

# Oxidative Stress in the Absence of Inflammation in a Mouse Model for Hepatitis C Virus-associated Hepatocarcinogenesis<sup>1</sup>

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## 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 after a long period, the core protein of HCV induces 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 before the development of HCC, we examined several parameters of oxidative stress and redox homeostasis in a mouse model of HCV-associated HCC. For young mice ages 3–12 months, there was no significant difference in the levels of hydroperoxides of phosphatidylcholine (PCOOH) and phosphatidylethanolamine in liver tissue homogenates between transgenic and nontransgenic control mice. In contrast, the PCOOH level was increased by 180% in old core gene transgenic mice > 16 months old. Concurrently, there was a significant increase in the catalase activity, and there were 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 the 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 may thereby contribute to or facilitate, at least in part, the development of HCC in HCV infection.

## INTRODUCTION

Despite the overwhelming evidence from epidemiological studies connecting chronic HCV<sup>3</sup> infection and the development of HCC (1, 2), the precise mechanism of hepatocarcinogenesis in HCV infection remains unclear. HCV may contribute to the development of HCC by facilitating the accumulation of genetic damage as a result of continuous cell death followed by regeneration in the course of chronic

hepatitis (3). If this is the case, HCV would only be indirectly associated with hepatocarcinogenesis. Another possibility is the direct involvement of HCV in hepatocarcinogenesis, whereby the product of the virus may be oncogenic and involved in cell transformation. 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 (4). 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- $\beta$  receptor, or dead box protein (5–7), 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 (8, 9). ROS are endogenous oxygen-containing molecules formed as normal products during aerobic metabolism (10). ROS include a number of species such as superoxide, hydroxyl, and peroxy 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 (11, 12). 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 (13). Most markers of oxidative injury used reflect free radical attack on polyunsaturated fatty acids, with the classical route of attack involving lipid peroxidation, which generates hydroperoxides and endoperoxides.

In the present study, we explored the oxidant/antioxidant status in the liver of a transgenic mouse model of HCC in HCV infection, before development of HCC, by sequential quantification of hydroperoxide level, GSH level, and catalase activity and comparison of these parameters with those of age-matched nontransgenic control mice. We found an age-dependent increase in oxidative stress in the livers of transgenic mice that 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 (14). 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<sub>4</sub>) was administered to mice *i.p.* at a dose of 0.5 ml/kg body weight. Because HCC develops preferentially in male transgenic mice, we used male mice that were heterozygously transgenic for the core gene, and we used nontransgenic littermates of the transgenic mice as controls. Transgenic mice carrying the HCV envelope genes

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<sup>3</sup> The abbreviations used are: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; PCOOH, phosphatidylcholine; ROS, reactive oxygen species; PEOOH, phosphatidylethanolamine; CL, chemiluminescence; HPLC, high-performance liquid chromatography; GSH, glutathione; GSSG, oxidized glutathione; GPx, glutathione peroxidase; mtDNA, mitochondrial DNA; AP-1, activator protein 1.

under the same regulatory region as that in the core gene transgenic mice were used as control (15). Obese mice were obtained by treating the mice with monosodium L-glutamate (16). 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  $-70^{\circ}\text{C}$ , because storage inevitably results in an increase in peroxide products (17). Liver samples (100 mg) were homogenized in 2 ml of 0.15 M NaCl solution. Four ml of chloroform/methanol (2:1, v/v) containing 90 nM butylated hydroxytoluene as an antioxidant were added to 1 ml of homogenate and mixed vigorously for 1 min. The mixture was centrifuged at  $1000 \times g$  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 (18).

**Determination of PCOOH and PEOOH Hydroperoxides.** Hydroperoxide products of PCOOH and PEOOH in liver total lipids were determined by CL-HPLC, as described previously (18, 19). The system consisted of a Jasco HPLC system (Japan Spectroscopic Co., Tokyo, Japan) and postcolumn CL detection. The HPLC column was a Jasco Finepak SIL NH2-5 (5  $\mu\text{m}$ ;  $250 \times 4.6$  mm), the column mobile phase was 2-propanol/methanol/water (135:45:20, v/v/v), and the flow rate was 1.0 ml/min. Postcolumn CL detection was carried out using a CLD-100 detector (Tohoku Electronic Industries Co., Sendai, Japan), which employs a cooled photomultiplier tube, to suppress photomultiplier tube dark current and improve the S:N ratio of HPLC analysis. A mixture of luminol and cytochrome *c* in 50 mM borate buffer (pH 10.0) was used as a hydroperoxide-specific postcolumn CL reagent (18, 19). Calibration was carried out using authentic PCOOH as a standard, as described previously (18). Protein concentrations were determined by Lowry's method using BSA as the protein standard.

**Determination of Catalase Activity and GSH Level.** Catalase activity was measured spectrophotometrically (at 240 nm) by following the decrease in absorbance of hydrogen peroxide after the addition of 0.1 ml of supernatant to 0.9 ml of 15 nM  $\text{H}_2\text{O}_2$  in 50 mM phosphate buffer (pH 6.8; Ref. 20). Reduced GSH and GSSG levels were measured as described previously (21). Briefly, tissues were homogenized with Physcotron (Niti-on, Tokyo, Japan) after the addition of 4 ml/g wet tissue of 5% trichloroacetic acid containing 5 mM  $\text{Na}_2\text{EDTA}$  and centrifuged at  $1850 \times g$  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 3000 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 were injected onto the HPLC column. The total amount of GSH was computed by adding the amounts obtained for GSH and GSSG.

**Spontaneous *in Situ* Liver Surface CL.** The spontaneous *in situ* surface CL of the liver was monitored using a photon counter (Johnson Research Foundation, Philadelphia, PA) with a model 9658 photomultiplier responsive over the range of 350–850 nm as described elsewhere (22, 23). CL in the intact organ is the result of different photoemissive reactions: ROS and lipid radicals are primary contributors to tissue CL (22, 23). Intrinsic low-level light emission by living tissues is measured by a 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, which is placed as close as possible to the organ surface. To avoid CL from other organs, the mouse is covered with aluminum foil, and only the liver is exposed to the photomultiplier. Emission was expressed in counts/s/cm<sup>2</sup> liver surface. Spectral analysis of liver CL was performed with cutoff Kodak Wratten filters (Eastman Kodak, Rochester, NY) as described elsewhere (24).

**Electron Microscopy.** For standard electron microscopic techniques, mouse liver was perfused with 1.6% glutaraldehyde (TAAB Laboratory Equipment, Reading, United Kingdom), excised, and fixed at  $4^{\circ}\text{C}$  for 1 h.

**DNA Isolation and Analysis of mtDNA.** Total DNA was isolated from the liver as described previously (25). Detection of mtDNA deletion present between the direct repeats of mtDNA (direct repeat 17 corresponding to bp 979–5650 of mouse mtDNA; Ref. 26) was performed by PCR with primers

TAAGTCGTAACAAGGTAAGC (bp 979–998) and GATGGTGGTAG-GAGTCAAAA (bp 5650–5631). Amplification was carried out in a thermal cycler for a total of 35 cycles consisting of  $94^{\circ}\text{C}$  for 40 s,  $50^{\circ}\text{C}$  for 30 s, and  $72^{\circ}\text{C}$  for 2 min in 100  $\mu\text{l}$  of the reaction mixture containing 200  $\mu\text{M}$  deoxynucleotide triphosphates, 1.0  $\mu\text{M}$  each of the primers,  $1 \times$  PCR buffer [50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM  $\text{MgCl}_2$ , 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, whereas 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 the means  $\pm$  SE. 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  $>16$  months old (4). In the present study, analyses were performed on two groups of mice: (a) young mice (3–12-month-old mice); and (b) old mice (mice  $>6$  months old).

**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 (27). Direct determination of the primary products of oxidative attack has been shown to be the most accurate measure of lipid peroxidation (28). We chose to determine the levels of PCOOH and PEOOH by the CL-HPLC method because these are the most reliable parameters for lipid peroxidation (18, 19). We first determined the levels of PCOOH and PEOOH in the liver of young HCV core gene transgenic and nontransgenic control mice ages 3–12 months. There was no significant difference in the levels of PCOOH ( $0.89 \pm 0.16$  versus  $0.83 \pm 0.08$  nmol/g protein;  $P = 0.46$ ) or PEOOH ( $0.43 \pm 0.11$  versus  $0.42 \pm 0.14$  nmol/g protein;  $P = 0.90$ ) in these mice, as shown in Fig. 1A. In contrast, in older mice that were  $>16$  months old, when the core gene transgenic mice start to develop HCC (4, 29), the levels of both PCOOH ( $2.30 \pm 0.42$  versus  $0.83 \pm 0.19$  nmol/g protein;  $P < 0.01$ ) and PEOOH ( $1.04 \pm 0.32$  versus  $0.42 \pm 0.15$  nmol/g protein;  $P < 0.05$ ) were significantly higher than those in nontransgenic control mice (Fig. 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-month-old simple obese mice, which

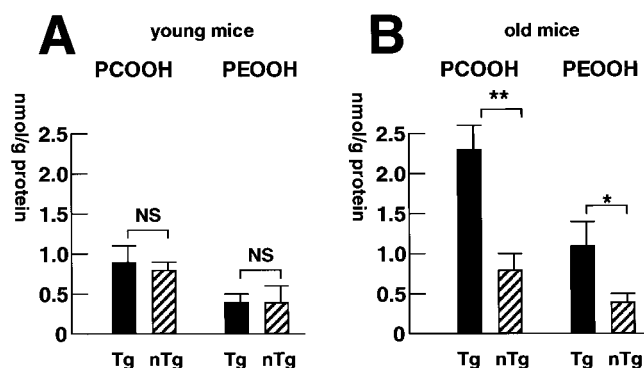


Fig. 1. Levels of lipid peroxidation in core gene transgenic and control mice. A, young mice ages 3–12 months. B, old mice  $>16$  months of age. The hydroperoxide products of PCOOH or PEOOH were determined in liver tissue homogenates of transgenic mice (Tg) and nontransgenic control mice (nTg). The data are the means  $\pm$  SE ( $n = 5$  in each group). NS, not statistically significant. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

showed a moderate grade of hepatic steatosis, were not significantly different from those in the livers of nontransgenic control mice ( $0.98 \pm 0.33$  versus  $0.83 \pm 0.19$  nmol/g protein;  $P = 0.41$ ). The levels of PCOOH in the livers of 16-month-old 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 nontransgenic control mice ( $0.79 \pm 0.27$  versus  $0.83 \pm 0.19$  nmol/g protein;  $P = 0.82$ ). This indicates that not the regulatory feature of protein expression driven by the hepatitis B virus 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.

**Antioxidative State in HCV Core Gene Transgenic Mice.** Measurement of ROS is not complete without the measurement of defense systems that protect against ROS. In the assessment of ROS *in vivo*, the balance between generation and elimination is more important than the measurement of any single component (17). In the livers of young mice, we determined: (a) catalase activity; (b) total GSH; and (c) GSH levels. Catalase is the enzyme that generates  $H_2O$  and  $O_2$  by metabolizing hydrogen peroxide ( $H_2O_2$ ) that is produced by the action of superoxide dismutase. Reduced GSH plays a central role in cellular defense against oxidative stress (30) by regulating the intracellular concentration of ROS via a reaction catalyzed by GPx. The catalase activity was  $149.8 \pm 22.1$  units/100 mg liver in transgenic mice, whereas it was  $104.2 \pm 18.4$  units/100 mg liver in nontransgenic control mice ( $P < 0.01$ ; Fig. 2A). The total GSH level (GSH + GSSG) was significantly lower in transgenic mice than in nontransgenic control mice ( $2.62 \pm 0.59$  versus  $3.89 \pm 0.58$   $\mu$ mol/g liver;  $P < 0.01$ ; Fig. 2B). In addition, the ratio of GSH:total GSH was significantly lower in transgenic mice than in nontransgenic control mice ( $0.832 \pm 0.051$  versus  $0.908 \pm 0.016$ ;  $P < 0.05$ ; Fig. 2C).

These results for the antioxidative 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 GSH system. Therefore, to directly measure free radical formation, we took advantage of the method for measuring spontaneous *in situ* liver surface CL (Fig. 3A) and compared the levels of CL in the livers of core gene transgenic mice and nontransgenic control mice at a young age (6 months). As shown in Fig. 3B, spontaneous liver surface CL was significantly higher in transgenic mice by approximately 170% compared to that in normal control mice ( $4968 \pm 488$  versus  $1858 \pm 682$  arbitrary units;  $P < 0.01$ ).

**Mitochondrial Morphology and DNA Damage.** Because mtDNA is more sensitive to oxidative damage than nuclear DNA (31),

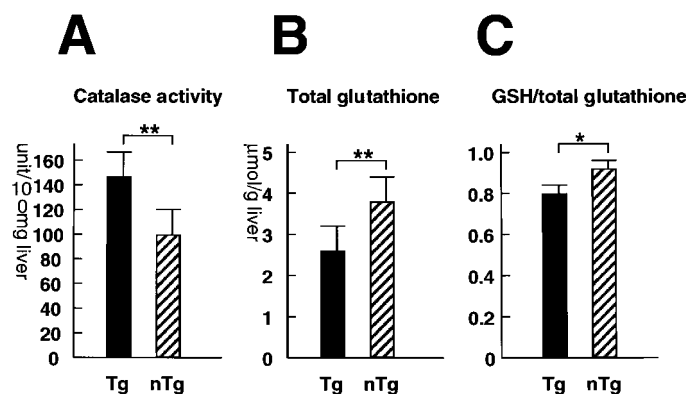


Fig. 2. Antioxidant systems in young core gene transgenic and control mice. A, catalase activity; B, total GSH level; C, the ratio of reduced GSH:total GSH. The data are the means  $\pm$  SE ( $n = 5$  in each group). Tg, transgenic mice; nTg, nontransgenic control mice. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

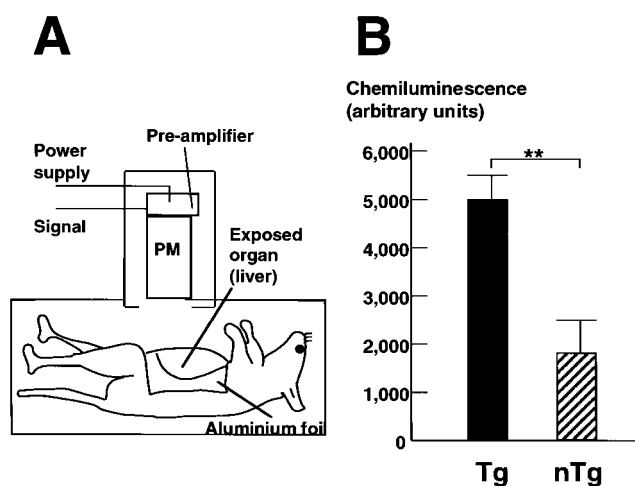


Fig. 3. Spontaneous *in situ* liver surface CL. A, system for the measurement of whole organ CL *in situ* photomultiplier (PM). B, levels of ROS as determined by the spontaneous surface CL method in 6-month-old mice. The data are the means  $\pm$  SE ( $n = 5$  in each group). Tg, transgenic mice; nTg, nontransgenic control mice. \*\*,  $P < 0.01$ .

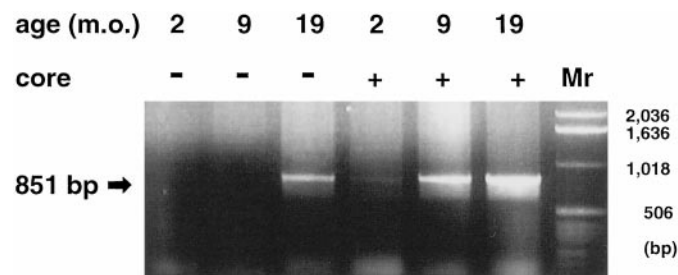


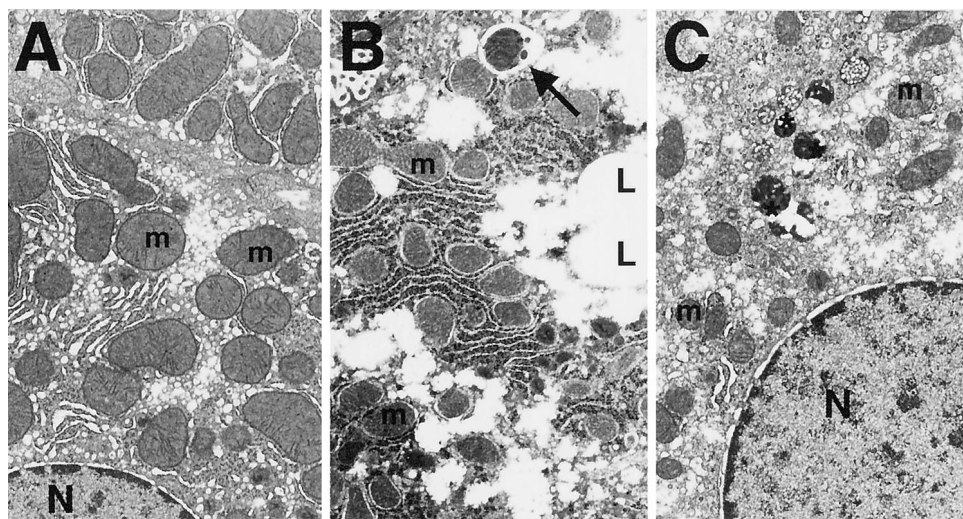
Fig. 4. PCR detection of deletion in liver mtDNA. Total hepatic DNA from core gene transgenic (core-positive, +) and nontransgenic control (core-negative, -) mice was subjected to PCR amplification. The arrow indicates the position of the PCR deletion product of 851 bp. Mr, molecular weight standard.

we investigated 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 (26). As shown in Fig. 4, this deletion was not detectable in 2- or 9-month-old nontransgenic control mice but was detectable at the age of 2 months and prominent at the age of 9 months in core gene transgenic mice. The deletion was more prominent in 19-month-old transgenic mice, whereas only a weak band indicating deletion was detectable in 19-month-old nontransgenic control mice, which may be the result of aging.

Electron microscopy showed no clear evidence of swelling or degeneration of mitochondria in 3-month-old transgenic mice, but the disruption of the double structure of the mitochondrial membrane was observed in mitochondria from the livers of transgenic mice (Fig. 5, A and B). In transgenic mice  $> 12$  months of age, hepatocytes exhibited scattered residual bodies or lipofuscin granules and secondary lysosomes carrying various cytoplasmic organelles (Fig. 5, B and C). These structures are found in senescent cells *in vivo* and are recognized as morphological signs of damage induced by ROS (32, 33).

**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 (34–36). To assess this possibility in our mouse model, we administered alcohol to mice and examined whether or not alcohol enhanced the effect of the core protein on the levels of hydroperoxide in mouse liver. The PCOOH level was determined in the livers of 6-month-old transgenic and nontransgenic control mice with or without adminis-

Fig. 5. Ultrastructural manifestation in the livers of core gene transgenic mice. A, a nontransgenic control mouse (age, 6 months); B, a transgenic mouse (age, 16 months); C, a transgenic mouse (age, 16 months). The *arrow* indicates the secondary lysosome containing mitochondrial remnants (B). Lipofuscin granules are indicated by *asterisks* (B). *m*, mitochondrion; *N*, nucleus; *L*, lipid droplet.



tration of 5% ethanol for 3 weeks. Nontransgenic control mice fed with a 5% ethanol diet for 3 weeks showed only a slight increase in PCOOH levels compared with those not fed an ethanol diet. In contrast, transgenic mice exhibited an approximate 200% increase in PCOOH levels compared with nontransgenic control mice fed with the same 5% ethanol diet ( $3.11 \pm 0.54$  versus  $1.02 \pm 0.27$  nmol/g protein;  $P < 0.01$ ; Fig. 6A) or transgenic mice not fed an ethanol diet ( $3.11 \pm 0.54$  versus  $0.88 \pm 0.28$  nmol/g protein;  $P < 0.01$ ; Fig. 6A).

We then administered  $\text{CCl}_4$  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  $\text{CCl}_4$  treatment increased PCOOH levels in mice, and the levels were higher in transgenic than in nontransgenic control mice, the difference was not statistically significant ( $2.96 \pm 1.03$  versus  $2.07 \pm 0.48$ ;  $P = 0.12$ ; Fig. 6B).

## DISCUSSION

In the present study, we demonstrated a striking intracellular increase in lipid peroxidation, 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 (4). 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 (4). 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 (37–40), and ROS have been suggested to play a role in the development of liver cancer (41, 42). 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 (43, 44). 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 at levels similar to those in the livers of chronic hepatitis C patients (4), 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 peroxidation. Lipid peroxidation 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 (28). Determining hydroperoxide products, particularly PCOOH or PEOOH, is one of the most reliable methods for evaluating lipid peroxidation (18), in contrast to measuring the level of thiobarbituric acid reactive substances, which is troublesome due to a number of artifacts and nonspecific reactions (28).

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 CL 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 CL 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 was terminated when the mice became older.

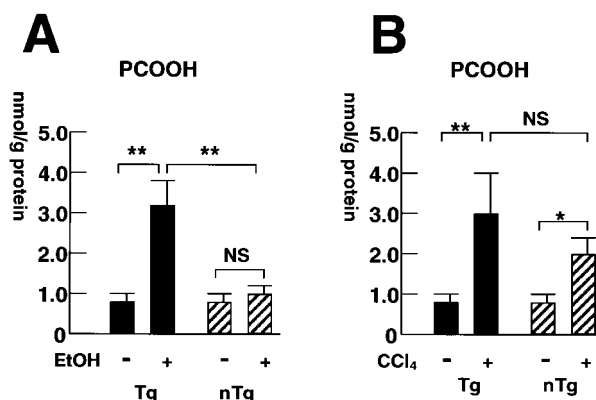


Fig. 6. Induction of hydroperoxide products by alcohol and carbon tetrachloride. A, hydroperoxide products of PCOOH determined in liver tissue homogenates from mice fed an ethanol-containing diet. B, PCOOH determined in liver tissue homogenates from mice treated with carbon tetrachloride. The data are the means  $\pm$  SE ( $n = 5$  in each group). Tg, transgenic mice; nTg, nontransgenic control mice; EtOH, ethanol. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; NS, not statistically significant.

Catalase and GPx play important roles in cellular antioxidant defense by reducing the levels of hydroperoxides, which can otherwise be converted to highly reactive hydroxyl radicals through the metal-mediated Fenton reaction (45). 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 (46, 47), but this was not observed in the livers of the parental mouse strain C57BL/6 mouse used in this study (Fig. 1, A and B), a finding that is harmonious with the very low incidence of spontaneous liver tumors in this strain (48, 49).

Starting from the age of 2 months, there was a significantly higher incidence of mtDNA deletion in the livers of transgenic mice than in the livers of nontransgenic control mice, which may be associated with the increase in production of ROS. mtDNA is known to be 10–15-fold more sensitive to oxidative damage than nuclear DNA (31, 50). 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 (51–53). 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. The search for gross chromosomal alterations in HCC that developed in transgenic mice is currently under way. 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 (13). It would therefore be interesting to know whether or not the intracellular signaling pathways, such as nuclear factor  $\kappa$ B or AP-1, are activated. In this mouse model, AP-1, but not nuclear factor  $\kappa$ B, is activated.<sup>4</sup>

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 (34–36). In these studies, alcohol is one of the independent cofactors 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 greater increase in hydroperoxide levels in CCl<sub>4</sub>-treated transgenic mice than in CCl<sub>4</sub>-treated nontransgenic control mice: the difference, however, was not statistically significant, which may be due to the fact that CCl<sub>4</sub> is a very strong inducer of ROS (54). 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 differ, 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 (55, 56).

In this mouse model for HCV-associated hepatocarcinogenesis, the development of HCC is preceded by hepatic steatosis, which is one of the histological characteristics of hepatitis C (57). Steatosis in core gene transgenic mice is age dependent and characterized by the appearance of micro- and macrovesicular fat droplets (4). The pathogenesis of steatosis in these mice is not completely clear, but both the  $\beta$ -oxidation of fatty acids in mitochondria and secretion of triacyl-

glycerol from the liver were found to be impaired.<sup>5</sup> 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 yet clear 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,<sup>5</sup> which leads to the ROS overproduction (58). This may explain how excessive ROS are 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 and so forth. (59). Therefore, a 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 be responsible in part 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.

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