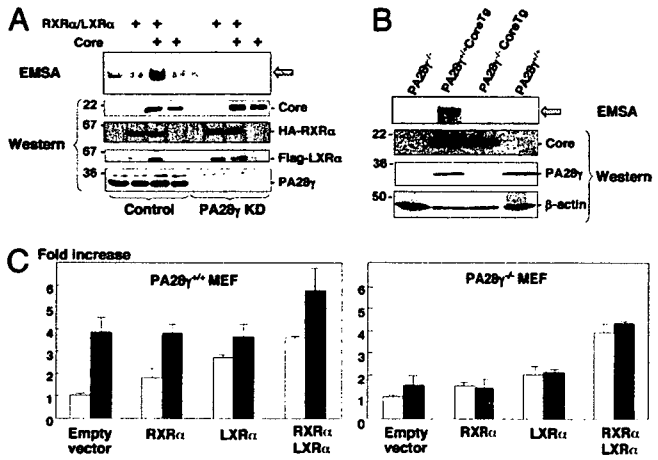

Fig. 5. PA28γ is required for HCV core-dependent activation of the *srebp-1c* promoter. (*A*) Effect of PA28γ knockdown on the LXRα/RXRα–DNA complex. FLAG-LXRα and HA-RXRα were expressed in FLC4 (control) or PA28γ-knockdown (PA28γ KD) cells together with or without HCV core protein. Cells were harvested at 48 h posttransfection, and nuclear extracts were mixed with the reaction buffer for EMSA. (*Upper*) The resulting mixtures were subjected to PAGE and blotted with horseradish peroxidase-streptavidin. The mobility shift of the LXRE probe is indicated by an arrow. (*Lower*) Expression of HCV core, HA-RXRα, FLAG-LXRα, and PA28γ in cells was detected by immunoblotting. (*B*) Effect of PA28γ knockout on the LXRα/RXRα–DNA complex in the mouse liver. (*Upper*) Nuclear extracts were prepared from the livers of 2-month-old PA28γ$^{-/-}$, PA28γ$^{+/+}$CoreTg, PA28γ$^{-/-}$CoreTg, and PA28γ$^{+/+}$ mice and subjected to EMSA. The mobility shift of the LXRE probe is indicated by an arrow. (*Lower*) The expression of HCV core, PA28γ, and β-actin in the livers of the mice was detected by immunoblotting. (*C*) Effect of HCV core protein on *srebp-1* promoter activity in PA28γ-knockout fibroblasts. A plasmid encoding firefly luciferase under the control of the *srebp-1c* promoter was transfected into MEFs prepared from PA28γ$^{+/+}$ (*Left*) or PA28γ$^{-/-}$ (*Right*) mice together with a plasmid encoding a *Renilla* luciferase. An empty plasmid or plasmids encoding mouse RXRα or LXRα were also cotransfected into the cells together with (gray bars) or without (white bars) a plasmid encoding HCV core protein. Luciferase activity under the control of the *srebp-1c* promoter was determined, and it is expressed as the fold increase in relative luciferase activity after standardization with the activity of *Renilla* luciferase.

tiates *srebp-1c* promoter activity in an LXRα/RXRα-dependent manner.

HCV Core Protein Activates the *srepb-1c* Promoter in an LXRα/RXRα- and PA28γ-Dependent Manner. To examine whether PA28γ is required for HCV core-induced enhancement of *srebp-1c* promoter activity in human liver cells, a PA28γ-knockdown human hepatoma cell line (FLC4 KD) was prepared. Enhancement of binding of the LXRE probe to LXRα/RXRα by coexpression of HCV core protein and LXRα/RXRα in FLC4 cells was diminished by knockdown of the PA28γ gene (Fig. 5*A*). Furthermore, formation of the LXRα/RXRα–LXRE complex was enhanced in the livers of PA28γ$^{+/+}$CoreTg mice but not in those of PA28γ$^{-/-}$, PA28γ$^{+/+}$, or PA28γ$^{-/-}$CoreTg mice (Fig. 5*B*). The expression of the HCV core protein in the mouse embryonic fibroblasts (MEFs) of PA28γ$^{+/+}$ mice induced the activation of the mouse *srebp-1c* promoter through the endogenous expression of LXRα and RXRα (Fig. 5*C Left*). Further enhancement of the activation of the *srebp-1c* promoter by HCV core protein in PA28γ$^{+/+}$ MEFs was achieved by the exogenous expression of both LXRα and RXRα. However, no enhancing effect of HCV core protein on *srebp-1c* promoter activity was observed in PA28γ$^{-/-}$ MEFs (Fig. 5*C Right*). These results support the notion that HCV core protein enhances the activity of the *srepb-1c* promoter in an LXRα/RXRα- and PA28γ-dependent manner.

Table 1. HCC in mice at 16–18 months of age

Mouse and sex	Total no. of mice	No. of mice developing HCC	Incidence, %
PA28γ$^{+/+}$CoreTg			
Male	17	5	29.4
Female	28	3	10.7
PA28γ$^{+/-}$			
Male	16	0	0
Female	4	0	0
PA28γ$^{-/-}$			
Male	23	0	0
Female	13	0	0
PA28γ$^{-/-}$CoreTg			
Male	15	0	0
Female	21	0	0

PA28γ Plays a Crucial Role in the Development of HCC in PA28γ$^{+/+}$ CoreTg Mice. The incidence of hepatic tumors in male PA28γ$^{+/+}$ CoreTg mice older than 16 months was significantly higher than that in age-matched female PA28γ$^{+/+}$CoreTg mice (6). We reconfirmed here that the incidence of HCC in male and female PA28γ$^{+/+}$CoreTg mice at 16–18 months of age was 29.4% (5 of 17 mice) and 10.7% (3 of 28 mice), respectively. To our surprise, however, no HCC developed in PA28γ$^{-/-}$CoreTg mice (males, 15; females, 21), although, as expected, no HCC was observed in PA28γ$^{+/-}$ (males, 16; females, 4) and PA28γ$^{-/-}$ mice (males, 23; females, 13) (Table 1). These results clearly indicate that PA28γ plays an indispensable role in the development of HCC induced by HCV core protein.

Discussion

HCV core protein is detected in the cytoplasm and partially in the nucleus and mitochondria of culture cells and hepatocytes of transgenic mice and hepatitis C patients (6, 23, 24, 26). Degradation of HCV core protein was enhanced by deletion of the C-terminal transmembrane region through a ubiquitin/proteasome-dependent pathway (27). We previously reported (18) that PA28γ binds directly to HCV core protein and then enhances degradation of HCV core protein in the nucleus through a proteasome-dependent pathway because HCV core protein was accumulated in nucleus of human cell line by treatment with proteasome inhibitor MG132. In this work, accumulation of HCV core protein was observed in nucleus of hepatocytes of PA28γ$^{-/-}$CoreTg mice (Fig. 1*D*). This result directly demonstrates that HCV core protein migrates into the nucleus and is degraded through a PA28γ-dependent pathway. However, HCV core protein accumulated in the nucleus because knockout of PA28γ gene abrogated the ability to cause liver pathology, suggesting that interaction of HCV core protein with PA28γ in the nucleus is prerequisite for the liver pathology induced by HCV core protein. We have previously shown (18) that HCV core protein is degraded through a PA28γ-dependent pathway, and Minami *et al.* (28) reported that PA28γ has a cochaperone activity with Hsp90. Therefore, degradation products of HCV core protein by means of PA28γ-dependent processing or correct folding of HCV core protein through cochaperone activity of PA28γ might be involved in the development of liver pathology. We do not know the reason why knockout of the PA28γ gene does not affect the total amount of HCV core protein in the liver of the transgenic mice. PA28γ-dependent degradation of HCV core protein may be independent of ubiquitination, as shown in SRC-3 (21), whereas knockdown of PA28γ in a human hepatoma cell line enhanced the ubiquitination of HCV core protein [supporting information (SI) Fig. 6], suggesting that lack of PA28γ suppresses a ubiquitin-independent degradation but enhances a ubiquitin-dependent degradation of HCV core protein. Therefore, the total amount of HCV

core protein in the liver of the mice may be unaffected by the knockout of the PA28γ gene.

Our results suggest that the interaction of HCV core protein with PA28γ leads to the activation of the *srebp-1c* promoter along an LXRα/RXRα-dependent pathway and the development of liver steatosis and HCC. HCV core protein was not included in the LXRα/RXRα–LXRE complex (Fig. 3*A*), suggesting that HCV core protein indirectly activates the *srebp-1c* promoter. Cytoplasmic HCV core protein was shown to interact with Sp110b, which is a transcriptional corepressor of RARα-dependent transcription, and this interaction leads to the sequestering of Sp110b in the cytoplasm, resulting in the activation of RARα-dependent transcription (29). The sequestration of an unidentified corepressor of the LXRα/RXRα heterodimer in the cytoplasm by HCV core protein may also contribute to the activation of the *srebp-1c* promoter. Although the precise physiological function of PA28γ-proteasome activity in the nucleus is not known, PA28γ has previously been shown (21) to regulate nuclear hormone receptors by means of the degradation of its coactivator SRC-3 and to participate in the fully Hsp90-dependent protein refolding (28). It appears reasonable to speculate that degradation or refolding of HCV core protein in a PA28γ-dependent pathway might be involved in the modulation of transcriptional regulators of various promoters, including the *srebp-1c* promoter. Saturated or monounsaturated fatty acids have been shown to enhance HCV RNA replication in Huh7 cells containing the full-length HCV replicon (7). The up-regulation of fatty acid biosynthesis by HCV core protein may also contribute to the efficient replication of HCV and to the progression of HCV pathogenesis.

Expression of HCV core protein was reported to enhance production of reactive oxygen species (ROS) (30), which leads to carbonylation of intracellular proteins (31). Enhancement of ROS production may trigger double-stranded DNA breaks and result in the development of HCC (30, 32, 33). HCV core protein could enhance the protein carbonylation in the liver of the transgenic mice in the presence but not in the absence of PA28γ (SI Fig. 7), suggesting that PA28γ is required for ROS production induced by HCV core protein. Development of HCC was observed in PA28γ$^{+/+}$CoreTg mice but not in PA28γ$^{-/-}$CoreTg mice (Table 1). Enhancement of ROS production by HCV core protein in the presence of PA28γ might be involved in the development of HCC in PA28γ$^{+/+}$CoreTg mice.

It is well known that resistant viruses readily emerge during the treatment with antiviral drugs targeting the viral protease or replicase, especially in the case of infection with RNA viruses. Therefore, antivirals targeting the host factors that are indispensable for the propagation of viruses might be an ideal target for the development of antiviral agents because of a lower rate of mutation than that of viral genome, if they have no side effects to patients. Importantly, the amino acid sequence of PA28γ of mice is identical to that of human, and mouse PA28γ is dispensable because PA28γ knockout mice exhibit no abnormal phenotype except for mild growth retardation. Therefore, PA28γ might be a promising target for an antiviral treatment of chronic hepatitis C with negligible side effects.

In summary, we observed that a knockout of the PA28γ gene from PA28γ$^{+/+}$CoreTg mice induced the accumulation of HCV core protein in the nucleus and disrupted the development of both steatosis and HCC. Activation of the *srebp-1c* promoter was up-regulated by HCV core protein both *in vitro* and *in vivo* through a PA28γ-dependent pathway, suggesting that PA28γ plays a crucial role in the development of liver pathology induced by HCV infection.

Materials and Methods

Histology and immunohistochemistry, real-time PCR, and detection of proteins modified by ROS are discussed in *SI Materials and Methods*.

Plasmids and Reagents. Human PA28γ cDNA was isolated from a human fetal brain library (18). The gene encoding HCV core protein was amplified from HCV strain J1 (genotype 1b) (34) and cloned into pCAG-GS (35). Mouse cDNAs of RXRα and LXRα were amplified by PCR from the total cDNAs of the mouse liver. The RXRα and LXRα genes were introduced into pEF-FLAGGspGBK (36) and pcDNA3.1 (Invitrogen, Carlsbad, CA), respectively. The targeting fragment for human PA28γ knockdown (GGATCCGGTGGATCAGGAAGTGAAGTTCAAGAGA-CTTCACTTCCTGATCCACCTTTTTTGGAAAAGCTT) was introduced into the BamHI and HindIII sites of pSilencer 4.1 U6 hygro vector (Ambion, Austin, TX). Mouse anti-FLAG (M2) and mouse anti-β-actin antibodies were purchased from Sigma (St. Louis, MO). Rabbit polyclonal antibody against synthetic peptides corresponding to amino acids 70–85 of PA28γ was obtained from AFFINITI (Exeter, U.K.). Horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit IgGs were purchased from ICN Pharmaceuticals (Aurora, OH). Rabbit anti-HCV core protein was prepared by immunization with recombinant HCV core protein (amino acids 1-71), as described in ref. 24. Mouse monoclonal antibody to HCV core protein was kindly provided by S. Yagi (37). The plasmid for expression of HA-tagged ubiquitin was described in ref. 27.

Preparation of PA28γ-Knockout HCV CoreTg Mice. The generation of C57BL/6 mice carrying the gene encoding HCV core protein genotype 1b line C49 and that of PA28γ$^{-/-}$ mice have been reported previously (22, 25). Both strains were crossbred with each other to create PA28γ$^{-/-}$CoreTg mice. PA28γ$^{-/-}$CoreTg mice were identified by PCR targeted at the PA28γ or HCV core gene (22, 25). Using 1 μg of genomic DNA obtained from the mouse tail, the PA28γ gene was amplified by PCR with the following primers: sense, PA28-3 (AGGTGGATCAGGAAGTGAAGCTCAA); and antisense, PA28γ-5cr (CACCTCACTTGTGATCCGCTCTCT-GAAAGAATCAACC). The targeted sequence for the PA28γ-knockout mouse was detected by PCR using the PA28-3 primer and the PAKO-4 primer (TGCAGTTCATTCAGGGCACCGGA-CAG). The transgene encoding HCV core protein was detected by PCR as described in ref. 25. The expression of PA28γ and HCV core protein in the livers of 6-month-old mice was confirmed by Western blotting with mouse monoclonal antibody to HCV core protein, clone 11-10, and rabbit antibody to PA28γ. Mice were cared for according to the institutional guidelines. The mice were given ordinary feed, CRF-1 (Charles River Laboratories, Yokohama, Japan), and they were maintained under specific pathogen-free conditions.

All animal experiments conformed to the Guidelines for the Care and Use of Laboratory Animals, and they were approved by the Institutional Committee of Laboratory Animal Experimentation (Research Institute for Microbial Diseases, Osaka University).

Preparation of Mouse Embryonic Fibroblasts. MEFs were prepared as described in ref. 22. MEFs were cultured at 37°C under an atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% FBS, penicillin, streptomycin, sodium pyruvate, and nonessential amino acids.

Transfection and Immunoblotting. Plasmid vectors were transfected into the MEFs and 293T cells by liposome-mediated transfection by using Lipofectamine 2000 (Invitrogen). The amount of HCV core protein in the liver tissues was determined by an ELISA as described in ref. 37. The cell lysates were subjected to SDS/PAGE (12.5% gel), and they were then transferred onto PVDF membranes. Proteins on the membranes were treated with specific antibody and Super Signal Femto (Pierce, Rockford, IL). The results were then visualized by using an LAS3000 imaging system (Fuji Photo Film, Tokyo, Japan). The method of immunoprecipitation test is described in ref. 18.

Reporter Assay for *srebp-1c* Promoter Activity. The genomic DNA fragment encoding the *srebp-1c* promoter region (located from residues −410 to +24) was amplified from a mouse genome. The fragment was introduced into the KpnI and HindIII sites of pGL3-Basic (Promega, Madison, WI), and it was designated as pGL3-srebp-1cPro. The plasmids encoding RXRα and LXRα were transfected into MEFs together with pGL3-srebp-1cPro and a control plasmid encoding *Renilla* luciferase (Promega). The total DNA for transfection was normalized by the addition of empty plasmids. Cells were harvested at 24 h posttransfection. The ligand of RXRα, 9-*cis*-retinoic acid (Sigma), and that of LXRα, 22(*R*)-hydroxylcholesterol (Sigma) were added at a final concentration of 5 μM each to the culture medium of 293T cells transfected with pGL3-srebp-1cPro together with expression plasmids encoding RXRα, LXRα, and HCV core protein at 24 h posttransfection. Cells were harvested 24 h after treatment. Luciferase activity was measured by using the dual-luciferase reporter assay system (Promega). Firefly luciferase activity was standardized with that of *Renilla* luciferase, and the results are expressed as the fold increase in relative luciferase units.

Electrophoresis Mobility Shift Assay (EMSA). EMSA was carried out by using a LightShift Chemiluminescent EMSA kit (Pierce) according to the manufacturer's protocol. Nuclear extract of the cell lines and liver tissue was prepared with an NE-PER nuclear and cytoplasmic extraction reagent kit (Pierce). Briefly, double-stranded oligonucleotides for EMSA were prepared by annealing both strands of each LXRE of the *srebp-1c* promoter (5′-GGACGCCCGCTAGTAACCCCGGC-3′) (16). Both strands were labeled at the 5′ ends with biotin. The annealed probe was incubated for 20 min on ice with nuclear extract (3 μg of protein) in a reaction buffer containing 10 mM Tris·HCl (pH 7.5), 50 mM KCl, 1 mM DTT, 0.05 μg/μl poly(dI-dC), 2.5% glycerol, 0.05% Nonidet P-40, and 0.1 nM labeled probe, with or without 1 mM nonlabeled probe. The resulting mixture was subjected to PAGE (5% gel) at 120 V for 30 min in 0.5× TBE. The DNA–protein complex was transferred to a Hybond N+ membrane (Amersham, Piscataway, NJ), incubated with horseradish peroxidase-conjugated streptavidin, and visualized by using an LAS3000 imaging system.

Statistical Analysis. The results are expressed as the mean ± SD. The significance of differences in the means was determined by Student's *t* test.

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Review

Drug resistance in antiviral treatment for infections with hepatitis B and C viruses

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Treatments for infections with hepatitis B and C viruses have recently developed markedly, and range from nonspecific interferon-based treatments to specific antiviral treatments, such as those that inhibit hepatitis virus-coded protein production or activity. These developments have contributed to the achievement of excellent enhancement of the antiviral effect. On the other hand, the development of specific antiviral therapies has created unprecedented problems. Antiviral drug-resistant strains of viruses have emerged, leading to a poor prognosis for infected patients. Clarification of the mechanisms underlying the emergence of such resistance to drugs will be useful for the treatment of such patients. In this review, we outline pathological conditions associated with hepatitis B and C viruses and their treatments, and discuss the current situation and mechanisms underlying the emergence of antiviral drug-resistant strains.

Key words: Interferon, lamivudine, entecavir, adefovir, ribavirin

Introduction

Infections with hepatitis B and C viruses (HBV and HCV, respectively) are a problem worldwide. Many patients are infected with HBV and HCV, and these infections are not only a major medical burden but also a socioeconomic burden because of their possible progression from chronic hepatitis to cirrhosis and hepatocellular carcinoma (HCC) if left untreated.[1,2] The treatments against these hepatitis viruses have been mainly nonspecific, based on the use of interferon (IFN). Specific antiviral drugs developed recently that inhibit hepatitis viral replication, for example, by inhibition of reverse transcriptase in HBV, have shown remarkable efficacy.[3,4] However, these treatments have also turned out to be a double-edged sword because they have led to the emergence of strains resistant to these drugs. The emergence of antiviral drug-resistant strains of these viruses may hinder the development of treatments against them.

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HBV infection

HBV belongs to the family *Hepadnaviridae*. HBV is a DNA virus that has an approximately 3.2-kb circular incomplete double-stranded DNA genome. When it replicates, HBV forms DNA by using its own reverse transcriptase with RNA as a replicative intermediate. Worldwide, the distribution of HBV carriers and the incidence of HCC are closely correlated. The number of HBV carriers in Japan is estimated to be approximately one million, many of whom became infected via perinatal mother-to-child transmission. Hepatitis B e antigen (HBeAg) is a protein produced by the wild-type HBV strain as it replicates that appears in the blood. Generally, the presence of HBeAg indicates an abundance of the virus, and the detection of anti-HBe antibody often indicates a marked decrease in the HBV load.

HBV carriers are HBeAg-positive asymptomatic carriers (AsC) in the early stages of HBV infection. Their blood HBV level is very high in these stages. They can develop symptoms of hepatitis at any time of their life, but generally do between adolescence and their early 30s. At that time, the HBV load decreases markedly, and symptoms of hepatitis improve in 2 to 3 years in 80%–90% of HBV patients. Most of these patients then become negative for HBeAg and positive for the anti-HBe antibody. The annual incidence of seroconversion from being HBeAg-positive to anti-HBe antibody-

positive has been reported to be approximately 5%.[5,6] However, symptoms of hepatitis persist in about 10%–20% of these patients even after seroconversion; such patients often develop cirrhosis and HCC.[7]

Treatment of HBV infection

There are several choices for treatment of chronic hepatitis B, but they all have limited efficacy. HBV elimination [i.e., serum HB surface antigen (HBsAg)-negative status] is difficult in HBV carriers. Seroconversion from HBeAg-positive to anti-HBe antibody-positive is generally the goal of HBV infection treatment. It should also be understood that many HBV AsCs do not need to or often must not undergo treatment with any anti-HBV agent.

In approximately 80% of patients with chronic hepatitis B, seroconversion occurs spontaneously. Consequently, chronic hepatitis B does not progress to cirrhosis in most chronic hepatitis B patients. The 15%–20% of HBV carriers who do not show seroconversion ultimately develop cirrhosis and/or HCC and should undergo antiviral treatment for chronic hepatitis B. IFN and reverse transcriptase inhibitors, including lamivudine, adefovir dipivoxil, and entecavir, have been approved for the treatment of chronic hepatitis B. Among these, lamivudine was the first used for chronic hepatitis B patients.

Lamivudine recipients are more likely than placebo recipients to show a histological response (52% vs. 23%, $P < 0.001$), the disappearance of HBeAg in serum (32% vs. 11%, $P = 0.003$), a sustained decrease in serum HBV DNA level to undetectable levels (44% vs. 16%, $P < 0.001$), and a sustained normalization of the serum alanine aminotransferase (ALT) level (41% vs. 7 %, $P < 0.001$) after 52 weeks of treatment.[8]

Entecavir has an antiviral effect comparable to that of lamivudine. In one study of HBeAg-positive patients,[9] histological improvement after 48 weeks occurred in 226 of 314 patients in an entecavir-treated group (72%) and in 195 of 314 patients in a lamivudine-treated group (62%, $P = 0.009$). More patients in the entecavir-treated group than in the lamivudine-treated group had undetectable serum HBV DNA levels according to a polymerase chain reaction (PCR) assay (67% vs. 36%, $P < 0.001$), and normalization of ALT levels (68% vs. 60%, $P = 0.02$). However, the seroconversion rate was not very high in either group.

Emergence of antiviral resistance in HBV infection

The treatment efficacy against HBV infection has improved, as mentioned above. The primary reason for chronic hepatitis B becoming resistant to treatment is the emergence of drug-resistant strains.

Mechanisms underlying development of resistance to IFN-based treatments

IFNs are administered in a wide range of doses according to various protocols for chronic hepatitis B. Such variation in protocols must be taken into account in determining the therapeutic efficacy of such drugs. Viral factors potentially involved in drug resistance include the HBV genotype and the presence of HBeAg, a precore gene mutation, or a core promoter mutation. HBeAg negativity, HBV genotypes C and D, precore gene 1896 mutation, and 1762/1764 mutations in the core promoter have been reported to be responsible for a poor response to IFN treatment. Strictly, these factors do not indicate "drug resistance" to IFN. The acquisition of the precore gene 1896 mutation also decreases the efficacy of IFN treatment.[10]

Mutation of the HBV polymerase domain

The HBV polymerase (*Pol*) gene is shown in Fig. 1. The Pol/RT region of this gene encodes HBV reverse transcriptase (RT). In HBV gene replication, reverse transcription from pregenomic RNA to viral gene DNA occurs, and reverse transcriptase is used in this process. The Pol/RT region is further divided into five domains from A to E. The Pol/RT active center is reported to be in domain C, domains B and E have been reported to be important for RNA template binding, and domains A and D for binding to nucleic acids.

Nucleic acid analog formulations are incorporated into the viral genome involved in reverse transcription instead of nucleic acids to inhibit viral replication competitively. However, if a Pol/RT gene mutation emerges, the nucleic acid analog formulation administered may be unable to inhibit viral replication because of interference with its binding to RT. Acquisition of this mutation of the Pol/RT gene thus results in the emergence of a drug-resistant viral strain. This mutation is shown in Fig. 1.

Resistance to lamivudine

Among nucleic acid analog formulations, lamivudine (LAM) has been used longest for the treatment of chronic hepatitis B , and its use continued until resistance to this drug emerged. Many factors underlying this resistance have been identified.

LAM resistance is caused by a mutation in which the 204th methionine (M) in the polymerase domain is replaced by valine (V) or isoleucine (I) (rtM204V/I). Because the site containing the 204th methionine is referred to as the YMDD motif, this mutation is also called the YVDD mutation or YIDD mutation. Another mutation associated with rtM204V/I is caused by replacement of the 180th leucine (L) by M (rtL180M).

rtM204V/I was detected in 10% of HBeAg-positive patients with chronic hepatitis B 24 weeks after the start

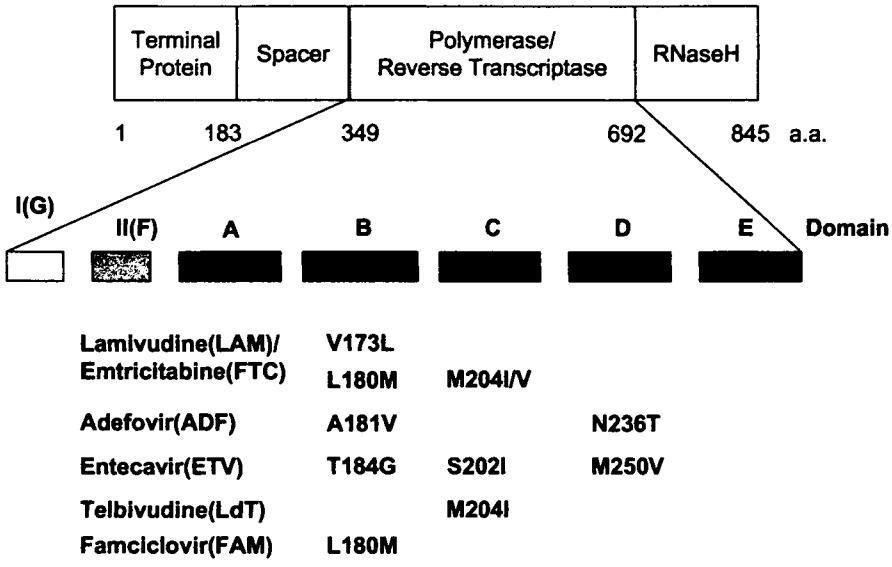

Fig. 1. Structure of the hepatitis B virus polymerase/reverse transcriptase gene and amino acid substitutions associated with resistance to nucleoside analogs. *a.a.*, amino acids

of LAM administration, in 24% after 52 weeks, and in 65% after 5 years.[4] In Japan, in an extensive study on the clinical course of both HBeAg-positive and HBeAg-negative chronic hepatitis B patients, the rate of emergence of LAM-resistant strains was 50%, 5 years after the start of LAM administration.[11] Not all patients having rtM204V/I develop hepatitis. Although the incidence of amino acid mutation of polymerase is high in patients with hepatitis, no specific mutation has been observed.[12]

The case of a patient who was LAM-resistant despite the absence of mutation in the YMDD motif was reported recently. The 181st alanine (A) in the Pol domain was replaced by threonine (T) (rtA181T) in this patient. An experiment using chimeric mice bearing human hepatocytes demonstrated that this mutation causes LAM resistance.[13]

Resistance to adefovir dipivoxil

The use of adefovir dipivoxil (ADV) in combination with LAM has been approved in Japan for patients showing LAM resistance. The emergence of ADV-resistant viral strains was first studied with regard to the administration of ADV alone. ADV resistance occurs in approximately 6% of patients 3 years after the start of ADV administration.[14] The replacement of the 236th asparagine (N) in the Pol domain by T (rtN236T) and that of the 181st A in the Pol domain by V (rtA181V) were observed in ADV-resistant strains.

Following the administration of ADV to patients exhibiting LAM resistance, strains with rtA181V/T or rtN236T emerged in 18% of the patients 1 year after the start of ADV administration.[15] rtA181T was also observed in LAM-resistant strains in the absence of mutation in the YMDD motif. It was reported recently that some patients exhibiting LAM resistance are non-responders to ADV from the start of ADV administration. These resistant strains had the rtI233V mutation.[16]

Resistance to entecavir

Administration of entecavir (ETV) for more than 52 weeks to patients with LAM-resistant chronic hepatitis B leads to the emergence of resistant viral strains in 1.4% of patients,[17] although no results have been reported for long-term administration of ETV. The amino acid sequence of the RT domain in resistant viral strains was analyzed in two patients, and strains with rtM250V and rt169T were found in one patient and strains with rtT184G and rtS2021 in the other.[21] In Japan, strains with rtS202G and rtL2691 have been detected.[22] It is notable that all of the patients who acquired ETV resistance were resistant to LAM. Thus, initial treatment with ETV alone may be less likely to lead to ETV-resistant viral strain emergence, but this hypothesis should be confirmed in future studies (Table 1).

HCV infection

HCV is a positive-strand RNA virus belonging to the family *Flaviviridae*. Approximately 170 million HCV carriers or patients are estimated to be persistently infected with HCV worldwide, and approximately 1.8 million in Japan. HCV is transmitted to humans by direct contact with infected or contaminated blood. The routes of infection include transfusion of contaminated or HCV-tainted blood or blood products, which have now been eliminated in Japan, sharing needles among drug abusers, acupuncture, and tattoos. Acupuncture and

Table 1. Clinical trial of entecavir for the treatment of chronic hepatitis B

No.	Authors	HBeAg	Lam resistance at baseline	ETV resistance at baseline	ETV resistance at end point (1 year)	Biochemical rebound (1 year)	Reference No.
1	Chang et al.	Positive	No		0/339	6/339 (2%)	9
2	Sherman et al.	Positive	Yes	11/186 (6%)	7/134 (5%)		17
3	Colonno et al.	Positive	No		0/354	6/354 (2%)	18
		Negative			1/325 (0.3%)	8/325 (2%)	
4	Lai et al.	Negative	No		0/211	5/211 (2%)	19
5	Chang et al.	Positive	Yes	6/181 (3%)	2/181 (1%)		20
		Negative					

HBeAg, hepatitis B e antigen; LAM, lamivudine; ETV, entecavir

depilation are invasive treatments and should be considered to involve risk for infection with HCV unless disposable medical instruments are used.[23]

HCV infection develops into persistent infection at a very high rate, becoming persistent in 70%–80% of patients with acute HCV infection. Generally, patients develop acute hepatitis 2 to 3 months after their initial infection with HCV. However, many patients are unaware of the onset of hepatitis because of the mild subjective symptoms and mild jaundice, if any. Although 20%–30% of patients developing acute hepatitis recover from the disease spontaneously, acute hepatitis develops into chronic hepatitis (by definition, hepatitis persisting for more than 6 months) in the remaining 70%–80% of patients. Then, chronic hepatitis enters an inactive phase that lasts 10–15 years. Serum ALT level, which indicates the destruction of hepatocytes, is within normal limits during the inactive phase, but viral growth continues.

Chronic hepatitis enters an active phase after 10–15 years in many patients, although there are marked individual differences. Serum ALT level increases to about two to three times the normal level when chronic hepatitis enters the active phase. Once chronic hepatitis C enters the active phase, it will not improve spontaneously. If the disease is left untreated, the risk of progressing from chronic hepatitis to cirrhosis increases. Hepatitis C characteristically progresses gradually but steadily.[24] The risk of developing HCC is high among patients with cirrhosis. The risk of HCC development in cirrhosis patients is 5%–7%.[25] Patients infected with HCV should be diagnosed during the inactive phase of chronic hepatitis C and start treatment for HCV elimination (antiviral treatment) as soon as the hepatitis enters the active phase.

Treatment of HCV infection

HCV infection is treated mainly with IFN-based drugs. The treatment efficacy is evaluated 6 months after the end of IFN-based drug administration. If HCV-RNA is not detected by the sensitive RT-PCR test, the patient is considered to show a sustained virological response (SVR), indicating that HCV has been virtually eliminated.

At present, polyethylene glycol-interferon (Peg-IFN) treatment in combination with ribavirin plays a key role in the treatment of HCV infection. Peg-IFN, an IFN molecule covalently bonded to Peg, is a sustained-release formulation. It needs to be injected only once weekly from the start of treatment, whereas conventional IFN preparations require administration three times weekly. Administration of Peg-IFN alone is more effective than that of a conventional IFN-based drug alone, but the administration of Peg-IFN in combination with ribavirin is even more effective.[26–28] An SVR rate of approximately 50% can be expected even in cases of chronic hepatitis infected with a high viral load of HCV of genotype 1, and an SVR rate of approximately 60% can be generally expected. Peg-IFN is usually administered for 48 consecutive weeks. It is important to continue the treatment for 48 weeks, although the dose may be reduced if adverse drug reactions appear. In addition, extending the administration period to a total of 72 weeks recently proved effective in patients who became HCV-negative after 12 weeks of treatment.[29]

There is a long history of treatment with IFN alone: treatment of non-A, non-B hepatitis with IFN alone dates back to around 1985, before the discovery of HCV. A nationwide survey conducted by the Study Group of the Ministry of Health, Labour and Welfare of Japan in 1995 showed that the SVR rate for treatment with IFN alone for 6 months (administration of 6 to 10 million units) was approximately 30% in all patients. However, in patients with the genotype 1 HCV, which is the major genotype worldwide and in about 70% of Japanese HCV patients, particularly those with high viral loads (determined as an HCV-RNA load of 100 KIU/ml or more), SVR was obtained in only about 2%–7%. The efficacy of treatment with IFN alone is thus low. Hence, Peg-IFN in combination with ribavirin

Table 2. Relationship between the ISDR and the response to interferon treatment for chronic hepatitis patients with genotype 1 hepatitis C virus infection

No.	Authors	Interferon	Ribavirin	Relationship between ISDR and viral load	Relationship between ISDR and response	Ethnicity	Ref. no.
1	Enomoto et al.	α	No	Yes	Yes	Japanese	30
2	Kurosaki et al.	β	No	Yes	Yes	Japanese	31
3	Chayama et al.	α	No	ND	Yes	Japanese	32
4	Zeuzem et al.	α	No	No	No	German	33
5	Squadrito et al.	α	No	No	No	French	34
6	Hofgartner et al.	α	No	ND	No	American	35
7	Khorsi et al.	α	No	ND	No	French	36
8	Saiz et al.	α	No	ND	Yes	Spanish	37
9	Frangeul et al.	α	No	ND	No	French	38
10	Odeberg et al.	α	No	ND	No	Sweden	39
11	Chung et al.	α	No	ND	No	American	40
12	Ibarrola et al.	α	Yes	ND	No	Spanish	41
13	Sarrazin et al.	α	Yes	ND	Yes	German	42
14	McKechnie et al.	α	No	ND	No	English	43
15	Yoshioka et al.	α	No	ND	Yes	Japanese	44
16	Stratidaki et al.	α	No	ND	No	American	45
17	Murphy et al.	α	Yes	ND	No	American	46
18	Cappiello et al.	PEGα	Yes	ND	No	Italian	47
19	Aslan et al.	α	No	ND	No	Turkish	48
20	Murayama et al.	α	Yes	ND	Yes	Japanese	49

ISDR, interferon sensitivity determining region; ND, not described; PEG, pegylated

is the first choice for patients with intractable disease, as mentioned above.

Emergence of antiviral resistance in HCV infection

In the treatment of chronic hepatitis C with IFN alone or IFN (or Peg-IFN) in combination with ribavirin, HCV-RNA does not disappear in some patients, particularly in those with genotype 1 HCV. Approximately 10% of genotype 1 HCV patients with high viral loads never become HCA-RNA-negative during the period of treatment with IFN (or Peg-IFN) in combination with ribavirin.

Not only host factors but also viral factors have been identified as causes for the nonelimination of HCV. The HCV genotype is a typical viral factor, and patients infected with genotype 1 or 4 are more resistant to treatment than those with genotype 2 or 3.

Another reported factor is the interferon sensitivity-determining region (ISDR) in NS5A, a region consisting of 40 amino acids, first reported by Enomoto et al.[30] ISDR is contained in the binding site of interferon α-inducible RNA-dependent protein kinase (PKR). Mutation in ISDR may cause dysfunction in the binding between the NS5A protein and PKR, leading to a decrease in viral protein translation. In Japan, a close correlation between IFN treatment efficacy and mutation in ISDR in genotype 1b HCV patients was found.[31,32] In Europe and the United States, however, the correlation between amino acid mutation in ISDR and IFN treatment efficacy is not clear even in patients infected with HCV genotype 1 (Table 2).

In addition, mutation of the PKR/eIF2α phosphorylation homology domain of the E2 domain has been reported to correlate with IFN-based drug efficacy, but this needs further clarification.

Ribavirin-resistant viral strain

Ribavirin shows low anti-HCV activity in some patients even when it is administered alone, and chronic hepatitis C has been treated with ribavirin alone. The structure of a ribavirin-resistant viral strain that has emerged has been studied. Mutation of the 415th amino acid (F415Y) in the RNA-dependent RNA polymerase (RdRp) domain of NS5B was detected in strains infecting patients treated with ribavirin alone who became ribavirin-resistant.[50] This mutation was considered to be related to IFN treatment efficacy in patients with genotype 1a HCV. In a study using a replicon, mutations of the 404th and 442nd amino acids (G404S and E442G) were detected.[51]

Conclusions

An overview of the mechanisms underlying the emergence of drug-resistant HBV and HCV strains has been given above. The emergence of drug-resistant strains of HBV in particular has posed problems. This resistance has resulted from the development of a wide range of

drugs for HBV, ranging from nonspecific IFN-based drugs to viral protein-specific RT inhibitors. Although no serious problem has arisen to date as regards HCV, specific anti-HCV drugs such as protease inhibitors and RNA polymerase inhibitors are beginning to be developed, so the emergence of drug-resistant viral strains is expected to be a major problem. Indeed, the emergence of a strain resistant to VX950, an HCV protease inhibitor with high antiviral activity, following a short period of administration of this drug has already been reported.[52] HBV and HCV do not seem to be very easy to eliminate.

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DYSFUNCTION OF ENERGY METABOLISM IN HEPATIC CARCINOGENESIS

Hepatitis C virus contributes to hepatocarcinogenesis by modulating metabolic and intracellular signaling pathways

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Key words
hepatitis C virus, hepatocarcinogenesis, intracellular signaling transduction, oxidative stress, transgenic mouse.

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Abstract

Persistent infection with hepatitis C virus (HCV) is a major risk factor for development of hepatocellular carcinoma (HCC). However, it remains controversial in the pathogenesis of HCC associated with HCV as to whether the virus plays a direct or an indirect role. The studies using transgenic mouse models, in which the core protein of HCV has an oncogenic potential, indicate that HCV is directly involved in hepatocarcinogenesis, albeit other factors such as continued cell death and regeneration associated with inflammation would also play a role. The downstream events of the core protein are segregated into two components. One is the augmented production of oxidative stress along with the activation of scavenging system, including catalase and glutathione, in the putative pre-neoplastic stage with steatosis in the liver. Thus, oxidative stress production in the absence of inflammation by the core protein would partly contribute to the development of HCC. The generation of oxidative stress is estimated to originate from mitochondrial dysfunction in hepatocytes by HCV infection. The other component is the alteration of intracellular signaling cascade of mitogen-activated protein kinase and activating factor (AP)-1, leading to the activation of cell cycle control. The combination of these pathways, collective with HCV-associated alterations in lipid and glucose metabolism, would lead to the frequent development of HCC in persistent HCV infection. These results suggest that there would be a mechanism for hepatocarcinogenesis in persistent HCV infection that is distinct from those for the other cancers. Similar to the pathogenesis of other cancers, the accumulation of a set of genetic aberrations may also be necessary for a multistage development of HCC. However, HCV core protein, to which an oncogenic potential is ascribed, may allow some of the multiple steps to be bypassed in hepatocarcinogenesis. Therefore unlike for other cancers, HCV infection may be able to cause HCC in the absence of a complete set of genetic aberrations. Such a scenario, 'non-Vogelstein-type' carcinogenesis, would explain the rare feature of hepatocarcinogenesis in HCV infection, the extraordinarily high incidence and the multicentric nature of HCC development.

Introduction

Hepatitis C virus (HCV) infects hundreds of millions of people persistently, and induces a spectrum of chronic liver disease worldwide.[1] It impacts on society in a number of domains including the medical, sociological and economic. Hepatocellular carcinoma (HCC) has become the major cause of death in individuals persistently infected with HCV. In particular, HCV has been given increasing attention because of its wide and deep penetration in the community, coupled with a very high incidence of HCC in persistent HCV infection. Once liver cirrhosis is established in hosts infected with HCV, HCC develops at a yearly rate of 5–7%.[2] Knowledge on the mechanism of HCC development in chronic HCV infection therefore is urgently required for the prevention of HCC.

Hepatocellular carcinoma frequently develops in persistent HCV infection

How HCV induces HCC is not clear yet, despite the fact that more than 70% of patients with HCC in Japan are infected with HCV.[1,3,4] Hepatitis C virus infection is also common in patients with HCC in other countries albeit to a lesser extent. These lines of evidence obligate hepatologists to the considerable task of determining the role of HCV in hepatocarcinogenesis. Inflammation induced by HCV, manifesting in various forms of hepatitis, should be considered in a study on the carcinogenic capacity of hepatitis viruses. It has been proposed repeatedly that the necrosis of hepatocytes due to chronic inflammation and ensuing regeneration enhances mutagenesis in host cells, the accumulation of which culminates in HCC. This theory presupposes an indirect involvement of hepatitis

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viruses in HCC via hepatic inflammation. However, this leaves specialists in hepatology with a serious question: can inflammation per se result in the development of HCC in such a high incidence or multicentric nature in HCV infection? The secondary role of HCV would have to be weighed against an extremely rare occurrence of HCC in patients with autoimmune hepatitis in whom severe inflammation in the liver persists indefinitely.

This background and reasoning led to the suggestion that HCC may be induced, at least in part, by viral proteins. This possibility has been evaluated by introducing genes of HCV into hepatocytes in culture with little success. One of the difficulties in using cultured cells is the carcinogenic capacity of HCV, if any, which would be weak and would take a long time to manifest itself. It takes 30–40 years for HCC to develop in individuals infected with HCV. Another constraint common to studies on carcinogenesis is the development of HCC by transformed cells that might have gone out of growth control and escaped surveillance of the host. Should this be the case, the analysis of transformed cells would not be sufficient for solving the mystery of carcinogenesis. On the basis of these points, we chose to investigate carcinogenesis in chronic viral hepatitis using transgenic mouse technology.

HCV core protein has an oncogenic activity in transgenic mouse

Transgenic mouse lines with sections of the HCV genome were engineered by introducing genes excised from the cDNA of the HCV genome of genotype 1b.[5,6] The mouse lines were from a C57BL/6 strain, which is known for a rare spontaneous occurrence of HCC.[7] Three different transgenic mouse lines have been established, which carry the core gene, envelope genes or non-structural genes (Fig. 1), respectively, under the same transcriptional control element. Among these mouse lines, only the transgenic mice carrying the core gene develop HCC in two independent lineages.[6] The envelope gene transgenic mice do not develop HCC, despite high expression levels of both E1 and E2 proteins.[8,9] The transgenic mice carrying the entire non-structural genes have developed no HCC.

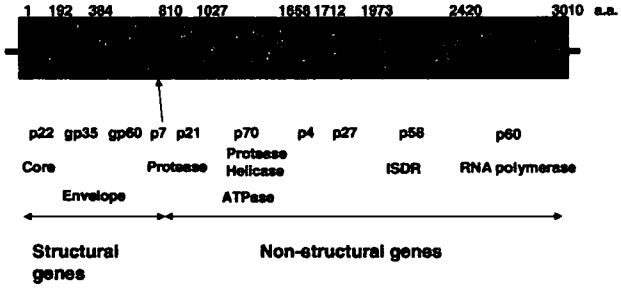

Figure 1 Structure of the hepatitis C virus (HCV) genome. The HCV genome consists of structural and non-structural regions. The structural region consists of the core, envelope and p7 genes. The non-structural region cones enzyme proteins of NS3 to NS5B. Among the three different transgenic mouse lines established, which carry the core, envelope and non-structural region, respectively, only the transgenic mice carrying the HCV core gene develop hepatocellular carcinoma (HCC) after an early phase with hepatic steatosis in two independent lineages. The mice transgenic for the envelope genes or non-structural genes do not develop HCC. ISDR, interferon-sensitivity determining region.

The transgenic mice carry the core gene and express the core protein of an expected size, approximately 21 kDa, the level of which in the liver is similar to that in the liver of chronic hepatitis C patients. Early in life, these mice develop hepatic steatosis, which is one of the histologic characteristics of chronic hepatitis C, along with lymphoid follicle formation and bile duct damage.[10] Thus, the core gene transgenic mouse model well reproduces this feature of chronic hepatitis C. Of note, significant inflammation is not observed in the liver of this animal model. Late in life, these transgenic mice develop HCC. Most hepatic nodules have a pathology characterized by 'nodule in nodule', and HCC with a low degree of differentiation develops within adenoma as well as within HCC with a higher degree of differentiation.[6] Although numerous lipid droplets are found in cells forming adenoma, as in non-tumorous cells, they are rarely observed in HCC cells. These histological features closely resemble those observed in HCC developing in chronic hepatitis C patients, in whom prominent lipid droplets are found in small, well-differentiated HCC and its precursors; poorly differentiated HCC without lipid droplets develops from within differentiated HCC.[6] Notably, the development of steatosis and HCC has been reproduced in other HCV transgenic mouse lines, which harbor the entire HCV genome or structural genes including the core gene.[11] These outcomes indicate that the core protein per se of HCV has an oncogenic potential when expressed *in vivo*.

Sequence to the core protein expression in the liver

It is difficult to clarify the mechanism of carcinogenesis even for our simple model in which only the core protein is expressed in otherwise normal liver tissues. There is a notable feature of the localization of the core protein in hepatocytes: although the core protein predominantly exists in the cytoplasm associated with lipid droplets, it is also present in the mitochondria and nuclei.[6,12] On the basis of this finding, the pathways related to these two organelles, the mitochondria and nuclei, were analyzed.

One activity of the core protein is an increased production of oxidative stress in the liver. We note that the production of oxidative stress is increased in our transgenic mouse model in the absence of inflammation in the liver (hepatitis). This reflects overproduction of reactive oxygen species (ROS) in the liver, or predisposition to it, which is staged by the HCV core protein without any intervening inflammation.[13,14] The overproduction of oxidative stress results in the generation of deletions in the mitochondrial DNA, an indicator of genetic damage. Thus, the core protein induces oxidative stress overproduction in the absence of inflammation, and may, at least in part, contribute to hepatocarcinogenesis in HCV infection (Fig. 2). If inflammation is induced in the liver with the HCV core protein, the production of oxidative stress is escalated to an extent that cannot be scavenged by a physiological antagonistic system. This indicates that the inflammation in chronic HCV infection would be different to that produced in other

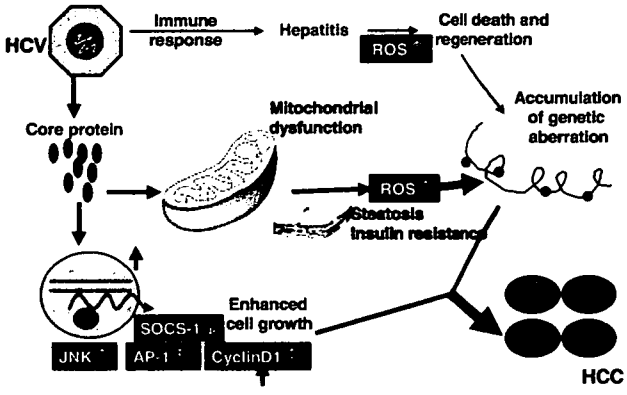

Figure 2 Molecular pathogenesis of liver disease in hepatitis C virus (HCV) infection. Induction of oxidative stress together with hepatic steatosis by the HCV core protein would play a pivotal role in the development of hepatocellular carcinoma (HCC). Alterations in cellular gene expressions, such as tumor necrosis factor-α (TNF-α) or suppressor of cytokine signaling-1 (SOCS-1), and those in the intracellular signaling pathways including c-Jun N-terminal kinase (JNK) would be coaccelerators to hepatocarcinogenesis in HCV infection. ROS, reactive oxygen species.

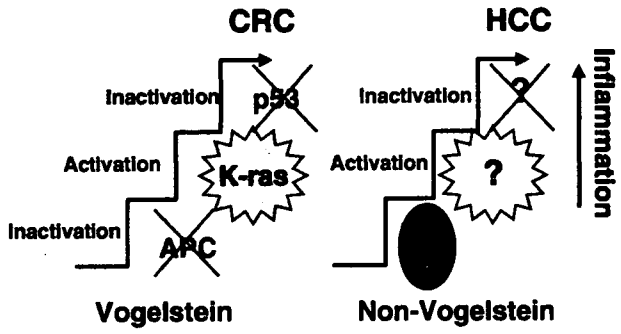

Figure 3 Mechanism of hepatitis C virus (HCV)-associated hepatocarcinogenesis. Multiple steps are required in the induction of all cancers; it would be mandatory for hepatocarcinogenesis that genetic mutations accumulate in hepatocytes. However, in HCV infection, some of these steps may be skipped in the development of hepatocellular carcinoma (HCC) in the presence of the core protein. The overall effects achieved by the expression of the core protein would be the induction of HCC, even in the absence of a complete set of genetic aberrations, required for carcinogenesis. By considering such a 'non-Vogelstein-type' process for the induction of HCC, a plausible explanation may be give for many unusual events occurring in HCV carriers. CRC, colorectal cancer; APC, *adenomatous polyposis coli*.

types of hepatitis, such as autoimmune hepatitis. The basis for the overproduction of oxidative stress may be ascribed to the mitochondrial dysfunction.[13,15] The function of the electron transfer system of the mitochondrion is suggested in association with the presence of the HCV core protein.[16] Hepatic steatosis in hepatitis C may work as fuel for oxidative stress overproduction.[14,17,18]

Other possible pathways would be the alteration of the expression of cellular genes, interacting with cellular proteins, and modulation of intracellular signaling pathways (Fig. 2). For an example, tumor necrosis factor (TNF)-α and interleukin-1β have been found to be transcriptionally activated.[19] The core protein has also been found to interact with some cellular proteins, such as retinoid X receptor (RXR)-α, which play pivotal roles in cell proliferation and metabolism.[20] The mitogen-activated protein kinase (MAPK) cascade is also activated in the liver of the core gene transgenic mouse model. The MAPK pathway, which consists of three routes, c-Jun N-terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK), is involved in numerous cellular events including cell proliferation. In the liver of the core gene transgenic mouse model prior to HCC development, only the JNK route is activated. Downstream of the JNK activation, transcription factor activating factor (AP)-1 activation is markedly enhanced.[19,21] Far downstream, both the mRNA and protein levels of cyclin D1 and CDK4 are increased (Fig. 2). The suppression by HCV core protein of the suppressor of cytokine signaling (*SOCS*)-*1*, a tumor suppressor gene, may also contribute to hepatocarcinogenesis. Thus, the HCV core protein modulates the intracellular signaling pathways and gives an advantage to hepatocytes for cell proliferation.

Such an effect of the core protein on the MAPK pathway, combined with that on oxidative stress, may explain the extremely high incidence of HCC development in chronic hepatitis C (Fig. 2).

Hepatocarcinogenesis in HCV infection: A mechanism distinct from those in other cancers

The results of our studies on transgenic mice have indicated a carcinogenic potential of the HCV core protein *in vivo*; thus, HCV may be directly involved in hepatocarcinogenesis. In research studies of carcinogenesis, the theory by Kinzler and Vogelstein has gained popularity.[22] They have proposed that the development of colorectal cancer is induced by the accumulation of a complete set of cellular gene mutations. They have deduced that mutations in the *adenomatous polyposis coli* gene for inactivation, those in *K-ras* for activation and those in the *p53* gene for inactivation accumulate, which cooperate toward the development of colorectal cancer.[22] Their theory has been extended to the carcinogenesis of other cancers as well, called 'Vogelstein-type' carcinogenesis (Fig. 3).

On the basis of the results for the induction of HCC by the HCV core protein, we would like to introduce a mechanism different from that of Kinzler and Vogelstein for hepatocarcinogenesis in HCV infection. We do allow a multistage process in the induction of all cancers; it would be mandatory for hepatocarcinogenesis that many mutations accumulate in hepatocytes. Some of these steps, however, may be skipped in the development of HCC in HCV infection to which the core protein would contribute (Fig. 3). The overall effects achieved by the expression of the viral protein would be the induction of HCC, even in the absence of a complete set of genetic aberrations, required for carcinogenesis.

By considering such a 'non-Vogelstein-type' process for the induction of HCC, a plausible explanation may be given for many unusual events happening in HCV carriers.[23] Now it does not seem so difficult as before to determine why HCC develops in persistent HCV infection at an outstandingly high incidence. Our theory may

also give an account of the non-metastatic and multicentric de novo occurrence characteristics of HCC, which would be the result of persistent HCV infection.

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Pathogenesis of HCV-associated HCC: Dual-pass carcinogenesis through activation of oxidative stress and intracellular signaling

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Overwhelming lines of epidemiological evidence have indicated that persistent infection with hepatitis C virus (HCV) is a major risk toward development of hepatocellular carcinoma (HCC). It remains controversial, however, in the pathogenesis of HCC associated with HCV, whether the virus plays a direct role or merely an indirect one. The studies using transgenic mouse models by us and others, in which the core protein of HCV has oncogenic potential, indicate that HCV is directly involved in hepatocarcinogenesis, albeit other factors such as continued cell death and regeneration associated with inflammation would play a role, as well. The downstream events of the core protein are segregated into two components. One is the augmented production of oxidative stress along with the activation of scavenging system including catalase and glutathion (GSH) in the putative preneoplastic stage with steatosis in the liver. Thus, oxidative stress production in the absence of inflammation by the core protein would partly contribute to the development of HCC. The generation of oxidative stress is estimated to originate from mitochondrial dysfunction in hepatocytes by HCV infection. The other is the alteration of intracellular signaling cascade of MAPK (JNK), AP-1, cyclin D1, and CDK4. The combination of these pathways, collective with HCV-associated alterations in lipid and glucose metabolism, would lead to the frequent development of HCC in persistent HCV infection. Our results suggest that there would be a mechanism for hepatocarcinogenesis in persistent HCV infection that is distinct from those for other cancers. Similar to the pathogenesis of other cancers, the accumulation of a set of genetic aberrations may also be necessary for multistage development of HCC. However, HCV core protein, to which an oncogenic potential is ascribed, may allow some of the multiple steps to be bypassed in hepatocarcinogenesis. Therefore, unlike other cancers, HCV infection can elicit HCC in the absence of a complete set of genetic aberrations. Such a scenario, "non-Vogelstein-type" carcinogenesis, would explain the unusually high incidence and multicentric nature of HCC development in HCV infection.

Key words: hepatitis C virus, hepatocarcinogenesis, intracellular signaling transduction, oxidative stress, transgenic mouse

INTRODUCTION

WORLDWIDE, HEPATITIS C virus (HCV) infects hundreds of millions of people persistently, and induces a spectrum of chronic liver diseases.[1] Hence, it affects society in a number of domains including medical, sociological, and economic. Hepatocellular carcinoma (HCC) has become the most frequent cause of death in individuals persistently infected with HCV. In particular, HCV has been given increasing attention because of its wide and deep penetration in the community, coupled with a very high incidence of HCC in persistent HCV infection. Once liver cirrhosis is established in hosts infected with HCV, HCC develops at a yearly rate of 5–7%.[2] Knowledge of the mechanism of HCC development in chronic HCV infection, therefore, is imminently required for the prevention of HCC.

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UNIQUENESS OF HCC DEVELOPMENT IN HCV INFECTION

HOW HCV INDUCES HCC is not yet clear, despite the finding that more than 70% of patients with HCC in Japan are infected with HCV.[1,3,4] HCV infection

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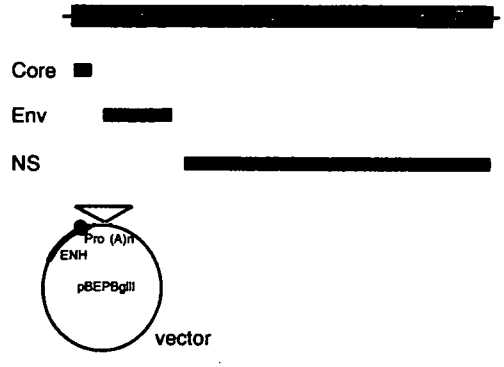

Figure 1 Hepatitis C virus (HCV) transgenic mouse lines. Among the three different transgenic mouse lines established, only the transgenic mice carrying the HCV core gene develop hepatocellular carcinoma (HCC) after an early phase with hepatic steatosis in two independent lineages. The mice transgenic for the envelope genes or non-structural genes do not develop HCC. core, core genes, env, envelope genes; NS, non-structural genes.

is also common in patients with HCC in other countries, albeit to a lesser extent. These lines of evidence obligate hepatologists to a considerable task of determining the role of HCV in hepatocarcinogenesis. Inflammation induced by HCV, manifesting in various forms of hepatitis, should be considered in a study on the carcinogenic capacity of hepatitis viruses. It has been proposed repeatedly that the necrosis of hepatocytes caused by chronic inflammation and ensuing regeneration enhances mutagenesis in host cells, the accumulation of which culminates in HCC. This theory presupposes an indirect involvement of hepatitis viruses in HCC *via* hepatic inflammation. However, this leaves specialists in hepatology with a serious question: can inflammation *per se* result in the development of HCC in such a high incidence or multicentric nature in HCV infection? The secondary role of HCV would have to be weighed against an extremely rare occurrence of HCC in patients with autoimmune hepatitis in whom severe inflammation in the liver persists indefinitely.

This background and reasoning lead to a possible activity of viral proteins for inducing HCC. This possibility has been evaluated by introducing genes of HCV into hepatocytes in culture with little success. A difficulty in using cultured cells is the carcinogenic capacity of HCV, if any, which would be weak and would take a long time to manifest. It takes 30–40 years for HCC to develop in individuals infected with HCV. Another constraint common to studies on carcinogenesis is the development of HCC by transformed cells that might have gone out of growth control and escaped surveillance of the host. Should this be the case, the analysis of transformed cells would not be sufficient for solving the mystery of carcinogenesis. On the basis of these viewpoints, we started tackling carcinogenesis in chronic viral hepatitis by transgenic mouse technology.

CORE PROTEIN OF HCV HAS ONCOGENIC ACTIVITY *IN VIVO*

AS ILLUSTRATED IN Figure 1, transgenic mouse lines with parts of the HCV genome were engineered by introducing the genes excised from the cDNA of the HCV genome of genotype 1b.[5,6] The background of the mouse lines is a C57BL/6 strain, which is known for a rare spontaneous occurrence of HCC.[7] Established are three different transgenic mouse lines, which carry the core gene, envelope genes, or non-structural genes, under the same transcriptional control element. Among these mouse lines, only the transgenic mice carrying the core gene develop HCC in two independent lineages (Fig. 1).[6] The envelope gene transgenic mice do not develop HCC, despite high expression levels of both E1 and E2 proteins.[8,9] The transgenic mice carrying the entire non-structural genes have not developed HCC.

The transgenic mice carrying the core gene express the core protein of an expected size, approximately 21 kDa, the level of which in the liver is similar to that in the liver of chronic hepatitis C patients. Early in life, these mice develop hepatic steatosis, which is a histologic characteristic of chronic hepatitis C, along with lymphoid follicle formation and bile duct damage.[10] Thus, the core gene transgenic mouse model well reproduces this feature of chronic hepatitis C. Of note, significant inflammation is not observed in the liver of this animal model. Late in life, these transgenic mice develop HCC. Most hepatic nodules disclose a pathology characterized by "nodule-in-nodule", and HCC with a low degree of differentiation develops within adenoma as well as within HCC with a higher degree of differentiation.[6] Although numerous lipid droplets are found in cells forming adenoma, as in non-tumorous cells, they are rarely observed in HCC cells. These histologic features

closely resemble those observed in HCC developing in chronic hepatitis C patients, in which prominent lipid droplets are found in small differentiated HCC and its precursors; poorly differentiated HCC without lipid droplets develops from within differentiated HCC.[6] Notably, the development of steatosis and HCC has been reproduced by other HCV transgenic mouse lines, which harbor the entire HCV genome or structural genes including the core gene.[11] These outcomes indicate that the core protein of HCV has an oncogenic potential when expressed *in vivo*.

MECHANISM OF HEPATOCARCINOGENESIS IN MOUSE MODEL FOR HCV-ASSOCIATED HCC

IT IS DIFFICULT to sort out the mechanism of carcinogenesis even for our simple model, in which only the core protein is expressed in otherwise normal liver tissue. There is a notable feature in the localization of the core protein in hepatocytes; while the core protein predominantly exists in the cytoplasm associated with lipid droplets, it is also present in the mitochondria and nuclei.[6,12] On the basis of this finding, the pathways related to these two organelles, the mitochondria and nuclei, were meticulously analyzed.

One activity of the core protein is an increased production of oxidative stress in the liver. The production of oxidative stress is increased in our transgenic mouse model in the absence of inflammation in the liver (hepatitis). This reflects a state of an overproduction of reactive oxygen species (ROS) in the liver, or predisposition to it, which is staged by the HCV core protein without any intervening inflammation.[13,14] The overproduction of oxidative stress results in the generation of deletions in the mitochondrial DNA, an indicator of genetic damage. Thus, the core protein induces oxidative stress overproduction in the absence of inflammation, and may, at least in part, contribute to hepatocarcinogenesis in HCV infection. If inflammation is induced in the liver with the HCV core protein, the production of oxidative stress is escalated to an extent that can no longer be scavenged by a physiologically antagonistic system. This indicates that the inflammation in chronic HCV infection would have a characteristic different in quality from those of other types of hepatitis, such as autoimmune hepatitis. The basis for the overproduction of oxidative stress may be ascribed to mitochondrial dysfunction.[13,15] The function of the electron transfer system of the mitochondrion is suggested in association with the presence of the HCV core protein.[16] Hepatic steatosis in hepatitis C may work as fuel for oxidative stress overproduction.[14,17,18]

Table 1 Biomolecular alterations with core protein expression observed in the transgenic mouse model

1.	Induction of cytokines including TNF-α and IL-1β[19]
2.	Activation of MAPK pathway and enhancement of AP-1 activation[19,20]
3.	Overproduction of oxidative stress or ROS in the absence of inflammation[13]
4.	Synergy of HCV core and alcohol in inducing oxidative stress and activating MAPK[13,20]
5.	Interaction of HCV core and RXR-α and PPAR-α[21]
6.	Induction of insulin resistance[17]
7.	Development of steatosis by inhibiting MTP activity[5,14,22]
8.	Interaction of HCV core and proteasome activator PA28γ[23]
9.	Inhibition of SOCS-1[24]

AP-1, activated protein-1; HCV, hepatitis C virus; IL-1β, interleukin-1β; MAPK, mitogen-activated protein kinase; MTP, microsomal triglyceride transfer protein; PPAR-α, peroxisome proliferator agonist receptor-α; ROS, reactive oxygen species; RXR-α, retinoid X receptor; SOCS-1, suppressor of cytokine signaling; TNF-α, tumor necrosis factor.

Other possible pathways are the alteration of the expression of cellular genes, interacting with cellular proteins, and modulation of intracellular signaling pathways (Table 1). For example, tumor necrosis factor (TNF)-α and interleukin-1β (IL-1β) have been found transcriptionally activated.[19] The core protein has also been found to interact with some cellular proteins, such as retinoid X receptor (RXR)-α, that play pivotal roles in cell proliferation and metabolism.[20] The mitogen-activated protein kinase (MAPK) cascade is also activated in the liver of the core gene transgenic mouse model. The MAPK pathway, which consists of three routes, c-Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK), is involved in numerous cellular events including cell proliferation. In the liver of the core gene transgenic mouse model prior to HCC development, only the JNK route is activated. Downstream in the JNK activation, transcription factor AP-1 activation is markedly enhanced.[19,21] Far downstream, both the mRNA and protein levels of cyclin D1 and CDK4 are increased. Thus, the HCV core protein modulates the intracellular signaling pathways and gives advantage for cell proliferation to hepatocytes.

Such an effect of the core protein on the MAPK pathway, combined with that on oxidative stress, may

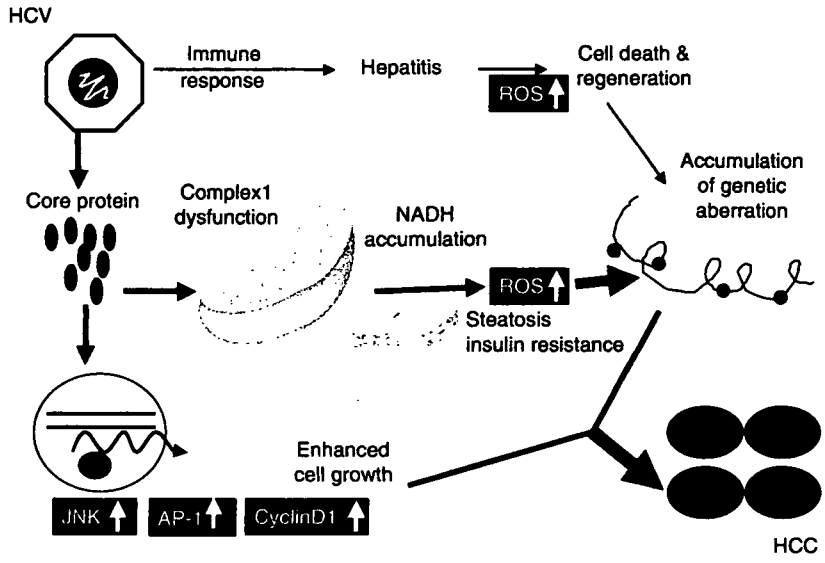

Figure 2 Mechanism of hepatitis C virus (HCV)-associated hepatocarcinogenesis. Inflammation should contribute to hepatocarcinogenesis by producing genetic aberrations via continual cell death and regeneration. In the case of HCV infection, the virus would contribute to hepatocarcinogenesis via two pathways: (i) the core protein acts on the function of mitochondrial electron transfer system, leading to the overproduction of oxidative stress. Inflammation may act synergistically with the core protein in inducing oxidative stress. The presence of steatosis and insulin resistance would enhance the production of oxidative stress; and (ii) modulation of cellular gene expression and signal transduction, which would give a growth advantage to hepatocytes. The combination of these alterations would escalate the development of hepatocellular carcinoma (HCC) in HCV infection. AP-1, activated protein-1; JNK, Jun N-terminal kinase; NADH, nicotinamide adenine dinucleotide; ROS, reactive oxygen species.

explain the extremely high incidence of HCC development in chronic hepatitis C.

HEPATOCARCINOGENESIS INDUCED BY HCV INFECTION: MECHANISM DISTINCT FROM OTHER CANCERS

THE RESULTS OF our studies on transgenic mice indicated a carcinogenic potential of the HCV core protein *in vivo*; thus, HCV may be directly involved in hepatocarcinogenesis.

In research studies of carcinogenesis, the theory by Kinzler and Vogelstein[25] has gained wide popularity. They proposed that the development of colorectal cancer is induced by the accumulation of a complete set of cellular gene mutations. They deduced that mutations in the *APC* gene for inactivation, those in *K-ras* for activation and those in the *p53* gene for inactivation accumulate, which cooperate toward the development of colorectal cancer.[25] The theory has been extended to the carcinogenesis of other cancers as well, called "Vogelstein-type" carcinogenesis (Fig. 2).

On the basis of results we obtained for the induction of HCC by the HCV core protein, we introduce a mechanism different from that of Kinzler and Vogelstein[25] for hepatocarcinogenesis in HCV infection. We allow multistages in the induction of all cancers; it would be mandatory for hepatocarcinogenesis that many mutations accumulate in hepatocytes. Some of these steps, however, may be skipped in the development of HCC in HCV infection to which the core protein would contribute (Fig. 3). The overall effects achieved by the expression of the viral protein would be the induction of HCC, even in the absence of a complete set of genetic aberrations, required for carcinogenesis.

By considering such a "non-Vogelstein-type" process for the induction of HCC, a plausible explanation may be given for many unusual events happening in HCV carriers.[26] Now it does not seem so difficult as before to determine why HCC develops in persistent HCV infection at an outstandingly high incidence. Our theory may also give an account of the non-metastatic and multicentric *de novo* occurrence characteristics of HCC, which would be the result of persistent HCV infection.

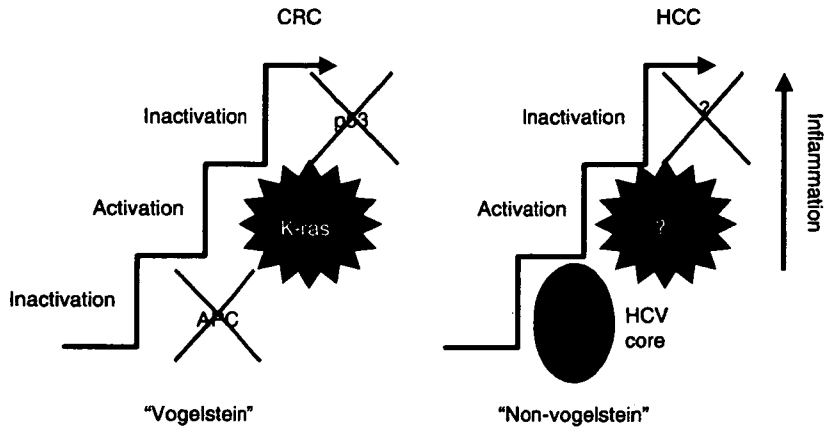

Figure 3 Hepatitis C virus (HCV)-associated hepatocarcinogenesis is a non-Vogelstein-type. Multiple steps are required in the induction of all cancers; it would be mandatory for hepatocarcinogenesis that genetic mutations accumulate in hepatocytes. However, in HCV infection, some of these steps may be skipped in the development of hepatocellular carcinoma (HCC) in the presence of core protein. Overall effects achieved by the expression of core protein would be the induction of HCC, even in the absence of a complete set of genetic aberrations, required for carcinogenesis. By considering such a "non-Vogelstein-type" process for the induction of HCC, a plausible explanation may be given for many unusual events in HCV carriers. CRC, colorectal cancer.

CONFLICT OF INTEREST

NO CONFLICT OF interest has been declared by the author.

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