0.25 mM PMSF, and 10 mg/ml aprotinin). The lysate was incubated for 3 h at 4°C with purified anti-PPARα antibody. The immune complexes were precipitated with *Staphylococcus aureus* protein A bound to agarose beads. After the precipitates had been washed in RIPA buffer, the labeled proteins were resolved by 10% SDS-PAGE and visualized by autoradiography.

Analysis of fatty acid uptake ability. Assays for fatty acid uptake were carried out according to a method reported by Graulet et al. (52) with minor modifications. Briefly, 3 mice in each group were fasted overnight. Livers were removed quickly, rinsed in ice-cold saline solution, and cut into 500-μm thick slices with an Oxford Vibratome (Oxford Laboratories). Approximately 150 mg of fresh liver (6–8 liver slices) was placed on stainless steel grids positioned in a 25-ml flask equipped with suspended plastic center wells (Kontes) and incubated in RPMI-1640 medium (Sigma-Aldrich) devoid of fatty acids for 2 h at 37°C. The medium was then replaced with fresh RPMI-1640 medium supplemented with an antibiotic-antimycotic cocktail and 0.8 mM [1-^{14}C]palmitic acid (4 mCi/mmol) (American Radiolabeled Chemicals) complexed to BSA (palmitic acid:albumin molar ratio of 4:1). After a 7-h incubation, the medium was collected and slices were washed with 2 ml of saline solution and homogenized in Tris buffer (25 mM Tris-HCl, pH 8.0; 50 mM NaCl). Fatty acid uptake ability was calculated as the sum of palmitic acid converted to CO_2 and ketone bodies with that incorporated into total cellular lipids after incubation. For measurement of CO_2 production by the liver slices, the center wells were placed into scintillation vials containing 4 ml of scintillation cocktail, and radioactivity was counted. For measurement of ketone body generation, aliquots of medium (500 μl) and liver homogenates (250 μl) were treated with ice-cold perchloric acid to make final concentrations of 200 mM and were centrifuged at 3,000 *g* for 20 min at 4°C. Aliquots of the supernatant containing the ketone bodies were introduced into the scintillation vials, and radioactivity was counted. Total cellular lipids were extracted from the liver homogenates according to a modified method developed by Folch et al. (53), collected into scintillation vials, and evaporated to dryness under an air stream; radioactivity was then counted. The experiment was repeated 3 times, and palmitic acid uptake ability was expressed as fold changes relative to that of *Ppara*$^{+/+}$ nontransgenic mice.

Other methods. To determine the hepatic content of lipids and lipid peroxides, lipids were extracted according to a method by Folch et al. (53). Triglycerides and free fatty acids were measured with a Triglyceride E-test kit and a NEFA C-test kit (Wako), respectively. Lipid peroxides (malondialdeyde and 4-hydroxyalkenals) were measured using an LPO-586 kit (OXIS International). Hepatic β-oxidation activity was determined as described previously (16). Hepatic caspase 3 activity was measured as described elsewhere (54). Plasma glucose and insulin levels were determined using a Glucose CII-test kit (Wako) and a mouse insulin ELISA kit (U-type, AKRIN-031; Shibayagi), respectively.

Statistics. Statistical analysis was performed with a 2-tailed Student's *t* test for quantitative variables or with a chi-square test for qualitative variables. Quantitative data are expressed as the mean ± SD. $P < 0.05$ was considered to be statistically significant.

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Hepatitis C virus core protein induces spontaneous and persistent activation of peroxisome proliferator-activated receptor α in transgenic mice: Implications for HCV-associated hepatocarcinogenesis

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Persistent infection of hepatitis C virus (HCV) can lead to a high risk for hepatocellular carcinoma (HCC). HCV core protein plays important roles in HCV-related hepatocarcinogenesis, because mice carrying the core protein exhibit multicentric HCCs without hepatic inflammation and fibrosis. However, the precise mechanism of hepatocarcinogenesis in these transgenic mice remains unclear. To evaluate whether the core protein modulates hepatocyte proliferation and apoptosis *in vivo*, we examined these parameters in 9- and 22-month-old transgenic mice. Although the numbers of apoptotic hepatocytes and hepatic caspase 3 activities were similar between transgenic and nontransgenic mice, the numbers of proliferating hepatocytes and the levels of numerous proteins such as cyclin D1, cyclin-dependent kinase 4 and c-Myc, were markedly increased in an age-dependent manner in the transgenic mice. This increase was correlated with the activation of peroxisome proliferator-activated receptor α (PPARα). In these transgenic mice, spontaneous and persistent PPARα activation occurred heterogeneously, which was different from that observed in mice treated with clofibrate, a potent peroxisome proliferator. We further demonstrated that stabilization of PPARα through a possible interaction with HCV core protein and an increase in nonesterified fatty acids, which may serve as endogenous PPARα ligands, in hepatocyte nuclei contributed to the core protein-specific PPARα activation. In conclusion, these results offer the first suggestion that HCV core protein induces spontaneous, persistent, age-dependent and heterogeneous activation of PPARα in transgenic mice, which may contribute to the age-dependent and multicentric hepatocarcinogenesis mediated by the core protein.

Key words: cell-cycle regulator; peroxisome; nuclear stabilization; heterogeneous PPARα activation

Hepatitis C virus (HCV) is one of the major causes of chronic hepatitis, and persistent infection with this virus can lead to a high incidence of hepatocellular carcinoma (HCC).[1,2] The prevalence of HCC because of chronic HCV infection has increased over the past two decades,[3,4] and chronic HCV infection has therefore been recognized as a serious disease. However, the precise mechanism of hepatocarcinogenesis during chronic HCV infection remains unclear.

Many experiments using cell culture systems have suggested the possibility that HCV core protein itself can modulate various cellular functions and can be directly linked to the development of HCV-related HCC.[5] For example, HCV core protein transforms rat embryo fibroblasts to a tumorigenic phenotype in cooperation with the H-*ras* oncogene,[6] suppresses *c-myc*-related apoptosis[7] and transcription of the *p53* gene,[8] interacts with a variety of proteins, including helicase, lymphotoxin-β receptor, or dead box protein, and modulates their functions.[9] We further established transgenic mouse lines carrying the HCV core gene, in which the core protein is constitutively expressed in the liver at levels similar to that found in chronic hepatitis C patients.[10] These mice exhibited multicentric hepatic adenomas, and developed HCCs in an age-dependent manner.[11] The livers of these mice were almost free of inflammation, necrosis and fibrosis,[10,11] suggesting that the core protein itself has a hepatocarcinogenic potential *in vivo*. However, the molecular mechanism of the development of HCC in the transgenic mice has not been fully understood.

In the livers of HCV core gene transgenic mice, an age-dependent increase in oxidative stress and resultant DNA damage were found,[12] and these effects may contribute to or facilitate the development of HCC. Another possible mechanism of hepatocarcinogenesis is continuous enhancement of hepatocyte proliferation. Cell proliferation and apoptosis are highly regulated processes for maintaining homeostasis in many organs, and during the carcinogenic process, sustained imbalance generally precedes cancer.[13,14] For example, in patients with chronic HCV infection, high hepatocyte proliferative activity relative to apoptosis may reliably predict a new development of HCC.[15] However, there is no information about whether or not hepatocyte proliferation accelerates persistently in mice carrying the HCV core gene, and no information about how the core protein promotes hepatocyte proliferation *in vivo*. In the current study, we began to examine changes in the parameters of hepatocyte proliferation and apoptosis in the transgenic mice.

Material and methods

Animals and treatments

HCV core gene transgenic mice on a C57BL/6N genetic background were produced as described earlier.[10] Because HCC developed preferentially in male transgenic mice,[11] 9- and 22-month-old male mice ($n = 8$ for either age group) were adopted. Sex- and age-matched nontransgenic mice ($n = 8$ for either age group) were used as controls. These mice were fed an ordinary diet and were treated in a specific pathogen-free state according to the institutional guidelines. For additional experiment, male wild-type mice fed a control diet containing 0.5% clofibrate for 2 weeks ($n = 8$) were used. All mice were killed by cervical dislocation and the livers were excised. When a hepatic tumor was present, it was removed and the remaining liver tissue was used. All experiments were performed in accordance with animal study protocols approved by the Shinshu University School of Medicine.

Abbreviations: AOX, acyl-CoA oxidase; CDK, cyclin-dependent kinase; DAB, 3,3'-diaminobenzidine; FITC, fluorescein isothiocyanate; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; L-FABP, liver-type fatty acid-binding protein; NEFA, nonesterified fatty acid; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; PMSF, phenylmethylsulfonyl fluoride; PPAR, peroxisome proliferator-activated receptor; PT, peroxisomal thiolase; RXR, retinoid X receptor; SDS, sodium dodecyl sulfate; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling.

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Preparation of hepatocyte nuclear fraction

Approximately 200 mg of liver tissues was transferred to a chilled Dounce homogenizer (Wheaton, Milliville, NJ) and homogenized on ice by 30 strokes in 1.2 mL of nuclei buffer [300 mM sucrose in 10 mM Tris-HCl, pH 7.4, 15 mM NaCl, 5 mM $MgCl_2$ and 0.25 mM phenylmethylsulfonyl fluoride (PMSF)]. The homogenate was filtered through gauze and centrifuged at 4,500*g* for 5 min at 4°C. The resulting pellet was resuspended, layered over 2 mL of nuclei buffer containing 2 M sucrose, and centrifuged at 23,000*g* for 1 hr at 4°C. The pellet obtained after ultracentrifugation was resuspended in 250 μL of nuclei buffer and used as the nuclear fraction. Preparation of nuclear fraction from isolated hepatocytes was performed as described elsewhere.[16]

Immunoblot analysis

Protein concentration was measured colorimetrically by a BCA™ Protein Assay kit (Pierce, Rockford, IL). For analysis of fatty acid-metabolizing enzymes and protein, whole liver lysate (10–20 μg protein) was subjected to 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.[17] For analysis of other proteins, hepatocyte nuclear fraction (100 μg protein) or whole liver lysate (200–300 μg protein) was subjected to electrophoresis. After electrophoresis, the proteins were transferred to nitrocellulose membranes, which were incubated with the primary antibody, followed by alkaline phosphatase-conjugated goat anti-rabbit or anti-mouse IgG. The origin of the primary rabbit polyclonal antibodies against fatty acid-metabolizing enzymes and protein was described earlier.[17] For immunoblot analysis of peroxisome proliferator-activated receptor α (PPARα), a polyclonal anti-mouse antibody[18] or commercial antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used. The antibodies against cell-cycle regulators and oncogene products were purchased commercially (Santa Cruz Biotech.).[19] Equal loading of the protein obtained from whole liver lysate and nuclear fraction was confirmed by reprobing the membranes with an antibody against β-actin and histone H1, respectively. The band intensity of nuclear PPARα was quantified densitometrically, normalized to that of histone H1, and subsequently expressed as the fold changes relative to that of 9-month-old nontransgenic mice.

mRNA analysis

Total liver RNA was extracted with an RNeasy Mini Kit™ (Qiagen, Valencia, CA). Five microgram of RNA was electrophoresed on 1.1 M formaldehyde-containing 1% agarose gels and transferred to nylon membranes by capillary blotting in 20× SSC buffer (3 M NaCl and 300 mM sodium citrate, pH 7.0) overnight. The membranes were hybridized with ^{32}P-labeled cDNA probes. The blots were exposed to a phosphorimager screen cassette and were analyzed using a Molecular Dynamics Storm 860 Phosphorimager system (Sunnyvale, CA). The origin of the cDNA probes has been described elsewhere.[17–19] Northern blot of β-actin was used as the internal control. The blot intensity was quantified, normalized to that of β-actin and subsequently expressed as the fold changes relative to that of 9-month-old nontransgenic mice.

Pulse-label and pulse-chase experiment

Parenchymal hepatocytes were isolated from transgenic and control mice by the modified *in situ* perfusion method.[20] After perfusion with 0.05% collagenase solution (Wako, Osaka, Japan), the isolated hepatocytes were washed thrice by means of differential centrifugation and the dead cells removed by density gradient centrifugation on Percoll (Amersham Pharmacia Biotech, Buckinghamshire, UK). The live hepatocytes were washed and suspended in William's E medium containing 5% fetal bovine serum. When the viability of the isolated hepatocytes exceeded 85% as determined by the trypan blue exclusion test, the following experiments were conducted. The isolated hepatocytes were washed twice and incubated in methionine-free medium containing 5% dialyzed fetal bovine serum for 1 hr at 37°C. The medium was replaced with the same medium containing 300 μCi/mL of [^{35}S]methionine (Amersham Pharmacia Biotech.). After 3-hr of incubation, the labeled medium was changed to the standard medium and the preparation was chased for 4, 8 or 16 hr. The labeled cells were washed, homogenized and centrifuged for preparation of the nuclear fraction. The levels of radioactivity in the homogenates of the pulse-labeled preparations were similar between the transgenic and the nontransgenic mice, suggesting that the [^{35}S]methionine uptake capacity in the former hepatocytes is similar to that in the latter. The nuclear fraction was lysed in RIPA buffer [10 mM Tris-HCl, pH 7.4, 0.2% sodium deoxycholate, 0.2% Nonidet P-40, 0.1% SDS, 0.25 mM PMSF, 10 μg/mL aprotinin]. The lysate was incubated for 3 hr at 4°C with purified anti-PPARα antibody. The immune complexes were precipitated with *Staphylococcus aureus* protein A bound to agarose beads. After the precipitates had been washed in RIPA buffer, the labeled proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. The nuclear fractions of the pulse-labeled preparations were also used for immunoblot analysis of PPARα.

Affinity chromatography for PPARα complex

All procedures were performed at 4°C. The nuclear fraction from the mouse liver was mixed with a 4-fold volume of a solution containing 12.5 mM potassium phosphate, pH 7.5, 25 mM NaCl, 0.25% Tween 20 and 0.1 mM PMSF. The mixture was briefly sonicated with a microsonicator, the Powersonic Model 50 (Yamato, Tokyo, Japan), and then centrifuged at 100,000*g* for 20 min. The supernatant was applied to an immobilized anti-PPARα IgG column (1.0 × 4.0 cm^2), prepared with the Affigel HZ Immunoaffinity kit^R (Bio-Rad, Hercules, CA) and equilibrated with 10 mM potassium phosphate, pH 7.5, 20 mM NaCl and 0.2% Tween 20. The solution was again passed through the column and this was repeated at least thrice. The column was washed and the elution performed with 150 mM sodium citrate, pH 3.0, and 200 mM NaCl, in a total volume of 2 mL. The eluate was resolved by 10 and 15% SDS-polyacrylamide gel electrophoresis for PPARα and the HCV core protein, respectively. The core protein expressed in COS cells was used as a positive marker.[21] The monoclonal antibody against the core protein was purchased commercially (ViroGen, Watertown, MA).

Cytochemical staining of peroxisomes

Liver peroxisome proliferation was evaluated by using 3,3′-diaminobenzidine (DAB) staining for catalase according to the method of Novikoff and Goldfischer with minor modifications.[22] Small pieces of liver were fixed with 2% glutaraldehyde in 100 mM sodium cacodylate buffer, pH 7.2, for 3 hr at 4°C, rinsed with sodium cacodylate buffer and cut into 100-μm sections with a Lancer^R Vibratome 1000 (Lancer, Bridgeton, MO). These sections were then incubated for 1 hr at 37°C in the DAB reaction medium (0.2% DAB tetrahydrochloride in 50 mM propanediol, pH 9.7, 5 mM KCN, 0.05% H_2O_2) and postfixed with 1% OsO_4 in 100 mM sodium phosphate, pH 7.4 for 1 hr. The sections were dehydrated through a graded series of ethanol and acetone treatments and embedded in Epok 812 (Oken, Tokyo, Japan). One micrometer sections were prepared, counterstained with 0.1% toluidine blue solution and examined by light microscopy. For electron microscopic examination, 0.1-μm sections were cut with a diamond knife, collected on grid meshes, stained with lead citrate and uranyl acetate and visualized with a JEM 1200EX II electron microscope (JEOL, Tokyo, Japan) at an accelerating voltage of 80 keV.

Morphometry of hepatic peroxisomes

Morphometric analysis of DAB-stained peroxisomes was carried out using electron photomicrographs. For each mouse, 10 independent fields in the pericentral area of liver lobuli were photomicrographed at an original magnification of 4,000×. At this magnification, peroxisomes smaller than 450 nm were clearly

identified. Peroxisomes were easily detected because of their high contrast because of the positive DAB reaction. In each frame, the number of peroxisomal profiles and the area of each individual profile were determined. The numerical density and volume density of peroxisomes were calculated using the following equations: numerical density (number/μm^2) = $N_P/(A_T - A_{empty})$, and volume density (%) = $A_{TP}/(A_T - A_{empty}) \times 100$, where N_P is the peroxisome number in the test area, A_T is the test area, A_{empty} is the area of the vascular and biliary lumens and that of the hepatocyte nuclei and lipid droplets, and A_{TP} is the area of total peroxisomal profiles in the test area. The area was measured with a Luzex AP image analyzer (Nireco, Tokyo, Japan).

Immunofluorescence staining

Liver samples were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), embedded in Tissue-Tek O.C.T compound™ (Sakura Finetek, Torrance, CA) and frozen. Frozen liver 5-μm sections were prepared, washed with PBS, blocked with bovine serum albumin for 1 hr and incubated overnight with rabbit polyclonal antibodies against cyclin D1 (1:50 dilution)[19] and PPARα (1:100 dilution),[18] and with mouse monoclonal antibody against proliferating cell nuclear antigen (PCNA) (1:100 dilution).[19] After 5 washes with PBS, these sections were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) or donkey anti-mouse IgG (Dako). The sections were mounted and viewed with an Olympus Fluoview confocal laser scanning microscope (Olympus, Tokyo, Japan). Two-thousand hepatocyte nuclei were examined for each mouse, and the number of hepatocyte nuclei stained with the antibodies against cyclin D1, PPARα and PCNA was counted and expressed as a percentage.

Assessment of apoptotic hepatocytes

Liver samples were cut into small pieces and then fixed in 4% paraformaldehyde in PBS. These samples were dehydrated, embedded in paraffin and cut into 4-μm sections. The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay was performed using a MEBSTAIN Apoptosis Kit II (Medical and Biological Laboratories, Nagoya, Japan). The number of apoptotic hepatocytes in 2,000 hepatocytes was counted for each mouse, and expressed as a percentage.

Other methods

Hepatic caspase 3 activity was measured as described elsewhere.[23] For analysis of the nuclear contents of nonesterified fatty acids (NEFAs), ~150 μL of the hepatocyte nuclear fraction, containing 1–2 mg of protein, was treated with a microsonicator. Lipid extraction was performed according to a modification of the method developed by Folch *et al.*[24] and the nuclear content of NEFAs was measured with a *NEFA* C-test kit™ (Wako).

Statistical analysis

Statistical analysis was performed by means of Student's *t*-test. The results are expressed as the mean ± standard deviation. A probability value of less than 0.05 was considered to be statistically significant.

Results

Accelerated hepatocyte proliferation in HCV core gene transgenic mice

To evaluate hepatocyte proliferative activity, PCNA-positive hepatocytes were counted in male transgenic mice and nontransgenic mice. Although hepatic inflammation and hepatocyte necrosis were not detected in either group, the numbers of PCNA-positive hepatocytes were significantly increased in the 9-month-old transgenic mice compared with the 9-month-old nontransgenic mice (Fig. 1*a*). The increase was more significant in the

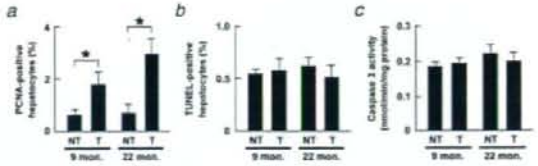

FIGURE 1 – Increase in hepatocyte proliferative activity. (*a*) The number of PCNA-positive hepatocytes. Two-thousand hepatocyte nuclei were examined for each mouse, and the number stained with anti-PCNA antibody was counted. Results are expressed as the mean ± standard deviation (n = 8). *, $p < 0.05$ between the transgenic mice and the nontransgenic mice; NT, nontransgenic mice; T, transgenic mice; 9 mon, 9-month-old mice; 22 mon, 22-month-old mice. (*b*) The number of apoptotic hepatocytes. The number of TUNEL-positive hepatocytes in 2,000 hepatocytes was determined for each mouse. Results are expressed as the mean ± standard deviation (n = 8). (*c*) Caspase 3 activity. Results are expressed as the mean ± standard deviation (n = 8).

22-month-old transgenic mice (Fig. 1*a*). The numbers of PCNA-positive hepatocytes in the 22-month-old transgenic mice corresponded with those in HCV polyprotein-expressing transgenic mice with HCC.[25] On the other hand, the parameters of apoptosis, *i.e.*, the numbers of TUNEL-positive hepatocytes and hepatic caspase 3 activity, remained unchanged between the 2 groups at the same ages (Figs. 1*b* and 1*c*). These results suggest that spontaneous hepatocyte proliferation occurs as early as the age of 9 months and persists for a long time in HCV core gene transgenic mice.

Simultaneous induction of cell-cycle regulators and oncogene products in HCV core gene transgenic mouse livers

To examine the changes in the expression of proteins associated with hepatocyte division, the livers of the 9- and 22-month-old mice were subjected to immunoblot analysis. The levels of many proteins including cell-cycle regulators [cyclin-dependent kinase (CDK) 1, 2 and 4, cyclin D1 and E, and PCNA], and oncogene products (c-Myc, c-Fos and c-Ha-Ras) were significantly higher in the 22-month-old transgenic mice than in the control mice (Fig. 2). The levels of CDK inhibitors such as p16 and p21 were similar between the 2 groups. Similar results were obtained from the 9-month-old transgenic mice (data not shown). Time course changes in the expression of key G1-S checkpoint regulators, cyclin D1 and CDK4, are shown in Figure 3*a*. The simultaneous increase in the expression of cyclin D1 and CDK4 in the transgenic mice was continuous and more pronounced with age. Northern blot analysis revealed that the increase of these proteins occurred at the transcriptional level (Figs. 3*b* and 3*c*). Thus, these results reveal that various proteins which accelerate cell-cycle progression were induced simultaneously, persistently and age-dependently in the transgenic mice.

Correlative induction of PPARα targets in HCV core gene transgenic mouse livers

As shown in Figure 2, the expression of many kinds of cell-cycle regulators and oncogene products is known to be induced by the functional activation of PPARα.[19,26–30] To investigate whether PPARα is activated in the livers of transgenic mice, the expression of representative PPARα target genes,[30] acyl-CoA oxidase (AOX), peroxisomal thiolase (PT) and liver-type fatty acid-binding protein (L-FABP), was examined. As demonstrated in Figure 3*a*, the levels of AOX, PT, and L-FABP were increased in the 9-month-old transgenic mice compared with the nontransgenic mice, and the increase was more pronounced in the 22-month-old transgenic mice. Northern blot analysis demonstrated that the increase in these PPARα targets was based on the increase in the transcriptional activity (Figs. 3*b* and 3*c*). The increase in the

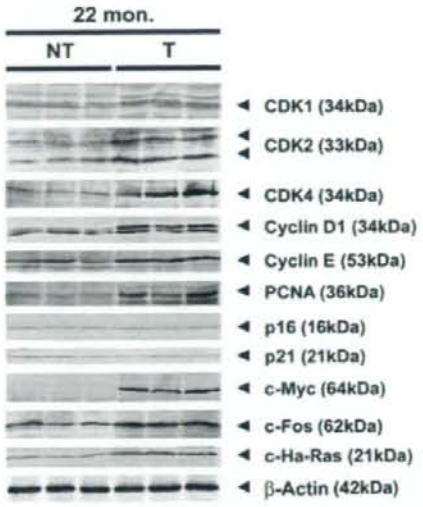

FIGURE 2 – Immunoblot analysis of cell-cycle regulators and oncogene products. Whole liver lysate (200 μg) was loaded in each lane. The band of β-actin was used as the loading control. The apparent molecular weight is indicated in parentheses. 22 mon, 22-month-old mice; NT, nontransgenic mice; T, transgenic mice.

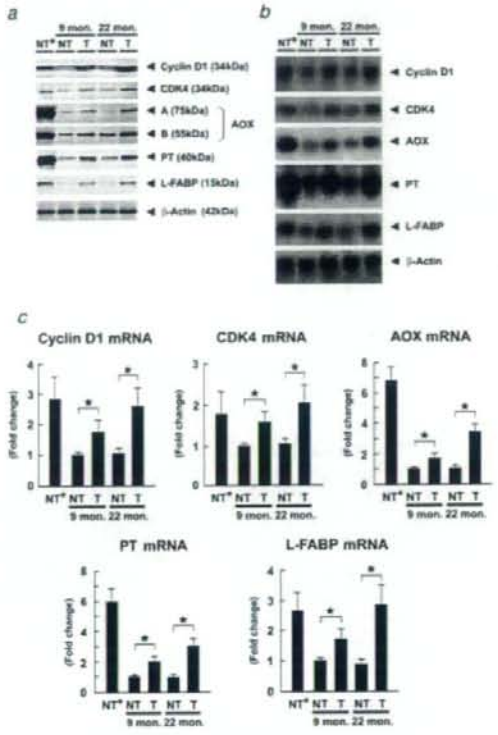

FIGURE 3 – Analysis of PPARα-regulated proteins. (*a*) Immunoblot analysis of cell-cycle regulators and fatty acid-metabolizing enzymes and proteins. Since no significant individual differences in the same mouse group were found in the preliminary experiments, 10 mg of liver pieces prepared from each mouse (n = 8/group) was mixed and homogenized. Whole liver lysate (200 μg for cyclin D1 and CDK4, and 20 μg for others) was loaded in each lane. The band of β-actin was used as the loading control. Results are representative of 4 independent experiments. The apparent molecular weight is indicated in parentheses. 9 mon, 9-month-old mice; 22 mon, 22-month-old mice; NT, nontransgenic mice; T, transgenic mice; NT*, nontransgenic mice treated with a control diet containing 0.5% clofibrate for 2 weeks; A and B, full-length and truncated AOX, respectively. (*b*) Northern blot analysis concerning the proteins in (*a*). Ten milligram of liver pieces from each mouse (n = 8/group) was mixed and homogenized, and total liver RNA was extracted. Hepatic RNA (5 μg) was separated on a denaturing gel, transferred to membranes and hybridized with the indicated ^{32}P-labeled cDNA probes. The blot of β-actin was used as the internal control. Results are representative of 4 independent experiments. (*c*) Quantification of hepatic mRNA levels. The mRNA level was quantified using a phosphorimager, normalized to that of β-actin, and subsequently normalized to that of 9-month-old nontransgenic mice. Results were obtained from 4 independent experiments and expressed as the mean ± standard deviation. Abbreviations are identical with those in (*b*). *, $p < 0.05$ between the transgenic mice and the nontransgenic mice.

mRNA expression of AOX, PT and L-FABP corresponded exactly with that of cyclin D1 or CDK4 (Figs. 3*b* and 3*c*). Therefore, these results demonstrate the strong correlation between continuous and age-dependent induction of cell-cycle regulators and functional activation of PPARα in these transgenic mice. Furthermore, the induction of these 5 proteins was also observed in wild-type mice treated with clofibrate, a potent PPARα activator; however, the degree of the induction of AOX and PT in the transgenic mice was smaller than that in the clofibrate-treated wild-type mice (Fig. 3), suggesting that the PPARα activation found in the transgenic mice was not as intense as that in the mice treated with clofibrate.

Histological evaluation of PPARα activation

An increase in the numbers of peroxisomes is associated with PPARα activation.[18] To determine whether peroxisome proliferation occurs in the HCV core gene transgenic mice, cytochemical staining for peroxisomal catalase was performed. A scattered distribution of hepatocytes with numerous peroxisomes was observed in the 9-month-old transgenic mice (Fig. 4*a*). Such hepatocytes were also found in the 22-month-old transgenic mouse livers (Fig. 4*a*). In contrast, almost all of the hepatocytes in the clofibrate-treated mice showed significant peroxisome proliferation (Fig. 4*a*). To quantitatively evaluate the degree of peroxisome proliferation, morphometric analysis of peroxisomes was conducted. The numerical density and volume density were significantly increased in the transgenic mice compared with those in the nontransgenic mice (Fig. 4*b*). The volume density, the most reliable parameter of peroxisome proliferation, was increased age-dependently in the transgenic mice, but the degree of the increase was not as prominent as that observed in mice with clofibrate administration (Fig. 4*b*). The finding that only some hepatocytes in the transgenic mice presented a marked peroxisome proliferation (Fig. 4*a*) is noteworthy, since it seems to correlate with the finding that intense expression of the core protein was observed only in particular hepatocytes.[10] These histological analyses reveal that spontaneous, continuous and age-dependent peroxisome proliferation and PPARα activation occur heterogeneously in the transgenic mouse livers, which is different from the response observed in the mice receiving clofibrate treatment.

Appearance of PPARα-, and cyclin D1-positive hepatocytes

We tried to detect abnormal hepatocytes to clarify the mechanism of hepatocarcinogenesis in the transgenic mice. On PPARα immunofluorescence staining, PPARα was primarily detected in the cytoplasm of the nontransgenic mice and the clofibrate-administered mice. Some hepatocytes having nuclei positively stained

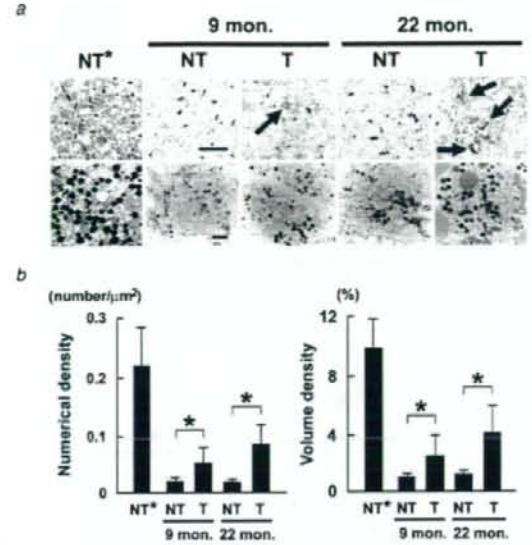

FIGURE 4 – Cytochemical staining for hepatic peroxisomes. (*a*) Light and electron photomicrographs of DAB-stained liver tissues. Peroxisomes are detected as darkly stained particles. The arrows in upper panels indicate hepatocytes showing profound peroxisome proliferation. The bars in the light and electron photomicrographs of 9-month-old nontransgenic mice indicate 50 and 2 μm, respectively. 9 mon, 9-month-old mice; 22 mon, 22-month-old mice; NT, nontransgenic mice; T, transgenic mice; NT*, nontransgenic mice treated with a control diet containing 0.5% clofibrate for 2 weeks. (*b*) Morphometric analysis of hepatic peroxisomes. The number of peroxisomes and the area of each individual peroxisome profile were measured in 10 photomicrographs for each mouse, and morphometric parameters such as numerical density and volume density were calculated. Results are expressed as the mean ± standard deviation ($n = 8$). Abbreviations are identical with those in (*a*). *, $p < 0.05$ between the transgenic mice and the nontransgenic mice.

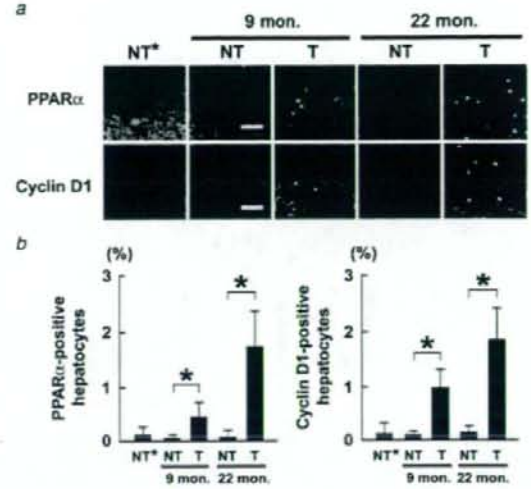

FIGURE 5 – Immunofluorescence staining for PPARα and cyclin D1. (*a*) Immunofluorescence staining using antibodies against PPARα and cyclin D1. The bars in the photomicrographs of 9-month-old nontransgenic mice indicate 50 μm. 9 mon, 9-month-old mice; 22 mon, 22-month-old mice; NT, nontransgenic mice; T, transgenic mice; NT*, nontransgenic mice treated with a control diet containing 0.5% clofibrate for 2 weeks. (*b*) The number of PPARα-, or cyclin D1-positive hepatocytes. Two-thousand hepatocyte nuclei were examined for each mouse, and the number of nuclei intensively stained with anti-PPARα or anti-cyclin D1 antibody was counted. Results are expressed as the mean ± standard deviation ($n = 8$). Abbreviations are identical with those of (*a*). *, $p < 0.05$ between the transgenic mice and the nontransgenic mice.

by anti-PPARα antibody were detected only in the transgenic mice (Fig. 5*a*). Similar to the case of PPARα, the hepatocytes having nuclei stained intensively by anti-cyclin D1 antibody were found only in the transgenic mice (Fig. 5*a*). A few hepatocytes stained by anti-CDK4 antibody were also observed only in the transgenic mice (data not shown). The frequency of appearance of PPARα-, or cyclin D1-positive hepatocytes was increased with age (Figs. 5*a* and 5*b*). Thus, the appearance of these specific hepatocytes in the transgenic mice seemed to be, at least in part, associated with sustained, age-dependent and heterogeneous PPARα activation in the transgenic mice.

Changes in PPARα levels

Since the expression of PPARα is known to be enhanced by its activation,[18,30] the quantitative change in PPARα was evaluated. The nuclear PPARα level in the transgenic mice was increased age-dependently, as expected (Figs. 6*a*, upper panel and 6*b*), but the PPARα level in the whole liver lysate remained unchanged (data not shown). The increase in nuclear PPARα in the transgenic mice was smaller than that in the clofibrate-treated wild-type mice (Figs. 6*a*, upper panel and 6*b*). Northern blot analysis revealed a higher PPARα mRNA level in the clofibrate-treated mice than in the controls, although this parameter in the transgenic mouse groups of each age was similar to that in the controls (Figs. 6*a*, lower panel and 6*b*). These results indicate that the increase in nuclear PPARα in the transgenic mice occurs mainly at the post-transcriptional level, which is distinct from that observed in the clofibrate-treated wild-type mice.

Stabilization of PPARα through a possible interaction with HCV core protein in hepatocyte nuclei

The increased stability of PPARα in hepatocyte nuclei is thought to be one of the possible causes of a disproportional increase in the nuclear PPARα level. To examine this possibility, a pulse-chase experiment was performed using isolated hepatocytes. The half-life of nuclear PPARα was ~7 hr in the control mice and 12.5 hr in the transgenic mice (Fig. 7*a*). In addition, the intensity of the labeled PPARα band (P in Fig. 7*a*, upper panels) in the control mice was similar to that in the transgenic mice. The finding that the [^{35}S]methionine uptake in the hepatocytes from the control mice was similar to that from the transgenic mice suggests that the increase in nuclear PPARα in the hepatocytes from the transgenic mice (Fig. 7*a*, lower right panel), as well as that *in vivo* (Fig. 6*a*, upper panel), is not because of the increased PPARα transfer into the nucleus.

In the transgenic mice, HCV core protein accumulated in the nuclei, as evidenced by immunoelectron microscopy,[11] suggesting a possible interaction of the core protein with PPARα in the nuclei. We therefore examined this possibility by anti-PPARα IgG affinity chromatography. When proteins combining with PPARα in hepatocyte nuclei were subjected to immunoblot analysis, the core protein was clearly detected (Fig. 7*b*). This result suggests the possibility of complex formation between the HCV core protein and PPARα, which is consistent with an interaction of the core protein with retinoid X receptor (RXR) α,[31] an essential heterodimeric partner of PPARα.[32] Thus, HCV core protein may

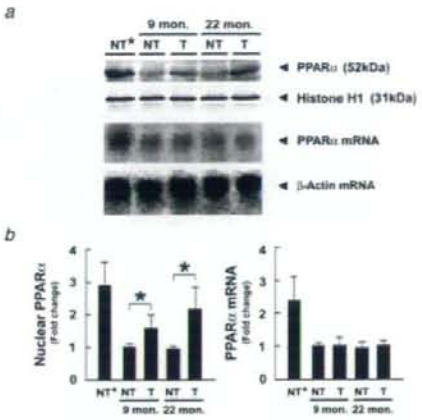

FIGURE 6 – Analysis of PPARα. (*a*) (Upper panels) Immunoblot analysis of nuclear PPARα. Since few individual differences in the same mouse group were found in the preliminary experiments, 30 mg of liver pieces from each mouse (n = 8/group) was mixed and homogenized to prepare the nuclear fraction. One-hundred microgram of nuclear protein was separated on 10% SDS-polyacrylamide gel, transferred to nitrocellulose membranes and reacted with antibody against PPARα. The band of histone H1 was used as the loading control. Results are representative of 4 independent experiments. The apparent molecular weight is indicated in parentheses. 9 mon, 9-month-old mice; 22 mon, 22-month-old mice; NT, nontransgenic mice; T, transgenic mice; NT*, nontransgenic mice treated with a control diet containing 0.5% clofibrate for 2 weeks. (Lower panels) Northern blot analysis of PPARα. A sample used in Figure 3*b* was adopted. Hepatic RNA (5 μg) was electrophoresed and hybridized with cDNAs for PPARα and β-actin, respectively. Results are representative of 4 independent experiments. (*b*) Quantification of nuclear PPARα levels and PPARα mRNA levels. The nuclear PPARα level was quantified densitometrically and normalized to the histone H1 level. The mRNA level of PPARα was quantified using a phosphorimager and normalized to that of β-actin. Values were subsequently normalized to those of 9-month-old nontransgenic mice. Results were obtained from 4 independent experiments and expressed as the mean ± standard deviation. Abbreviations are identical with those in (*a*). *, $p < 0.05$ between the transgenic mice and the nontransgenic mice.

directly or indirectly affect the stability of PPARα in hepatocyte nuclei.

Increase in PPARα ligands

PPARα is a ligand-activated transcription factor. Since the transgenic mice were fed a standard laboratory chow, endogenous substances such as NEFAs would serve as ligands of PPARα[33]; therefore, the contents of NEFAs in hepatocyte nuclei were compared between the 2 groups. The levels of NEFAs in hepatocyte nuclei in the transgenic mice were ~5 times higher than those in the control mice at the same age (Fig. 7*c*). This could account for the higher activation of PPARα in the transgenic mice than in the controls.

Discussion

A large number of variables are involved in the induction of HCC by HCV core protein. While the precise mechanism underlying hepatocarcinogenesis in HCV core gene transgenic mice cannot be fully elucidated from this study, our results could provide some clues to explain this phenomenon. We found spontaneous, persistent, age-dependent and heterogeneous PPARα activation in the transgenic mouse livers for the first time. This study thus advances our understanding of the association

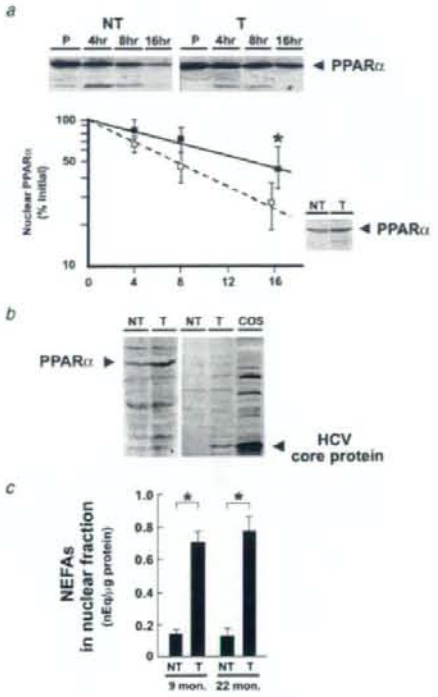

FIGURE 7 – Analyses of PPARα stability, interaction between PPARα with the core protein in hepatocyte nuclei, and nuclear contents of NEFAs. (*a*) Pulse-label and pulse-chase experiments for nuclear PPARα using isolated mouse hepatocytes. (Upper panels) Labeled PPARα bands on X-ray film. Pulse-label and pulse-chase experiments were performed as described in the Material and methods. NT, nontransgenic mice; T, transgenic mice; P, pulse-label; 4, 8, 16 hr, pulse-chase for 4, 8, 16 hr, respectively. (Lower left panel) Intensity plot of PPARα in 5 independent experiments. Values are normalized as a percentage of the values of the pulse-labeled band and expressed as the mean ± standard deviation. Open square, nontransgenic mice; black square, transgenic mice; *, $p < 0.05$ between the transgenic mice and the nontransgenic mice. (Lower right panel) Immunoblot analysis of an isolated hepatocyte nuclear fraction. NT, nontransgenic mice; T, transgenic mice. (*b*) Interaction between PPARα and HCV core protein in the nucleus. (Left panel) Immunoblot analysis (PPARα) of the eluate on anti-PPARα IgG affinity column chromatography. (Right panel) Immunoblot analysis (HCV core protein) of the same eluate. NT, nontransgenic mice; T, transgenic mice; COS, HCV core protein-overexpressing COS cell lysate. (*c*) Nuclear contents of NEFAs. The levels of NEFAs were measured using a hepatocyte nuclear fraction. Results are expressed as the mean ± standard deviation (n = 8). *, $p < 0.05$ between the transgenic mice and the nontransgenic mice; NT, nontransgenic mice; T, transgenic mice; 9 mon, 9-month-old mice; 22 mon, 22-month-old mice.

between HCV core protein-mediated hepatocarcinogenesis and persistent PPARα activation.

Hepatocyte proliferation is influenced by various factors, such as mitogenic chemicals, cytokines, growth factors and transcription factors. It has been reported that various kinds of cell-cycle regulators and oncogene products are induced by PPARα activation.[19,26–30] In particular, cyclin D1, CDK4, PCNA and c-Myc are potent and critical regulators of the G1-S checkpoint and cell-cycle progression,[13,14] and aberrant expression of these proteins is frequently detected in HCV-related HCC.[34–37] These key regulators are known to be induced in a PPARα-dependent manner in mice[19,30]; the continuous induction of these proteins and the

resultant acceleration of hepatocyte proliferation found in the transgenic mice may be attributed to persistent PPARα activation. In the current study, we demonstrated that there was a great variety of the intensity of PPARα activation among different hepatocytes (Fig. 4). This persistent and heterogeneous PPARα activation found especially in the transgenic mice may be linked with the age-dependent and multicentric hepatocarcinogenesis induced by the core protein.

It is well-known that the long-term administration of potent peroxisome proliferators such as fibrate drugs can induce hepatocarcinogenesis in rodents.[29] The findings observed in the transgenic mice markedly differ from those in mice with long-term treatment of peroxisome proliferators in several ways. Namely, the transgenic mice show no intense increase in AOX and PT (Fig. 3), no increase in PPARα mRNA (Fig. 6), heterogeneous peroxisome proliferation (Fig. 4) and age-dependent emergence of hepatocytes having nuclei stained intensively by anti-PPARα or anti-cyclin D1 antibody (Fig. 5). Therefore, the mode of PPARα activation and the mechanism of hepatocarcinogenesis caused by HCV core protein expression are indeed unique.

One of the mechanisms involved in the core protein-specific PPARα activation in mice is stabilization of PPARα in hepatocyte nuclei through a possible interaction with the core protein. In cultured cells expressing the core protein, it has been demonstrated that the core protein interacts with the PPARα-RXRα heterodimer and enhances the transcriptional activation mediated by PPARα regardless of the presence or absence of its ligands.[31] Since PPARα is ubiquitinated and degraded *via* the proteasome pathway,[38] it may be postulated that HCV core protein directly or indirectly influences the degradation pathway. It has been reported that the core protein binds to the proteasome activator PA28γ[39] which is known to combine with steroid receptor coactivator-3 and to accelerate its degradation.[40] Another possible mechanism is an increase in NEFAs in hepatocyte nuclei. The PPARα activation induced by the core protein enhances the expression of L-FABP,[30] which serves as a transporter of NEFAs into nuclei. Indeed, real-time confocal and multiphoton laser scanning microscopy has shown that L-FABP expression significantly increased the total uptake of medium- and long-chain fluorescent fatty acids into the nuclei of living cells.[41] Thus, increased L-FABP expression may facilitate the shuttling of NEFAs into hepatocyte nuclei for donating NEFAs to PPARα, leading to PPARα activation and further increase in L-FABP expression. Moreover, the binding of ligands causes conformational alternation of PPARα[42] and further stabilizes it in nuclei,[32] resulting in synergistic PPARα activation. Therefore, these findings concerning spontaneous and persistent PPARα activation induced by the core protein enable us to partially explain the precise molecular mechanism of hepatocarcinogenesis in HCV core gene transgenic mice.

The results obtained from the current study are consistent with the findings observed in chronically HCV-infected patients in several ways. That is, like the transgenic mice in the present study, chronically HCV-infected patients have been reported to show accelerated hepatocyte proliferation,[43] an increase in CDK4, cyclin D1 and E, PCNA, c-Myc and c-Fos,[34–37] and multicentric appearance of HCC.[44] Furthermore, it has been reported that a massive proliferation of peroxisomes was found in human nontumorous liver tissue adjacent to HCC.[45] Thus the earlier findings, including the unique function of HCV core protein *in vivo* and the diverse and significant roles of PPARα, may help to partially understand the onset and development of HCC in patients with chronic HCV infection. It has been demonstrated that the function of hepatic PPARα was impaired in patients with chronic HCV infection,[46] which is different from our results. Since HCC had not yet developed in the patients in the report, this discrepancy might derive from differences in the stage of the hepatocarcinogenic process.

The interpretation based on persistent activation of PPARα pertains to only one possible mechanism of hepatocarcinogenesis induced by the effects of HCV core protein. We cannot rule out the presence of other mechanisms. The exact relationship between PPARα activation and hepatocarcinogenesis may be elucidated by additional experiments in which PPARα activation is continuously inhibited in the same transgenic mice. Furthermore, the exact relationship may be confirmed when PPARα-null mice bearing the core protein gene do not represent development of HCC.

In conclusion, we demonstrated for the first time that spontaneous, persistent, age-dependent and heterogeneous activation of PPARα occurred in HCV core protein transgenic mice and caused continuous enhancement of hepatocyte proliferation, which may have contributed to the age-dependent and multicentric hepatocarcinogenesis observed in these mice. In addition, we observed nuclear stabilization of PPARα and an increase in NEFAs in the hepatocyte nuclei of the transgenic mice, which may have resulted in the HCV core protein-specific PPARα activation.

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Short Communication

Prevalence of hepatitis B virus infection in Japanese patients with HIV

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Patients with HIV infection are frequently infected with hepatitis viruses, which are presently the major cause of mortality in HIV-infected patients after the widespread use of highly active antiretrovirus therapy. We previously reported that approximately 20% of HIV-positive Japanese patients were also infected with hepatitis C virus (HCV). Hepatitis B virus (HBV) infection may also be an impediment to a good course of treatment for HIV-infected patients, because of recurrent liver injuries and a common effectiveness of some anti-HIV drugs on HBV replication. However, the status of co-infection with HIV and HBV in Japan is unclear. We conducted a nationwide survey to determine the prevalence of HIV–HBV co-infection by distributing a questionnaire to the hospitals belonging to the HIV/AIDS Network of Japan. Among the 5998 patients reported to be HIV positive, 377 (6.4%) were positive for the hepatitis B surface antigen. Homosexual men accounted for two-thirds (70.8%) of the HIV–HBV co-infected patients, distinct from HIV–HCV co-infection in Japan in which most of the HIV–HCV co-infected patients were recipients of blood products. One-third of HIV–HBV co-infected patients had elevated serum alanine aminotransferase levels at least once during the 1-year observation period. In conclusion, some HIV-infected Japanese patients also have HBV infection and liver disease. A detailed analysis of the progression and activity of liver disease in co-infected patients is needed.

Key words: co-infection, hepatitis B, HIV, liver disease.

INTRODUCTION

HEPATITIS B VIRUS (HBV) infection is a major public health problem worldwide, along with hepatitis C virus (HCV) and HIV infections. In the USA, the estimated prevalence of HBV is less than 1%, but approximately 1 million people are persistently infected.[1] The prevalence of HIV in the USA is also <1%, and the virus is estimated to have infected approximately 800 000 people.[2] Because of the common transmission routes, that is, parenteral transmission routes, many people with HIV infection are also infected with HBV. Among the HIV-positive people in the USA, the prevalence of HBV co-infection is 6–14%.[1,2] Before the introduction of highly active antiretroviral therapy (HAART) in 1996, most patients with HIV infection died of HIV-associated opportunistic infections, such as *Pneumocystis jiroveci* pneumonia and cytomegaloviral infection. Since the widespread use of HAART, the mortality associated with HIV infection has declined. However, the reduction in mortality due to opportunistic infection, has left patients co-infected with HIV and hepatitis viruses faced with the menace of progressive liver diseases due to HBV infection,[3,4] in addition to HCV infection.[5]

HBV co-infection or superinfection of HIV-infected patients leads to several problematic situations. First, HBV infection tends to develop into persistent infection in HIV-infected patients,[1,6,7] which is a rare event in healthy adults, although it substantially depends on the genotype of HBV.[8] It results in the acceleration of the development of cirrhosis and eventually hepatocellular carcinoma. Second, some nucleoside reverse transcriptase inhibitors (NRTI) used in HAART also have

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inhibitory effects on the replication of HBV.[9–12] A careless administration or discontinuation of NRTI on HIV–HBV co-infected patients may cause reactivation and/or aggravation of hepatitis B. In addition, the administration of anti-HBV drugs in HIV–HBV co-infection may lead to the development of drug resistance.[11,12] Third, liver injury occurs more frequently in patients on HAART who are co-infected with HIV and HBV than those infected with HIV only.[9,10]

Importantly, co-infection with HIV and HCV increases the morbidity and mortality of HIV-infected patients in Japan,[13] where the prevalence of HIV infection is increasing linearly, and is exceptionally high among developed countries.[14] There are more than 14 000 HIV-positive people in Japan as of 2006, according to the AIDS National Survey in Japan,[14] and approximately 0.8 million chronic HBV carriers.[15] However, the prevalence of co-infection with HIV and HBV in Japan has not been clarified to date. Therefore, we conducted a nationwide study by distributing a postal mail-based questionnaire to the hospitals belonging to the HIV/AIDS Network of Japan.

PATIENTS AND METHODS

IN THE QUESTIONNAIRE, the following information was obtained from the hospitals regarding the number of patients who visited the hospitals at least once between January and December in 2006: (i) the number of HIV-positive patients; (ii) the number of hepatitis B surface antigen (HBsAg)-positive patients among (i); (iii) the number of patients among (ii) who were determined at least once to have a serum alanine aminotransferase (ALT) level higher than 100 IU/L; (iv) the number of HIV-positive patients that contracted HIV from blood products; (v) the number of HBsAg-positive patients among (iv), (vi) the number of patients among (v) who were determined at least once to have a serum ALT level higher than 100 IU/L; (vii) the number of HIV-positive patients among homosexual men, (viii) the number of HBsAg-positive patients among (vii), (ix) the number of patients among (viii) who were determined at least once to have a serum ALT level higher than 100 IU/L; (x) the number of HIV-positive patients that contracted HIV through intravenous drug use (xi) the number of HBsAg-positive patients among (x), (xii) the number of patients among (xi) who had at least one determination of a serum ALT level more than 100 IU/L; (xiii) the number of HIV-positive patients whose transmission routes were classified as "others"; (xiv) the number of HBsAg-positive patients among (xiii); and (xv) the number of patients among (xiv) who were determined at least once to have a serum ALT level higher than 100 IU/L.

The questionnaire was sent to the 372 hospitals belonging to the HIV/AIDS Network of Japan by mail. Answers were mostly returned by mail and in some cases by fax. The list of the hospitals in the HIV/AIDS Network of Japan can be viewed at http://www.acc.go.jp/mLhw/mLhw_frame.htm.

RESULTS

THE QUESTIONNAIRE WAS sent to all 372 hospitals that were on the list of the hospitals in the HIV/AIDS Network of Japan in January 2006. Two hundred and seven hospitals (55.6%) responded within the indicated period. In total, 5998 patients were reported to be HIV positive. The collection rate of 55.6% was higher than that (47.8%) for a questionnaire HIV–HCV co-infection study carried out in 2003.[15] It may appear rather low, particularly considering the number of reported HIV-positive people in 2006, which was approximately 14 000, according to the AIDS National Survey in Japan.[14] However, not all of the HIV-positive people were going to hospitals, and the answers to the questionnaire were obtained from most of the major hospitals in the HIV/AIDS Network in big cities around Japan. This suggests that not all, but a majority of HIV-positive Japanese patients were enrolled in the study.

Among the 5998 patients reported to be HIV positive, 377 (6.3%) patients were positive for HBsAg (Table 1). Of these 377 patients, 122 (32.4%) had elevated serum ALT levels at least one time during the 1-year observation period.

The HBV prevalence rates, when fractionated by the routes of transmission, were as follows: among the 508 HIV-positive patients who contracted HIV from blood products, such as unheated concentrated coagulation factors, only 30 (5.9%) were HBsAg positive, which shows a marked contrast to the prevalence of HCV in this cohort (Fig. 1).[16] Among the 23 intravenous drug users, three (13.0%) were HBsAg positive. Among the 3213 HIV-positive patients who were homosexual men, 267 (8.3%) were HBsAg positive. In the remaining 2254 patients who were HIV-positive and whose route of HIV transmission was classified as "others", most contracted HIV heterosexually. This number (2254) showed a substantial increase from the 1316 obtained in the questionnaire for the HIV–HCV co-infection study in 2003, while the total number of HIV-positive patients increased from 4877 to 5998.[16] Among these, 77 (3.4%)

Table 1 Prevalence rates of hepatitis B virus infection among HIV-positive patients

Routes of transmission	No. patients	HBsAg positive (% in HIV positive according to route)	ALT >100 IU/L (% in HBsAg positive according to route)
Blood products	508 (5.9%)	30 (40.0%)	12
Homosexual men	3213 (8.3%)	267 (32.2%)	86
Drug addicts	23 (13.0%)	3 (66.7%)	2
Others (heterosexual etc.)	2254 (3.4%)	77 (28.6%)	22
Total	5998	377 (6.3%)	122 (32.4%)

ALT, serum alanine aminotransferase; HBsAg, hepatitis B surface antigen.

were HBsAg positive. In terms of the route of HIV infection, 267 (70.8%) of the 377 patients were homosexual men among the HIV–HBV co-infected patients. This shows a contrast to the status of HIV–HCV co-infection, in which the majority of HIV–HCV co-infected Japanese patients contracted both viruses from blood products.[16]

There were one or more HIV-positive patients in 154 (74.4%) of the 207 hospitals in the HIV/AIDS Network of Japan (Table 2). Twenty four (11.6%) of 207 hospitals had 20–49 HIV-positive patients, and 16 (7.7%) hospitals had 50 or more HIV-positive patients. There were one or more patients who were co-infected with HIV and HBV in 64 (30.9%) of the 207 hospitals. There were 10 or more HIV–HBV co-infected patients in nine (4.3%) hospitals, all of which had 50 or more HIV-positive patients (Table 2). HIV–HBV co-infected patients were concentrated in specific hospitals in big cities around Japan. In particular, in the Kanto area, HIV–HBV co-infected patients were concentrated in the HIV/AIDS Network hospitals in the Tokyo city area.

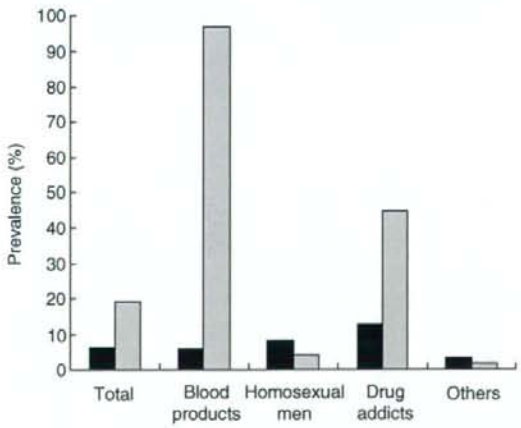

Figure 1 Prevalence rates of persistent hepatitis B virus and hepatitis C virus infections in the HIV-positive population sorted by the HIV risk group. (■), HBsAg, hepatitis B surface antigen; (▨), anti-HCV, antibody to hepatitis C virus. *Prevalence rates of anti-HCV are obtained from Koike K *et al.*[16]

DISCUSSION

ALONG WITH THE increase in the number of HIV-infected patients in Japan, co-infection with HIV and hepatitis viruses has become a major medical issue. HBV infection of HIV-positive patients raises several difficult problems: HBV infection tends to develop into persistent infection, even in adults; some NRTI used in HAART also have inhibitory effects on the replication of HBV, the improper administration, or discontinuation of which may lead to drug resistance; and HIV–HBV co-infected patients on HAART have liver injuries more frequently than HIV-monoinfected patients. It is important to determine the status of HBV infection in HIV-positive patients.

According to the statistics of the Ministry of Health, Labor, and Welfare of Japan, the number of reported HIV-positive people was slightly over 14 000 in 2006.[14] In the present study, 6.4% of HIV-positive patients were positive for HBsAg, the most reliable marker for ongoing HBV infection. It might have been advantageous if

Table 2 Number of hospitals categorized according to the number of patients infected with HIV and those co-infected with HIV and hepatitis B virus (HBV)

No. HIV (+)/ HBV (+)	No. HIV(+)				
	0	1–19	20–49	50+	Total
0	53	76	13	1	143
1–9	0	38	11	6	55
10+	0	0	0	9	9
Total	53	114	24	16	207

serum HBV-DNA levels were determined, but unfortunately, HBV-DNA level determination was not a routine laboratory test in most hospitals. In addition, considering that the antibody to the hepatitis B core antigen might be the only marker of ongoing HBV infection in some immuno-compromised patients, it would also be advantageous if this viral marker were available. These issues should be investigated in future studies. Comments from hospitals to the questionnaire included one indicating that not all HIV-positive patients underwent a test for serum HBsAg, suggesting the actual prevalence of HBsAg in HIV-infected patients might be higher than 6.4%.

In a previous questionnaire study of HIV–HCV co-infection, the prevalence of HCV infection among HIV-infected patients was 19.2%;[16] the prevalence of HBV infection (6.4%), is one-third of it. The lower positivity for HBsAg than for the anti-HCV antibody among those who contracted HIV through blood products accounts for this difference: almost all (96.9%) of the patients who contracted HIV through blood products were also anti-HCV antibody positive.[16] It should be noted that among the homosexual male patients who were HIV positive, 8.3% were HBsAg positive, which is twice as high as that of the anti-HCV antibody in these populations. A higher prevalence of HBV infection as a sexually transmitted infection than that of HCV[17] may explain the high prevalence of HBV infection in HIV-positive homosexual men. Similarly, a HBV prevalence of 3.4% in heterosexually transmitted HIV-positive patients is higher than that of the general Japanese population of the same age.[15]

Of the 377 patients who were HBsAg positive, 122 (32.4%) had elevated serum ALT levels at least once in the 1-year observation period. In this type of study using a questionnaire, it is difficult to obtain the details of patients' data, including age, body weight, and the degrees of liver injuries and fibrosis. If detailed items were included in the questionnaire, then the collection rate would be low. This time, to obtain a high collection rate, we asked whether the patients with HBsAg showed an elevated ALT level higher than 100 IU/L at least once during the 1-year observation period. We thereby do not have details on liver disease in HIV–HBV co-infected patients in the current study. Nonetheless, one-third of HIV–HBV co-infected patients have moderate liver injuries, either chronic hepatitis B or adverse effects of drugs, and are waiting for an aid for the amelioration of liver disease. A detailed analysis of the progression and activity of liver disease in HIV–HBV co-infected patients is expected.

The collection rate of the present questionnaire from the hospitals belonging to the HIV/AIDS Network was 55.6% (207 of 372). This was higher than that (47.8%) in the HIV–HCV co-infection questionnaire study carried out in 2003. The reason for this increase is not clear, but presumably the questionnaire conducted in 2003 has raised awareness among hospital staff regarding the relevance of hepatitis virus and HIV co-infection in clinical practice.

In the current study, both Japanese patients and those of other nationalities/ethnicities were included in the study. Although the ratio of newly diagnosed HIV-positive foreign people has been declining to approximately 10% in 2006, the one in total HIV positive still accounts for approximately 25% in Japan. Because the rates of the HBV carrier are different among countries, it is ideal to analyze the HBV prevalence separately according to the nationalities/ethnicities. However, in the current survey to the hospitals in HIV/AIDS Network of Japan, nationality/ethnicity was not itemized in order to make the questionnaire simple. If we would attempt to obtain such data under the approval of the ethical committee in each hospital, the response rate to questionnaire would be extremely lowered.

To establish measures that decrease the morbidity and mortality of HIV–HBV co-infected patients, it is essential to determine the current status of co-infection. In the present study, the number and transmission routes of HIV–HBV co-infected patients in Japan were determined for the first time, although detailed information on the severity and progression of liver disease in HIV–HBV co-infected patients has not been obtained yet. Undoubtedly, this will be the first step towards improving the prognosis and quality of life of Japanese patients co-infected with HIV and HBV.

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METABOLISM, CANCER AND GENETICS

Molecular basis for the synergy between alcohol and hepatitis C virus in hepatocarcinogenesis

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alcohol, hepatitis C virus, hepatocarcinogenesis, intracellular signal transduction, oxidative stress, transgenic mouse.

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Abstract

Overwhelming lines of epidemiological evidence have indicated that persistent infection with hepatitis C virus (HCV) is a major risk for the development of hepatocellular carcinoma (HCC). In addition, heavy alcohol use has been linked with earlier progression to HCC in chronic hepatitis C patients. However, in the pathogenesis of HCV-associated HCC, it still remains controversial as to whether the virus plays a direct or an indirect role, and as to how alcohol operates in the acceleration of HCC development. Several studies using transgenic mouse models, in which the core protein of HCV has an oncogenic potential, indicate that HCV is directly involved in hepatocarcinogenesis, although other factors such as continuous inflammation or environmental factors seem also to play a role. The downstream events of the HCV core protein expression in the transgenic mouse HCC model are segregated into two pathways. One is the augmented production of oxidative stress in the absence of inflammation along with the attenuation of some scavenging systems in the putative preneoplastic stage with steatosis in the liver. The other pathway is the alteration in cellular gene expression and intracellular signaling, including the mitogen-activated protein kinase cascade. The combination of these pathways would explain the unusually high incidence and multicentric nature of HCC development in HCV infection. In addition, alcohol feeding in this animal model further activated the two pathways synergistically with HCV, leading to an earlier development of HCC. Such a synergy would reveal the molecular basis for the acceleration of HCC development by alcohol in HCV infection.

Introduction

Hepatitis C virus (HCV) infects approximately 170 million people persistently worldwide, and induces a spectrum of chronic liver diseases, from chronic hepatitis, cirrhosis to hepatocellular carcinoma (HCC).[1] 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. It impacts the medical, sociological and economic domains of society. Once liver cirrhosis is established in hosts infected with HCV, HCC develops at a yearly rate of 5–7%,[2] resulting in the development of HCC in nearly 90% of HCV-associated cirrhosis patients in 15 years. In addition, the outstanding features in the mode of hepatocarcinogenesis in HCV infection (i.e. development of HCC in a multicentric fashion and a very high incidence), are not common in other malignancies except for hereditary cancers such as familial polyposis of the colon. Knowledge of the mechanism underlying HCC development in persistent HCV infection is therefore imminently required for the prevention of HCC.

However, alcohol has been known as an accelerating factor in the development of HCC in persistent HCV infection.[3–5] The pattern of the risk for HCC due to alcohol intake shows a continuous dose-effect curve without a definite threshold, although most studies have found that HCC risk increased only for alcohol consumption above 40–60 g of ethanol per day. Some evidence supports a positive interaction of alcohol intake, probably with HCV infection and possibly with HBV infection.[3] Synergistic interactions on the additive model were observed between heavy alcohol consumption and chronic hepatitis virus infection and diabetes mellitus.[4] However, it is unclear how alcohol causes the acceleration of HCC development in HCV infection.

How does HCV contribute to hepatocarcinogenesis?

How HCV is involved in hepatocarcinogenesis is not yet clear, despite the fact that nearly 80% of patients with HCC in Japan are persistently infected with HCV.[1,6,7] HCV infection is also common in patients with HCC in other countries, albeit to a lesser extent. These lines of evidence force us to determine the role of HCV in hepatocarcinogenesis. Inflammation induced by HCV should be considered in a study on hepatocarcinogenesis in hepatitis viral infection: necrosis of hepatocytes due to chronic inflammation followed by regeneration enhances genetic aberrations 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 context leaves us with a serious question: can inflammation alone result in the development of HCC in such a high incidence or is there a multicentric nature in HCV infection?

The other role of HCV would be weighed against an extremely rare occurrence of HCC in patients with autoimmune hepatitis in whom severe inflammation in the liver persists indefinitely, even after the development of cirrhosis. This background and reasoning led to a possible activity of viral proteins for inducing neoplasia. This possibility has been evaluated by introducing HCV genes 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. In fact, it takes 30–40 years for HCC to develop in individuals infected with HCV. On the basis of these viewpoints, we started to investigate carcinogenesis in chronic hepatitis C, *in vivo*, by transgenic mouse technology.

Transgenic mouse studies revealed an *in vivo* oncogenic activity of HCV core protein

Transgenic mouse lines with parts of the HCV genome were engineered by introducing the genes from cDNA of the HCV genome of genotype 1b.[8,9] Three different transgenic mouse lines were 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.[9] The envelope gene transgenic mice do not develop HCC, despite high expression levels of both E1 and E2 proteins.[10,11] 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, and the intrahepatic level of the core protein 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 histological characteristics of chronic hepatitis C, along with lymphoid follicle formation and bile duct damage.[12] Thus, the core gene transgenic mouse model well reproduces the feature of chronic hepatitis C. Of note, any pictures of significant inflammation are not observed in the liver of this animal model. Late in life, these transgenic mice develop HCC. 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.[13–15] These outcomes indicate that the core protein per se of HCV has an oncogenic potential when expressed *in vivo*.

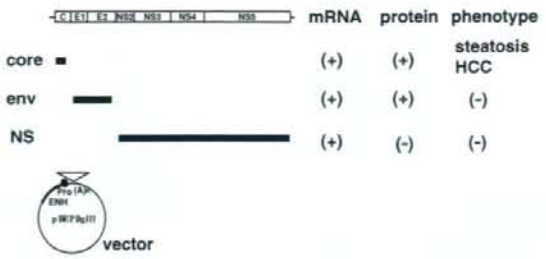

Figure 1 Transgenic mouse lines carrying the hepatitis C virus (HCV) genome. Three different types of transgenic mouse lines, carrying the core gene, envelope genes or non-structural genes of HCV, respectively, were established under the control of the same regulatory elements. Among these mouse strains, only the transgenic mice carrying the HCV core gene developed 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 did not develop HCC. env, envelope genes; NS, non-structural genes.

Oxidative stress overproduction and MAPK activation as consequences to the core protein expression in the liver

It is difficult to determine 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 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.[9,16] 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. We would like to draw particular attention to the fact that 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 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.[17,18] The overproduction of oxidative stress results in the generation of deletions in the mitochondrial DNA, an indicator of genetic damage. In addition, analysis of the anti-oxidant system revealed that some anti-oxidative molecules are not increased despite the overproduction of ROS in the liver of core gene transgenic mice; hemeoxygenase-1 and glutathione peroxidase are not augmented whereas catalase and glutathione *S*-transferase levels are increased and enhanced by iron overloading (S Shinzawa *et al.*, unpubl. data, 2007). These results suggest that HCV core protein not only induces overproduction of ROS but also attenuates some of the anti-oxidant system, which may explain the mechanism underly-

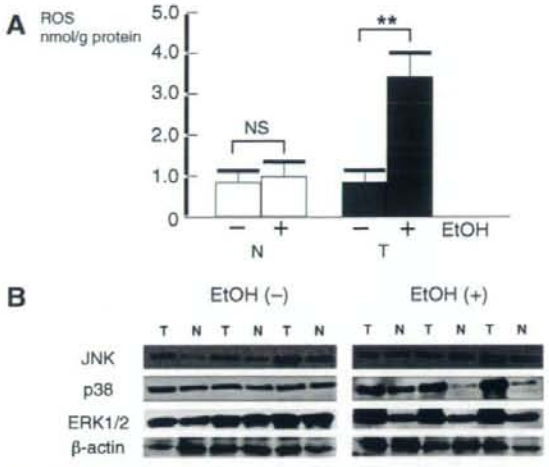

Figure 2 Alcohol administration enhances oxidative stress production and mitogen-activated protein kinase (MAPK) pathway activation in a synergistic fashion with hepatitis C virus (HCV) core protein. Administration of 5% alcohol for 3 weeks provoked an induction of reactive oxygen species (ROS) in HCV core gene transgenic mice, whereas it induced only a marginal increase in control mice, showing a synergy between the HCV core protein and ethanol in inducing ROS. Only the c-Jun N-terminal kinase (JNK) pathway is activated in the core gene transgenic mice before hepatocellular carcinoma (HCC) development, but feeding 5% alcohol for 3 weeks activated the other two pathways, p38 and ERK1/2, which was not observed in control mice. Thus, combining the effect of ethanol to that of the core protein resulted in the activation of all the MAPK pathways, among which only JNK was activated by the action of HCV core protein only in the absence of ethanol. ERK, extracellular signal-regulated kinase; EtOH, ethanol; N, non-transgenic control mouse; NS, statistically not significant; T, transgenic mouse. $^{**}P < 0.01$.

ing the production of a strong oxidative stress in HCV infection compared to other forms of hepatitis.

Thus, the core protein induces oxidative stress overproduction in the absence of inflammation, which may, at least in part, contribute to hepatocarcinogenesis in HCV infection. If inflammation were added to the liver with the HCV core protein, the production of oxidative stress would be escalated to an extent that cannot be scavenged any longer by a physiological antagonistic system. This indicates that the inflammation in chronic HCV infection would have a characteristic difference 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.[9,17] The dysfunction of the electron transfer system of the mitochondrion is suggested in association with the presence of the HCV core protein.[19] Hepatic steatosis in hepatitis C, which is also attributed to the action of the core protein,[8] may work as fuel for oxidative stress overproduction.[18,20,21]

Other possible pathways would be the alteration of the expression of cellular genes, interacting with cellular proteins, and modulation of intracellular signaling pathways. For example, tumor necrosis factor (TNF)-α and interleukin-1β have been found transcriptionally activated.[22] 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 lipid metabolism.[23] 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 protein (AP)-1 activation is markedly enhanced.[22,24] 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 confers an advantage for cell proliferation to hepatocytes. Interestingly, we found recently that a protein interacting with the core protein, proteasome activator 28γ (PA28γ), is indispensable for the core protein to exert its function for the development of steatosis, insulin resistance and HCC.[25,26]

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

Molecular basis for the synergy between alcohol and HCV infection in hepatocarcinogenesis

As described above, the production of oxidative stress is increased in the liver of aged HCV core gene transgenic mice in the absence of inflammation. In young mice, the increase in oxidative stress is apparently marginal. However, feeding 5% ethanol to mice for 3 weeks induced ROS in the liver of core gene transgenic mice, whereas it induced only a minimal increase in control mice, demonstrating a synergy between the core protein and ethanol in inducing ROS (Fig. 2a).[17] In contrast, only the JNK pathway is activated in the core gene transgenic mice before HCC development, but feeding 5% ethanol for 3 weeks activated the other two MAPK pathways, p38 and ERK1/2 in the core gene transgenic mice, the activation of which is not present in control mice (Fig. 2b). Thus, combining the effect of ethanol to that of the core protein provoked the activation of all the MAPK pathways, affording advantage to cell proliferation.[24]

In a long-term observation experiment, feeding 2% ethanol to the core gene transgenic mice for 9 months resulted in the acceleration of HCC development (Moriya K *et al.*, unpubl. data, 2007). Screening by the high-throughput immunoblot analysis revealed differential expression of proteins in the liver with or without ethanol feeding; some proteins, the levels of which were either increased or decreased by the effect of the core protein, such as Rho GTPase activating protein (GAP) or caspase-8, are down- or upregulated by the effect of ethanol feeding.

In summary, we postulate that the induction of oxidative stress, together with the activation of MAPK cascade, followed by AP-1 activation and cyclin D1 overexpression, plays a pivotal role in the development of HCC (Fig. 3). Alterations in cellular gene expressions, such as TNF-α or suppressor of cytokine signaling-1, and the

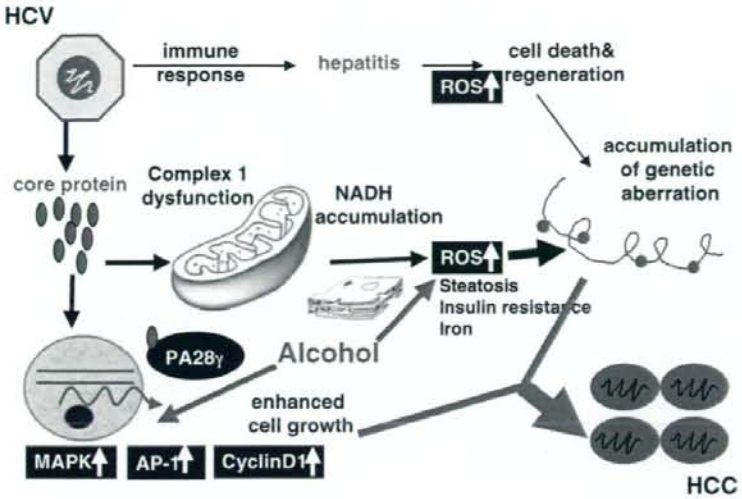

Figure 3 Molecular pathogenesis of hepatocellular carcinoma (HCC) development in hepatitis C virus (HCV) infection in association with alcohol. We postulate that induction of oxidative stress through the dysfunction in the mitochondrial electron transfer system, together with alterations in cellular gene expressions and the intracellular signaling pathways, including the mitogen-activated protein kinase (MAPK) cascade, play a pivotal role in the development of HCC. Alcohol activates both of these pathways and augments the development of HCC in HCV infection. AP-1, activating protein-1; NADH, nicotinamide adenine dinucleotide; PA28γ, proteasome activator 28γ; ROS, reactive oxygen species; SOCS-1, suppressor of cytokine signaling-1; TNF-α, tumor necrosis factor-α.

presence of steatosis and insulin resistance are co-accelerators to hepatocarcinogenesis in HCV infection. Finally, alcohol augments both of these pathways that are activated by the core protein, and further enhance the development of HCC in HCV infection (Fig. 3).

Conflict of interest

No conflict of interest has been declared by the authors.

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