Development of hepatocellular carcinoma in mouse model transgenic for hepatitis C virus core protein

C型肝炎ウイルスコア蛋白質発現トランスジェニックマウスにおける肝細胞癌の発症

森 屋 恭 爾

TABLE OF CONTENTS

Introduction

Worldwide, hepatitis B virus (HBV) and hepatitis C virus (HCV) infect hundreds of million people persistently and induce a spectrum of chronic liver diseases. Hence they impact the society in a number of domains including medical, sociologic and economic. Hepatocellular carcinoma (HCC) has become the most frequent cause of death in individuals persistently infected with HBV or HCV; in particular, HCV has gained increasing attention because of its wide and deep penetration into the community, coupled with a very high incidence of HCC in the persistent infection. Once liver cirrhosis is established in hosts infected with HCV, HCC develops at a yearly rate of 5-7%[1]. The knowledge on mechanism how HCC develops in chronic HCV infection, therefore, is urgently required for the prevention of HCC.

How hepatitis viruses induce HCC is not clear, as yet, despite the fact that more than 95% of patients with HCC in Japan are infected with HBV or HCV, or both[2,3,4] Infections with these two hepatitis viruses are common in patients with HCC in other countries, also, albeit to lesser extents. Evidence of concurrent or past HBV infection is demonstrated even in the remaining 5% of patients with HCC in Japan[3,5] which has been categorized in non-B, non-C HCC.

Inflammation induced by hepatitis viruses, manifesting itself in various forms of hepatitis, needs to be taken into considerations in the study for carcinogenic capacity of hepatitis viruses. It has been proposed repeatedly that necrosis of hepatocytes due to chronic inflammation and ensuing regeneration that follows would enhance mutagenesis in host cells, the accumulation of which might culminate in HCC. The theory presupposes an indirect involvement of hepatitis viruses in HCC via hepatic inflammation. It leaves us specialists in hepatology with a serious question, however;

1

can inflammation *per se* result in HCC in such a high incidence and a multicentric nature? The proposal for a secondary role of hepatitis viruses would have to be weighed against an extremely rare occurrence of HCC in patients with autoimmune hepatitis in whom severe inflammation of liver goes on indefinitely.

These backgrounds and reasoning lead one to a possible activity of viral proteins for inducing HCC. The possibility has been evaluated by introducing genes of HCV into hepatocytes in culture with little success. One of the difficulties with cultured cells is that a carcinogenic capacity of hepatitis viruses, if any, would be weak and take long to manifest itself. Actually, it takes 30-40 years for HCC to develop in individuals infected with HCV. Another constraint common to studies for carcinogenesis is the development of HCC by transformed cells that might have gone out of growth control and escaped surveillance of the host. Should it be the case, the analysis of transformed cells would not been sufficient for bringing the mystery of carcinogenesis into light. Based on these viewpoints, we started tackling the carcinogenesis in chronic HCV infection by the technology of transgenic mouse.

Transgenic mice are produced by introducing exogenous genes to fertilized eggs. The technology of transgenic mice crystallized in production of a phenotype, for the first time, in mice genetically engineered with the introduction of the gene of human growth hormone in 1985[6]. Since then, the technology has ignited explosion of enormous studies; it has become a standard technique at present. As of April 2001, a survey of the PubMed database by keywording "transgenic mouse" hits more than 17,000 publications; they will grow in an accelerated speed in the future.

The technology with transgenic mice involves the expression of exogenous genes in animals that prefers a dominant trait. A number of transgenic mice have

been produced for studies of many diseases by introducing various genes of viral origins in particular[7,8]. The virus represents a simple organism composed only of viral proteins and nucleic acids. In infected cells, viral genes are released from viral particles and they are then transcribed for replication and translation of viral proteins. Because these series of processes, including introduction of genes followed by transcription and translation, are faithfully mimicked in transgenic mice, they are suitable as a model of diseases induced by viral infections. The establishment of technology for transgenic mouse has offered incomparable opportunities for answering *in vivo* many questions raised in a variety of biological fields, which had been accomplished exclusively *in vitro* until the transgenic mouse entered the scene.

We have produced transgenic mice in which the core gene, envelope gene and non-structural gene of HCV were introduced primarily for studying the mechanism of hepatocarcinogenesis. It is of particular note that every viral gene is under the control of expression with the same enhancer and promoter. This has made it feasible to compare *in vivo* functions among various viral proteins. As salient results of our studies, HCV core protein has been found capable of inducing HCC due to carcinogenic potentials of the products of viral genes.

Infection with hepatitis C virus(HCV)has been identified as the major cause of post-transfusion non-A non-B hepatitis. And also it is known that the HCV infection is an important cause of cirrhosis and hepatocellular carcinoma.

HCV has a positive-sense, single-strand RNA genome approximately 10kbp in length, with similarities in genome organization and some sequence homology with pestiviruses.

The genomic structure was deduced from data obtained by sequence analysis and expression of the cloned DNA in vitro and vivo.The structual genes were located on

the 5'side of the genome and non-structual genes on the 3' side. The gene order starting from the 5'end was C, E1, E2/NS1, NS2, NS3, NS4,and NS5.The 5'non-coding region was about 340 bp.Specific antibodies against C,E1,E2/NS1, NS4/5 were detected in infected chimpanzees and humans.

In the case of pestiviruses,the core protein has an apparent molecular weight of 14kDa and is located carboxyterminal in the polyprotein.

For HCV,the putative core protein represents a polypeptide of about 21 kDa encoded in the 5'region of the HCV open reading frame.The core protein is highly conserved for HCV and pestiviruses,which points towards an important role of the primary sequence in formation of the viral core particle.

HCV core protein has been found several functions.HCV core protein is implicated in carcinogenesis as one of the causative factors.

We then established transgenic mouse model in which HCV core gene,and investigated the function of HCV gene in neoplastic transformation.

PART1;The Core Protein of Hepatitis C Virus

Induces Hepatocellular Carcinoma in Transgenic Mice

Abstract

Hepatitis C virus (HCV) is the major cause of chronic hepatitis worldwide, which finally leads to the development of hepatocellular carcinoma (HCC)[9,10]. However, the mechanism of hepatocarcinogenesis in chronic HCV infection is still unclear. The ability of the core protein of HCV to modulate gene transcription, cell proliferation and cell death[11-16] has been suggested as being involved in the pathogenesis of HCC. Here we report the development of HCC in two independent lines of HCV core gene transgenic mice which develope hepatic steatosis early in life as a histological feature characteristic of chronic hepatitis C[17]. After the age of 16 months, mice of both lines succumbed to hepatic tumors which first appeared as adenoma containing fat droplets in the cytoplasm. Then HCC, a more poorly differentiated neoplasia, developed from within adenoma, presenting a "nodule in nodule" feature without cytoplasmic fat droplets. This feature closely resembled the histopathological characteristics previously observed in the early stage of HCC in patients with chronic hepatitis C[18,19]. These results suggest that the HCV core protein has a major role in the development of HCC, and that these transgenic mice would provide good animal models for defining molecular events in hepatocarcinogenesis with HCV infection.

Despite the overwhelming evidence from clinical studies connecting chronic HCV infection and the development of HCC[9,10], the precise role of HCV in hepatocarcinogenesis remains unknown. It is postulated that HCV may promote the development of HCC by inducing continuous cell death followed by regeneration, whereby genetic damage accumulates to confer growth advantages to hepatocytes[20]. In this context, HCV has only an indirect association with hepatocarcinogenesis.

Another possibility is the direct involvement of HCV in hepatocarcinogenesis, wherein the product(s) of the virus may be involved in regulating cell proliferation. Recently, the core protein of HCV has been reported to transform fibroblasts with or without the cooperation of the *ras* oncogene[11,12] suppress the transcription of some genes[13,14] and modify the susceptibility of cultured cells to apoptotic signals[15,16]. The core protein, therefore, may be directly involved in hepatocarcinogenesis by perturbing the regulation of cell proliferation.   We tested this possibility by analyzing transgenic mice into which the HCV core gene had been introduced.

Materials and Methods

Production of transgenic mice.   Production of HCV core gene transgenic mice was previously described[17].   The core gene of HCV placed downstream of a transcriptional regulatory region from hepatitis B virus, which has been shown to allow high-level expression of genes in transgenic mice without interfering with mouse development[26], was introduced into C57BL/6 mouse embryos (Clea Japan Inc., Tokyo, Japan).   Mice were fed with ordinary chows (Funabashi Farms, Funabashi, Japan) and maintained in a specific pathogen-free state.

Antibodies and Western blotting.   The antibodies used in this study were an anti-core rabbit serum and an   anti-core mouse monoclonal antibody[17].   Western blotting of tissues from mouse or human liver was performed as described previously[26].

Histological and immunohistochemical methods. Tissue sections (5 μm thick) fixed in 10% neutral-buffered formalin or frozen were used for hematoxylin and eosin staining or immunostaining.   The HCV core protein was stained with anti-core rabbit serum.   For detection,   biotinylated anti-rabbit IgG followed by avidin-biotin peroxidase (Vector Labs, Inc., Burlingame, CA) was used. Specificity control of immunostaining was carried out as follows; liver tissues and other organs from a

normal littermate mouse were tested with immune serum. Normal rabbit sera were tested with transgenic mouse liver.

Electron microscopy. For standard electron microscopic techniques, mouse liver was perfused with 1.6% glutaraldehyde (TAAB Laboratory Equipment, Reading, UK), excised and fixed at 4°C for 1 h. For immunostaining, the liver tissues were fixed in 4% paraformaldehyde (Wako, Osaka, Japan). The grids were incubated with anti-core rabbit serum, followed by incubation with anti-rabbit IgG conjugated to gold particles. For controls, normal rabbit serum was used.

Results

Two independent transgenic mouse lines, C21 and C49, with a cDNA fragment covering the complete core gene of HCV genotype 1b (Fig. 1a) under an exogenous promoter were studied. Southern blotting of mouse genomic DNA revealed a distinct integration pattern for each lineage[17]. In both lineages, the expression of the core protein in the liver started at birth and continued for at least 19 months thereafter. The core protein levels in the transgenic mouse livers were examined and compared with those in liver samples from 42 patients with chronic hepatitis C (H. Fujie et al.,). The representative cases are shown in Fig. 1b. The intrahepatic levels of the core protein in human patients were almost comparable to those in the mice, allowing these transgenic mice to be appropriate models for study of the liver disease spectrum in human HCV infection. As we reported previously, these transgenic mice develop hepatic steatosis, which is one of the characteristic histopathological features of chronic hepatitis C, at as early an age as 3 months[17,21]. Steatosis, which is characterized by hepatocytes with fat droplets of various sizes in the cytoplasm, was slowly progressive toward the age of 12 months. However, no inflammation was observed in the liver during that period. The mean serum alanine aminotransferase (ALT) level in mice between the ages of 3 and 9 months was 90.4±83.3 IU/L in transgenic mice (n=12) and 80.3±48.7 IU/L in non-transgenic mice (n=6) (statistically not significant) .

To know the long-term consequences of HCV core gene expression, the transgenic mice from both the lineages were sacrificed for pathological examination at the ages of 3, 6, 9, 12 and 16 months. As was previously shown, the mice up to the age of 9 months showed only marked hepatic steatosis without inflammatory or neoplastic change (Fig. 2a). The mice sacrificed at the age of 12 months did not show inflammatory or neoplastic changes either. Surprisingly, however, gross hepatic nodules were observed in 16-month-old-mice (Fig. 2b). Microscopic examination revealed that these hepatic nodules, which compressed the neighboring non-tumorous liver parenchyma, were made up of eosinophilic cells with fat droplets in the cytoplasm (Fig. 2c). These nodules had the characteristics of hepatocellular adenoma[22].

We then sacrificed more transgenic mice between the ages of 16 and 19 months for further analysis of the hepatic lesions. Approximately one-fourth of the male transgenic mice were found to have hepatic nodules. Some of the hepatic nodules developed as well-differentiated HCC with trabecular features with cells containing fat droplets in the cytoplasm (Fig. 2d). Interestingly, most of the hepatic tumors exhibited a remarkable "nodule in nodule" characteristic, in which HCC developed in the midst of an adenoma or a better-differentiated HCC. As shown in Fig. 2e, a cluster of carcinoma cells (area 3) appeared in the middle of adenoma cells (area 2) which compressed the neighboring normal hepatocytes (area 1). It should be noted that fat droplets in the cytoplasm of the cells in the hepatocellular adenoma were as numerous as those in non-tumorous parts while those in HCC tissues had few fat droplets (Fig. 2f). This feature closely resembles the one observed in the development of HCC in human chronic hepatitis C, with small well-differentiated HCCs or their precursors occasionally showing marked fatty changes, and more poorly-differentiated HCCs without fatty changes developing from within them[18,19]. HCCs in the transgenic mice were generally composed of eosinophilic cells and showed trabecular features with mitotic figures (Fig. 2f). Immunohistochemical analysis demonstrated relatively higher levels of the core protein in hepatic tumors

compared to those in adjacent non-tumorous liver tissue (Fig. 2g and h). No infiltration of lymphocytes, suggestive of hepatitis, was observed. The mean serum ALT level in mice between the ages of 16 and 19 months was 136.2±116.3 IU/L in transgenic mice (n=18) and 121.8±91.9 IU/L in non-transgenic mice (n=10) (statistically not significant) .

The penetrance of HCC in the two lines of HCV core gene transgenic mice is shown in Table 1. The incidences of HCC in the male transgenic mice of the C21 and C49 lines, between the ages of 16 and 19 months, were 25.9% (7/27) and 30.8% (4/13), respectively. In contrast, none of non-transgenic littermate mice of the same age developed HCC, which is consistent with the very low incidence of hepatic tumors in the strain C57BL/6[23,24]. The incidences of HCC in the male C21 and C49 mice were significantly higher than in normal controls (p<0.01 and p<0.005, respectively). The low incidence in female transgenic mice appears to be consistent with the epidemiological data that men chronically infected with HCV are more prone to HCC than women[25].

Discussion

It should be noted that the transgenic mice which carry the envelope genes of HCV under the same transcription control region did not show neoplastic lesions in the liver[26]. Those mice had the entire envelope genes (both E1 and E2) and showed the expression of both proteins in substantial levels in the liver from birth to death. However, in contrast to the core-gene transgenic mice, no hepatic adenoma or HCC developed during their entire life-span of more than 24 months. Although several transgenic mice carrying HCV genes have been established and analyzed before[27-29], there is no evidence for the direct role of HCV in the induction of HCC. In those transgenic mice whose transgenes included the core gene, in which the expression of the core gene was very low[27,28] or was not detected with conventionally used anti-core antibodies[29], neoplastic changes in the liver would not be expected.

9

Immunoelectron microscopy revealed accumulation of the core protein in the nuclei and mitochondria (Fig. 2i), in addition to its association with lipid droplets[30]. The localization of the core protein in the nuclei in our mice supports the notion that the core protein may act as a transcriptional regulator and affect the proliferative ability of the cells[11-16], and thereby associated with hepatocarcinogenesis. Detection of the core protein in the mitochondria also may account for the electron microscopic finding of the disappearance of the double structure of mitochondrial membranes (Fig. 2j and k), which may cause steatosis through the impairment of fatty acid oxidation in mitochondria.

Our observations demonstrate that the expression of the HCV core gene results in progressive morphological and biochemical changes which finally lead to the development of HCC. Preexisting steatosis caused by the core protein may also be an important factor for the development of HCC[18,19], possibly by inducing the formation of lipid peroxidation products such as malondialdehyde[31]. The appearance of HCC with the "nodule in nodule" features suggests that some secondary hit(s) may be responsible for the development of malignant phenotype.

In conclusion, our results indicate that chronic hepatitis, with continuous cell death and regeneration, is not an absolute prerequisite for the development of HCC, and that HCV itself is directly involved in the development of HCC. Also, this transgenic mouse system would be an ideal animal model for defining molecular and pathological events in the course of hepatocarcinogenesis in HCV infection.

Figure Legends

Figure 1-1

Hepatitis C virus core gene transgenic mice: Transgene construct and expression.

a, A cDNA fragment covering the entire core gene was placed downstream of a transcriptional regulatory region from hepatitis B virus and introduced into mouse eggs[9].

C, core; E1, envelope 1; E2, envelope 2, NS, non-structural region.

b, Equivalent amounts of protein were separated through 12.5% SDS/PAGE and reacted with an anti-core mouse monoclonal antibody for immunodetection. Lane 1, positive control core protein expressed in HepG2 cells; lane 2, a liver tissue from a 9-month-old male non-transgenic mouse; lane 3, a liver tissue from a 9-month-old male transgenic mouse of the C21 line; lane 4, a liver tissue from a 17-month-old male transgenic mouse of the C49 line; lane 5, a liver tissue from a patient with chronic hepatitis C; lane 6, a liver tissue from another patient with chronic hepatitis C; lane 7, a liver tissue from a patient with chronic hepatitis B. Arrowhead indicates the position of the core protein.

Figure 1-2

Pathological changes in the liver of the HCV core transgenic mice.

a, Marked hepatic steatosis developed in a 12-month-old male transgenic mouse of the C21 line (x300). *b*, A gross finding of a hepatic nodule which developed in a 16-month-old male transgenic mouse of the C49 line (x3). *c*, A microscopic finding of a hepatocellular adenoma (T) which compresses the adjacent normal liver tissue (NT) (a 16-month-old male transgenic mouse of the C21 line, x120). *d*, Hepatocellular carcinoma (HCC) which developed in a 17-month-old male transgenic mouse of the C49 line. Note that carcinoma cells with the trabecular feature (T) have fat droplets in the cytoplasm and that acidophilic bodies are observed both in tumorous and non-tumorous tissues suggesting increased rate of apoptosis (x300). *e*, Development of HCC in a "nodule in nodule" fashion. HCC (area 3) developed from within an

adenoma (area 2) compressing the surrounding "normal" liver tissue (area 1) , the latter two of which have fat droplets in the cytoplasm while the former does not. (a 17-month-old male transgenic mouse of the C21 line, x125). *f,* A higher magnification of HCC cells shown in panel *e.* Note the trabecular feature and mitotic figure (arrow in the inset) (x300). *g,* Immunohistochemical staining of HCC with anti-core rabbit serum. HCC (T) showed a higher level of the core protein than the adjacent non-tumorous liver (NT). *h,* Control staining with preimmune rabbit serum. (*g* & *h,* a 16-month-old male transgenic mouse of the C49 line, x120). *i,* Electron microscopic analysis of the liver from an 18 month-old male transgenic mouse of the C21 line. Note disappearance of the double structure of the mitochondrial membranes (x15,000). *j,* Electron microscopic examination the liver of a 17 month-old non-transgenic littermate used as control (x15,000). *k,* Immunoelectron microscopy of the liver of a 12 month-old male transgenic mouse of the C49 line with anti-core rabbit serum. Accumulation of gold particles was observed chiefly in the nuclei (N) and mitochondria (M) (x15,000).

   Paraffin-embedded sections of liver were stained with hematoxylin-eosin (*a, c-f*). Frozen liver sections were immunostained with anti-core or preimmune rabbit serum and counterstained with methyl-green (*g* & *h*).
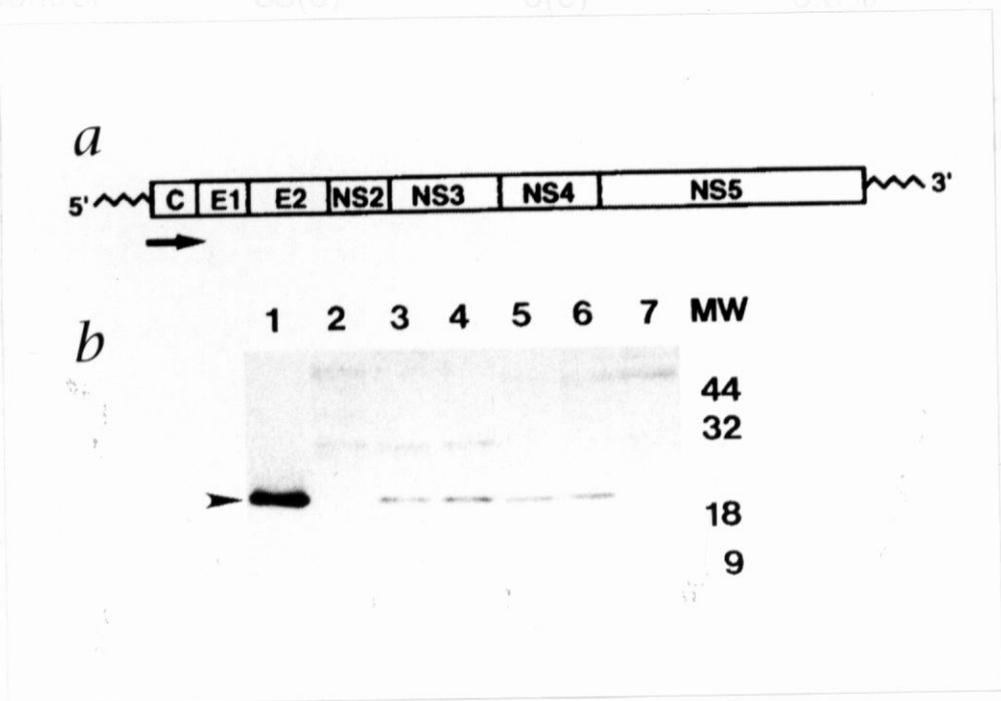
Table 1

Incidence of hepatic tumors in HCV core gene transgenic mice[1]

strain sex	mice number	mice with hepatic tumors	incidence
C21 male	27(25)	7(7)	25.9%*
C21 female	19(11)	0(0)	0.0%†
C49 male	13 (12)	4(4)	30.8%**
C49 female	14 (8)	2 (2)	14.3%†
control male	36(0)	0(0)	0.0%
control female	33(0)	0(0)	0.0%

[1]Mice were sacrificed and analyzed at the age between 16 and 19 months.
*$p < 0.01$ compared to control male mice.
**$p < 0.005$ compared to control male mice.
†statistically not significant compared to control female mice.

( );Steatosis in mouse liver by microscopic study.

PART2;Increase in the Concentration of Carbon 18 Monounsaturated Fatty Acids in the Liver with Hepatitis C: Analysis in Transgenic Mice and Humans

Abstract

Steatosis is one of the histologic characteristics of chronic hepatitis C, and is well reproduced in a transgenic mouse model for hepatocellular carcinoma (HCC), in which the core protein of hepatitis C virus (HCV) plays a pivotal role in inducing steatosis and HCC. In the present study, the lipid composition in the liver of the HCV core gene transgenic mice as well as in those of chronic he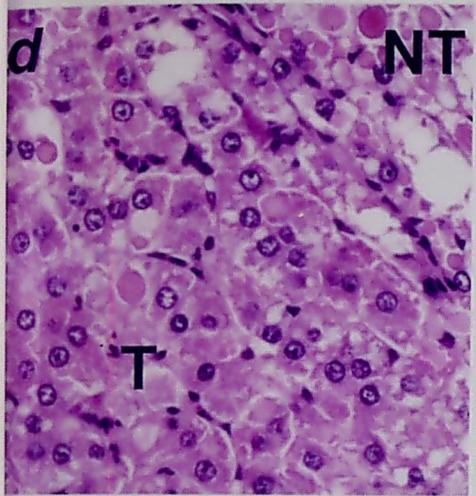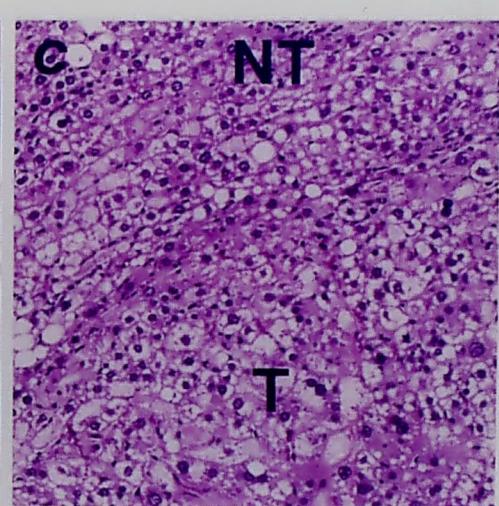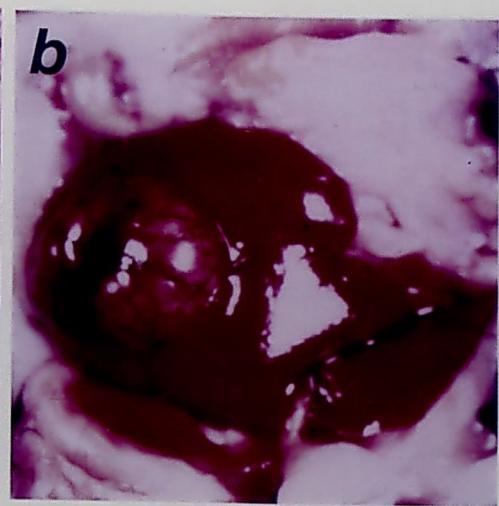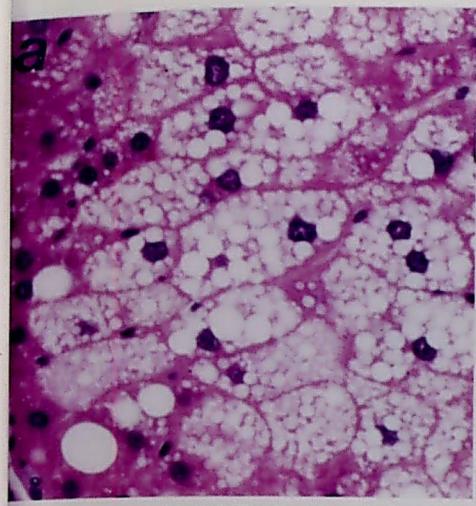patitis C patients was determined. The concentration of carbon 18 monounsaturated (C18:1) fatty acids, such as oleic and vaccenic acids, which are known to increase membrane fluidity leading to higher cell division rates, significantly increased in the livers of transgenic mice compared to non-transgenic control mice. The concentration of C18:1 fatty acids also significantly increased in the livers of chronic hepatitis C patients compared to subjects without HCV infection. These results suggest that HCV may affect a specific pathway in the lipid metabolism and cause steatosis in the liver.

How hepatocellular carcinoma (HCC) develops in patients with hepatitis C virus (HCV) infection has not been clarified, despite strong evidence from epidemiological studies associating HCV infection and development of HCC[9,10]. Recently, the core protein of HCV has been shown to induce HCC in transgenic mice, suggesting a direct involvement of HCV in the development of HCC in chronic hepatitis C[32]. In this mouse model for HCV-associated HCC, age-dependent hepatic steatosis was

observed early in life. This feature closely resembles the one observed in chronic hepatitis C patients, in whom steatosis is one of the histologic characteristics along with lymphoid follicle formation and bile duct damage[21,33,34]. The association of fatty change with the HCV core protein has also been supported by *in vitro* studies, where the introduced core gene induced formation of fat droplets in the cytoplasm[35,36]. In addition, the formation of fat droplets is associated with the development of small, early HCC; precursors for HCC or well-differentiated HCCs commonly have a number of lipid droplets in the cytoplasm, whereas de-differentiated HCCs which develop within differentiated tumors in a "nodule-in-nodule" fashion have very few lipid droplets[19,37]. This feature was also well reproduced in the HCV core gene transgenic mice[32]. Determination of the composition of lipid droplets accumulated in the livers of core gene transgenic mice, which are destined to develop HCC late in life, may lead to the elucidation of pathogenesis of liver diseases in HCV infection.

In the present study, we analyzed the composition of lipids that accumulated in the livers of transgenic mice and compared with those of non-transgenic littermates and non-transgenic obese control mice by chromatography. Moreover, we analyzed the composition of lipids in the livers of chronic hepatitis C patients and compared with that of subjects without HCV infection. Both the core gene transgenic mice and hepatitis C patients showed similar lipid profiles which are distinct from that of fatty liver due to simple obesity.

Materials and Methods

Transgenic mice. Production of HCV core gene transgenic mice has been described

previously[17]. Briefly, the core gene of HCV located downstream of a transcriptional regulatory region from hepatitis B virus was introduced into C57BL/6 mouse embryos (Clea Japan, Tokyo, Japan). Mice were cared for according to the National Institutes of Health guidelines, fed an ordinary chow diet (Funabashi Farms, Funabashi, Japan) and maintained in a specific pathogen-free state. Since HCC develops preferentially in male transgenic mice, male mice heterozygously transgenic for the core gene were used for analysis. Male non-transgenic littermate mice and non-transgenic obese mice were used as controls. Obese mice were obtained by the treatment of mice with monosodium-L glutamate as described previously[38]. At least eight mice were used in each experiment and the data were subjected to statistical analysis.

Patients. Sixteen patients (male:female=12:4) with HCC or metastatic liver tumor, who were admitted to our departments at the University of Tokyo Hospital, were studied. Eight patients were positive for anti-HCV, all of whom had HCC. Four out of the remaining eight patients had HCC, who were positive for hepatitis B surface antigen (HBsAg) but negative for anti-HCV. The remaining four patients, who were obese (body mass index≧28) and negative for both HBsAg and anti-HCV at the time of operation, underwent liver resection for metastatic liver tumors from colon cancer. The diagnosis of liver tumor was based on histologic, radiological and biochemical findings. Three of the eight anti-HCV-positive patients had a history of blood transfusion. None of the patients had a history of excessive alcohol intake. Non-cancerous liver tissues were obtained at the time of liver resection, cut into pieces and stored at -80°C until use. Serum samples were also collected at or prior to liver resection.

Histologically, four of the eight patients with anti-HCV had cirrhosis and the

remaining four had moderate to severe chronic hepatitis as the underlying liver disease. All four HBsAg-positive patients had moderate to severe chronic hepatitis, and all four with metastatic liver tumors showed no signs of hepatitis. The degree of hepatic steatosis was graded by the percentage of hepatocytes with fat droplets as follows: i) none; ii) mild, $\leq 10\%$; iii) moderate, $10\% <$ and $\leq 50\%$; and iv) severe, $50\% <$. Steatosis was severe in one, moderate in five and mild in two anti-HCV-positive patients; mild in two and none in two HBsAg-positive patients; and moderate in three and mild in one metastatic liver tumor patients. The experimental protocol was approved by the ethics review committee for human experimentation, and a written informed consent was obtained from each patient.

Reagents. Cholesterol esters and lipid standards were purchased from Sigma Chemical (St. Louis, MO), and glycogen and amyloglucosidase were obtained from Seikagaku Kogyo (Tokyo, Japan). Florisil (magnesium silicate) was obtained from Iwai Kagaku (Tokyo, Japan). Other chemicals were of analytical grade and purchased from Wako Chemicals, Tokyo, Japan.

Measurement of HCV core protein. The HCV core protein level was determined in liver tissues by the fluorescence enzyme immunoassay (FEIA) method as reported previously[39]. Briefly, 10 mg of liver tissues was homogenized in a solution containing 0.3% Triton-X100, 1.5% 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonic acid, and 15% sodium dodecyl sulfate. After incubating at 56°C for 30 min, the sample was centrifuged at 1,000 rpm for 5 min, and the supernatant was subjected to FEIA.

Preparation of liver homogenate and determination of total lipids. The liver tissues from mice or human patients were homogenized with 10 volumes of 0.6 mol/L

18

perchloric acid at 5°C for the determination of lipids. The homogenate was mixed with 10 volumes of chloroform/methanol solution (2:1) and the mixture was shaken for 5 min. The lower phase was washed twice with four volumes of saline, dried on anhydrous sodium sulphate and evaporated to complete dryness. The residue was dissolved in a chloroform/methanol (2:1) solution, and an aliquot was removed for measurement of the concentration of total lipids by Bragdon's method with potassium dichromate, using palmitic acid as a standard[40].

Fractionation of lipids. Lipids extracted from the liver homogenate were separated using a florisil column deactivated with 50% methanol[41]. Lipids suspended in 1 ml of *n*-hexane were applied to the florisil column (1x15 cm). Cholesterol esters were eluted with 50 ml of 15% dichloromethane in hexane, free cholesterol and triglycerides with 75% dichloromethane in hexane, monoglycerides with 20% methanol in dichloromethane and phospholipids with a chloroform: methanol: pyridine: acetic acid [9,10]mixture (3:2:1:1:3 by volume). The lipid content in each fraction was determined by Bragdon's method[40]. Since free cholesterol and triglycerides were not separated by this florisil column chromatography, these two lipids in the corresponding fractions were determined by an enzymatic method using commercial kits (Iyatro TC and Iyatro TG, Iyatron Co., Ltd., Tokyo, Japan) after dissolving in a 1% Triton X-100 solution.

Analysis of fatty acid composition in each class of lipids. The fatty acid composition of each lipid fraction was analyzed by gas chromatography[41]. After methanolysis by a modified Morrison and Smith method with boron trifluoride as the catalyst [41,42], fatty acid methyl esters were analyzed using a Shimadzu GC-7A gas chromatograph (Shimadzu Corp., Kyoto, Japan) equipped with a 30-m long x 0.3-mm

diameter support coated open tubular (SCOT)-type glass capillary column coated with ethylene glycol succinate. The protein concentration of the homogenates was measured by Lowry's method, using bovine serum albumin as a standard[43].

Electron microscopy. Using standard electron microscopic techniques, the mouse liver was perfused with 1.6% glutaraldehyde (TAAB Laboratory Equipment, Reading, UK), excised and fixed at 4°C for 1 h, as described previously[32].

Statistical analysis. Results are expressed as means±S.E. The significance of the difference of means was determined using Mann-Whitney's U-test.

Results

Steatosis and core protein level in transgenic mice. The level of core protein was significantly higher in mice with moderate to severe steatosis than in those with mild steatosis (P<0.001, Fig. 2-1), indicating a correlation between the grade of hepatic steatosis and the core protein level in transgenic mouse liver. Electron microscopy revealed a morphological mixture of micro- and microvesicular lipid droplets present throughout the cytoplasm of hepatocytes (Fig. 2-2). The nucleus and endoplasmic reticulum appeared intact but the double membrane structure of mitochondrion was disrupted. Secondary lysosomes carrying various cytoplasmic organelles were scattered in the cytoplasm (Fig.2- 2, arrows).

Lipid accumulation in transgenic mouse liver. Figure 2-3 shows the concentrations of lipids in liver tissues of core gene transgenic, non-transgenic littermate and non-transgenic obese mice. The triglyceride level significantly increased in the livers of transgenic mice compared to non-transgenic littermate mice

(38.6±39.7 vs. 3.2±1.7 mg/g liver, p<0.05), while the difference was not significant between transgenic mice and non-transgenic obese mice (38.6±39.7 vs. 19.5±19.7 mg/g liver, p=0.14). The free fatty acid level also significantly increased in transgenic mice compared to non-transgenic littermate mice (21.3±17.7 vs. 3.2±1.7 mg/g liver, p<0.05), while the difference was not significant between transgenic mice and non-transgenic obese mice (21.3±17.7 vs. 7.6±6.5 mg/g liver, p=0.059).

Fatty acid compositions of cholesterol esters, triglycerides and phospholipids in mouse liver. The fatty acid compositions of cholesterol esters, triglycerides and phospholipids were determined by gas chromatography in the livers of core gene transgenic mice, non-transgenic littermate and non-transgenic obese mice. The concentration of carbon 18 monounsaturated fatty acids (C18:1, oleic and vaccenic acids) significantly increased in transgenic mice. In the triglyceride fraction, the concentration of oleic acid was significantly higher in the core gene transgenic than in the non-transgenic littermates or non-transgenic obese mice (911.4±164.6 vs. 45.8±19.8 or 249.4±74.0 μmol/g liver, p<0.001). Similarly, the concentration of vaccenic acid was significantly higher in the core gene transgenic mice than in the non-transgenic littermate or non-transgenic obese mice (115.8±32.6 vs. 12.3±4.8 or 34.8±18.7 μmol/g liver, p<0.001). However, the concentration of palmitic acid was significantly higher in the non-transgenic obese mice than in the core gene transgenic mice (310.6±167.7 vs. 123.8±60.6 μmol/g liver, p<0.05), while there was no significant difference between the transgenic mice and non-transgenic littermate mice (123.8±60.6 vs. 72.6±43.8 μmol/g liver, p=0.14).

The fatty acid compositions of cholesterol esters, triglycerides and phospholipids in the mouse liver are summarized in Fig. 2-4. The proportion of C18:1

(oleic and vaccenic acids) in the triglyceride fraction was significantly higher in the transgenic mice than in the non-transgenic littermate and non-transgenic obese mice (42.3±11.2 vs. 27.3±11.4 or 27.2±12.5%, p<0.05). Similarly, the proportion of C18:1 fatty acids in the cholesterol ester fraction was significantly higher in the transgenic mice (29.4±10.4 vs. 17.9±8.4 or 14.4±7.9%, p<0.05), but no significant difference in the phospholipid fraction was observed. There was no significant difference in the proportions of C16:0, C16:1 and C18:0 fatty acids between transgenic mice and non-transgenic controls in each fraction.

Fatty acid compositions of cholesterol esters, triglycerides and phospholipids in human liver. The triglyceride level was higher in the chronic hepatitis C patients than in the subjects without HCV infection, but the difference was not significant (26.5±11.5 vs. 18.0±10.4 mg/g liver, p=0.104). The fatty acid compositions of cholesterol esters, triglycerides and phospholipids in the livers of the subjects with and without HCV infection are summarized in Fig. 2-5. Similar to the results in mice, the proportion of C18:1 fatty acids (oleic and vaccenic acids) in the triglyceride fraction was significantly higher in the chronic hepatitis C patients than in the subjects without HCV infection (36.0±9.2 vs. 24.1±9.9%, p<0.05). The fatty acid compositions observed in the fatty liver patients (increase of C16:0 and C18:0) were compatible with the previous reports on fatty liver patients[44,45]. There was no significant difference in the proportion of C18:1 fatty acids in the cholesterol ester fraction (29.0±12.8 vs. 24.3±11.2%, p=0.29) between these two groups (Fig. 2-5).

Discussion

Steatosis has been strongly associated with chronic hepatitis C by a body of evidence consisting of clinicopathologic[21,33,34] and experimental studies, both *in vivo* [17,32,46] and *in vitro* [35,36].We recently showed the direct interaction of HCV core protein with retinoid X receptor alpha(RXR alpha), a transctiptional regulator that controls many aspects of cell proliferation,differentiation,and lipid metabolism[47]. The core protein binds to the DNA-binding domain of RXR alpha ,leading to increase the DNA binding of RXRalpha to its responsive element.

The expression level of the core protein and the grade of steatosis have also been correlated in the liver of chronic hepatitis C patients[48]. However, the composition of fatty acids that accumulated in the livers of chronic hepatitis C patients, which may result from disturbances in the lipid metabolism by HCV, has not been studied yet. The current study, to our knowledge, is the first report on the effect of HCV infection on the composition of lipids in the liver. The proportion of C18:1 fatty acids, such as oleic and vaccenic acids, significantly increased in the triglyceride and cholesterol-ester fractions in the livers of HCV core gene transgenic mice. This finding is of a great importance because the proportion of C18:1 fatty acids also increased in the livers of patients who suffered from chronic hepatitis C. It should be emphasized that, in contrast to HCV-associated steatosis, the proportion of palmitic acid (C16:0) increased instead of that of the C18:1 fatty acids in the livers of mice with simple obesity. Although these simple obesity mice were generated through hypothalamic damage by the administration of monosodium-L glutamate[38], the composition of lipid in these mice shows a similar pattern to the one observed in human fatty liver due to

simple obesity[44,45] . Thus, the accumulation of C18:1 fatty acids may be due to the effect of a specific action of the HCV core protein.

In our animal model for HCV pathogenesis, steatosis is solely a consequence of the expression of the core protein in the liver, which has also been supported by cell culture studies[35,36]. In the case of human chronic hepatitis C, the pathogenesis of steatosis may be complicated and one cannot exclude the possibility of the effect of inflammation on lipid metabolism. However, the coincidence of dominant accumulation of C18:1 fatty acids in the livers of transgenic mice and hepatitis C patients suggests that HCV may also play a role in the alteration of lipid metabolism in chronic hepatitis C patients.

Quantitative determination of the core protein in the liver of transgenic mice revealed a correlation between the grade of steatosis and the level of core protein, further supporting the causative role of the core protein in the pathogenesis of hepatic steatosis in transgenic mice. The range of the core protein level in the transgenic mouse liver, which was determined by the FEIA method, was within that in the liver of chronic hepatitis C patients[49] . Together with the results of Western blot analysis[32] core gene transgenic mice is, therefore, not a result of overexpression but an expression of the core protein at a level similar to that in the livers of chronic hepatitis C patients.

The relevance of the alteration in the lipid metabolism in the liver with hepatitis C, and that of the predominance of C18:1 fatty acids, are not yet clear. However, it is noteworthy that a higher level of oleic acid accounts for increased membrane fluidity, leading to increased cell metabolism and higher cell division rates[50-53] . It is also interesting to note that stearic acid (C18:0) inhibits the growth of cancer cells and oleic

acid abrogates this effect [54]. Therefore, the predominance of C18:1 fatty acids might induce hepatocytes to grow or divide actively, thereby associated with hepatocarcinogenesis. The mechanism underlying steatosis in the core gene transgenic mice is not thoroughly understood yet, but both β-oxidation of fatty acids in the mitochondrion and secretion of triglycerides from the liver were found to be impaired (Moriya K, et al., unpublished data).Core protein may lead to reduction in microsomal triglyceride transfer protein activity.

In conclusion, the same lipid profile, i.e., C18:1 fatty acid predominance, is present in the steatotic livers of HCV core gene transgenic mice and chronic hepatitis C patients. This suggests that HCV may affect a specific pathway in the lipid metabolism and cause hepatic steatosis in HCV-infected liver.

Figure Legends

Fig. 2-1

Steatosis and intrahepatic core protein level in transgenic mice.

The level of hepatitis C virus core protein was determined by the fluorescence enzyme immunoassay method, and was correlated with the grade of steatosis. The data are means±S.E., n=10 in each group. ***$p < 0.001$.

Fig. 2-2

Morphological study of lipid accumulation in the transgenic mouse liver by electron microscopy.

A number of lipid droplets of various sizes (L) were present in hepatocytes from a core gene transgenic mouse (6 m.o.). While the nuclei were intact (N), the double-structured mitochondrial membrane was disrupted (m) and the secondary lysosomes were observed to contain remnants of mitochondria (arrows). (x5,000)

Fig. 2-3

Lipid contents in the mouse liver.

Lipids extracted from liver tissues of transgenic (Tg, hatched bars), non-transgenic littermate (nTg, black bars) or non-transgenic obese mice (nTg obese, dotted bars) were fractionated using a florisil column.

The data are means±S.E., n=8 in each group. *$p < 0.05$; NS, not statistically significant. CE, cholesterol esters; TG, triglycerides; PL, phospholipids; FFA, free fatty acids.

Fig. 2-4

Fatty acid composition (relative composition, %) in the mouse liver.

The fatty acid composition of each lipid fraction from liver tissues of transgenic (Tg, hatched bars), non-transgenic littermate (nTg, black bars) or non-transgenic obese mice (nTg obese, dotted bars) was analyzed by gas chromatography. The sum of percentages does not become 100 due to the presence of small fractions.

The data are means±S.E., n=8 in each group. *p<0.05. CE, cholesterol esters; TG, triacylglycerol; PL, phospholipids.

Fig. 2-5

Fatty acid composition (relative composition, %) in the human liver.

The fatty acid composition of each lipid fraction from liver tissues of patients with chronic hepatitis C (HCV+, hatched bars) or subjects without HCV infection (HCV-, black bars) was analyzed by gas chromatography. The sum of percentages does not become 100 due to the presence of small fractions.

The data are means±S.E., n=8 in each group. HCV, hepatitis C virus; *p<0.05. CE, cholesterol esters; TG, triacylglycerol; PL, phospholipids.

Fig. 2-1

HCV core protein level (Arbitorary unit)

Grade of steatosis

mild (<10%)   moderate (10-50%)   marked (50%<)

Fig. 2-2

Fig. 2-3

mg/g liver

CE   TG   PL   FFA

Tg
nTg
nTg obese

Fig. 2-4

(%)

C16:0 C16:1 C18:0 C18:1   C16:0 C16:1 C18:0 C18:1   C16:0 C16:1 C18:0 C18:1
CE   TG   PL

HCV+
HCV-

Fig. 2-5

(%)

C16:0 C16:1 C18:0 C18:1   C16:0 C16:1 C18:0 C18:1   C16:0 C16:1 C18:0 C18:1
CE   TG   PL

Tg
nTg
nTg obese

PART3;Oxidative Stress in the Absence of Inflammation in a Mouse Model for Hepatitis C Virus-Associated Hepatocarcinogenesis

Abstract

The mechanism of hepatocarcinogenesis in hepatitis C virus (HCV) infection is still undefined. One possibility is the involvement of oxidative stress, which can produce genetic mutations as well as gross chromosomal alterations and contribute to cancer development. We recently showed that the core protein of HCV induces, a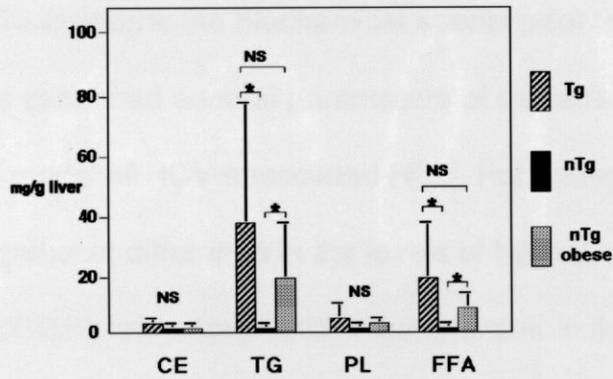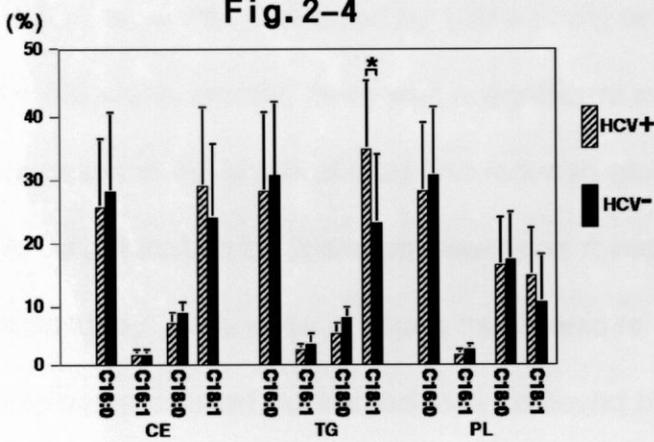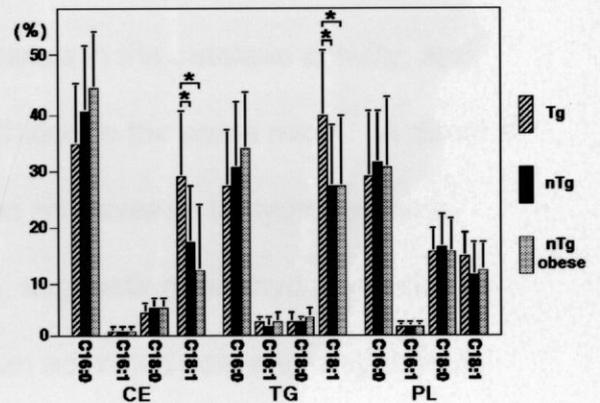fter a long period, hepatocellular carcinoma (HCC) in transgenic mice with marked hepatic steatosis but without inflammation, indicating a direct involvement of HCV in hepatocarcinogenesis. To elucidate the biochemical events prior to the developmentof HCC, we examined several parameters of oxidative stress and redox homeostasisin a mouse model of HCV-associated HCC. For young mice aged 3 to 12 months,there was no significant difference in the levels of hydroperoxides of phosphatidylcholine (PCOOH) and phosphatidylethanolamine in liver tissue homogenates between transgenic and non-transgenic control mice. In contrast, PCOOH level was increased by 180% in old core gene transgenic mice aged over 16 months. Concurrently, there was a significant increase in the catalase activity, and decreases in the levels of total and reduced glutathione in the same mice. A direct *in situ* determination by chemiluminescence revealed an increase in hydroperoxide products by 170% even in young transgenic mice, suggesting that hydroperoxides were overproduced but immediately removed by an activated scavenger system in young mice. Electron microscopy revealed lipofuscin granules, secondary lysosomes

carrying various cytoplasmic organelles and disruption of the double membrane structure of mitochondria, and PCR analysis disclosed a deletion in mitochondrial DNA. Interestingly, alcohol caused a marked increase in PCOOH level in transgenic mice, suggesting synergism between alcohol and HCV in hepatocarcinogenesis. The HCV core protein thus alters the oxidant/antioxidant state in the liver in the absence of inflammation, and thereby may contribute to or facilitate, at least partly, the development of HCC in HCV infection.

Recently, the core protein of HCV has been shown to induce HCC in transgenic mice, and has been suggested to play a central role in the development of HCC in chronic hepatitis C[32]. However, it still remains unclear how the core protein operates in the development of HCC: modulation of certain cellular gene products such as helicase, lymphotoxin-β receptor or dead box protein[55-57], as shown in cell culture systems, may contribute to hepatocarcinogenesis. Another possibility is the induction of oxidative stress.

Endogenous oxidants generated by multiple intracellular pathways are an important class of naturally occurring carcinogens[58,59]. Reactive oxygen species (ROS) are endogenous oxygen-containing molecules formed as normal products during aerobic metabolism[60]. ROS include a number of species such as superoxide, hydroxyl, and peroxyl radicals, and certain nonradicals such as singlet oxygen and hydrogen peroxide that can be easily converted into radicals. ROS can induce genetic mutations as well as chromosomal alterations and thus contribute to cancer development in multistep carcinogenesis [61,62]. Moreover, a number of recent studies have demonstrated that ROS at submicromolar levels act as novel intra- and

intercellular secondary messengers and thus modulate various aspects of cellular functions including proliferation, apoptosis and gene expression[63]. Most markers of oxidative injury utilized reflect free radical attack on polyunsaturated fatty acids, with the classical route of attack involving lipid peroxidation,

In the present study, we explored the oxidant/antioxidant status in the liver of a transgenic mouse model of HCC in HCV infection, prior to development of HCC, by sequential quantification of hydroperoxide level, glutathione level and catalase activity and comparison of these parameters with those of age-matched non-transgenic control mice.   We found an age-dependent increase in oxidative stress in the livers of transgenic mice which develop HCC in the absence of inflammation as a consequence of core protein expression.

Materials and Methods

Transgenic mice.   The production of HCV core gene transgenic mice has been described previously[17] .   Briefly, the core gene of HCV placed downstream of a transcriptional regulatory region from the hepatitis B virus was introduced into C57BL/6 mouse embryos (Clea Japan, Tokyo, Japan). Mice were cared for according to institutional guidelines, fed an ordinary chow diet (Funabashi Farms, Funabashi, Japan) and maintained in a specific pathogen-free state.   For the ethanol loading experiment, mice were fed a diet containing 5% ethanol (Oriental Yeast Co., Ltd., Tokyo, Japan) for 3 weeks.   For the induction of inflammation, carbon tetrachloride (CCl_4) was administered to mice i.p. at a dose of 0.5 ml/kg weight.   Since HCC develops preferentially in male transgenic mice, we used male mice which were

31

heterozygously transgenic for the core gene, and as controls we used non-transgenic littermates of the transgenic mice. Transgenic mice carrying the HCV envelope genes under the same regulatory region as that in the core gene transgenic mice were used as control[64]. Obese mice were obtained by treating the mice with monosodium-L glutamate[38]. At least five mice were used in each experiment and the data were subjected to statistical analysis.

Chemicals. Unless otherwise stated, all chemicals were of reagent quality and purchased from Wako Chemicals, Tokyo, Japan.

Lipid extraction from the liver. For determination of hydroperoxide levels, liver tissues were used immediately after sacrifice of the mice and never used after storage, even at -70°C, because storage inevitably results in an increase in peroxide products[65]. Liver samples (100mg) were homogenized in 2 ml of 0.15 M NaCl solution. To 1 ml of homogenate, 4 ml of chloroform/methanol (2:1, v/v; containing 90 nM butylated hydroxytoluene as an antioxidant) was added and mixed vigorously for 1 min. The mixture was centrifuged at 1,000 g for 15 min. Then, the lower chloroform layer was collected and evaporated to dryness under nitrogen stream. The liver total lipids were dissolved in an appropriate amount of chloroform/methanol (2:1, v/v) and subjected to analysis[66].

Determination of phosphatidylcholine and phosphatidylethanolamine hydroperoxides. Hydroperoxide products of phosphatidylcholine (PCOOH) and phosphatidylethanolamine (PEOOH) in liver total lipids were determined by chemiluminescence (CL)-high performance liquid chromatography (HPLC), as previously described [66,67]. The system consisted of a Jasco HPLC system (Japan Spectroscopic Co., Tokyo, Japan) and post-column chemiluminescence detection.

The HPLC column was a Jasco Finepak SIL NH2-5 (5µm, 250 X 4.6mm), the column mobile phase was 2-propanol/methanol/water (135:45:20, v/v/v) and the flow rate was 1.0 ml/min. Post-column chemiluminescence detection was carried out using a CLD-100 detector (Tohoku Electronic Industries Co., Sendai, Japan), which employs a cooled photomultiplier tube (PMT), to suppress PMT dark current and improve the S/N ratio of HPLC analysis. As a hydroperoxide-specific post-column chemiluminescence reagent, a mixture of luminol and cytochrome C in 50 mM borate buffer (pH 10.0) was utilized [66,67]. Calibration was carried out using authentic PCOOH as a standard, as described previously[66]. Protein concentrations were determined by Lowry's method using bovine serum albumin as the protein standard.

Determination of catalase activity and glutathione level. Catalase activity was measured spectrophotometrically (at 240 nm) by following the decrease in absorbance of hydrogen peroxide after addition of 0.1 ml of supernatant to 0.9 ml of 15 nM H_2O_2 in 50 nM phosphate buffer, pH 6.8 [68]. Reduced glutathione (GSH) and oxidized glutathione (GSSG) levels were measured as described previously[69]. Briefly, tissues were homogenized with Physcotron (Niti-on, Tokyo, Japan) after adding 4 ml/g wet tissue of 5% trichloroacetic acid containing 5 mM Na_2EDTA, and were centrifuged at 1,850x g for 10 min. After the addition of 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole and ethyl acetate to the supernatant liquid followed by vigorous shaking, the sample was centrifuged at 3,000 rpm for 5 min. Then the solution was reacted with ammonium 7-fluoro-2,1,3-benzoxadiazole under the reduction of disulfides with tri-*n*-butylphosphine in acetonitrile. After cooling on ice water, the solution was adjusted to pH 2 with HCl, and 10 ml of the acidic solution was injected onto the HPLC column. The total amount of glutathione was computed by

adding the amounts obtained for GSH and GSSG.

Spontaneous *in situ* liver surface chemiluminescence. The spontaneous *in situ* surface chemiluminescence of the liver was monitored using a photon counter (Johnson Research Foundation, Philadelphia, PA) with a model 9658 photomultiplier responsive over the range of 350 to 850 nm as described elsewhere[70,71]. Chemiluminescence in the intact organ is the result of different photoemissive reactions: reactive oxygen species and lipid radicals are primary contributors to tissue chemiluminescence[70,71]. Intrinsic low-level light emission by living tissues is measured by very sensitive photomultiplier. A mouse is fixed in a light-tight chamber supplied with necessary physiological equipment, and the liver is exposed to the photomultiplier placed as close as possible to the organ surface. To avoid chemiluminescence from other organs, the mouse is covered with aluminium foil and only the liver is exposed to the photomultiplier. Emission was expressed in counts per second per square centimeter of liver surface (cps/cm^2). Spectral analysis of liver chemiluminescence was performed with cut-off Kodak Wratten filters (Eastman Kodak, Rochester, NY) as described elsewhere [72].

Electron microscopy. For standard electron microscopic techniques, mouse liver was perfused with 1.6% glutaraldehyde (TAAB Laboratory Equipment, Reading, UK), excised and fixed at 4°C for 1 h.

DNA isolation and analysis of mitochondrial DNA. Total DNA was isolated from the liver as described previously [3]. Detection of mitochondrial DNA (mtDNA) deletion present between the direct repeats of mtDNA (direct repeat 17 corresponding to bp 979-5650 of mouse mtDNA) [73] was performed by PCR with the primers TAAGTCGTAACAAGGTAAGC (bp 979-998) and GATGGTGGTAGGAGTCAAAA (bp

5650-5631). Amplification was carried out in a thermal cycler for a total of 35 cycles consisting of 94°C for 40 s, 50°C for 30 s and 72°C for 2 min, in 100 μl of the reaction mixture containing 200 μM dNTPs, 1.0 μM each of the primers and 1X PCR buffer [50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl$_2$ and 0.001% (w/v) gelatin] and 2 units of Ampli-Taq polymerase (Perkin Elmer Cetus Corp., Norwalk, CT). The PCR product of the intact repeat is 4671 bp long, while the predicted size of the deletion between the direct repeats is 3821 bp, producing a PCR deletion product of 851 bp. The PCR products were analyzed by electrophoresis in a 2% agarose gel.

Statistical analysis. Results are expressed as means±S.E. The significance of the difference in means was determined by Student's *t*-test.

RESULTS

The HCV core gene transgenic mice express the core protein in the liver at levels similar to those in the liver of chronic hepatitis C patients, develop steatosis without inflammation early in life, and finally develop HCC when they are aged over 16 months[32]. In the present study, analyses were performed on two groups of mice: young mice aged 3 to 12 months and old mice aged over 16 months.

Lipid peroxidation in HCV core gene transgenic mice. We investigated the levels of lipid peroxidation as a measure of accumulated oxidative damage to membrane lipids. Lipid peroxidation is important because it amplifies the free radical production process and its products could lead to cellular and tissue damage[74]. Direct determination of the primary products of oxidative attack has been shown to be the most accurate measure of lipid peroxidation[75]. We selected to determine the

levels of PCOOH and PEOOH by the CL-HPLC method, since these are the most reliable parameters for lipid peroxidization[75,76]. We first determined the levels of PCOOH and PEOOH in the liver of young HCV core gene transgenic and non-transgenic control mice aged 3 to 12 months. There was no significant difference in the levels of PCOOH (0.89 ± 0.16 vs. 0.83 ± 0.08 nmol/g protein, p=0.46) or PEOOH (0.43 ± 0.11 vs. 0.42 ± 0.14 nmol/g protein, p=0.90) in these mice as shown in Figure 3-1A.   In contrast, for older mice aged over 16 months, when the core gene transgenic mice start to develop HCC (32, 75), both the levels of PCOOH (2.30 ± 0.42 vs. 0.83 ± 0.19 nmol/g protein, p<0.01) and PEOOH (1.04 ± 0.32 vs. 0.42 ± 0.15 nmol/g protein, p<0.01) were significantly higher than those in non-transgenic control mice (Fig. 3-1B). There was a correlation between ROS generation and the level of the core protein when old core gene transgenic mice were analyzed (data not shown). The levels of PCOOH in the livers of 16-m.o. simple obese mice, which showed a moderate grade of hepatic steatosis, were not significantly different from those in the livers of non-transgenic control mice (0.98 ± 0.33 vs. 0.83 ± 0.19 nmol/g protein, p=0.41). The levels of PCOOH in the livers of 16-m.o. transgenic mice expressing the HCV envelope proteins under the same regulatory region as that in the core gene transgenic mice were not significantly elevated compared to non-transgenic control mice (0.79 ± 0.27 vs. 0.83 ± 0.19 nmol/g protein, p=0.82). This indicates that not the regulatory feature of protein expression driven by the HBV regulatory region used in the current study but the expressed protein itself, i.e., the core protein, is responsible for the excessive production of ROS in the mouse liver.

Anti-oxidative state in HCV core gene transgenic mice.   Measurement of ROS is not complete without the measurement of defense systems that protect against ROS.

36

In the assessment of ROS *in vivo*, the balance between generation and elimination is more important than the measurement of any single component [67]. We determined, in the livers of young mice, 1) catalase activity, 2) total glutathione and 3) GSH levels. Catalase is the enzyme that generates H_2O and O_2 by metabolizing hydrogen peroxide (H_2O_2) which is produced by the action of superoxide dismutase. Reduced glutathione (GSH) plays a central role in cellular defense against oxidative stress[77], by regulating the intracellular concentration of ROS via a reaction catalyzed by glutathione peroxidase (GPx). The catalase activity was 149.8 ± 22.1 units/100 mg of liver in transgenic mice, whereas it was 104.2 ± 18.4 units/100 mg of liver in non-transgenic control mice ($p < 0.01$, Fig. 3-2A). The total glutathione level (GSH+GSSG) was significantly lower in transgenic mice than in non-transgenic control mice (2.62 ± 0.59 vs. 3.89 ± 0.58 µmol/g of liver, $p < 0.01$, Fig. 3-2B). In addition, the ratio of GSH to total glutathione was significantly lower in transgenic mice than in non-transgenic control mice (0.832 ± 0.051 vs. 0.908 ± 0.016, $p < 0.05$, Fig. 3-2C).

These results for the anti-oxidative system suggest that ROS are overproduced in the livers of transgenic mice even at a young age, but are promptly scavenged by concomitantly activated antioxidants such as catalase and the glutathione system. Therefore, to directly measure free radical formation, we took an advantage of the method for measuring spontaneous *in situ* liver surface chemiluminescence method (Fig. 3-3A), and compared the levels of chemiluminescence in the livers of core gene transgenic mice and non-transgenic control mice at young age (6 m.o.). As shown in Figure 3-3B, spontaneous liver surface chemiluminescence was significantly higher in transgenic mice by approximately 170% compared to in normal control mice

(4,968±488 vs. 1,858±682 arbitrary units, p<0.01).

Mitochondrial morphology and DNA damage. Because mtDNA is more sensitive to oxidative damage than nuclear DNA[78], we investigated for the effects of increased ROS production on mtDNA damage. For this purpose, hepatic DNA was analyzed by PCR and screened for large 3821-bp deletions associated with direct sequence repeats normally present in aging mice[74]. As shown in Fig.3- 4, this deletion was not detectable in non-transgenic control mice aged 2 or 9 months, but was detectable at the age of 2 months and was prominent at the age of 9 months in core gene transgenic mice. The deletion was more prominent in transgenic mice aged 19 months, while only a weak band indicating deletion was detectable in 19-m.o. non-transgenic control mice, which may be the result of aging.

Electron microscopy showed no clear evidence of swelling or degeneration of mitochondria in transgenic mice aged 3 months, but the disruption of the double structure of the mitochondrial membrane was observed in mitochondria from the livers of transgenic mice (Fig. 3-5A and 3-5B). In transgenic mice over the age of 12 months, hepatocytes exhibited scattered residual bodies or lipofuscin granules and secondary lysosomes carrying various cytoplasmic organelles (Fig. 3-5B and 3-5C). These structures are found in senescent cells *in vivo* and are recognized as morphologic signs of damages induced by ROS [79,80].

Effects of alcohol and inflammation on lipid peroxidation. In human chronic hepatitis C, alcohol intake has been documented as a synergistic factor for the acceleration of hepatocarcinogenesis[80-82]. To assess this possibility in our mouse model, we administered alcohol to mice and examined whether or not alcohol enhances the effect of the core protein on the levels of hydroperoxide in mouse liver.

The PCOOH level was determined in the livers of transgenic and non-transgenic control mice aged 6 months with or without administration of 5% ethanol for 3 weeks. Non-transgenic control mice fed with a 5% ethanol diet for 3 weeks showed only a slight increase in PCOOH levels compared to those not fed an ethanol diet. In contrast, transgenic mice exhibited an approximate 200% increase in PCOOH levels compared to non-transgenic control mice fed with the same 5% ethanol diet (3.11 ± 0.54 vs. 1.02 ± 0.27, $p < 0.01$, Fig. 3-6A) or transgenic mice not fed an ethanol diet (3.11 ± 0.54 vs. 0.88 ± 0.28, $p < 0.01$, Fig. 3-6A).

We then administered carbon tetrachloride (CCl_4) to mice (0.5 ml/kg body weight), to examine the possibility that inflammation and the core protein may act synergistically to induce oxidative stress in the liver. Although CCl_4 treatment increased PCOOH levels in mice and the levels were higher in transgenic than non-transgenic control mice, the difference was not statistically significant (2.96 ± 1.03 vs. 2.07 ± 0.48, $p = 0.12$, Fig.3- 6B).

Discussion

In the present study, we demonstrated a striking intracellular increase in lipid peroxidization, as revealed by the determination of PCOOH and PEOOH levels, in the livers of transgenic mice destined to develop HCC in the latter half of their lives[32]. Antioxidant systems were also activated in accordance with the extent of oxidative stress. It should be emphasized that such alteration in the oxidant/antioxidant state occurred in the absence of inflammation in the livers of transgenic mice[32]. In patients with HCV infection, increased levels of lipid peroxidation represented by the

production of derivatives such as malondialdehyde or 4-hydroxynonenal have been demonstrated in the liver, serum or leukocytes [84-87], and ROS have been suggested to play a role in the development of liver cancer[88,89]. In humans, however, it is not clear whether ROS production is triggered by the action of HCV *per se* or by inflammation which is instrumental in producing ROS in a variety of organs[90,91]. In our transgenic mouse model for viral hepatocarcinogenesis, ROS overproduction occurred in the absence of any inflammation, suggesting that the HCV core protein *per se*, which was expressed in similar levels to those in the livers of chronic hepatitis C patients[32], is sufficient to induce oxidative stress in the mouse liver. This is very important in that it leads to the idea that the presence of HCV itself may induce the production of ROS in human liver and render hepatocytes susceptible to DNA damage, the accumulation of which may lead to malignant transformation.

We determined the levels of PCOOH and PEOOH in the liver as a measure of the extent of lipid peroxidization. Lipid peroxidization is the most reliable marker of excessive ROS activity *in vivo*, and generates hydroperoxides, endoperoxides, long-lived aldehydes and the end-products malondialdehyde, ethane and pentane[75]. Determining hydroperoxide products, particularly PCOOH or PEOOH, is one of the most reliable methods for evaluating lipid peroxidation[66], in contrast to measuring the level of thiobarbituric acid reactive substances (TBARS), which is troublesome due to a number of artifacts and non-specific reactions[75].

HCV core gene transgenic mice showed an increase in hydroperoxide levels in an age-dependent manner. Hydroperoxide levels were increased in old transgenic mice but not in young mice as determined by the CL-HPLC method, whereas the determination of hydroperoxide by the spontaneous *in situ* liver surface

chemiluminescence method revealed the elevation of hydroperoxide levels also in young mice. This is probably due to the difference in the two methods used. Because the spontaneous *in situ* liver surface chemiluminescence method can be used to detect hydroperoxides generated in the liver in real time before elimination by the antioxidant system, there could be a discrepancy between determinations using this method and CL-HPLC of homogenized tissues. In fact, the antioxidant systems such as catalase and GPx were also activated in young mice, suggesting that, at a young age, the generation of hydroperoxides was counterbalanced by the antioxidant systems, but the balance terminated when the mice became older. Catalase and GPx play important roles in cellular antioxidant defense by reducing the levels of hydroperoxides, which otherwise can be converted to highly reactive hydroxyl radicals through the metal-mediated Fenton reaction[92]. Thus, the production of ROS was initiated in the young mice and became evident in the livers of the older core gene transgenic mice. It has been suggested that the generation of ROS increases in the brains or livers of old normal rats [93,94], but this was not observed in the livers of the parental mouse strain C57BL/6 mouse used in this study (Figs. 1A and B), a finding which is harmonious with the very low incidence of spontaneous liver tumors in this strain[22,23].

Starting from the age of 2 months, there was a significantly higher incidence of mtDNA deletion in the livers of transgenic mice than non-transgenic control mice, which may be associated with the increase in the production of ROS. MtDNA is known to be 10-15-fold more sensitive to oxidative damage than nuclear DNA[78,95]. Enhanced ROS generation and the progressive accumulation of mtDNA damage have been described in aging rodents and human livers in degenerative diseases

associated with oxidative liver damage[96-98] . Increased ROS generation in the livers

of core gene transgenic mice may lead to premature oxidative damage of hepatic

mtDNA and play a part in the development of HCC. Search for gross chromosomal

alterations in HCC developed in the transgenic mice is currently underway. Recent

studies have demonstrated that ROS at submicromolar levels act as intracellular

secondary messengers and thus regulate various aspects of cellular functions

including proliferation, apoptosis and gene expression [63] .It would therefore be

interesting to know whether or not the intracellular signaling pathways, such as NF-κB

or AP-1, are activated. In this mouse model, not NF-κB but AP-1 is activated[1].

In the current study, a synergy was revealed between the core protein and

alcohol administration with respect to the generation of hydroperoxide in mouse liver.

This is of great interest because a synergy between excessive alcohol intake and

HCV infection has been documented in the development of HCC in chronic hepatitis

C patients[81-83] . In these studies, alcohol is one of the independent co-factors

accelerating the development of HCC in chronic hepatitis C patients. Our result may

explain this synergy in chronic hepatitis C patients. In addition, there was a higher

increase in hydroperoxide levels in CCl_4-treated transgenic mice than in CCl_4-treated

non-transgenic control mice: the difference, however, was not statistically significant,

which may be due to the fact that CCl_4 is a very strong inducer of ROS[99]. These

results indicate that the HCV core protein not only induces ROS production in

hepatocytes but also predisposes hepatocytes to the generation of more ROS when

stimulated by ROS-inducing factors such as alcohol or inflammation. In this sense,

ROS production in hepatitis C might be different, by the presence of the HCV core

protein, from that in hepatitis caused by other factors; e.g., autoimmune chronic active

hepatitis, which is not associated with HCC development [100,101].

In this mouse model for HCV-associated hepatocarcinogenesis, the development of HCC is preceded by hepatic steatosis, which is one of the histologic characteristics of hepatitis C[21] . Steatosis in core gene transgenic mice is age-dependent, and characterized by the appearance of micro- and macrovesicular fat droplets[95]. The pathogenesis of steatosis in these mice is not completely clear, but both the β-oxidation of fatty acids in mitochondria and secretion of triacylglycerol from the liver were found to be impaired[2]. Because no significant elevation was observed in the levels of hydroperoxide in steatite liver tissues from mice with simple obesity, not steatosis but the core protein *per se* or steatosis with the core protein may play a central role in the generation of ROS in the transgenic mouse liver. It is not clear yet how the core protein induces ROS overproduction. However, we recently obtained some evidence in the core gene transgenic mice suggesting an impairment of the mitochondrial electron transfer system[2], which leads to the ROS overproduction[102]. This may explain how excessive ROS is produced in the liver of HCV core gene transgenic mice. This may also explain the mechanism of synergy between alcohol and the core protein in inducing ROS. Alcohol has been shown to induce ROS in the liver through acetaldehyde formation and mitochondrial damage, etc[103]. Therefore, low dose of alcohol, which is not enough to induce ROS by itself, can be synergistic with the core protein.

In conclusion, our results indicate that the HCV core protein induces ROS in the liver in the absence of inflammation, which may, in part, be responsible for the development of HCC in this mouse model as well as in chronic hepatitis C patients. Other mechanisms, such as modulation of cellular gene expression by the core

protein or interaction of the core protein with cellular proteins, may also play a role in the development of HCC.

The promotion of increases in free radical formation with time in the livers of HCV core protein transgenic mice ,but without inflammation,could help explain why hepatocellular carcinoma expression mostly occures after extended periods of exposure to hepatitis C.Alcohol intake   reduced the time delay before increases in free radical formation became significant.As inflammation and steatosis are associated with alcohol induced liver damage,increased liver damage in HCV infected individuals imbibing alcohol may explain why they frequently present sooner with hepatocellular carcinoma than those individuals consuming significantly less alcohol

FIGURE LEGENDS

Fig.3-1.   Levels of lipid peroxidation in core gene transgenic and control mice.

A, Young mice aged 3 to 12 months.

B, Old mice aged over 16 months.

The hydroperoxide products of phosphatidylcholine (PCOOH) or phosphatidylethanolamine (PEOOH) were determined in liver tissue homogenates of transgenic mice (Tg) and non-transgenic control mice (nTg).

The data are means±S.E., n=5 in each group. NS, not statistically significant.

Fig. 3-2.   Antioxidant systems in young core gene transgenic and control mice.

A, Catalase activity; **B**, total glutathione level; **C**, the ratio of reduced/total glutathione levels.

The data are means±S.E., n=5 in each group. Tg, transgenic mice; nTg, non-transgenic control mice.   *$p < 0.05$, **$p < 0.01$.

Fig. 3-3.   Spontaneous *in situ* liver surface chemiluminescence.

A, System for the measurement of whole organ chemiluminescence *in situ* photomultiplier (PM).

B, Levels of reactive oxygen species as determined by the spontaneous surface chemiluminescence method in mice aged 6 months.

The data are means±S.E., n=5 in each group. Tg, transgenic mice; nTg, non-transgenic control mice.   **$p < 0.01$.

Fig.3- 4. PCR detection of deletion in liver mitochondrial DNA.

Total hepatic DNA from core gene transgenic (core+) and non-transgenic control (core-) mice was subjected to PCR amplification. The arrow indicates the position of the PCR deletion product of 851 bp.　Mr, molecular weight standard; bp, bases pairs.

Fig. 3-5. Ultrastructural manifestation in the livers of core gene transgenic mice.

A, A non-transgenic control mouse (aged 6 months); **B**, a transgenic mouse (aged 16 months); **C**, a transgenic mouse (aged 16 months).

The arrow indicates the secondary lysosome containing mitochondrial remnants (B).

Lipofuscin granules are indicated by asterisks (C).

m, mitochondrion; N, nucleus; L, lipid droplet.

Fig.3- 6. Induction of hydroperoxide products by alcohol and carbon tetrachloride.

A, Hydroperoxide products of phosphatidylcholine (PCOOH) determined in liver tissue homogenates from mice fed an ethanol-containing diet.

B, PCOOH determined in liver tissue homogenates from mice treated with carbon tetrachloride.

The data are means$\pm$S.E., n=5 in each group. Tg, transgenic mice; nTg, non-transgenic control mice; EtOH, ethanol. *$p<0.05$, **$p<0.01$, NS, not statistically significant.
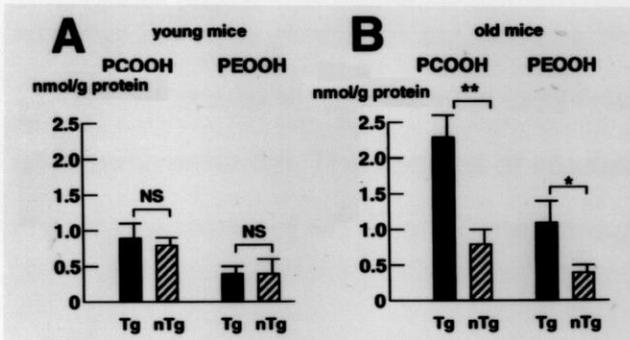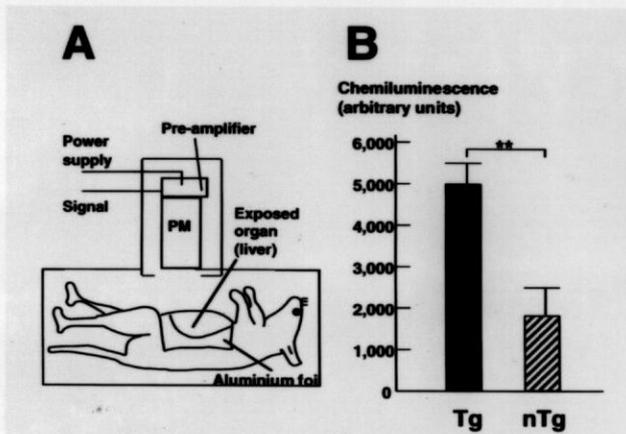
Fig. 3-1

A
young mice

PCOOH PEOOH

nmol/g protein

NS

NS

Tg nTg Tg nTg

B
old mice

PCOOH PEOOH

nmol/g protein

**

*

Tg nTg Tg nTg

Fig. 3-2

A
Catalase activity
unit/100 mg liver

**

Tg nTg

B
Total glutathione
μmol/g liver

**

Tg nTg

C
GSH/total glutathione

*

Tg nTg

Fig. 3-3

A

Power supply
Pre-amplifier
Signal
PM
Exposed organ (liver)
Aluminium foil

B
Chemiluminescence (arbitrary units)

**

Tg nTg

Fig. 3-4

age (m.o.)	2	9	19	2	9	19	Mr
core	−	−	−	+	+	+	

851 bp →

2,036
1,636
1,018
506
(bp)

Fig. 3-5

A B C

N L L

Fig. 3-6

A
PCOOH
nmol/g protein

** **

NS

EtOH − + − +
Tg nTg

B
PCOOH
nmol/g protein

** NS

*

CCl₄ − + − +
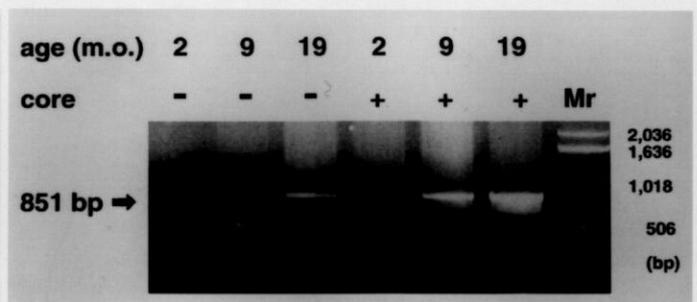Tg nTg
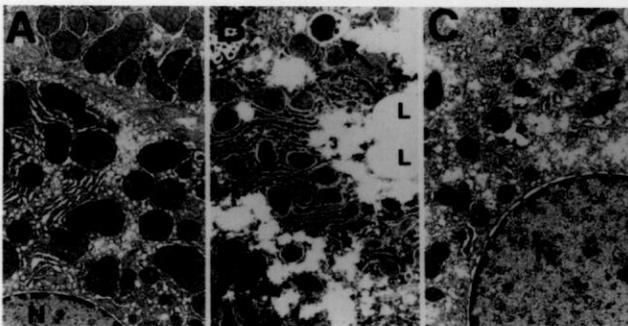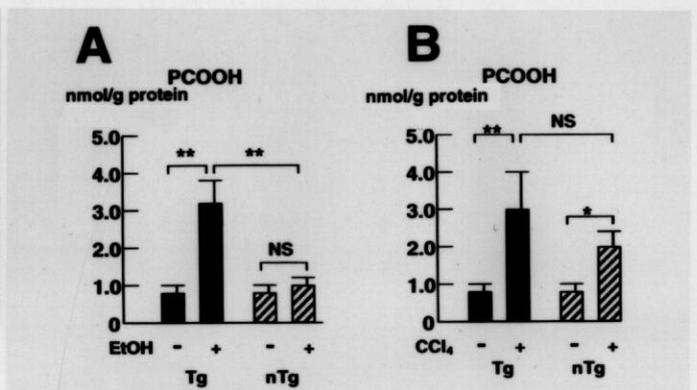
Summary

In the pathogenesis of HCC associated with HCV infection,it remains controversial whether the virus plays a direct role or indirect role. Owing to the establishment of transgenic mouse model, we now understand that the core protein of HCV has oncogenic potential. The findings of studies indicate that radicals may have some part in carcinogenesis of HCV core gene transgenic mice.

Acknowledgments

References

1. Ikeda K., Saitoh S., Suzuki Y., Kobayashi M., Tsubota A., Koida I, . Disease progression and hepatocellular carcinogenesis in patients with chronic viral hepatitis: a prospective observation of 2215 patients. J Hepatol 1988; 28: 930-938.

2. Kiyosawa K., Sodeyama T., Tanaka E., Gibo Y., Yoshizawa K., Nakano Y., Furuta S.,Akahane Y.,Nishioka K.,Purcell RH. Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. Hepatology 1990; 12: 671-675.

3.Yotsuyanagi H., Shintani Y., Moriya K., Fujie H., Tsutsumi T., Kato T., Nishioka K.,Takayama T.,Makuuchi M.,Iino S.,Kimura K.,Koike K. Virological analysis of non-B, non-C hepatocellular carcinoma in Japan: frequent involvement of hepatitis B virus. J Infect Dis 2000; 181: 1920-1928.

4.Shintani Y., Yotsuyanagi H., Moriya K., Fujie H., Tsutsumi T., Takayama T.,Makuuchi M.,Kimura S.,Koike K. Significance of hepatitis B virus DNA detected in hepatocellular carcinoma in patients with hepatiits C. Cancer 2000; 88: 2478-24864.

5.Koike K., Yasuda K., Yotsuyanagi H., Moriya K., Hino K., Kurokawa K.,Iino S. Dominant replication of either virus in dual infection with hepatitis viruses B and C. J Med Virol 1995; 45: 236-239.

6. Hammer RE, Brinster RL, Rosenfeld MG, Evans RM, Mayo KE. Expression of human growth hormone-releasing factor in transgenic mice results in increased somatic growth. Nature 1985; 315:413-416.

7.Hanahan D. Dissecting multistep tumorigenesis in transgenic mice. Ann Rev Genet 1988; 22: 479-519.

8. Chisari FV. Hepatitis B virus transgenic mice: insights into the virus and the disease. Hepatology 1955; :1316-1325.

9. Saito, I.Miyamura T,Ohbayashi A,Harada H,Katayama T,Kikuchi S,Watanabe Y,Koi S,Onji M,Ohta Y,Choo Q,Houghton M,Kuo G. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. Proc. Natl. Acad. Sci. USA 87,6547-6549 (1990).

10. Simonetti, R.G.,Camma C.,Fiorello F.,Cottone M.,Rapicetta M.,Marino L.,Fiorentimo G.,Craxi A.,Ciccaglione A.,Giusepetti R. Hepatitis C virus infection as a risk factor for hepatocellular carcinoma in patients with cirrhosis. Ann. Intern. Med. 116, 97-102 (1992).

11. Ray, R.B., Lagging, L.M. Meyer, K. & Ray, R. Hepatitis C virus core protein cooperates *ras* and transforms primary rat embryo fibroblasts to tumorigenic phenotype. J. Virol. 70, 4438-4443 (1996).

12. Chang, J.,Yang SH.,Cho YG.,Hwang SB.,Hahn YS.,Sung YC. Hepatitis C virus core from two different genotypes has an oncogenic potential but is not sufficient for transforming primary rat embryo fibroblasts in cooperation with the H-*ras* oncogene. J. Virol. 72, 3060-3065 (1998).

13. Kim, D.W., Suzuki, R., Harada, T., Saito, I. & Miyamura, T. Trans-suppression of gene expression by hepatitis C viral core protein. Jpn. J. Med. Sci. Biol. 47, 211-220 (1994).

14. Ray, R.B., Steele, R., Meyer, K. & Ray, R. Transcriptional repression of p53 promoter by hepatitis C virus core protein. J. Biol. Chem. 272, 10983-10986 (1997).

15. Ruggieri, A., Harada, T., Matsuura, Y. & Miyamura, T. Sensitization to Fas-mediated apoptosis by hepatitis C virus core protein. Virology 229, 68-76 (1997).

16. Ray, R.B., Meyer, K. & Ray, R. Suppression of apoptotic cell death by hepatitis C virus core protein. Virology 226, 176-182 (1996).

17. Moriya, K., Yotsuyanagi H., Shintani Y., Fujie H., Ishibashi K., Matsuura Y.,Miyamura T.,Koike K. . Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. J. Gen. Virol. 78,1527-1531 (1997).

18. Sugihara, S.,Nakashima O.,Kojiro M.,Majima Y.,Tanaka M.,Tanikawa K. The morphologic transition in hepatocellular carcinoma. Cancer 70, 1488-1492 (1992).

19. Nakashima, O., Sugihara, S., Kage, M. & Kojiro, M. Pathomorphologic characteristics of small hepatocellular carcinoma: a special reference to small hepatocellular carcinoma with distinct margins. *Hepatology* **22**, 101-105 (1995).

20. Hino, O. & Kajino, K. Hepatitis virus-related hepatocarcinogenesis. Intervirology 37, 133-135 (1994).

21. Bach, N., Thung, S.N. & Schaffner F. The histological features of chronic hepatitis C and autoimmune chronic hepatitis: a comparative analysis. Hepatology 15, 572-577 (1992).

22. Frith, C.H. & Ward, J.M. A morphological classification of proliferative and neoplastic hepatic lesions in mice. J. Environ. Pathol. Toxicol. 3, 329-351 (1979).

23. Smith, G.S., Walford, R.L. & Mickey, M.R. Lifespan and incidence of cancer and other diseases in selected long-lived inbred mice and their F1 hybrids. J. Natl. Cancer Inst. 50, 1195-1213 (1973).

24. Nakamura, K., Kuramoto, K., Shibasaki, K., Sumiya, S. & Ohtsubo, K. Age-related incidence of spontaneous tumors in SPF C57BL/6 and BDF1 mice. Exp. Anim. 41, 279-285 (1992).

25. Caporaso, N.Romano M.,Marmo R.,de Sio I.,Morisco F.,Minerva A.,Coltorti M. Hepatitis C virus infection is an additive risk factor for development of hepatocellular carcinoma in patients with cirrhosis. J. Hepatol. 12, 367-371 (1991).

26. Koike, K. Moriya, K.,Ishibnashi K., Yotsuyanagi H., Shintani Y., Fujie H., Kurokawa K.,Matsuura Y.,Miyamura T., Sialadenitis resembling Sjogren's syndrome in hepatitis C virus envelope gene transgenic mice. Proc. Natl. Acad. Sci. USA 94, 233-236 (1997).

27. Kawamura, T.Furusaka A.,Koziel MJ.,Chung RT.,Wang TC.,Schmidt EV.,Liang TJ. Transgenic expression of hepatitis C virus structural proteins in the mice. Hepatology 25, 1014-1021 (1997).

28. Matsuda J.,Suzuki M.,Nozaki C.,Shinya N.,Tashiro K.,Mizuno K.,Uchinuno Y.,Yamamura K. Transgenic mouse expressing a full-length hepatitis C virus cDNA. Jpn. J. Cancer Res. 89, 150-158 (1998).

29. Pasquinelli, C.Shoenberger JM.,Chung J.,Chang KM.,Guidotti LG.,Selby M.,Berger K.,Lesniewski R.,HoughtonM.,Chisari FV. Hepatitis C virus core and E2 protein expression in transgenic mice. Hepatology 25, 719-727 (1997).

30. Moriya, K., Fujie H., Yotsuyanagi H., Shintani Y.,Tsutsumi T.,., Matsuura Y.,Miyamura T.,Kimura S.,Koike K. Subcellular localization of hepatitis C virus structural proteins in the liver of transgenic mice. Jpn. J. Med. Sci. Biol. 50, 169-177 (1997).

31. Paradis, V. Mathrin P.,Kollinger M.,Imbert-Bismut F.,Charlotte F.,Piton A.,Opolon P.,Holstage A.,Poynard T.,Bedossa P.In situ detection of lipid peroxidation in chronic hepatitis C: correlation with pathological features. J. Clin. Pathol. 50, 401-406 (1997).

32. Moriya, K., Fujie, H., Shintani, Y., Yotsuyanagi, H., Tsutsumi, T., Ishibashi, K., Matsuura, Y., Kimura, S., Miyamura, T., Koike, K. (1998). The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. Nature Med. 4,1065-1067.

33. Lefkowitch, J.H., Schiff, E.R., Davis, G.L., Perrillo, R.P., Lindsay, K., Bodenheimer, H.C. Jr., Balart, L.A., Ortego, T.J., Payne, J., Dienstag, J.L., Gibas, A., Jacobson, I.M., Tamburro, C.H., Carey, W., O'brien, C., Sampliner, R., Van Thiel, D.H., Feit, D., Albrecht, J., Meschievitz, C, Sanghivi, B., Vaughan, R.D. and the Hepatitis International Therapy Group. (1993). Pathological diagnosis of chronic hepatitis C: a multicenter comparative study with chronic hepatitis B. Gastroenterology 104,595-603.

34. Czaja, A.J., Carpenter, H.A., Santrach, P.J., Moore, S.B. (1998). Host- and

disease-specific factors affecting steatosis in chronic hepatitis C. J. Hepatol. 29,198-206.

35. Moradpour, D., Englert, C., Wakita, T., Wands, J.R. (1996). Characterization of cell lines allowing tightly regulated expression of hepatitis C virus core protein. Virology 222,51-63.

36. Barba, G., Harper, F., Harada, T., Kohara, M., Goulinet, S., Matsuura, Y., Eder, G., Schaff, Z., Chapman, M.J., Miyamura, T., Brechot, C. (1997). Hepatitis C virus core protein shows a cytoplasmic localization and associates to cellular lipid storage droplets. Proc. Natl. Acad. Sci. U S A 94,1200-1205.

37. Sugihara, S., Nakashima, O., Kojiro, M., Majima, Y., Tanaka, M., Tanikawa, K. (1992). The morphologic transition in hepatocellular carcinoma. Cancer 70,1488-1492.

38. Yoshida, T., Yoshioka, K., Sakane, N., Umekawa, T., Kondo, M. (1995). Probucol prevents the progression of fatty liver in MSG obese mice. Exp Clin Endocrinol Diabetes 103,119-122.

39. Aoyagi, K., Ohue, C., Iida, K., Kimura, T., Tanaka, E., Kiyosawa, K., Yagi, S. (1999). Development of a simple and highly sensitive enzyme immunoassay for hepatitis C virus core antigen. J. Cli.n Microbiol. 37,1802-1808.

40. Bragdon, J.H. (1960) Total lipids in serum. In Lipids and the Steroid Hormones in Clinical Medicine (Sunderman, F.W. ed.). pp9-14, Lippincott , Philadelphia, PA.

41. Todoroki, T., Imai, K., Matsumoto, K., Kano, S. (1983). Initial deactivation of florisil adsorbent for column chromatographic separation of lipids. Analyst 108,1267-1269.

42. Morrison, W.R., Smith, L.M. (1964). Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride-metahnol. J. Lipid. Res. 5,600-608.

43. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. (1951). Protein measurement with Folin ohenol reagent. J. Biol. Chem. 193,265-275.

44. Laurell, S., Lundquist, A. (1971). Lipid composition of human liver biopsy specimens. Acta. Med. Scand.189, 65-68.

45. Pollesello, P., Masutti, F., Croce, L.S., Toffanin, R., Eriksson, O., Paoletti, S., Hockerstedt, K., Tiribelli, C. (1993). 1H NMR spectroscopic studies of lipid extracts from human fatty liver. Biochem. Biophys. Res. Commun. 192, 1217-1222.

46. Lemon, S.M., Lerat, H., Weinman, S.A., Honda, M. (2000). A transgenic mouse model of steatosis and hepatocellular carcinoma associated with chronic hepatitis C virus infection in humans. Trans. Am. Clin. Climatol. Assoc. 111,146-156.

47. Tsutsumi, T., Suzuki,T.,Shimoike,T.,Suzuki,R., Moriya, K., Shintani, Y.,Fujie, H., Matsuura, Y., Koike, K., Miyamura, T. (2002). Interaction of hepatitis C virus core protein with retinoid X receptor alpha modulates its transcriptional activity. Hepatology.35(4):937-946,

48. Fujie, H., Yotsuyanagi, H., Moriya, K., Shintani, Y., Tsutsumi, T., Takayama, T., Makuuchi, M., Matsuura, Y., Miyamura, T., Kimura, S., Koike, K. (1999). Steatosis and intrahepatic hepatitis C virus in chronic hepatitis. J. Med. Virol. 59,141-145.

49. Koike. K. Molecular mechanism of hepatocarcinogenesis in hepatitis viral infection. Oncology in press.

50. Ntambi, J.M. (1999). Regulation of stearoyl-CoA desaturase by polyunsaturated fatty acids and cholesterol. J. Lipid. Res. 40,1549-1558.

51 Li, J., Ding, S..F, Habib, N.A., Fermor, B.F., Wood, C.B., Gilmour, R.S. (1994). Partial characterization of a cDNA for human stearoyl-CoA desaturase and changes

in its mRNA expression in some normal and malignant tissues. Int. J. Cancer 57,348-352.

52. Habib, N.A., Wood, C.B., Apostolov, K., Barker, W., Hershman, M.J., Aslam, M., Heinemann, D., Fermor, B., Williamson, R.C., Jenkins, W.E. (1987). Stearic acid and carcinogenesis. Br. J. Cancer 56,455-458.

53. Fermor, B.F., Masters, J.R., Wood, C.B., Miller, J., Apostolov, K., Habib, N.A. (1992). Fatty acid composition of normal and malignant cells and cytotoxicity of stearic, oleic and sterculic acids in vitro. Eur. .J Cancer 28A,1143-1147.

54. Gleeson, R.P., Ayub, M., Wright, J.T., Wood, C.B., Habib, N.A., Soutter, W.P., Sullivan, M.H., White, J.O. (1990). Fatty acid control of growth of human cervical and endometrial cancer cells. Br. J. Cancer 61,500-503

55. You, L.R., Chen, C.M., Yeh, T.S., Tsai, T.Y., Mai, R.T., Lin, C.H., Lee, Y.H. Hepatitis C virus core protein interacts with cellular putative RNA helicase. J. Virol., 73: 2841-2853, 1999.

56. Matsumoto, M., Hsieh, T.Y., Zhu, N., VanArsdale, T., Hwang, S.B., Jeng, K.S., Gorbalenya, A.E., Lo, S.Y., Ou, J.H., Ware, C.F., Lai, M.M. Hepatitis C virus core protein interacts with the cytoplasmic tail of lymphotoxin-beta receptor. J. Virol., 71: 1301-1309, 1997.

57. Mamiya, N., Worman, H.J.. Hepatitis C virus core protein binds to a DEAD box RNA helicase. J. Biol. Chem., 274: 15751-15756, 1999.

58. Cerutti, P.A., Trump, B.F. Inflammation and oxidative stress in carcinogenesis. Cancer Cells, 3: 1-7, 1991.

59. Dreher, D., Junod, A.F. Role of oxygen free radicals in cancer development. Eur. J. Cancer, 32: 30-38, 1996.

60. Chance, B., Sies, H., Boveris, A. Hydroperoxide metabolism in mammalian organs. Physiol. Rev., 59: 527-605, 1979.

61. Cerutti, P.A. Oxy-radicals and cancer. Lancet, 344: 862-863, 1994.

62. Wiseman, H., Halliwell, B. Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer.   Biochem. J., 313: 17-29, 1996.

63. Adler, V., Yin, Z., Tew, K.D., Ronai, Z. Role of redox potential and reactive oxygen species in stress signaling. Oncogene, 18: 6104-6111, 1999.

64. Koike, K., Moriya, K., Ishibashi, K.,   Matsuura, Y., Suzuki, T., Saito, I., Iino, S., Kurokawa, K., Miyamura, T. Expression of hepatitis C virus envelope proteins in

65. Punchard, N.A., Kelly, F.J. Introduction. *In:* N.A. Punchard and F.J. Kelly (eds.), Free Radicals, pp1-8. Oxford: IRL Press, 1996.

66. Miyazawa,T.,Yasuda, K., and Fujimoto, K. Chemiluminescence-high performance liquid chromatography of phosphatidylcholine hydroperoxide. Anal. Lett., 20: 915-925, 1987.

67. Miyazawa, T., Suzuki, T., Fujimoto, K., and   Yasuda, K. Chemiluminescent simultaneous determination of phosphatidylcholine hydroperoxide and phosphatidylethanolamine hydroperoxide in the liver and brain of the rat.   J. Lipid Res., 33: 1051-1059, 1992.

68. Aebi, H.   Catalase.   In: H. Bergmeyer (eds.), Methods of Enzymatic Analysis, pp673-684. New York: Academic Press, 1974.

69. Toyo'oka, T., Furukawa, F., Suzuk,i T., Saito, Y., Takahashi, M., Hayashi, Y., Uzu, S., Imai, K. Determination of thiols and disulfides in normal rat tissues and hamster pancreas treated with N-nitrosobis(2-oxopropyl)amine using 4-(aminosulfonyl)-7-

fluoro-2,1,3-benzoxadiazole and ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate. Biomed. Chromatogr., 3: 166-172, 1989.

70. Boveris, A., Cadenas, E., Reiter, R., Filipkowski, M., Nakase, Y., Chance, B. Organ chemiluminescence: noninvasive assay for oxidative radical reactions. Proc. Natl. Acad. Sci. U S A, 77: 347-351, 1980.

71. Cadenas, E., Sies, H. Low-level chemiluminescence as an indicator of singlet molecular oxygen in biological systems. Methods Enzymol., 105: 221-231, 1984.

72. Cadenas, E., Wefers, H., Sies, H. Low-level chemiluminescence of isolated hepatocytes. Eur. J. Biochem., 119: 531-536, 1981.

73. Tanhauser, S.M., Laipis, P.J. Multiple deletions are detectable in mitochondrial DNA of aging mice. J. Biol. Chem., 270: 24769-24775, 1995.

74. Esterbauer, H., Schaur, R.J., Zollner, H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic. Biol. Med., 11: 81-128, 1991.

75. Brown, R.K., Kelly, F.J. Peroxides and other products. *In:* N.A. Punchard and F.J. Kelly (eds.), Free Radicals, pp119-131. Oxford: IRL Press, 1996.

76. Koike, K.   Transgenic mouse models of viral hepatitis: insight into viral hepatocarcinogenesis.   Viral Hepatitis Rev., 5: 177-203, 1999.

77. Halliwell, B., Gutteridge, J.M. Role of free radicals and catalytic metal ions in human disease: an overview.   Methods Enzymol., 186: 1-85, 1990.

78. Lee, C.M., Weindruch, R., Aiken, J.M. Age-associated alterations of the mitochondrial genome. Free Radic. Biol. Med., 22: 1259-1269, 1997.

79. Collins, V.P., Arborgh, B., Brunk, U., Schellens, J.P. Phagocytosis and degradation of rat liver mitochondria by cultivated human glial cells. Lab. Invest., 42:

209-216, 1980.

80. Rubin, H., Chow, M., Yao, A. Cellular aging, destabilization, and cancer. Proc. Natl. Acad. Sci. U S A, 93:1825-1830, 1990.

81. Ikeda, K., Saitoh, S., Koida, I., Arase, Y., Tsubota, A., Chayama, K., Kumada, H., Kawanishi, M. A multivariate analysis of risk factors for hepatocellular carcinogenesis: a prospective observation of 795 patients with viral and alcoholic cirrhosis. Hepatology, 18: 47-53, 1993.

82. Yoshihara, H., Noda, K., Kamada, T . Interrelationship between alcohol intake, hepatitis C, liver cirrhosis, and hepatocellular carcinoma. Recent Dev. Alcohol, 14: 457-469, 1998.

83. Donato, F., Tagger, A., Chiesa, R., Ribero, M.L., Tomasoni, V., Fasola, M., Gelatti, U., Portera, G., Boffetta, P., Nardi, G. Hepatitis B and C virus infection, alcohol drinking, and hepatocellular carcinoma: a case-control study in Italy. Brescia HCC Study. Hepatology, 26: 579-584, 1997.

84. Farinati, F., Cardin, R., D'Errico, A., De Maria, N., Naccarato, R., Cecchetto, A., Grigioni, W. Hepatocyte proliferative activity in chronic liver damage as assessed by the monoclonal antibody MIB1 Ki67 in archival material: the role of etiology, disease activity, iron, and lipid peroxidation. Hepatology, 23: 1468-1475, 1996.

85. Paradis, V., Mathurin, P., Kollinger, M., Imbert-Bismut, F., Charlotte, F., Piton, A., Opolon, P., Holstege, A., Poynard, T., Bedossa, P. In situ detection of lipid peroxidation in chronic hepatitis C: correlation with pathological features. J. Clin. Pathol., 50: 401-406, 1997.

86. Romero, M.J., Bosch-Morell, F., Romero, B., Rodrigo, J.M., Serra, M.A., Romero, F.J. Serum malondialdehyde: possible use for the clinical management of chronic

hepatitis C patients. Free Radic. Biol. Med. 25: 993-997, 1998.

87. Boya, P., de la Pena, A., Beloqui, O., Larrea, E., Conchillo, M., Castelruiz, Y., Civeira, M.P., Prieto, J. Antioxidant status and glutathione metabolism in peripheral blood mononuclear cells from patients with chronic hepatitis C.   J. Hepatol., 31: 808-814, 1999.

88. Reddy, J.K., Rao, M.S. Oxidative DNA damage caused by persistent peroxisome proliferation: its role in hepatocarcinogenesis. Mutat. Res., 214: 63-68, 1989.

89: Perera, M.I., Betschart, J.M., Virji, M.A., Katyal, S.L., Shinozuka, H. Free radical injury and liver tumor promotion. Toxicol. Pathol., 15: 51-59, 1987.

90. Hensley, K., Robinson, K.A., Gabbita, S.P., Salsman, S., Floyd, R.A. Reactive oxygen species, cell signaling, and cell injury. Free Radic. Biol. Med., 28: 1456-1462, 2000.

91. Chapple, I.L. Reactive oxygen species and antioxidants in inflammatory diseases. J. Clin. Periodontol., 24: 287-296, 1997.

92. Winterbourn, C.C. Toxicity of iron and hydrogen peroxide: the Fenton reaction. Toxicol. Lett., 82-83: 969-9674, 1995.

93. de Haan, J.B., Cristian,o F., Iannello, R.C., Kola, I. Cu/Zn-superoxide dismutase and glutathione peroxidase during aging.   Biochem. Mol. Biol. Int., 35: 1281-1297, 1995.

94. Miyazawa, T., Suzuki, T., Fujimoto, K. Age-dependent accumulation of phosphatidylcholine hydroperoxide in the brain and liver of the rat.   Lipids.. 28:789-793, 1993.

95. Shigenaga, M.K., Hagen, T.M., Ames, B.N. Oxidative damage and mitochondrial decay in aging. Proc. Natl. Acad. Sci. U S A, 91:10771-10778, 1994.

96. Sastre, J., Pallardo, F.V., Pla, R., Pellin, A., Juan, G., O'Connor, J.E., Estrela, J.M., Miquel, J., Vina, J. Aging of the liver: age-associated mitochondrial damage in intact hepatocytes. Hepatology, 24: 1199-1205, 1996.

97. Mansouri, A., Gaou, I., Fromenty, B., Berson, A., Letteron, P., Degott, C., Erlinger, S., Pessayre, D. Premature oxidative aging of hepatic mitochondrial DNA in Wilson's disease. Gastroenterology, 113: 599-605, 1997.

98. Fromenty, B., Grimbert, S., Mansouri, A., Beaugrand, M., Erlinger, S., Rotig, A., Pessayre, D. Hepatic mitochondrial DNA deletion in alcoholics: association with microvesicular steatosis. Gastroenterology, 108: 193-200, 1995.

99. Alexeyeva, I.N., Berezovsky, V.A., Bryzgina, T.M., Pavlovich, S.I., Alexyuk, L.I., Makogon, N.V., Gotsulyak, Ya.N., Martinova, T.V. Effects of carbontetrachloride and chenodeoxycholic acid on hepatic monooxygenase system, lipid peroxidation and some immunologic parameters in rats. Acta Physiol. Pharmacol. Bulg., 22: 45-50, 1996.

100. Ryder, S.D., Koskinas, J., Rizzi, P.M., McFarlane, I.G., Portmann, B.C., Naoumov, N.V., William,s R. Hepatocellular carcinoma complicating autoimmune hepatitis: role of hepatitis C virus. Hepatology, 22: 718-722, 1995.

101. Burroughs, A.K., Bassendine, M.F., Thomas, H.C., Sherlock, S. Primary liver cell cancer in autoimmune chronic liver disease. Br. Med. J., 282: 273, 1981

102. Fridovich, I. Hypoxia and oxygen toxicity. Adv. Neurol., 26:255-259, 1979.

103. Lumeng, L., Crabb, D.W. Alcoholic liver disease. Curr. Opin. Gastroenterol., 16:208-218, 2000.