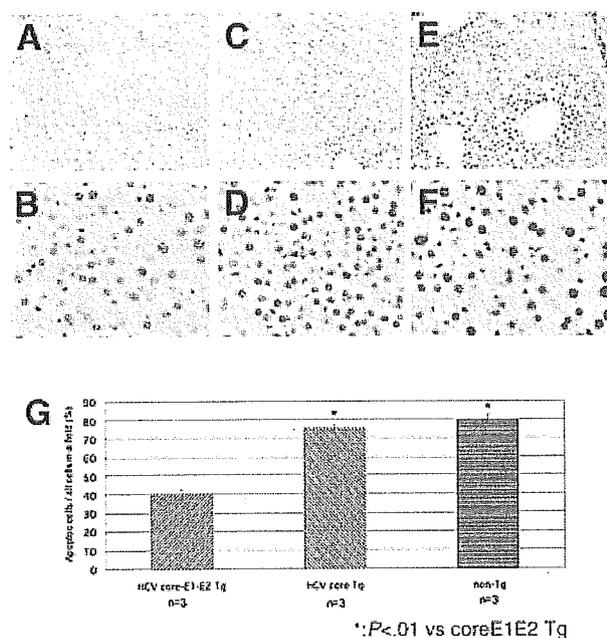
Fig. 7. Apoptotic index in Fas-induced apoptosis in HCV transgenic mouse models. (A) core-E1-E2 Tg, 10×, (B) core-E1-E2 Tg, 40×, (C) core Tg, 10×, (D) core Tg, 40×, (E) non-Tg, 10×, (F) non-Tg. 40×, (apoptosis positive: **brown** nuclei, apoptosis negative: **blue-green** nuclei), (G) Statistical analysis of Apoptotic index. Al was significantly lower in core-E1-E2 Tg than core Tgs and non Tgs. Note there was no difference of apoptosis induction in mesenchymal cells, which do not express HCV proteins, in all groups.

levels of HCV RNA and/or protein expression. The data from Pasquinelli et al. differ from others in transgene construct, in that MUP was used as the promoter.[10] These may have led to a level of protein insufficient for developing HCC.

The experiment by our group was unique in that we used FVB transgenic mice.[2] We used the albumin promoter as did Lerat et al.[9] The transgenic mice developed by Kawamura et al. demonstrated high core expression,[2] whereas those by Lerat et al. showed much lower core protein expression, suggesting that levels of core protein expression alone do not explain the HCC phenotype, and that the FVB background may have a protective role in hepatocarcinogenesis. Indeed, saline-treated control animals expressing the core and core-E1-E2 construction on the FVB×C57Bl/6 background both failed to develop HCC, unless they were exposed to a chemical carcinogen. Thus, it appears that mouse genetic background plays a critical role in explaining the disparate outcomes between these transgenic models.

The present study demonstrates that DEN initiates tumor development and that HCV core+E1+E2 proteins act to further accelerate tumor growth. Sell et al. reported a similar function of HBV in hepatocarcinogen-

esis.[11] They showed that mice transgenic for HBV large envelope protein are at increased risk for adenoma and HCC if exposed to DEN at several months of age. These data suggest that DEN- and HBs antigen-induced liver damage act synergistically to produce HCC in transgenic mice. Nontransgenic mice exposed to DEN at the same age showed no morphological alterations. Subsequently, Huang et al. suggested that a strong and sustained proliferative response in hepatocytes in mice transgenic for HBV occurs after the onset of hepatocellular injury and precedes the development of HCC.[12] Our data differ from that of Huang et al. in that hepatocyte mitotic and proliferation indices were not increased in transgenic mice exposed to DEN. However, the apoptotic index of hepatocytes in DEN-treated HCV core+E1+E2 transgenic mice was significantly reduced compared to DEN-treated core transgenic mice or nontransgenic mice. Together, these findings suggest that HCV and HBV envelope proteins work synergistically with chemical carcinogens to induce HCCs through different mechanisms.

The relationship between HCV E1 and E2 proteins and apoptosis was confirmed by double staining of core-expressing, apoptotic hepatocytes and by our *in vivo* studies demonstrating the suppressive effect of HCV core-E1-E2 on Fas-induced apoptosis. There are contradictory reports regarding the effect of HCV envelope proteins on apoptosis. Honda et al. reported that liver samples from HCV core-E1-E2 transgenic mice showed higher Fas-mediated cell damage compared with non transgenic mice.[13] Dumoulin et al. reported that HCV core protein or HCV E2 protein individually do not prevent TNF-α or Fas induced-apoptosis in transient transfected HepG2 cells.[14] Lasarte et al. reported that a recombinant adenovirus encoding HCV core and E1 proteins protects liver cells from cytokine-induced hepatocellular damage in experimental models of TNF-mediated hepatic injury.[15] These data suggest the possibility that E1 with or without E2 has an antiapoptotic effect.

Notably, none of our mouse lines developed significant steatosis. Our findings suggested that HCV related HCC does not exclusively employ steatosis as a precondition; rather, HCV only acts as a "second hit" on the backdrop of chemically induced genetic injury. Given data that DEN can generate reactive oxygen species (ROS) and enhance oxidative stress,[16-19] it is tempting to speculate that DEN and steatosis, another known cause of ROS, may act in similar manners to predispose hepatocytes to genotoxic injury and malignant transformation.

In summary, HCV E1 and/or E2, possibly in conjunction with core protein, act as tumor accelerators in a DEN-based model of hepatocarcinogenesis and appear to do so by suppressing apoptosis. These data suggest a pre-

viously unrecognized and unexpected property of the viral envelope glycoproteins.

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Therapeutic efficacy of decreased nitrite production by bezafibrate in patients with primary biliary cirrhosis

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Background. The therapeutic efficacy of bezafibrate, a hypolipidemic drug, has been shown in patients with primary biliary cirrhosis (PBC) in some pilot studies; however, little is known regarding the mechanism of action of bezafibrate in PBC. This study was conducted to evaluate the therapeutic efficacy, as well as to gain insight about the possible mechanism of action, of bezafibrate in PBC. ***Methods.*** Sixteen patients with PBC were administered with bezafibrate (400 mg/day) either with ($n = 10$) or without ursodeoxycholic acid (UDCA; $n = 6$). The peripheral blood of these patients was collected before and at different times after therapy commencement, and antigen-presenting dendritic cells (DCs) were then cultured. The DCs were enriched and cultured with *Staphylococcus aureus* Cowan strain-1 for 48 h to evaluate their capacity to produce nitrite. ***Results.*** One month after the start of bezafibrate therapy, the serum levels of alkaline phosphatase ($P = 0.0005$), γ-glutamyl transpeptidase ($P = 0.0006$), total cholesterol ($P = 0.0072$), and immunoglobulin M ($P = 0.0281$) were decreased significantly compared to those before patients started bezafibrate therapy. The levels of nitrite produced by DCs decreased in all patients with PBC within 1 month of commencement of bezafibrate therapy. Moreover, decreased nitrite production by DCs was also seen when nitrite production was evaluated 1 year after the start of bezafibrate therapy. ***Conclusions.*** This study reconfirms the therapeutic efficacy of bezafibrate in patients with PBC, including those with UDCA-resistant PBC. Downregulation of nitrite production by DCs may have some relationship with the therapeutic efficacy of bezafibrate; however, further study will be needed to clarify whether or not the antiinflammatory activity of bezafibrate is mediated through nitrite production.

Introduction

Primary biliary cirrhosis (PBC) is a chronic cholestatic liver disease characterized by destruction of intralobular bile ducts, positivity for antimicrondrial antibodies (AMA) in the serum, and increases of serum alkaline phosphatase (ALP) and immunoglobulin M (IgM).[1] PBC is basically an "extraparenchymal" autoimmune disease of the liver, because it affects biliary ductules in portal tracts, although cases of overlap PBC and autoimmune hepatitis also exist.[2] Patients with PBC show humoral and cellular responses to the inner lipid domain of the E2 component of pyruvate dehydrogenase complex (PDC), a well-conserved enzyme located on the inner mitochondrial membrane.

The PBC autoantigen, PDC, is located on the inner surface of the inner mitochondrial membrane and is therefore normally separated from the estracellular immune system by three membranes. However, PDC-like epitopes are present on the surfaces of biliary epithelial cells (BECs) within, and in freshly cultured liver samples from patients with established PBC.[3,4] Although the mechanism of PDC transport from the inner mitochondrial domain to the surface of the BECs is not known, several factors may be important in this regard. In PBC, apoptosis of BECs in the early stage of PBC may expose the cells of the immune system to PDC. Indeed, several apoptogenic proteins, including cytochrome c, are released from the intermediate mitochondrial space at an early stage during the induction of apoptois.[5]

Recently, several studies have shown that nitrosative stress resulting from increased nitric oxide (NO) synthesis may contribute to the pathogenesis of chronic

inflammatory diseases, including PBC. Increased levels of NO in the sera, and increased expression of inducible nitric oxidase synthase (iNOS) at the site of damaged BECs have been reported in PBC.[6-8] Indeed, NO may be involved in the pathogenesis of several autoimmune diseases by causing damage to mitochondria and inducing several proinflammatory cytokines.[9]

From the therapeutic viewpoint, PBC represents a unique autoimmune disease, because immune suppressive agents are ineffective in PBC, although immune suppressor drugs are widely used, effectively, for other autoimmune diseases. Ursodeoxycholic acid (UDCA; 3α 7βdihydroxy-5β-cholanic acid), which constitutes only 3% of total human bile acid and the major bulk of black bear's bile, is being increasingly used for the treatment of cholestatic liver disease, including PBC. In PBC, UDCA improves serum liver chemistry, may delay disease progression to severe fibrosis or cirrhosis, and may prolong transplant-free survival.[10] However, many patients with PBC are resistant to UDCA. Recently, bezafibrate, a hypolipidemic drug, has been used for therapy in PBC patients, including those who are UDCA-resistant. Some pilot studies have reported that therapy with bezafibrate resulted in improved liver function test results in PBC patients.[11-13] However, the therapeutic potential of bezafibrate should be assessed by conducting more studies in different groups of PBC patients. Moreover, almost nothing is known regarding the mechanism underlying the therapeutic activity of bezafibrate in PBC patients.

This study was conducted to gain insights about (1) the therapeutic efficacy of bezafibrate and (2) the mechanism of action of bezafibrate in PBC. The therapeutic efficacy of bezafibrate was studied by treating two groups of PBC patients with bezafibrate. Some UDCA-resistant PBC patients were treated with bezafibrate along with UDCA, and other PBC patients were treated with only bezafibrate. Next, the mechanism of action of bezafibrate was investigated by evaluating the impact of bezafibrate therapy on nitrite production by antigen-presenting dendritic cells (DCs), because we previously reported the increased production of NO by DCs from patients with PBC.[14]

Patients and methods

Patients and therapy with bezafibrate

A total of 16 patients with PBC attending the Third Department of Internal Medicine, Ehime University School of Medicine, Ehime, Japan, were enrolled in this study. The diagnosis of PBC was based on the presence of typical clinical, serum biochemical, serological, and liver histological findings. All patients were female

(mean age ± SD, 58 ± 13 years) and their serum was positive for AMA. Liver histology was staged according to the classification of Scheuer et al.[15] Briefly, stage I was defined as portal inflammation confined to portal triads; stage II was characterized as portal and periportal inflammation without septal fibrosis or bridging necrosis; in stage III, lobular fibrosis and/or bridging necrosis were present; and stage IV corresponded to cirrhosis. All patients had elevated levels of serum ALP and γ-glutamyl transpeptidase (γ-GTP) before the start of bezafibrate therapy.

Ten of the 16 patients had been receiving UDCA, for 1–10 years, before enrollment in this clinical trial; however, therapy with UDCA had not resulted in normalization of ALP in these patients, and they were regarded as having UDCA-resistant PBC. These 10 UDCA-resistant PBC patients were given both UDCA and bezafibrate. The remaining 6 patients were given only bezafibrate. Bezafibrate was given at a dose of 400 mg/day. None of the patients were receiving any other drugs, including immune suppressive drugs, during the study period. The clinical profiles of the patients just prior to the start of bezafibrate treatment are shown in Table 1. The median levels of ALP in the sera were significantly higher in patients with UDCA-resistant PBC (995 IU/l; interquartile range, 679 IU/l) compared to patients receiving only bezafibrate (589 IU/l; interquartile range, 171 IU/l; $P = 0.0393$). The other liver function test parameter did not show any significant difference between these two groups.

Informed consent was obtained from all patients after an explanation of the nature and purpose of the study, and the Institutional Ethics Committee of Ehime University Hospital approved the protocol.

Isolation and functional analyses of dendritic cells (DCs)

The method of enrichment of DCs from peripheral blood has been described in detail by us.[14] In short, peripheral blood mononuclear cells (PBMCs) were collected from heparinized fresh blood by centrifuging on a Ficoll-Conray column (Daiichi Pharmaceutical, Tokyo, Japan). The PBMCs (5–20 × 10^6) were suspended in 3.0 ml of RPMI 1640 (Iwaki, Chiba, Japan) plus 10% fetal calf serum (Filtron, Brooklyn, Australia), containing streptomycin and penicillin (Gibco, Grand Island, NY, USA), and adhered on a plastic surface for 2h, after which the nonadherent cells were washed out by gentle washing and the adherent cells were cultured for an additional 8 days with granulocyte monocyte colony-stimulating factor (800 U/ml; Genzyme, Cambridge, MA, USA) and interleukin-4 (500 U/ml; Genzyme). This procedure, reproducibly, gave growing DC aggre-

Table 1. Clinical characteristics of patients with PBC

	Total	UDCA-resistant PBC patients	Patients treated with only bezafibrate
Numbers	16	10	6
Age, years	59.5 (15)	61 (9)	60 (18)
Sex	All female	All female	All female
Alkaline phosphatase (104–338 IU/l)[a]	713 (626)	995 (679)*	589 (171)
γ-glutamyl transpeptidase (6–71 IU/l)[a]	230 (199)	248 (258)	201 (32)
Leucine aminopeptidase (35–71 IU/l)[a]	162 (95)	205 (251)	143 (106)
Immunoglobulin M (35–220 mg/dl)[a]	448 (286)	448 (292)	424 (318)
Total cholesterol (113–233 mg/dl)[a]	254 (88)	257 (70)	254 (112)
Total bilirubin (0.1–1.1 mg/dl)[a]	0.75 (0.4)	0.8 (0.3)	0.65 (0.40)
Histological evaluation			
Scheuer's stage I	10	6	4
Scheuer's stage II	2	1	1
Scheuer's stage III	3	3	0
Therapeutic history			
Prior therapy with UDCA	10	10	0
No specific therapy	6		

*$P = 0.0393$ compared to patients treated with only bezafibrate
Data values are shown as medians (interquartile ranges)
Liver biopsy was not available in one patient treated with only bezafibrate
[a] Normal range

gates at 5–7 days, and these were dislodged by gentle pipetting.

Morphologically, DCs were identified by the presence of thin motile cytoplasmic processes or veils on phase-contrast microscopy. The levels of expression of HLA DR, CD86, and CD83 on DCs were analyzed using fluorescein isothiocyanate-conjugated mouse anti-human major histocompatibility class II antigens (HLA-DR; clone Immu-357; Immunotech, Marseille Cedex, France), phycoerythrin-conjugated mouse anti-human CD83 (clone HB15A; Immunotech), and phycoerythrin-conjugated mouse anti-human CD86 (clone 2331; FUN-1; Pharmingen, San Diego, CA, USA), respectively. Subclass-matched respective antibodies were used as controls. DCs expressed moderate levels of HLA DR and CD86, and almost no CD83. DCs stimulated allogenic T cells in an allogenic mixed leukocyte reaction in a dose-dependent manner (data not shown).

Estimations of nitrite

DCs (1 million) were cultured with *Staphylococcus aureus* Cowan strain I (0.0075%) at 37°C for 48h, exactly according to our previous report.[14] After the end of the cultures, the culture supernatants were centrifuged five times and the clear supernatants were preserved. The levels of nitrite in the culture supernatants were measured by a commercial kit (Griess Assay Kit NO kit-C, Wako, Osaka, Japan), as described.[14] Aliquots of culture supernatants were incubated with Griess reagent (1% sulfanilamide, 0.1% naphthyle-

thylenediamine dihydrochloride, and 2.5% H_3PO_4) at room temperature for 10min. Color development due to enzymatic reaction was estimated with an enzyme-linked immunosorbent assay reader at 540nm. Concentrations of nitrite in the samples were calibrated with a reference standard of sodium nitrite, supplied with the kit, and the levels of nitrite were expressed as in micromoles per milliter of supernatant.

Statistical analysis

The levels of ALP, γ-GTP, IgM, and cholesterol in the sera were calculated as medians; with interquartile ranges shown in parentheses. The amounts of nitrite in the culture supernatants of DCs were expressed similarly. Statistical comparison was done by the paired *t*-test when data were normally distributed. When the distribution was skewed, the Wilcoxon signed-rank test was used. *P* values of less than 0.05 were considered to be statistically significant. Statistical calculations were performed using the Stat View (version 5.0; Abacus Concepts, Berkeley, CA, USA) statistical computer software program.

Results

Decline of liver enzymes within 1 month of commencement of bezafibrate therapy

The levels of ALP in PBC patients before and 1 month after the start of bezafibrate administration are shown

Table 2. Marked improvement of liver function test parameters in PBC patients due to intake of bezafibrate

Liver function test parameters	Before the start of bezafibrate therapy	One month after the start of bezafibrate therapy	P values*
Alkaline phosphatase (IU/l)	713 (626)	394 (266)	$P = 0.0005$
γ-Glutamyl transpeptidase (IU/l)	230 (199)	131 (112)	$P = 0.0006$
Total cholesterol (mg/dl)	254 (88)	217 (88)	$P = 0.0072$
Immunoglobulin M (mg/dl)	448 (286)	328 (231)	$P = 0.0281$
Total bilirubin (mg/dl)	0.75 (0.4)	0.7 ± 0.5	$P = 0.3465$

*P values compare the data at 1 month after the start of bezafibrate therapy with those before the start of bezafibrate therapy
Sixteen patients with PBC were treated with bezafibrate; the parameters of liver function tests before and 1 month after the start of therapy are tabulated. Data values are shown as medians (interquartile ranges)
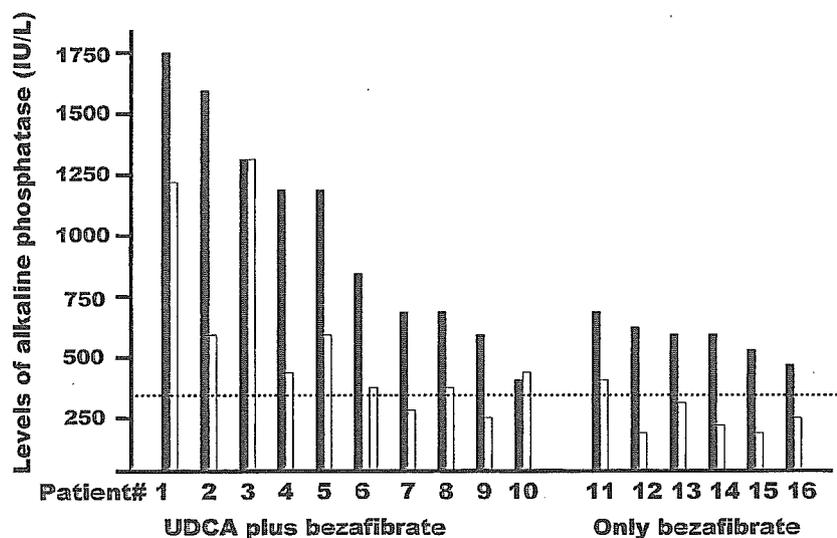

Fig. 1. Therapy of patients with primary biliary cirrhosis (PBC) with bezafibrate for 1 month caused decreased levels of alkaline phosphatase in the sera. Ten patients (patients 1–10) were treated with ursodeoxycholic acid (*UDCA*) prior to enrollment in this study, but all of these patients showed elevated levels of serum alkaline phosphatase (UDCA-resistant PBC) before receiving bezafibrate. These 10 patients received both UDCA and bezafibrate. Six patients (patients 11–16) received only bezafibrate. The levels of alkaline phosphatase before starting bezafibrate therapy are shown by *black bars* and those 1 month after the start of bezafibrate therapy are shown by *open bars. Dotted horizontal line* shows normal level

in Fig. 1. Of the total of 16 patients with PBC, 10 were UDCA-resistant. The levels of ALP were reduced in 8 of these 10 patients within 1 month of therapy commencement. One of the 2 patients in whom ALP was not reduced was Scheuer stage III and the other was Scheuer stage I. In 2 UDCA-resistant PBC patients, the levels of ALP decreased to the normal level within 1 month after commencement of bezafibrate therapy (patients 7 and 9; Fig. 1). The effect of bezafibrate on serum ALP was more pronounced in PBC patients who received only bezafibrate (patients 11–16; Fig. 1). The levels of ALP were reduced in all 6 of these patients, and in 5 of these patients, the levels of ALP decreased to the normal range (Fig. 1).

The effect of bezafibrate on serum levels of liver enzymes and blood biochemistry is shown in Table 2. The mean levels of ALP ($P = 0.0005$), γ-GTP ($P = 0.0006$), total cholesterol ($P = 0.0072$), and IgM ($P = 0.0281$) in the sera were significantly decreased within 1 month of the start of bezafibrate therapy compared to their levels

before intake of bezafibrate. The levels of serum bilirubin remained almost unchanged before and at 1 month after bezafibrate therapy.

Sustained improvements of liver function test parameters due to 12 months of bezafibrate therapy

Although intake of bezafibrate for 1 month resulted in decreased levels of liver enzymes in most patients with PBC, it was important to evaluate the long-term impact of bezafibrate on liver enzymes in these patients. This long-term impact was evaluated in ten patients with PBC who were treated with bezafibrate for 12 months. The levels of liver enzymes before and after 12 months of bezafibrate therapy are shown in Fig. 2. Patients with PBC taking bezafibrate for 12 months showed significantly lower levels of ALP ($P = 0.0069$), γ-GTP ($P = 0.0051$), IgM ($P = 0.0077$), and total cholesterol ($P = 0.0173$) in the sera compared to the levels
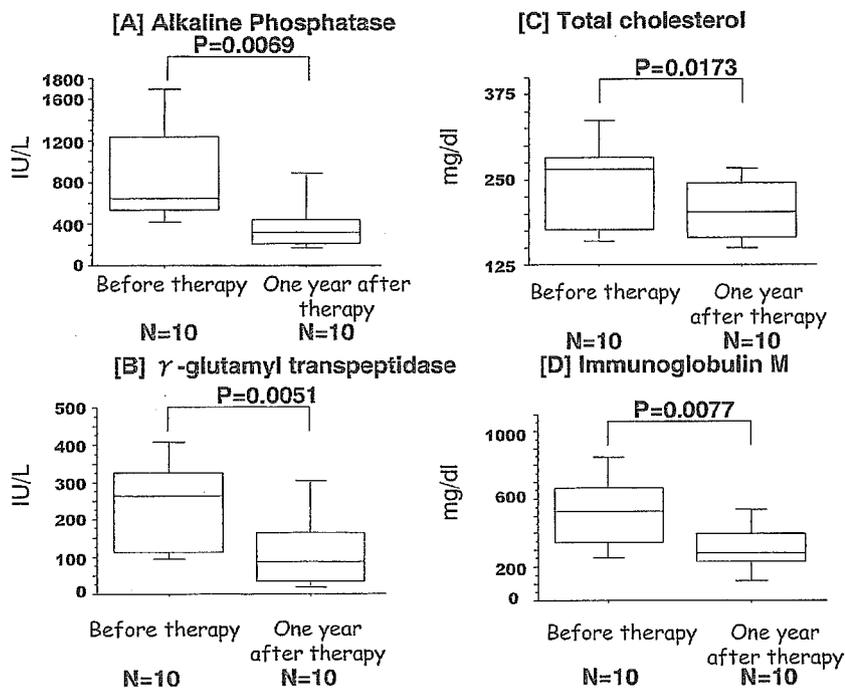
[A] Alkaline Phosphatase
P=0.0069

IU/L
1800
1600
1200
800
400
0

Before therapy   One year after
                      therapy
N=10   N=10

[B] γ-glutamyl transpeptidase
P=0.0051

IU/L
500
400
300
200
100
0

Before therapy   One year
                  after therapy
N=10   N=10

[C] Total cholesterol
P=0.0173

mg/dl
375
250
125

Before therapy   One year
                  after therapy
N=10   N=10

[D] Immunoglobulin M
P=0.0077

mg/dl
1000
600
200
0

Before therapy   One year
                  after therapy
N=10   N=10

Fig. 2A–D. Long-term effect of bezafibrate on blood biochemistry in patients with PBC. Ten patients with PBC were treated with daily administration of bezafibrate for 12 months. The levels of alkaline phosphatase (**A**), γ-glutamyl transpeptidase (**B**), total cholesterol (**C**), and immunoglobulin M (**D**) before the start of bezafibrate intake and after 12 months of bezafibrate intake are shown as medians (interquartile ranges). P values indicate differences in these parameters between before therapy and after 1 year of therapy

of these parameters before the start of bezafibrate therapy. The improvement in the biochemical parameters of liver function was seen in all ten of these patients with PBC, including the patients with Scheuer stage III.

Decreased production of nitrite by dendritic cells due to bezafibrate therapy

Although we found good therapeutic effects of bezafibrate in PBC patients, including those who were UDCA-resistant, one of the main purposes of this study was to develop insights regarding the mechanism underlying the subjective and biochemical improvements due to bezafibrate therapy. We emphasized the production of nitrite by antigen-presenting DCs from PBC patients, because nitrite is associated with the progression of various autoimmune diseases, and we have already shown increased nitrite production by DCs from PBC patients.

As shown in Fig. 3, nitrite was detected in the culture supernatants of DCs from all 16 patients with PBC before the start of bezafibrate therapy. The levels of nitrite in the PBC patients were 8.2 (2.5) μM/ml (n = 16). However, the levels of nitrite produced by DCs were almost same in the 10 UDCA-resistant PBC patients, at 8.2 (1.9) μM/ml, and in the 6 PBC patients who had not received UDCA, at 8.3 (4.6) μM/mL. On the other hand, very low levels of nitrite were detected in the culture

supernatants of DCs from 7 of 10 normal control subjects.

Interestingly, the levels of nitrite produced by DCs were decreased in all 16 patients with PBC after 1 month of therapy with bezafibrate (Fig. 3), being significantly lower 1 month after therapy commencement compared to before the start of therapy (P = 0.0005). This was seen in all patients with PBC and was not dependent on whether or not there was a previous history of UDCA intake (UDCA-resistant PBC patients). Decreased nitrite production was also seen in the 2 patients with PBC who did not show improvement of serum ALP (Fig. 1).

We checked the levels of nitrite produced by DCs in the ten patients with PBC who received bezafibrate for 12 months. The levels of nitrite produced by DCs before and after 12 months of bezafibrate therapy are shown in Fig. 4. The levels of nitrite produced by DCs were significantly lower 12 months after the commencement of bezafibrate therapy compared to levels before the start of bezafibrate therapy (P = 0.0093).

Discussion

There is no curative therapy for PBC. The present goals of therapy are to improve serum liver chemistry, delay disease progression to severe fibrosis and cirrhosis, and prolonge transplant-free survival.[1-3] At present, UDCA

P=0.0005

Before
Therapy
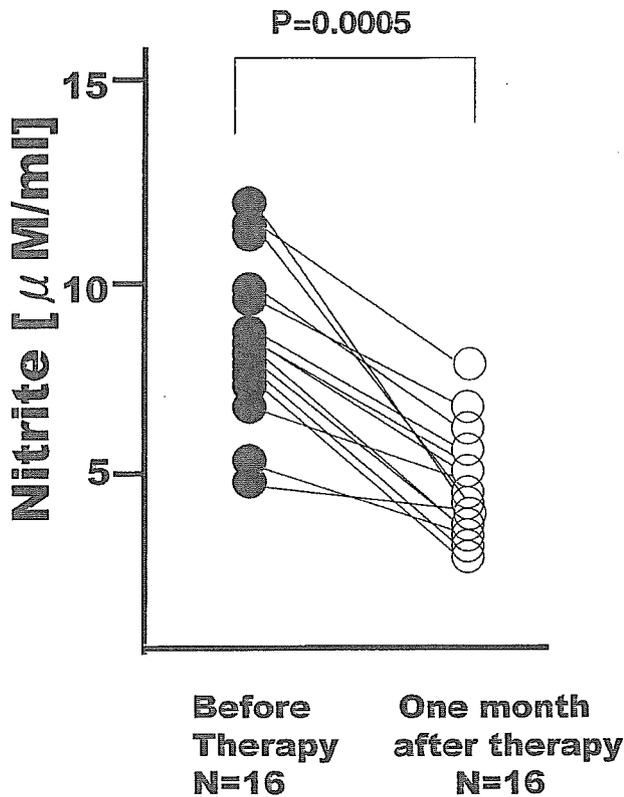N=16

One month
after therapy
N=16

Fig. 3. Decreased production of nitrite by dendritic cells (DCs) from patients with PBC, due to intake of bezafibrate for 1 months. The levels of nitrite produced by DCs from each patient with PBC before intake of bezafibrate are shown by *black circles* and those after 1 month of bezafibrate intake are shown by *open circles*
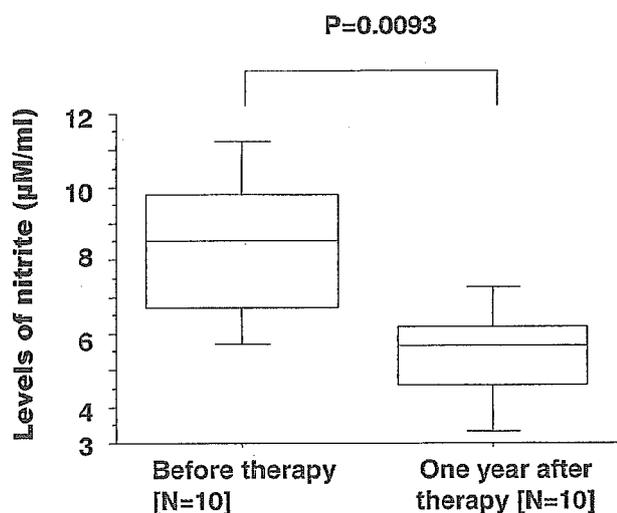
P=0.0093

Before therapy
[N=10]

One year after
therapy [N=10]

Fig. 4. The levels of nitrite produced by DCs after 1 year of bezafibrate therapy were significantly lower than those before the start of therapy. Data values are shown as medians (interquartile ranges). *P* value indicates the difference between before therapy and after 1 year of therapy

is the drug of first choice for the treatment of PBC. However, many patients are resistant to UDCA, and their biliary enzymes do not decrease significantly after UDCA therapy. These patients are regarded as having UDCA-resistant PBC.

In this study, administration of bezafibrate to PBC patients resulted in reduced levels of serum ALP, γ-GTP, and IgM. In some patients with PBC, the levels of ALP were normalized within 1 month of therapy commencement. In particular, the role of bezafibrate in UDCA-resistant PBC patients inspired considerable optimism for the wider usage of this drug for PBC patients. The therapeutic efficacy of bezafibrate in PBC patients is interesting, and is of profound clinical importance. In addition to the present study, some pilot studies have also reported the therapeutic efficacy of bezafibrate in PBC patients.[11-13]

It is now important to elucidate the mechanisms underlying the therapeutic effects of bezafibrate. Understanding of the mechanism underlying the therapeutic potential of bezafibrate in PBC is important for developing a better therapeutic regimen for PBC and also for developing other bezafibrate-like drugs for PBC. Bezafibrate is a hypolipidemic drug, and its antilipidemic properties are related to the promotion of beta-oxidation and the suppression of acetyl coenzyme A (CoA) carboxylase activity in the liver.[16,17] Bezafibrate also facilitates the expression of multidrug resistant protein 2 genes in mice. Bezafibrate may play a role in the suppression of inflammatory responses, because it is a ligand for peroxisome proliferator-activated receptor (PPAR)-α, β.[18] However, the roles of beta-oxidation, acetyl CoA, and PPAR, if any, are not clear in PBC. Thus, it may be postulated that the antilipidemic activity of bezafibrate and the reduction of liver enzymes in PBC may be regulated by different pathways.

We evaluated nitrite production by DCs from PBC patients before and after bezafibrate therapy. Intake of bezafibrate caused downregulation of nitrite production by DCs from all patients with PBC within 1 month of therapy commencement. Most importantly, the nitrite-producing capacity of DCs remained lower for the entire duration of bezafibrate intake. In this study, we measured nitrite production by DCs. However, many other cells, such as macrophages and parenchymal cells, can also produce nitrite. Further study is warranted to evaluate whether or not bezafibrate is able to downregulate nitrite production by other cells. This would provide insights about the systemic role of bezafibrate in PBC.

Several studies have shown that No may have a role in the pathogenesis of PBC. NO may cause direct damage to the mitochondria of BECs in PBC patients and may expose the immune system to PDC, the antigen of

the inner mitochondrial domain. In fact, overproduction of NO in the serum,[7,8] increased expression of iNOS, and the increased formation and accumulation of nitrotyrosine in liver tissues in PBC[6] have been reported. We have also reported increased NO production by DCs from patients with PBC.[14] The present study shows that bezafibrate treatment resulted in the improvement of biochemical parameters in PBC, and the downregulation of nitrite production by DCs. However, one of the major limitations of this study is its inability to clarify whether these two events are interrelated or independent. Moreover, it is necessary to address the relative contribution of the antiinflammatory properties of bezafibrate and the decreased production of nitrite during the clinical improvement in PBC patients. Finally, the role of bezafibrate in other possible causes of autoimmunity, such as the production of excessive proinflammatory cytokines, deserves further evaluation.

In summary, this study provided two important findings regarding bezafibrate therapy in patients with PBC. First, it showed that bezafibrate was effective in PBC patients, including those who were UDCA-resistant. However, long-term follow up of these patients would provide more insights about the therapeutic utility of bezafibrate. Secondly, we showed that bezafibrate therapy resulted in the downregulation of nitrite produced by DCs in patients with PBC. As UDCA also downregulates the production of NO from macrophages, taking this knowledge together with the present findings it appears that anti-nitrite agents may act synergistically, providing the therapeutic effects of UDCA or bezafibrate for treating PBC.

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Original Paper

Intervirology

Infectious Source Factors Affecting the Severity of Sexually Transmitted Acute Hepatitis due to Hepatitis B Virus Genotype C

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Abstract
Objective: The aim of this study was to identify clinical features and virological aspects of infectious sources that are related to the severity of sexually transmitted acute hepatitis B virus (HBV) infection in patients, especially in cases of genotype C. *Methods:* Nineteen patients with acute HBV infection, 10 classified with severe acute hepatitis (SH) (prothrombin time; PT <40%) and 9 with typical acute hepatitis (AH) (PT >40%), and their infectious sources (all were sexual partners) were studied. Infectious source factors were analyzed in relation to the severity of hepatitis in the patients' partners. *Results:* The nucleotide homology of HBV-DNA between each pair was ≥98.9%. Sixteen were infected with HBV genotype C. Among the 16 infectious sources, age, numbers with elevated alanine aminotransferase (ALT, 7/9 vs. 1/7), anti-HBe positivity (8/9 vs. 1/7) and core promoter mutations at nt 1762 (7/9 vs. 1/7), nt 1764 (8/9 vs. 1/7) and precore mutation at nt 1896 (8/9 vs. 1/7) were significantly higher in the sources of SH than in those of AH. *Conclusion:* Higher age, elevated ALT, anti-HBe positivity and core promoter/precore mutations were possible risk factors for an infectious source of the severe form of sexually transmitted acute hepatitis due to HBV genotype C.

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Introduction

Hepatitis B virus (HBV) is one of the most common endemic viruses in the world, with more than 300 million people chronically infected. HBV causes a variety of liver diseases, including self-limiting acute hepatitis, fulminant hepatitis, chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. Acute HBV infection induces acute hepatitis, and 1–2% of those patients develop fulminant hepatitis.

Both vertical and horizontal means are known for HBV transmission, and the former can be well prevented by injection with immunoglobulin containing a high titer of antibody to the hepatitis B surface antigen (HBsAg) combined with HBV vaccine to newborns [1]. Among the infectious routes of horizontal HBV transmission, post-transfusion hepatitis has decreased dramatically by the

screening of donated blood [2, 3], and sexual transmission or transmission from partners has become the most common route in adults, in both western and eastern countries [4–9]. To diminish the occurrence of fulminant hepatitis B in adults, it is important to understand the risk factors of the infectious source that have a relationship with the severity of sexually transmitted hepatitis in patients; however, those factors are not well known. The purpose of this study was to determine infectious source factors that are related to the severity of sexually transmitted hepatitis in patients.

Patients and Methods

Patients and Infectious Sources

Nineteen patients with acute HBV infection and their sexual partners (which included spouses), who were proven to be positive for HBsAg, were enrolled. All patients and infectious sources were Japanese living in the western part of Japan. The patients with acute HBV infection included all available patients with known infectious sources attended in our hospitals between 1995 and 2003. Patients with acute HBV infection were divided into 2 groups according to the level of prothrombin time (PT), as PT or level of clotting factors are important markers for estimating the severity of liver disease [10, 11]. The severe acute hepatitis (SH) group was composed of 10 patients who showed severe liver dysfunction with prolonged PT (PT activity percentage <40%), and the self-limited typical acute hepatitis (AH) group was composed of 9 patients who had a PT activity percentage greater than 40%. Among the 10 patients in the SH group, 6 were diagnosed with fulminant hepatitis which was defined as severe liver dysfunction, showing PT of less than 40%, with the presence of hepatic encephalopathy within 8 weeks from the first appearance of symptoms [12], while the other 4 were diagnosed with acute hepatitis severe type that was defined as acute hepatitis, with PT of less than 40%, without hepatic encephalopathy. The ratio of SH to AH was high in this study, because all patients with SH and their sexual partners or spouses were cooperative and willing to be involved in this study, whereas some patients with AH and some of their sexual partners with AH rejected to be involved in this study.

The diagnostic criteria for acute HBV infection were positivity for IgM type antibody to anti-hepatitis B core (HBc) and for anti-HBc with a low titer (<90% in 200-fold diluted serum). Only patients with acute HBV infection who were negative for IgM type antibody to hepatitis A virus (IgM-anti-HA), antibody to hepatitis C virus (anti-HCV) and HCV-RNA were included in this study.

Sexual partners (n = 19) who were proven to be positive for serum HBsAg and suspected of being the infectious sources were the main subjects of this study. Age, sex, diagnosis, liver function tests, HBV markers and HBV sequences of the core promoter to the precore region were analyzed. Serological and biochemical tests were performed between 1 and 4 weeks from the onset of acute HBV infection. Diagnoses were based on the results of biochemical and serological tests, except for 5 subjects who were diagnosed by histological examination (3 with chronic hepatitis, 1 with liver cirrhosis, 1 with hepatocellular carcinoma). Subjects with normal liver function test results were diagnosed as asymptomatic carriers, whereas those with

elevated transaminase and delay in an indocyanine green clearance test without signs of portal hypertension were diagnosed with chronic hepatitis.

Acute phase serum samples (taken less than 2 weeks after onset) from patients with acute HBV infection were obtained and stored at −80°, until used. Serum samples from the sexual partners were obtained within 4 weeks from the onset of acute HBV infection and were also stored at −80°.

The purpose of this study was explained to all patients or their families as well as the sexual partners or spouses. Written informed consent was obtained from all of the subjects who were involved in this study.

Serological Markers

HBsAg (AxSYM HBsAg, Dainabot, Tokyo, Japan), anti-HBc (AxSYM HBc, Dainabot), IgM-anti-HBc (AxSYM HBc-M, Dainabot), hepatitis B e antigen (HBeAg)(AxSYM HBeAg, Dainabot), antibody to HBeAg (anti-HBe)(AxSYM HBeAb, Dainabot), IgM-anti-HA (AxSYM HA-M, Dainabot), anti-HCV (Ortho Diagnostics, Tokyo, Japan) and HCV-RNA (Amplicor TM HCV, Roche Diagnostics, Mannheim, Germany) were assayed, using commercial kits. Viral load was estimated by the level of HBV-DNA or serum DNA polymerase activity. Quantification of HBV-DNA was done either by the transcription-mediated amplification (TMA) method (GEN-PROBE Inc., San Diego, Calif., USA), a branched DNA probe assay (Daiichi-Kagaku, Tokyo, Japan) or the solution hybridization method (Toray Industries, Inc., Tokyo). Serum DNA polymerase activity was measured using Kaplan's method, with some modifications [13]. As this was a retrospective study, the assays of the viral load were not identical among the patients who had been admitted to several different hospitals; moreover, the amount of stored sera were not enough.

Sequencing of Core Promoter and Precore Region

DNA was extracted from sera. Briefly, 50 μl of each serum sample was incubated with lysis buffer containing proteinase K. DNA was extracted using a phenol-chloroform solution and precipitated with ethanol, and HBV-DNA was amplified by polymerase chain reaction (PCR). For the amplification of the core promoter and precore regions, a semi-nested PCR was performed using primers P1, F3 and P4 (P1: 5′-AAGGACTGGGAGGAGTTGGGGGA-3′, nt 1725–1747; P4: 5′-GATACAGAGCAGAGGCGGTGT-3′, nt 2015–1995; F3: 5′-GTCAGAAGGCAAAAAAGAGAG-3′, nt 1966–1946; P1 and P4 for first round PCR, and P1 and F3 for second round PCR). Direct sequencing was done using a commercially available kit (Big-Dye Terminator Cycle Sequencing FS Ready Reaction Kit, Applied Biosystems, Alameda, Calif., USA) with P1 and F3 used as the sequencing primers [14].

The accuracy of the sequences was ensured by identification of the sequence data of the genome obtained by the sense sequencing primer (P1) and that obtained by the anti-sense sequencing primer (F3).

HBV Genotyping

The HBV genotype was determined, based on the restriction fragment length polymorphism patterns of the S gene sequence [15].

Statistical Analysis

Statistical analyses were performed using Wilcoxon's Rank test and Fisher's exact test. p values of less than 0.05 were considered statistically significant.

Table 1. Clinical features of patients with acute hepatitis B virus infection[a]

Patient	Diag-nosis	Sex/age	T. bil mg/dl	ALT IU/l	PT %	HBeAg/Ab	Viral load DNA-P, cpm	Viral load HBV-DNA	Geno-type	Mutation nt 1762T	1764A	1896A
SH1	FH	M/51	23.6	1,296	9	–/+	10		C	–	–	–
SH2	FH	M/44	14.9	7,126	29	–/+	10		C	–	+	+
SH3	FH	M/40	33.4	4,895	19	–/+	3		C	+	+	+
SH4	FH	F/25	7.3	4,664	26	–/+	NE		C	+	+	+
SH5	FH	F/44	15.5	4,775	14	–/+	1		C	+	+	+
SH6	AHs	M/24	8.9	10,880	18	–/+	119		C	+	+	+
SH7	AHs	F/29	10.8	4,908	22	–/+	56		C	+	+	+
SH8	AHs	F/27	7.9	2,139	36	–/+	NE		C	+	+	+
SH9	AHs	F/26	6.4	2,850	30	–/+	2		C	+	+	+
AH1	AH	M/25	24.4	2,170	55	–/+	980		C	–	–	–
AH2	AH	M/22	2.1	3,040	90	+/–	NE		C	–	–	–
AH3	AH	M/41	16.8	1,082	64	–/+		<0.7 mEq/ml	C	–	–	–
AH4	AH	F/40	4.9	797	98	–/+		5.6 LGE/ml	C	–	–	–
AH5	AH	M/26	9.6	4,080	68	–/+	222		C	–	+	+
AH6	AH	F/28	1.6	2,892	54	–/+		6.3 LGE/ml	C	–	–	–
AH7	AH	M/26	2.5	3,039	75	–/+	NE		C	–	–	–

a SH = Severe acute hepatitis; FH = fulminant hepatitis; AHs = acute hepatitis severe type; AH = typical acute hepatitis; T. bil = total bilirubin; ALT = alanine aminotransferase; PT = prothrombin time; DNA-P = DNA polymerase; NE = not examined; LGE = log genome equivalents.

Table 2. Clinical features of infectious sources of patients with acute HBV infection[a]

Infectious sources	Transmitted patient	Clinical diagnosis	Sex/age	T. bil mg/dl	ALT IU/l	HBeAg/Ab	Viral load DNA-P, cpm	Viral load HBV-DNA	Geno-type	Mutation nt 1762T	1764A	1896A
IS1	SH1	ASC	F/53	0.5	16	–/+	25		C	–	–	–
IS2	SH2	CH	F/40	0.6	54	–/+	232		C	–	+	+
IS3	SH3	CH	F/49	0.7	64	–/+	4.5		C	+	+	+
IS4	SH4	CH	M/23	0.4	33	–/+	NE		C	+	+	+
IS5	SH5	LC	M/57	2.2	96	–/+	6		C	+	+	+
IS6	SH6	CH	F/21	0.1	79	+/–	9,846		C	+	+	+
IS7	SH7	CH	M/22	0.8	129	–/+	4		C	+	+	+
IS8	SH8	LC	M/38	0.7	70	–/+		7.2 LGE/ml	C	+	+	+
IS9	SH9	HCC	M/45	0.5	114	–/+	68		C	+	+	+
IS10	AH1	ASC	F/22	0.5	22	+/–	1,500		C	–	–	–
IS11	AH2	ASC	F/20	0.3	34	+/–	NE		C	–	–	–
IS12	AH3	ASC	F/28	0.3	11	+/–		>3,800 mEq/ml	C	–	–	–
IS13	AH4	ASC	M/39	0.3	24	+/–		510 pg/ml	C	–	–	–
IS14	AH5	CH	F/19	0.5	20	–/+	222		C	+	+	+
IS15	AH6	CH	M/28	0.3	549	+/–		220 mEq/ml	C	–	–	–
IS16	AH7	ASC	F/22	0.5	10	+/–		8.5 LGE/ml	C	–	–	–

a IS = Infectious source; SH = severe acute hepatitis; AH = typical acute hepatitis; ASC = asymptomatic carrier; CH = chronic hepatitis; LC = liver cirrhosis; HCC = hepatocellular carcinoma; T. bil = total bilirubin; ALT = alanine aminotransferase; DNA-P = DNA polymerase; NE = not examined; LGE = log genome equivalents.

Fig. 1. Comparison of HBV nucleotide sequence in patients with acute HBV infection and their infectious sources. SH = Severe acute hepatitis; AH = typical acute hepatitis; IS = infectious source. * From Kobayashi and Koike [49].

Results

Features of Patients with Acute HBV Infection

Sixteen patients with acute HBV infection were infected with genotype C, 2 with genotype D and 1 with genotype A. Table 1 shows clinical features of 16 patients with acute HBV genotype C infection, with data from the early stage of onset of hepatitis. There were no differences for age and sex distribution between the SH and AH groups. One patient with fulminant hepatitis (SH2) died, 1 with fulminant hepatitis (SH3) received a liver transplant and the other 14 were alive at the time of this study.

Among those with genotype C HBV infection, core promoter mutations (nt 1762T, nt 1764A) and a precore mutation (nt 1896A) were significantly higher in patients with SH (7/9 vs. 0/7, p < 0.05; 8/9 vs. 1/7, p < 0.01; 8/9 vs. 1/7, p < 0.01, respectively).

Features of Infectious Sources

Among all 19 infectious sources, 2 were infected with genotype D, 1 had genotype A and 16 had genotype C. Five of them were spouses and 14 were sexual partners. The HBV genotype was identical in all 19 pairs. Table 2 shows data of the infectious source subjects with genotype

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Infectious Source Factors of AH-B

Table 3. The comparison of clinical features of infectious sources of patients with acute genotype C HBV infection[a]

	Infectious sources of patients with		p value
	SH	AH	
Number	9	7	
Age, median (range), years	40 (21–57)	22 (19–39)	0.037
Sex, M/F	5/4	2/5	0.358
Diagnosis			0.035
ASC	1	5	
CH, LC, HCC	8	2	
T. bil			0.999
Within normal range, <1.2 mg/dl	8	7	
Elevated ≥ 1.2 mg/dl	1	0	
ALT			0.041
Within normal range (<40 IU/l)	2	6	
Elevated (≥ 40 IU/l)	7	1	
HBeAg/anti-HBe state			0.009
HBeAg +	1	6	
Anti-HBe +	8	1	
Mutation in core promoter region			
nt 1762T	7	1	0.041
nt 1764A	8	1	0.009
Mutation in precore region			
nt 1896A	8	1	0.009

[a] ASC = Asymptomatic carrier; CH = chronic hepatitis; LC = liver cirrhosis; HCC = hepatocellular carcinoma; T. bil = total bilirubin; ALT = alanine aminotransferase; SH = severe acute hepatitis; AH = typical acute hepatitis.

C infection. Among the 9 sources of SH, 1 was diagnosed as an asymptomatic carrier and the other 8 had chronic liver diseases (5 with chronic hepatitis, 2 with liver cirrhosis, 1 with hepatocellular carcinoma). Of the 7 sources of AH, 5 were asymptomatic carriers and 2 had chronic hepatitis.

Comparison of HBV between Partner Pairs

Nucleotide sequences of all subjects infected with genotype C between nt 1755 and nt 1937 (183 bases) were analyzed, and their sequences are partially shown in figure 1. A comparison of the nucleotide sequences revealed that the nucleotide homology between each pair was ≥98.9%, and the same nucleotide substitutions at the same positions were seen in each pair. Therefore, the sexual partners were confirmed as the respective infectious sources.

Features of Infectious Sources Infected with Genotype C

A comparison of clinical features between 9 infectious sources of SH and 7 infectious sources of AH is shown in table 3. Statistical differences were observed for age, diagnosis, ALT, HBeAg/Ab and mutations at nt 1762, nt 1764 and nt 1896. Age ($p < 0.05$), ratio of patients with chronic liver diseases ($p < 0.05$), elevated ALT ($p < 0.05$), anti-HBe ($p < 0.01$) and the mutations at nt 1762T ($p < 0.05$), nt 1764A ($p < 0.01$) and nt 1896A ($p < 0.01$) were significantly higher in the infectious sources of SH than in those of AH. Among 6 of the infectious sources diagnosed as asymptomatic carriers, 1 was a source of SH and positive for anti-HBe, while the other 5 were infectious sources of AH and positive for HBeAg. Multivariate analysis was not possible in this study because the number of patients was restricted.

Discussion

Previous studies concerning factors related to the severity of acute HBV infection were mainly performed by analyzing virological aspects of patients. Many reports, mainly from Asia, have shown that the G to A mutation at nt 1896 in the precore region that induces translational