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Interferon reduces somatic mutation of mitochondrial DNA in liver tissues from chronic viral hepatitis patients

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SUMMARY. We recently reported that the genetic instability resulting in the high rate of mitochondrial DNA (mtDNA) mutation in noncancerous liver tissue is consistent with the multicentric hepatocarcinogenesis detected clinically. Interferon (IFN) has been reported to reduce hepatocarcinogenesis in individuals with hepatitis virus infection. Liver biopsy specimens were obtained from 26 patients with chronic hepatitis C virus (HCV) infection before and after IFN therapy (total dose: 252 million units). The mean (±SD) age of the study population was 45 ± 9 years and 13 (50%) were male [mode of acquisition: blood transfusion (27%), unknown (73%); viral load: 5.2 ± 1.1 k copies/mL; duration of infection: 17 ± 9 years (65%), unknown (35%); genotype: I (4%), II (80%), III (8%), IV (8%); alcohol intake: positive (31%), negative (69%)]. DNA samples were extracted from the specimens and subjected to direct sequencing. The mtDNA mutation frequency in the D-loop was increased in liver specimens from individuals with HCV infection compared with 21 controls (2.5 *vs* 0.6, $P < 0.001$). IFN therapy decreased the mtDNA mutation (mean difference = 0.7, $P < 0.001$) and the decreased number of mtDNA mutations was positively correlated with suppression of the total histological activity index score (mean difference = 1.3, $P < 0.01$). These results clearly indicate that the mutational rate of mtDNA is strongly associated with IFN therapy. Thus, analysis of mtDNA could provide a new criterion for the therapeutic evaluation of the effect of IFN, and may be useful for the prediction of risk of carcinogenesis.

Keywords: chronic hepatitis, hepatocarcinogenesis, interferon, mitochondrial DNA, oxidative stress.

INTRODUCTION

Persistent infection with hepatitis C virus (HCV) can ultimately result in the development of hepatocellular carcinoma (HCC). Thus, unlike other types of cancer, HCC is usually preceded by chronic inflammation for 20–40 years, although development of HCC can be rapid, particularly in individuals with viral hepatitis and high plasma levels of alanine aminotransferase [1]. Chronic inflammation induced by hepatitis viruses thus plays an important role in hepatocarcinogenesis. The extent of oxidative stress is increased in the liver of individuals infected with hepatitis viruses [2,3], and reactive oxygen species are important inducers of DNA mutations [4].

Mutations accumulate to a greater extent in mitochondrial DNA (mtDNA) than in nuclear DNA, predominantly because mitochondria generate substantial amounts of reactive oxygen species, while they lack histone-like nucleoproteins [5]. Because, expression of the entire mitochondrial genome is necessary for the maintenance of mitochondrial functions, including electron transport, small changes in the mtDNA sequence can result in profound impairment of such functions, thereby enhancing generation of free radicals, which in turn accelerates the rate of DNA mutation.

We have previously reported that the frequency of mtDNA mutations is markedly increased in both noncancerous and cancerous liver specimens from individuals with HCC [6], and showed that the frequency of mtDNA mutations in HCC tissue was greater than that previously described for other types of cancers [7–9]. Furthermore, the extent of accumulated mtDNA mutations in HCC tissues showed a positive correlation with the degree of malignancy. The genetic instability that results in the high rate of mtDNA mutation in noncancerous liver tissues is consistent with the multicentric hepatocarcinogenesis that is detected clinically.

It is suggested that the treatment of hepatitis patients with interferon (IFN) reduces the incidence of HCC [10,11]. Normalization of the plasma levels of alanine aminotransferase by IFN treatment reduces the rate of hepatocarcinogenesis, even

Abbreviations: HAI, histological activity index; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon; mtDNA, mitochondrial DNA.

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if the hepatitis virus is not eliminated [12]. Thus, we hypothesized that mtDNA in the liver might be affected by hepatitis virus infection and IFN therapy. The present work, demonstrates that HCV infection increases mtDNA mutation in the liver, so the treatment of HCV-infected patients with IFN significantly decreases the frequency of mtDNA mutation.

MATERIALS AND METHODS

Tissue specimens

We analysed liver mtDNA in 26 patients with chronic HCV infection who had received IFN-β (a total dose of 252 million units/patient) for 2 months. The mean (±SD) age of the study population was 45 ± 9 years and 13 (50%) were male [mode of acquisition: blood transfusion (27%), unknown (73%); viral load: 5.2 ± 1.1 k copies/mL; duration of infection: 18 ± 9 years (65%), unknown (35%); genotype: I (4%), II (80%), III (8%), IV (8%); alcohol intake: positive (31%), negative (69%)]. The liver specimens were obtained from these patients by needle biopsy before and after IFN therapy. The histological activity index (HAI) score of the liver specimens was evaluated by Knodell's scoring system. Twenty-one control liver samples were obtained from individuals (age: 57 ± 12 years; male/female: 60/40%) without viral infection, but with liver disease other than HCC (including hepatolithiasis and colorectal carcinoma with liver metastasis).

This study was performed in accordance with the Helsinki Declaration of 1975 (1983 revision) and was approved by the Ethics Committee of Osaka City University Medical School and Osaka City Medical Center. Informed consent was obtained from each patient.

PCR amplification

Freshly obtained liver specimens were frozen, micro dissected using a cryostat, and digested with proteinase K (0.1 mg/mL) in the presence of 1% sodium dodecyl sulphate (SDS). DNA was extracted using phenol–chloroform and ethanol precipitation, as described previously [6]. Each DNA sample (50 ng) was amplified by the polymerase chain reaction (PCR) with the use of overlapping sets of primers to screen the entire mitochondrial genome. PCR (initial incubation at 94 °C for 5 min followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min) was performed in a final volume of 50 μL, using a GeneAmp PCR system 9600 (Perkin–Elmer).

The generation of large PCR products excluded the possibility of nuclear pseudogenes complicating the analysis [13]. In addition, the primers were selected to avoid such co-amplification by analysis of cell lines devoid of mtDNA.

Sequence analysis

Aberrant PCR products were purified with a QIAquick PCR purification kit (Qiagen) and sequenced with an Applied Biosystems DNA sequencer (Perkin-Elmer) and a Dye Terminator Cycle Sequencing FS Ready Reaction kit (Applied Biosystems). The entire mtDNA sequence was examined for one control subject and for one chronic hepatitis type C patient, and the sequence of the displacement (D)-loop (nucleotides 100–600) was examined for the remaining 20 control subjects and 25 individuals with chronic HCV infection. All mutations were confirmed by repeated analysis of DNA extracted from the specimens.

Statistics

The Student's *t*-test was used to analyse differences in the number of mtDNA mutations between the groups of controls and chronic hepatitis. The significance of differences between median values was evaluated by the Paired *t*-test in the number of mtDNA mutations and the changes of HAI score before and after IFN treatment. A P value of <0.05 was considered significant.

RESULTS

The entire mitochondrial genomes of liver specimens obtained from an individual with chronic hepatitis type C before, just after, and 3 years after IFN treatment, and of one control liver specimen were amplified by PCR and sequenced manually. The mtDNA mutations detected are indicated in Fig. 1. Compared with a mtDNA sequence deposited in GenBank (accession no.J01415), the mtDNA sequence obtained from the liver specimen of the control subject contained three single-base variants, all of which were located in the D-loop. The mtDNA sequences obtained from the chronic hepatitis type C specimen before IFN therapy contained 24 mutations, only half of which were detected in the mtDNA from specimens obtained just after and 3 years after IFN treatment. Consistent with previous observations [6–9], most of the mutations detected in this present study were homoplasmic.

The greatest frequency of mtDNA mutations was apparent in the D-loop, especially in the region between nucleotides 100 and 600. All mtDNA samples contained a G → A transition at nucleotide 263, a T → C transition at nucleotide 489, and a C insertion between nucleotides 311 and 312. We then compared the number of mutations in the D-loop region (between nucleotides 100 and 600), excluding the three mutations (variants) common to all samples, among control liver specimens and tissues from individuals with chronic hepatitis type C obtained before and after IFN treatment (Figs 2 & 3). The individuals with hepatitis type C virus had much more mtDNA mutations in D-loop than control (2.8 *vs* 0.5, $P < 0.01$). The mean difference in the number of mtDNA mutations was 0.7 for specimens obtained before and just after IFN therapy ($P < 0.001$).

The decreased number of mtDNA mutations in D-loop (?mtDNA mutation) was positively correlated with the

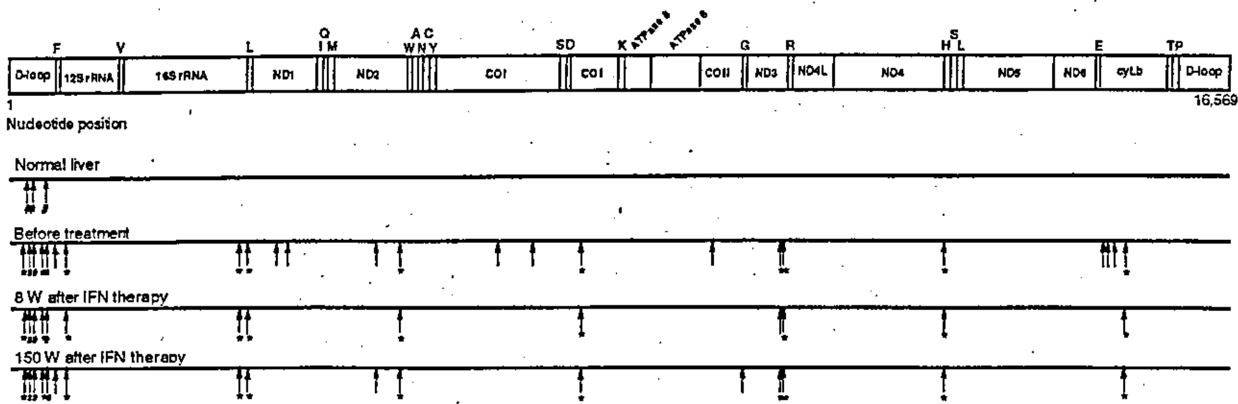

Fig. 1 Location of mtDNA mutations in specimens of control liver and liver tissues from a patient with chronic hepatitis C virus (HCV) infection. Arrows indicate the positions of mutations in mtDNA from liver specimens obtained from a control subject and from chronic HCV infection patients before and after interferon (IFN) treatment. The three common mutations (#) and the identical sites of mutations in the liver specimens before and after IFN therapy (*) are indicated. ND1, NADH (reduced nicotinamide adenine dinucleotide) dehydrogenase 1; COI, cytochrome c oxidase I; ATPase 8, ATP synthase 8; Cyt b, cytochrome b; F, V, L, I, Q, M, W, A, N, C, Y, S, D, K, G, R, H, E, T and P, tRNAs for phenylalanine, valine, leucine, isoleucine, glutamine, methionine, tryptophan, alanine, asparagine, cysteine, tyrosine, serine, aspartate, lysine, glycine, arginine, histidine, glutamate, threonine, and proline, respectively.

decrease (mean difference = 1.3, $P < 0.01$) in the total HAI score (?Activity), as shown in Fig. 4.

The variation of the mtDNA mutations were a C deletion at nucleotide 164, a G insertion between 184 and 185, a G → A transition at 207, a C insertion between 303 and 304, a C → T transition at 317, 320 and 530, and a deletion of GA at 514–515.

DISCUSSION

The present work, shows that IFN treatment markedly decreases the number of liver mtDNA mutations in individuals with chronic HCV infection, and that this change is correlated with a concomitant decrease in total HAI score.

Although each hepatocyte contains hundreds of mitochondria and each mitochondrion contains one to 10 genomes [14], most of the mutations identified in mtDNA were homoplasmic. Tumour cell mitochondria have been reported to proliferate preferentially when tumour cells are fused with normal cells [7,15], suggesting the presence of a mechanism that stimulates the replication of tumour-associated mutant mtDNA molecules. The D-loop region of mtDNA is important in the replication and expression of mitochondrial genome, because it contains both a leading-strand replication origin and transcriptional promoter regions [16]. Thus, mutations within the D-loop might affect the rate of DNA replication by modulating its interaction with trans-acting factors.

Mitochondria that undergo replication appear to acquire DNA damage more readily, resulting in an accumulation of mutations, compared with those maintained under resting conditions. Our data suggest that mtDNA mutations accu-

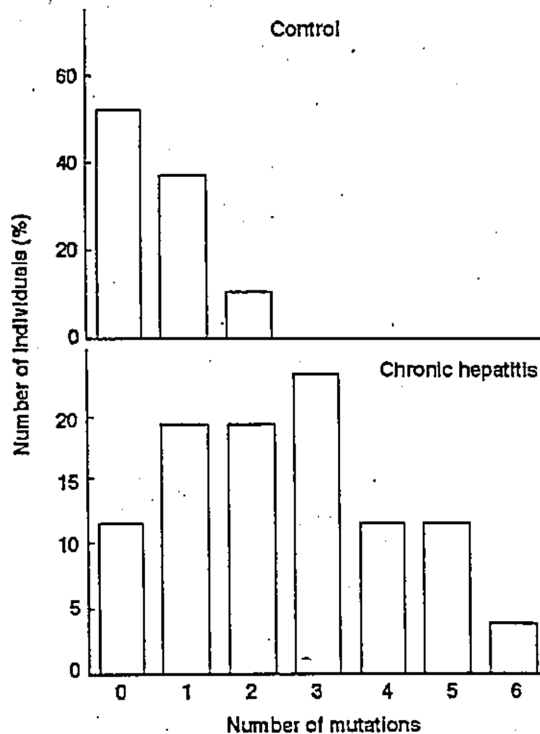

Fig. 2 The number of mtDNA mutations in the D-loop from control livers and liver specimens from patients with chronic hepatitis C virus (HCV) infection. The number of mutations in the D-loop (nucleotides 100–600), with the exception of the three mutations common to all subjects, was compared among the control liver specimens ($n = 21$) and liver tissue obtained from chronic HCV infection patients before interferon therapy ($n = 26$).

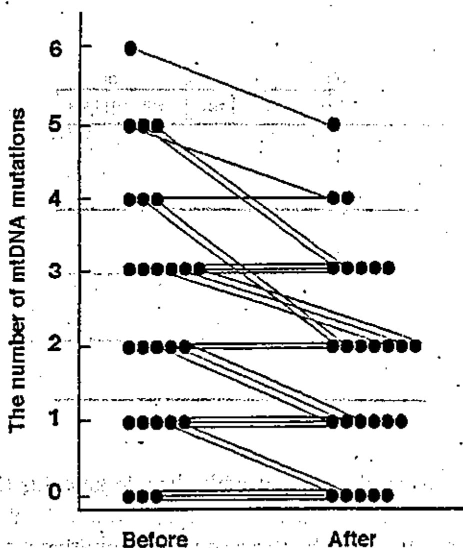

Fig. 3 Changes in the number of mtDNA mutations in the D-loop from liver specimens from patients with chronic hepatitis C virus (HCV) infection before and just after interferon (IFN) therapy. The number of mutations in the D-loop (nucleotides 100–600), with the exception of the three mutations common to all subjects, was compared among the liver tissue obtained from chronic HCV infection patients before and just after IFN therapy ($n = 26$).

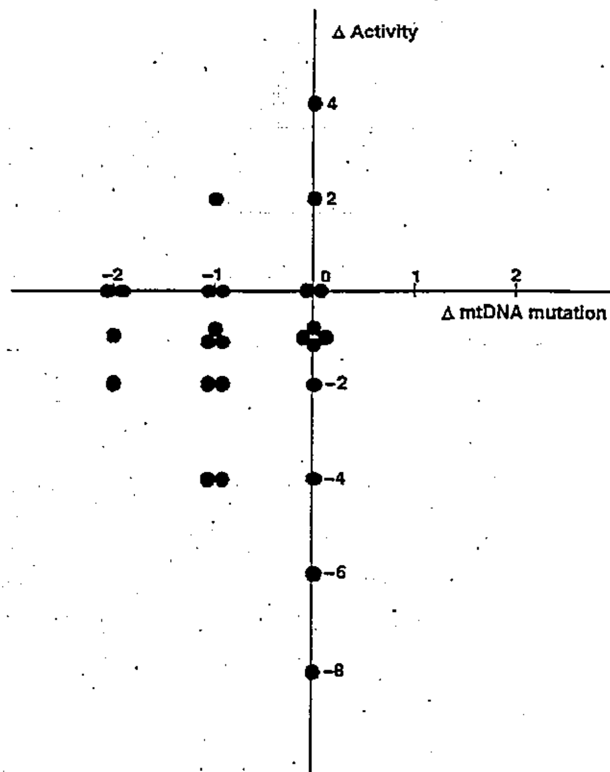

Fig. 4 The relationship between changes in histological activity index (HAI) score and the number of mtDNA mutations. Changes in the number of mtDNA mutations in the D-loop were compared with the changes in HAI score (?Activity) before and after interferon (IFN) treatment for each subject ($n = 26$). (?mtDNA mutation) = (the number of mtDNA mutations after IFN therapy) – (the number of mtDNA mutations before IFN therapy); and (?Activity) = (HAI score after IFN therapy) – (HAI score before IFN therapy).

mulate during the neoplastic transformation of hepatocytes. In mtDNA harbouring certain mutations might generate abnormal RNAs or proteins, the latter of which may promote leakage of electrons from the mitochondrial electron transport chain. The amounts of endogenously produced reactive oxygen species, such as superoxide and related free radicals, might thus be increased in cells with mutant mtDNA, and the resulting oxidative modification of DNA may contribute to the early stages of hepatocarcinogenesis.

Although activated leucocytes infiltrate into inflammatory liver tissue, the number of mitochondria in infiltrated inflammatory cells is significantly smaller than in hepatocytes. Thus, the contribution of contaminating inflammatory cells to the increased mtDNA mutation frequency observed in HCC tissues is likely to be negligible. Furthermore, mtDNA preparations from liver tissues in individuals with chronic HCV infection were compared with those from paired blood samples. No mutations were found in the blood samples, except for the three common mutations in the D-loop.

Despite our attempts to detect deletions in mtDNA with the use of multiple primers for PCR analysis, no deletion was found in liver tissues from individuals with chronic HCV infection, suggesting that hepatocytes with large mtDNA deletions might be eliminated, while cells and/or mitochondria without such deletions preferentially undergo proliferation.

The high frequency of mtDNA mutations in the liver of patients with chronic hepatitis suggests that hepatocytes in such patients continuously undergo malignant transformation during inflammation induced by HCV infection. The observation that most of the mutations detected were homoplasmic in nature indicates that the mutated mtDNA had become dominant in the liver. Given the clonal nature and large number of mtDNA copies, the unusually high rate of mtDNA mutation in the liver of patients with chronic HCV infection indicates genomic instability, which is likely to contribute to hepatocarcinogenesis. Therefore, analysis of mutations in mtDNA could provide a new criterion for the therapeutic evaluation of the effect of IFN, and may be useful for prediction of the risk of carcinogenesis.

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Previous or Occult Hepatitis B Virus Infection in Hepatitis B Surface Antigen-Negative and Anti-Hepatitis C-Negative Patients with Hepatocellular Carcinoma

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Abstract
Purpose. We investigated the clinical and virologic findings in hepatitis B surface antigen (HBsAg)-negative and anti-hepatitis C virus antibody (anti-HCV)-negative patients with hepatocellular carcinoma (HCC) to investigate the role of previous or occult hepatitis B virus (HBV) infections in the development of HCC.
Methods. We examined sera and HCC samples from 40 HBsAg-negative and anti-HCV-negative patients. Sera were tested for some viral markers, and genomic DNA was extracted from the HCC samples. HBx RNA was also extracted from the HCC and amplified by a polymerase chain reaction with reverse transcription (RT-PCR).
Results. Hepatocellular carcinomas from five patients with anti-HBc (group 1, 25 patients) and nine patients without anti-HBc (group 2, 15 patients) were examined for HBx RNA. HBx RNA was detected in four of the five HCC samples from group 1 and in four of the nine HCC samples from group 2.
Conclusion. These findings suggested that previous or occult hepatitis B virus infection is common in HBsAg-negative and anti-HCV-negative patients with HCC.

Key words Hepatocellular carcinoma · Hepatitis B virus · Hepatitis C virus · Occult infection · Hepatitis Bx RNA

Introduction

The major causes of hepatocellular carcinoma (HCC) are hepatitis B virus (HBV) and hepatitis C virus (HCV). In fact, in Japan, HB surface antigen (HBsAg) or anti-HCV antibody (anti-HCV) is detected in the sera of about 90% of patients with HCC.[1,2] In the other 10%, the cause of carcinogenesis and the relationship between viral hepatitis and HCC remain unclear. In patients infected with HBV, integration of the HBV genome and the production of HBx protein in the liver play important roles in the development of HCC.[3] HBV sequences have been found in HCC samples from patients without HBsAg,[4–15] possibly indicating an occult HBV infection.[16] Other studies have shown that HBV DNA is still present in blood or tissue after seroconversion to HBsAg-negativity in patients with chronic hepatitis B or transient HBV infection.[17–21] The role of occult HBV infection in chronic liver disease was recently clarified.[16] In Japan, the anti-HB core antibody (anti-HBc), which indicates present or past HBV infection, is detected in about 60% of HBsAg-negative and anti-HCV-negative patients with HCC.[1,2,14] However, the clinical features related to HBV infection in such patients have not been clarified.

The hepatitis G virus (HGV)/GB virus C and the TT virus (TTV), a novel DNA virus, were recently reported to cause liver disease.[22–24] However, few studies have investigated the prevalence of HGV and TTV in HBsAg-negative and anti-HCV-negative patients with HCC, and the role of these viruses in the development of HCC is still unclear.[1,25–30] We studied the clinical and virologic findings of HBsAg-negative and anti-HCV-negative patients with HCC to investigate the role of previous or occult HBV, HGV, and TTV infections in the development of HCC.

Patients and Methods

Patients

Between April 1990 and December 1999, 391 patients underwent liver resection for HCC at the Second

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Department of Surgery, Osaka City University Medical School. Sera from 40 of these patients were negative for both HBsAg and anti-HCV. We investigated the clinicopathologic findings and hepatitis markers in these 40 patients, comprised of 33 men and 7 women, with a mean age of 61 years (range 29–80). Clinicopathologic findings and hepatitis markers in these 40 patients were investigated. The medical and family history of chronic hepatitis B, and alcohol intake were established by interviews with the patients and their families. Clinical records were also reviewed if possible. Alcohol abuse was defined as the consumption of an estimated mean of 86 g or more of ethanol per day for at least 10 years, according to the Liver Cancer Study Group of Japan.[31]

We were able to use unfixed frozen sections of resected tissue specimens to detect HBx RNA, from five anti-HBc positive patients (group 1) and nine anti-HBc negative patients (group 2). There were no remarkable differences in clinicopathologic findings between the subjects of this study and other patients in either group.

This study was conducted in accordance with the Helsinki Declaration, adhering to the guidelines of the Ethics Committee of our institution. Informed consent was obtained from each patient.

Viral Markers in Serum and Tissue

Serum was obtained before surgery and frozen at −80°C until assayed. Sera was tested for anti-HCV by an enzyme-linked immunosorbent assay (Ortho Diagnostic Systems, Tokyo, Japan). Serum HCV RNA was examined by a polymerase chain reaction with reverse transcription (RT-PCR) using primers derived from a conserved 5′-untranslated region of the viral genome[32] and by a branched DNA probe method (>1.0 × 10^3 copy/ml, Quantiplex HCV-RNA, Chiron, Emeryville, CA, USA). Sera were also tested for HBsAg, anti-HBe antibody (anti-HBe), anti-HBs antibody (anti-HBs), and anti-HBc using an enzyme immunoassay (International Reagents, Kobe, Japan). A titer for anti-HBc with >70% inhibition was scored as positive, and was defined as high when there was greater than 70% inhibition after a 200-fold dilution of the test serum. We measured serum HBV DNA as described previously (>4.0 × 10^2 copy/ml).[33] Sera were tested for anti-hepatitis D virus by an enzyme immunoassay (Abbott Laboratories, North Chicago, IL, USA).

Hepatitis G virus/GB virus-C RNA was tested by a nested RT-PCR as described previously.[1] TT virus was tested by PCR using second- and third-generation primers as described previously.[30,34,35] For a semi-nested PCR assay (second generation), we used primers NG059 (outer, sense, 5′-ACAGACAGAGGAGAAGGCAACATG-3′), NG061 (inner, sense, 5′-GGCAACATGTTATGGATAGACTGG-3′), and NG063 (antisense, 5′-CTGGCATTTTACCATTTCCAAAGTT-3′). We also performed a single-round PCR assay (third generation) using primers T801 (sense, 5′-GCTACGTCACTAACCACG-3′) and T935 (antisense, 5′-CTBCGGTGTGTAAACTCACC-3′; B = mixture of G, C, and T).

Resected samples of HCC were frozen and stored at −80°C. Genomic DNA was extracted from both tissues by standard proteinase-K digestion, followed by phenol-chloroform extraction. RNA was also extracted from 100 mg of tissue by the acid guanidinium thiocyanate-phenol-chloroform method[36] followed by treatment with ribonuclease-free DNase (Stratagene Cloning Systems, La Jolla, CA, USA) at 37°C for 15 min. After inactivation of the DNase, the samples were incubated with reverse transcriptase (Gibco BRL, Gaithersburg, MD, USA) and 50 pmol of the downstream primer specific for HBx (1818–1799).[37] For the negative control, 10 µl of the cDNA and RNA samples (equivalent to 1 µg) were amplified in 50 µl of a reaction buffer containing 25 pmol of the two appropriate primers, deoxynucleotides, each at a concentration of 0.2 mmol/l, PCR buffer, and 2.5 units of Gold Taq polymerase (Roche Molecular Systems, Branchburg, NJ, USA). Thirty-five cycles of amplification were done (95°C for 30 s, 55°C for 60 s, and 72°C for 90 s) for the first PCR. With 2 µl of the first PCR product, a second PCR was done to detect HBx RNA.[14] Gel electrophoresis was done in 1.5% agarose and direct sequencing of the HBx gene was done with a DNA sequencing system (373A, Applied Biosystems, Tokyo, Japan) as described previously.[14]

Pathologic Examination

Resected specimens were cut into serial slices 5 mm thick, then fixed in 10% formalin, and stained with hematoxylin and eosin. The severity of active hepatitis (grade) and the degree of fibrosis (stage) in the noncancerous hepatic tissue was determined according to the definition of Desmet et al.[38] Grade 0 indicated no activity; grade 1, minimal activity; grade 2, a low level of activity; grade 3, a moderate level of activity; and grade 4, a high level of activity. Stage 0 indicated no fibrosis; stage 1, portal fibrous expansion (mild fibrosis); stage 2, portal-portal septa without architectural distortion (moderate fibrosis); stage 3, portocentral septa with architectural distortion (severe fibrosis); and stage 4, cirrhosis.

Table 1. Clinical features and serologic markers for hepatitis in HBsAg-negative and anti-HCV-negative patients with hepatocellular carcinoma

	Anti-HBc-positive (group 1, n = 25)	Anti-HBc-negative (group 2, n = 15)
Age (mean ± SD)	61.0 ± 8.3	60.4 ± 11.8
Sex (Male:Female)	22:3	11:4
History of chronic hepatitis B	15	0
Family history of chronic hepatitis B	8	1
History of blood transfusion	4	1
Alcohol abuse	9	7
Anti-HBe	12	0
Anti-HBs	15	0
HBV DNA	2	0
Anti-HDV	0	0
HCV RNA	0	0
HGV (GBV-C) RNA	2	0
TT virus DNA		
Second-generation primers	8	2
Third-generation primers	18	9
Associated liver disease		
Autoimmune hepatitis	1	1
Budd-Chiari syndrome	1	0

Anti-HBe, anti-hepatitis B e antibody; anti-HBs, anti-hepatitis B surface antibody; anti-HDV, anti-hepatitis D virus antibody; HGV, hepatitis G virus; GBV-C, GB virus C

Results

Sera from 40 of the total 391 patients were negative for HBsAg and anti-HCV (Table 1). The sera from 25 of these 40 patients were positive for anti-HBc (group 1). In group 1, the sera from 12 patients were positive for anti-HBe and the sera from 15 patients were positive for anti-HBs. The sera from the other 15 patients were negative for anti-HBe, anti-HBs, and anti-HBc (group 2). The sera from all 40 patients were negative for HCV RNA and anti-hepatitis D virus antibody. HGV RNA was detected in the sera from two patients in group 1 but none in group 2. TT virus DNA was detected in 10 of the 40 patients by second-generation primers, and in 27 of the 40 patients by third-generation primers.

Sera from two patients in group 1 were positive for HBV DNA and the titers for anti-HBc in these 2 patients were high. In group 1, eight patients had a family history of chronic hepatitis B, and 15 patients, including the 2 with HBV DNA and high titers of anti-HBc, had been treated for chronic hepatitis B (Table 1). These findings suggest strongly that HCC was detected after seroconversion to HBsAg-negativity during chronic hepatitis B in at least 15 patients in group 1. Figure 1 shows the typical clinical course of one patient in whom HCC was detected after seroconversion to HBsAg-negativity. In group 2, HBV DNA was not detected in the sera of any patients. None of the group 2 patients had a history of chronic hepatitis B, and only one patient had a family history of chronic hepatitis B. Nine patients in group 1 and seven patients in group 2 had a history of alcohol abuse. Four patients in group 1 (two of whom had signs of chronic hepatitis B)

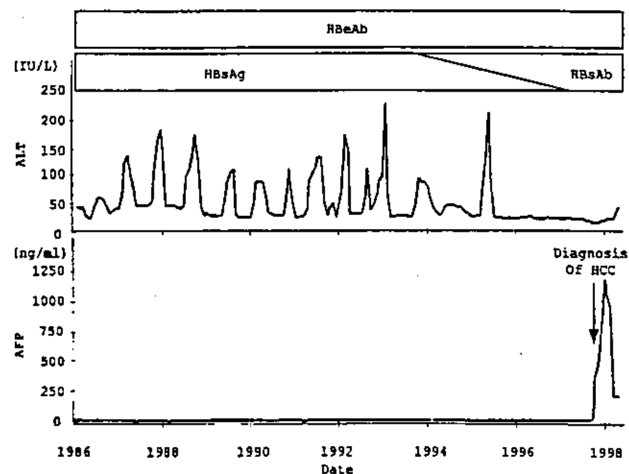

Fig. 1. Typical clinical course of one patient in whom HCC was detected after seroconversion of hepatitis B surface antigen. *HBeAg*, hepatitis B e antigen; *HBsAg*, hepatitis B surface antigen; *HBsAb*, hepatitis B surface antibody; *AFP*, α-fetoprotein; *ALT*, alanine aminotransferase. *Arrow* indicates the diagnosis of HCC. HBsAg disappeared with the remission of ALT fluctuations. HCC was detected after seroconversion of HBsAg

Table 2. Laboratory test results and histologic findings of noncancerous hepatic tissue from HBsAg-negative and anti-HCV-negative patients with hepatocellular carcinoma

	Anti-HBc-positive (group 1, n = 25)	Anti-HBc-negative (group 2, n = 15)
AFP (>20 ng/ml)	9	5
PIVKA II (>40 ng/ml)	12	8
Albumin (g/dl)	3.6 (3.2/4.1)	3.9 (3.8/4.1)
AST (IU/l)	49 (28/76)	44 (31/73)
ALT (IU/l)	52 (34/76)	45 (26/70)
$ICGR_{15}$ (%)	12.3 (5.5/23.6)	11.0 (8.3/19.0)
HAI score		
Grade (≧3)	1	3
Stage (≧3)	13	6
Tumor differentiation		
Well differentiated	2	2
Other	23	13

Laboratory test results are expressed as the median with 10th and 90th percentiles
AFP, α-fetoprotein; PIVKA II, protein-induced vitamin K absence or antagonist II; AST, aspartate aminotransferase; ALT, alanine aminotransferase; $ICGR_{15}$, indocyanine green retention rate at 15 min; HAI score, histologic activity index score, defined by Desmet et al.[38]; Grade, the severity of active hepatitis in the noncancerous hepatic tissue; Stage, the degree of fibrosis in the noncancerous hepatic tissue

Table 3. HBx RNA in hepatocellular carcinoma and noncancerous hepatic tissue

Patient no.	Hepatocellular carcinoma HBx RNA	Noncancerous hepatic tissue HBx RNA
Group 1		
5	+	+
10	−	−
20	+	+
24	+	+
25	+	+
Group 2		
2	−	−
3	−	−
4	+	−
5	−	−
6	+	+
7	−	−
8	−	−
13	+	+
14	+	+

and one patient in group 2 had a history of blood transfusion. One patient in group 1 and one patient in group 2 had autoimmune hepatitis, and one patient in group 1 had Budd-Chiari syndrome. Although the serum albumin concentration was significantly higher in group 2 than in group 1 (P = 0.002), there were no differences in other laboratory test results or histologic findings of the cancerous and noncancerous hepatic tissues (Table 2).

Resected specimens from five patients in group 1 and nine patients in group 2 were tested for HBx RNA (Table 3 and Fig. 2). HBx RNA was detected in four of the five group 1 samples of HCC and in four of the nine group 2 samples of HCC.

HBx RNA was also detected in two of six patients in group 2 with severe fibrosis or cirrhosis. The other four of these six patients were heavy drinkers.

Discussion

The clinical significance of previous HBV infection in HCC development, especially in patients with HCV-related HCC, has been the subject of many recent studies.[14,39–43] However, there have been few reports on the clinical features and significance of previous or occult HBV infection in HBsAg-negative and anti-HCV-negative patients with HCC. Negative HBsAg and

Fig. 2A–C. Nucleotides from HCC samples (*C*) and noncancerous hepatic tissues (*N*). **A** HBx DNA amplification by nested polymerase chain reaction (PCR). **B** β-Actin mRNA amplification as the housekeeping gene. **C** HBx mRNA amplification by nested reverse transcription (*RT*)-PCR. The extracted RNA was treated with reverse transcriptase [RT (+) or not RT (−)]. Only samples treated with RT were amplified. The expected molecular weights of the PCR products are given as base pairs (*bp*), on the left. *P*, cloned HBx gene as the positive control; *M*, size marker

positive anti-HBc usually indicates seroconversion in chronic hepatitis B or previous transient HBV infection. In this study, 15 of 25 patients with anti-HBc (group 1) had a history of chronichepatitis B, and the sera from 2 of these 15 patients were positive for HBV DNA and the titer of anti-HBc was high. These findings indicate that HBsAg seroconversion occurred before HCC was detected in at least 15 patients. In fact, the detection of HCC after seroconversion has been reported before.[44] In patients with chronic hepatitis B, screening for signs of HCC is necessary even after the disappearance of serum HBsAg. Although four patients in group 1 and one patient in group 2 had a history of blood transfusion, the route of HBV infection could not be identified in most patients.

Occult HBV and its oncogenic potential are considered a consequence of the potential of this virus to be integrated into the host genome. HBx RNA was detected in the HCC samples from four of the five group 1 patients and four of the nine group 2 patients, which suggests that many, if not most HBsAg-negative and anti-HCV-negative patients with HCC have been infected with HBV. Free episomal HBV genomes or viral particles may persist in the liver during occult infection. Other investigators have reported that Southern hybridization of genomic DNA in HCC tissues enabled the detection of HBV DNA in some HBsAg-negative and anti-HCV-positive patients.[12,15] HBV DNA might have been integrated in the early stage of hepatocarcinogenesis, but then lost during clonal evolution.[15] On the other hand, HBx-coding plasmids can transform NIH 3T3 cells,[45] and HBx protein induces the transformation of liver cells in transgenic mice.[46] HBx protein binds to and inactivates wild-type p53 protein.[47,48] HBx protein also transactivates viral and cellular genes through transcription regulatory factors such as AP-1, AP-2, NF-κB, and CREB.[48,49] Hepatitis B virus DNA was not detected in normal liver tissue without serum markers for viral hepatitis and liver cancer, in donor for living-related liver transplants.[50] These findings indicate that HBx RNA expression, which is necessary for producing the protein, is critical in the development of HCC in patients with occult HBV infection, although the reason that HBx RNA is present in HCC tissue is still unclear. In six of our group 2 patients with severe fibrosis or cirrhosis, HBx RNA was detected in two, and the other four were heavy drinkers, passively contributing to their hepatocarcinogenesis.[51]

Several investigators have reported a poor or weak association between HGV/GBV-C infection and HCC.[1,25–28] Accordingly, we detected HGV RNA in only 2 of the 40 patients. Thus, HGV did not contribute strongly to the development of HCC in patients without HBsAg and anti-HCV. TT virus was recently cloned from patients with post-transfusion hepatitis of unknown etiology;[24] however, TTV DNA is detected commonly in the adult population of Japan, indicating that it is an unlikely causative agent of chronic liver disease.[30,35] In this study, TTV was detected in 10 patients by second-generation primers and in 27 patients by third generation primers. Moreover, the prevalence of TTV was similar to that in patients with chronic liver disease without HCC and in the healthy population.[30,35] These findings indicate that TTV is not related to hepatocarcinogenesis.

In conclusion, HBx RNA was often detected in HCC samples from HBsAg-negative and anti-HCV-negative patients, suggesting that previous or occult HBV infection may be critical in the development of HCC. Hepatitis G virus and TTV are not causative agents for HCC in HBsAg-negative and anti-HCV-negative patients.

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L-Carnitine Inhibits Hepatocarcinogenesis via Protection of Mitochondria

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Hepatocellular carcinoma is usually preceded by chronic inflammation. However, the molecular mechanism in hepatocarcinogenesis is not well known. Recently, we reported that mitochondrial dysfunction plays an important role in hepatocarcinogenesis via the production of free radicals. Furthermore, we proved that L-carnitine effectively protects mitochondrial function *in vivo*. Therefore, we investigated whether long-term administration of L-carnitine could prevent hepatitis and subsequent hepatocellular carcinoma in Long-Evans Cinnamon rats that are often analyzed as a model of hepatocarcinogenesis. The results indicated that oxidative stress elicited from abnormally accumulated copper increased the amount of free fatty acids, thereby inducing mitochondrial dysfunction, resulting in cell death and enhanced secondary generation of reactive oxygen species, which were significantly inhibited by carnitine treatment. Finally, the occurrence of placental glutathione S-transferase-positive foci as a marker for preneoplastic lesions and hepatocarcinogenesis were significantly inhibited by L-carnitine. These facts suggest that mitochondrial injury plays an essential role in the development of hepatocarcinogenesis and that the clinical use of carnitine has excellent therapeutic potential in individuals with chronic hepatitis.
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Key words: Long-Evans Cinnamon rats; chronic hepatitis; free radical; mitochondria; carcinogenesis; L-carnitine

Hepatocellular carcinoma is usually preceded by chronic inflammation caused by persistent infection with hepatitis viruses. Although a lot of effort has been made to elucidate the mechanism of hepatocarcinogenesis, the detailed molecular mechanism in it is still not well known.

It is well known that the extent of oxidative stress is increased in the livers of individuals infected with hepatitis viruses,[1,2] and reactive oxygen species (ROS) are important inducers of both tissue injury and DNA damage.[3] Because mitochondrial DNA (mtDNA) lacks histones and related protective systems, mutations accumulate to a greater extent in it than in nuclear DNA.[4] Synthesis of mitochondrial proteins necessary for the maintenance of mitochondrial functions depends on both nuclear DNA and mtDNA.[5] Thus, oxidative injury to mtDNA causes mutations in mitochondrial genes and/or results in the generation of abnormal proteins in the electron transport system.[6,7] Furthermore, mitochondrial membrane injury could also be induced via lipid peroxidation reactions in the presence of ROS.[8] Mitochondria are the major source of reactive oxygen species even in their normal state. When their function is impaired, the amount of endogenously produced ROS due to promoting leakage of electrons from the mitochondrial electron transport chain, such as superoxide and related free radicals, may thus be markedly increased.[9,10] These highly reactive compounds can act as initiators and/or promoters, cause DNA damage, activate procarcinogens and inactivate anti-oncogene.[11,12] Therefore, the resulting injury to mitochondria underlying chronic inflammation may contribute to the early stages of hepatocarcinogenesis. Thus, we hypothesized that hepatocarcinogenesis might be inhibited by protection of mitochondria during the process of chronic inflammation.

Long-Evans Cinnamon (LEC) rats with a deletion in the copper transporting P-type ATPase gene[13] homologous to the Wilson's disease gene (ATP7b)[14] are characterized by the excessive accumulation of copper in the liver due to a deficiency in copper incorporation into ceruloplasmin in the Golgi apparatus and biliary copper excretion.[15] The mutant rats spontaneously develop hepatitis with jaundice at 4 months after birth, followed by death in 50% due to fulminant hepatitis.[16] The rats that recover from fulminant hepatitis exhibit chronic hepatitis, cholangiofibrosis and, eventually, hepatocellular carcinoma and cholangiocarcinoma at a high incidence after about 1 year.[16] LEC rats are a unique and useful animal model for clarifying the mechanism of, and developing treatment strategies for, hepatocarcinogenesis in humans because the natural history of liver disease in LEC rats closely resembles that of human hepatic disease and the characteristics of liver cancer formation based on inflammation. The striking ultrastructural and functional abnormal alterations in mitochondria are also observed in the hepatocytes of LEC rats.[16–18] Furthermore, the severity of ultrastructural alterations in mitochondria correlates with the degree of icterus displayed by the rats,[17] suggesting that hepatic mitochondria are important targets in copper-induced toxicity in LEC rats. Therefore, in this study, we used LEC rats to investigate the role of mitochondria in the process of hepatocarcinogenesis.

L-carnitine is a natural nutrient and essential for the β-oxidation of fatty acids in mitochondria to generate ATP.[19,20] We previously reported that L-carnitine effectively prevents mitochondrial injury deriving from oxidative damage *in vivo*.[21] In fact, there are also several reports showing that carnitine effectively inhibited mitochondrial injury induced by oxidative stress and mitochondria-dependent apoptosis.[22–25] It is thought that carnitine shows its protective effects on mitochondria and on whole cells by inhibiting free fatty acid-induced mitochondrial membrane damage and/or its secondary effects.[22,26,27]

Using the hypothesis that oxidative injury induced during the process of hepatocarcinogenesis in LEC rats might be inhibited by carnitine, we investigated whether this agent could prevent inflammatory damage and hepatocarcinogenesis in LEC rats.

Material and methods

L-carnitine, a GPT-UV test kit, a T-Bil assay kit, an NEFA assay kit and an ABC kit were purchased from Wako Pure Chemical (Osaka, Japan). An *in situ* apoptosis detection kit was purchased from Takara Shuzo (Kusatsu, Japan). Other reagents used were of the highest grade commercially available.

Animal experiments

Male LEC rats were fed laboratory chow and water *ad labitum* from 6 weeks and used for experiments. Animals were administered L-carnitine contained in drinking water at a concentration of

Abbreviations: 4-HNE, 4-hydroxy-2-nonenal; 8-OHdG, 8-hydroxydeoxyguanosine; ALT, alanine aminotransferase; AST, asparate aminotransferase; FFA, free fatty acid; GST-P, glutathione S-transferase placental form; HO-1/2, heme oxygenase-1/2; HSP-70, heat shock protein 70; JVS, juvenile visceral steatosis; LEC, Long-Evans Cinnamon; Mn-SOD, manganese-superoxide dismutase; mtDNA, mitochondrial DNA; OCTN-2, organic cation transporter number 2; RCI, respiratory control index; ROS, reactive oxygen species; T-Bil, total bilirubin; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

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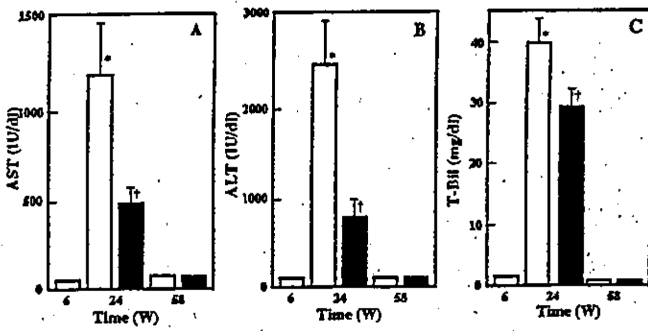

FIGURE 1 – Effects of carnitine on hepatic dysfunction. Animals were orally administered carnitine (1 mg/ml in drinking water; 100 mg/kg body weight) long term from 6 weeks. At the indicated times of 6, 24 and 58 weeks, plasma levels of ALT (*a*), AST (*b*) and T-Bil (*c*) were measured as described in text. Open columns, untreated group; closed columns, carnitine-treated group. Data are expressed as mean ± SD ($n = 7$). Asterisk, $p < 0.01$ *vs.* untreated group at 6 weeks; dagger, $p < 0.01$ *vs.* untreated group at 24 weeks.

1 mg/ml (100 mg/kg/day) over a long term until sacrificed. Blood was taken regularly every 1–2 months, and plasma was obtained for analysis. All experiments were approved by the Animal Care and Use Committee of Osaka City University Medical School.

Hepatic function analysis

Plasma levels of asparate aminotransferase (AST), alanine aminotransferase (ALT) and total bilirubin (T-Bil) were determined by standard method using GPT-UV test kit (for AST and ALT) and a T-Bil assay kit.

Histologic analysis

At 6, 24 and 58 weeks after perfusion with ice-cold 0.9% NaCl solution, the liver specimens were obtained from LEC rats, fixed in 10% formalin and embedded in paraffin. Thin sections of tissue specimens (5 μm) were stained with hematoxylin and eosin. Pathologic examination was carried out under light microscopy.

Measurement of carnitine and free fatty acid (FFA) levels

The total carnitine (free carnitine + acylcarnitine) levels in the serum and livers of LEC rats were measured using a total carnitine assay kit (Kainos, Japan).

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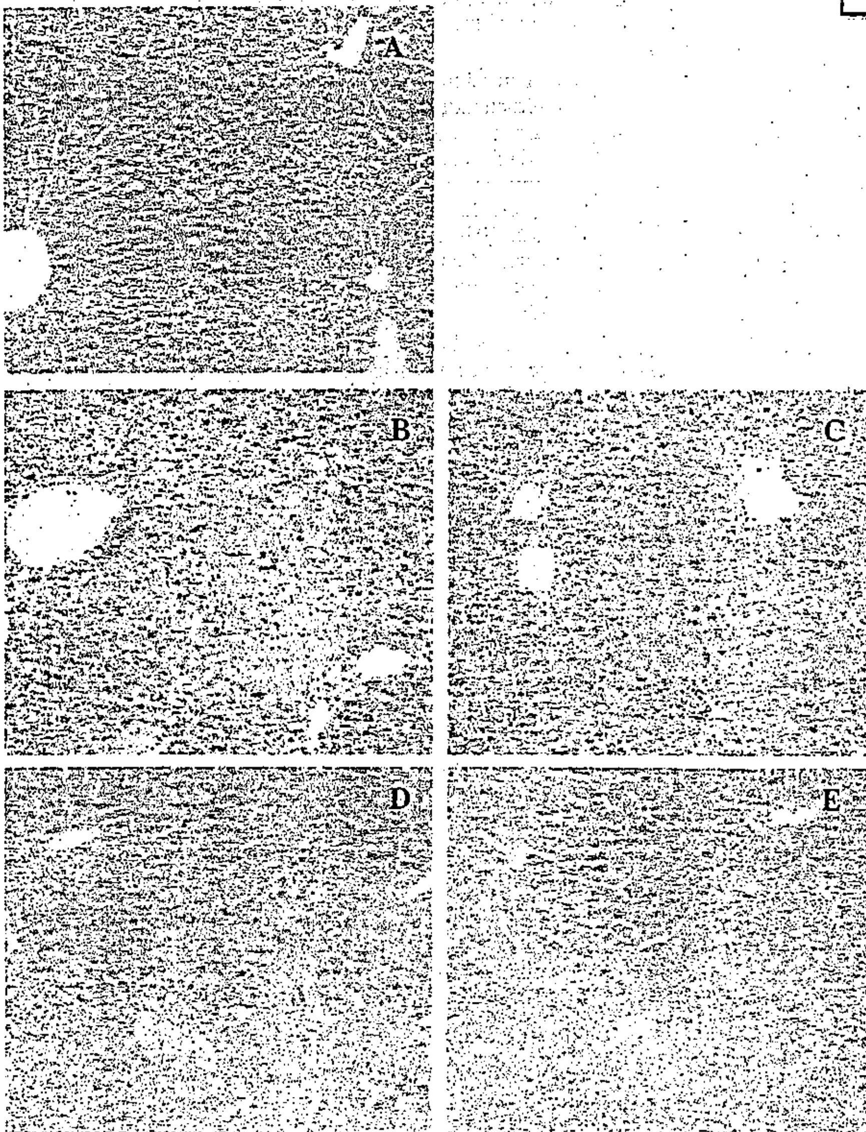

FIGURE 2 – Effects of carnitine on hepatic structure. Effects of carnitine on the structure of the liver were examined by hematoxylin and eosin staining at 6 (*a*), 24 (*b* and *c*) and 58 (*d* and *e*) weeks. Other conditions were the same as in Figure 1. (*a*, *b* and *d*), untreated group; (*c* and *e*), carnitine-treated group. Data show one typical result of 4 separate experiments. Magnification: 100×.

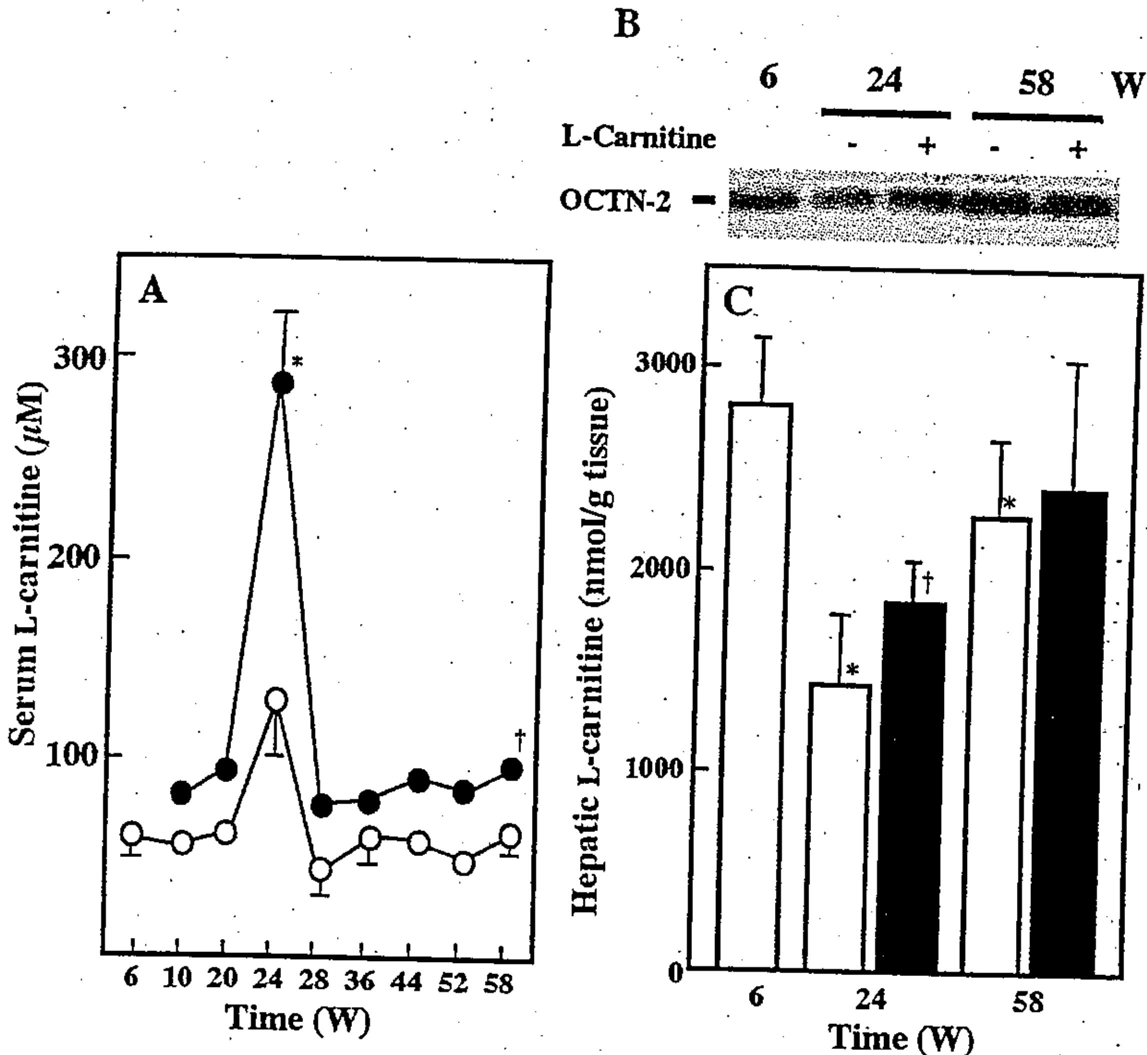

FIGURE 3 – Effects of carnitine on the serum and hepatic levels of total carnitine. The total carnitine levels in the serum (*a*) and livers (*c*) of LEC rats were measured. The hepatic expression of OCTN-2 is shown in (*b*). Other conditions were the same as in Figure 1. Open circles and columns, untreated groups; closed circles and columns, carnitine-treated groups. Data are expressed as mean ± SD ($n = 7$). (*a*) asterisk, $p < 0.01$ *vs.* untreated group at 24 weeks; dagger, $p < 0.01$ *vs.* untreated group at 58 weeks. (*c*) asterisk, $p < 0.01$ *vs.* untreated group at 6 weeks; dagger, $p < 0.05$ *vs.* untreated group at 24 weeks.

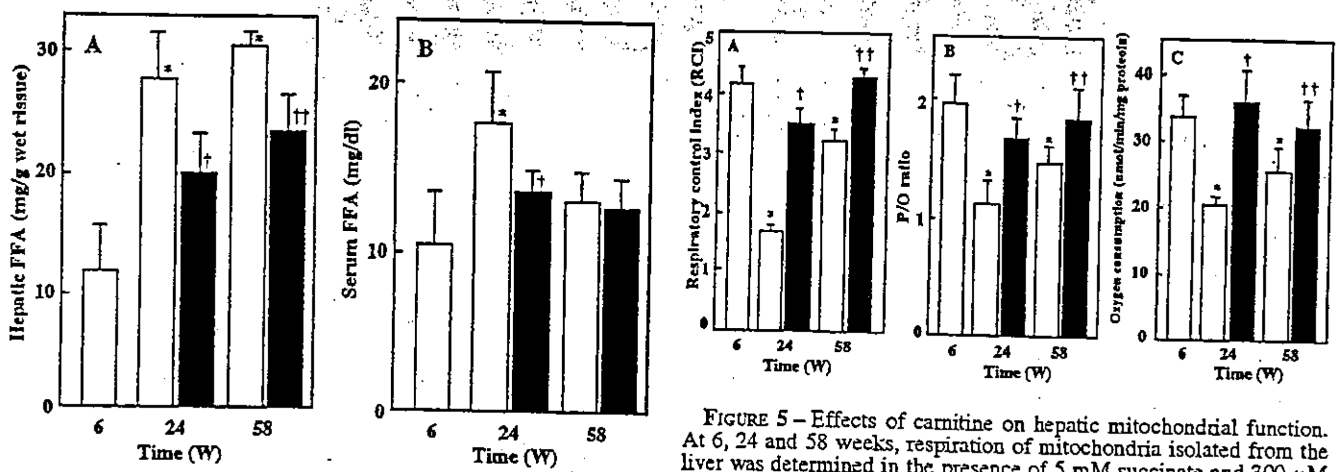

FIGURE 4 – Effects of carnitine on hepatic and serum levels of FFA. At 6, 24 and 58 weeks, the levels of FFA were measured in the livers (*a*) and serum (*b*). Open columns, untreated group; closed columns, carnitine-treated group. Data are expressed as mean ± SD ($n = 7$). Asterisk, $p < 0.01$ *vs.* untreated group at 6 weeks; dagger, $p < 0.05$ *vs.* untreated group at 24 weeks; double dagger, $p < 0.05$ *vs.* untreated group at 58 weeks.

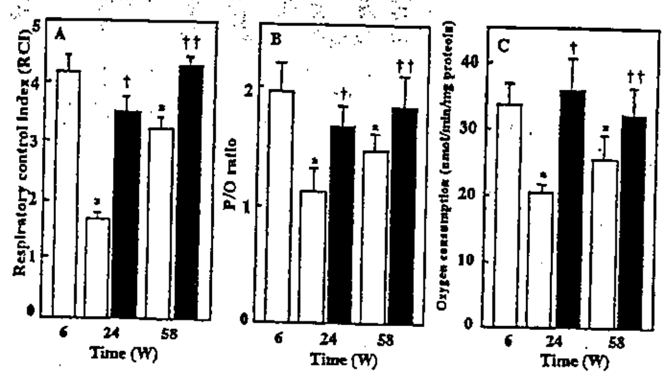

FIGURE 5 – Effects of carnitine on hepatic mitochondrial function. At 6, 24 and 58 weeks, respiration of mitochondria isolated from the liver was determined in the presence of 5 mM succinate and 300 μM ADP and followed by analysis of RCI (*a*), P/O ratio (*b*) and O_2 consumption (*c*). Open columns, untreated group; closed columns, carnitine-treated group. Data are expressed as mean ± SD ($n = 7$). Asterisk, $p < 0.01$ *vs.* untreated group at 6 weeks; dagger, $p < 0.05$ *vs.* untreated group at 24 weeks; double dagger, $p < 0.05$ *vs.* untreated group at 58 weeks.

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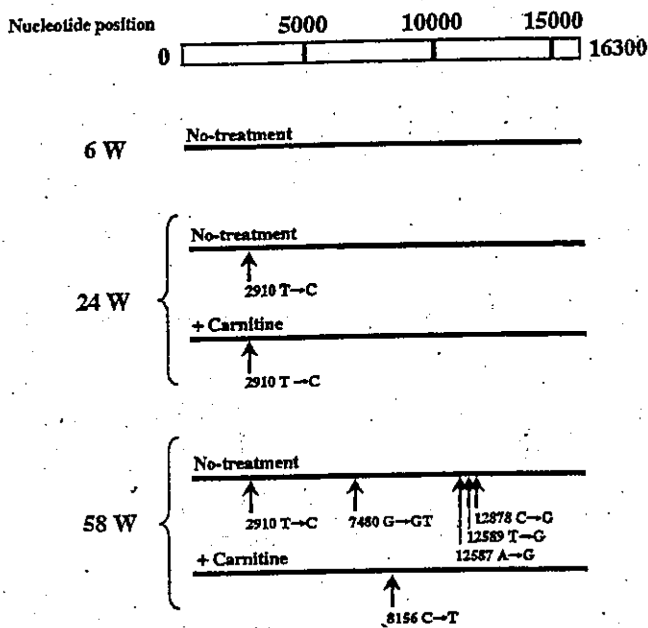

FIGURE 6 – Effects of carnitine on hepatic mtDNA. At 6, 24 and 58 weeks, DNA was extracted from noncancerous liver tissue, and the entire sequence of mtDNA was analyzed. Arrows indicate positions of mutations in mtDNA.

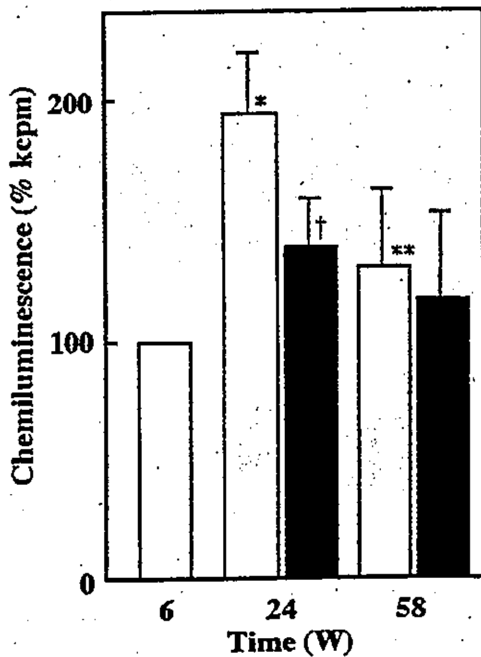

FIGURE 7 – Effects of carnitine on ROS production of hepatic mitochondria. At 6, 24 and 58 weeks, ROS production of isolated liver mitochondria was determined in the presence of 5 mM succinate and 300 μM ADP. Open columns, untreated group; closed columns, carnitine-treated group. Data are expressed as mean ± SD ($n = 7$). Asterisk, $p < 0.01$ *vs.* untreated group at 6 weeks; double asterisk, $p < 0.05$ *vs.* untreated group at 6 weeks; dagger, $p < 0.01$ *vs.* untreated group at 24 weeks.

Western blot analysis

Liver specimens were homogenized and their cytosolic fractions (each 10 μg protein) were subjected to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto nitrocellulose sheets using a Pharmacia semidry blot system (2 mA/cm^2 for 1 hr in 0.2 M Tris-glycine buffer; Pharmacia, Gaithersburg, MD) for detection of organic cation transporter number 2 (OCTN-2). The sheets were incubated in TBS solution (140 mM NaCl, 50 mM Tris-HCl, pH 7.2) containing 0.1% Tween-20 and 5% low-fat milk powder for 12 hr at 4°C. Then, the sheets were incubated with an anti-OCTN-2 antibody (1:1,000 in TBS solution with 0.5% low-fat milk powder) for 12 hr at 4°C. The incubated sheets were washed 5 times to eliminate nonspecific binding of the antibody. After incubation with a horseradish peroxidase-conjugated anti-IgG antibody (1:1,000 in TBS with 0.5% low-fat milk powder) at 25°C for 1 hr, immunoreactive spots were detected by ECL (Amersham, Buckinghamshire, U.K.).

Measurement of ffa levels

The FFA level[illegible] the serum and livers of LEC rats were analyzed by an N[illegible] assay kit.

Analysis of mitochondrial respiration

Mitochondria in noncancerous liver tissue were isolated according to the method described previously[28] using a medium containing 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4) and 0.1 mM EDTA. EDTA was omitted in the final wash and the mitochondrial samples were suspended in 0.25 M sucrose containing 10 mM Tris-HCl, pH 7.4, at 10–20 mg protein/ml and stored at 4°C until use. Protein concentration was determined by the bicinchoninic acid method[29] using bovine serum albumin as a standard.

Respiration of liver mitochondria was determined polarographically using a Clark-type oxygen electrode fitted to a 2 ml water-jacketed closed chamber at 25°C.[30] Isolated mitochondria were suspended in the reaction medium (1 mg protein/2 ml) consisting of 0.2 M sucrose, 10 mM KCl, 1 mM $MgCl_2$, 2 mM sodium phosphate and 10 mM Tris-HCl (pH 7.4). Oxygen consumption was monitored in the presence of 5 mM succinate and 300 μM ADP. The respiratory control index (RCI) and ADP/O ratio were analyzed to test the function of mitochondria.

Sequence analysis

Noncancerous liver tissue was frozen, microdissected with a cryostat and digested with proteinase K (0.1 mg/ml) in the presence of 1% SDS. DNA was extracted using a nucleic acid purification kit (MagExtractor Genome, Toyobo, Osaka, Japan). Each DNA sample (50 ng) was subjected to amplification by PCR using overlapping sets of primers to screen the entire mitochondrial genome. PCR (an initial incubation at 94°C for 5 min, followed by 40 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 3 min) was performed in a final volume of 50 μl with a GeneAmp PCR system 9600 (Perkin Elmer, Oak Brook, IL). The generation of large PCR products excluded the possibility of nuclear pseudogenes complicating the analysis.[31] In addition, the primers were selected to avoid such coamplification by analysis with cell lines devoid of mtDNA.

Aberrant PCR products were purified with a QIAquick PCR purification kit (Qiagen, Chatsworth, CA) and sequenced with an Applied Biosystems DNA sequencer and a Dye Terminator Cycle Sequencing FS Ready Reaction kit (Applied Biosystems, Foster City, CA). The entire sequence of mtDNA was examined for noncancerous liver tissue from each one of the untreated rats at 6, 24 and 58 weeks and carnitine-treated rats at 24 and 58 weeks, respectively. All mutations were confirmed by repeated analysis of DNA extracted from the noncancerous liver tissue.

Analysis of mitochondrial generation of ROS

Because mitochondria contain a large amount of Mn-SOD that effectively dismutates the superoxide radical, a highly sensitive chemiluminescence probe, L-012, was used for the analysis of ROS.[32] Isolated liver mitochondria (0.5 mg protein/ml) were incubated in the reaction medium containing 5 mM succinate and 300 μM ADP in the presence of 100 μM L-012 at 25°C. Chemiluminescence intensity was recorded continuously using Luminescence Reader BLR-201 (Aloka, Tokyo, Japan).

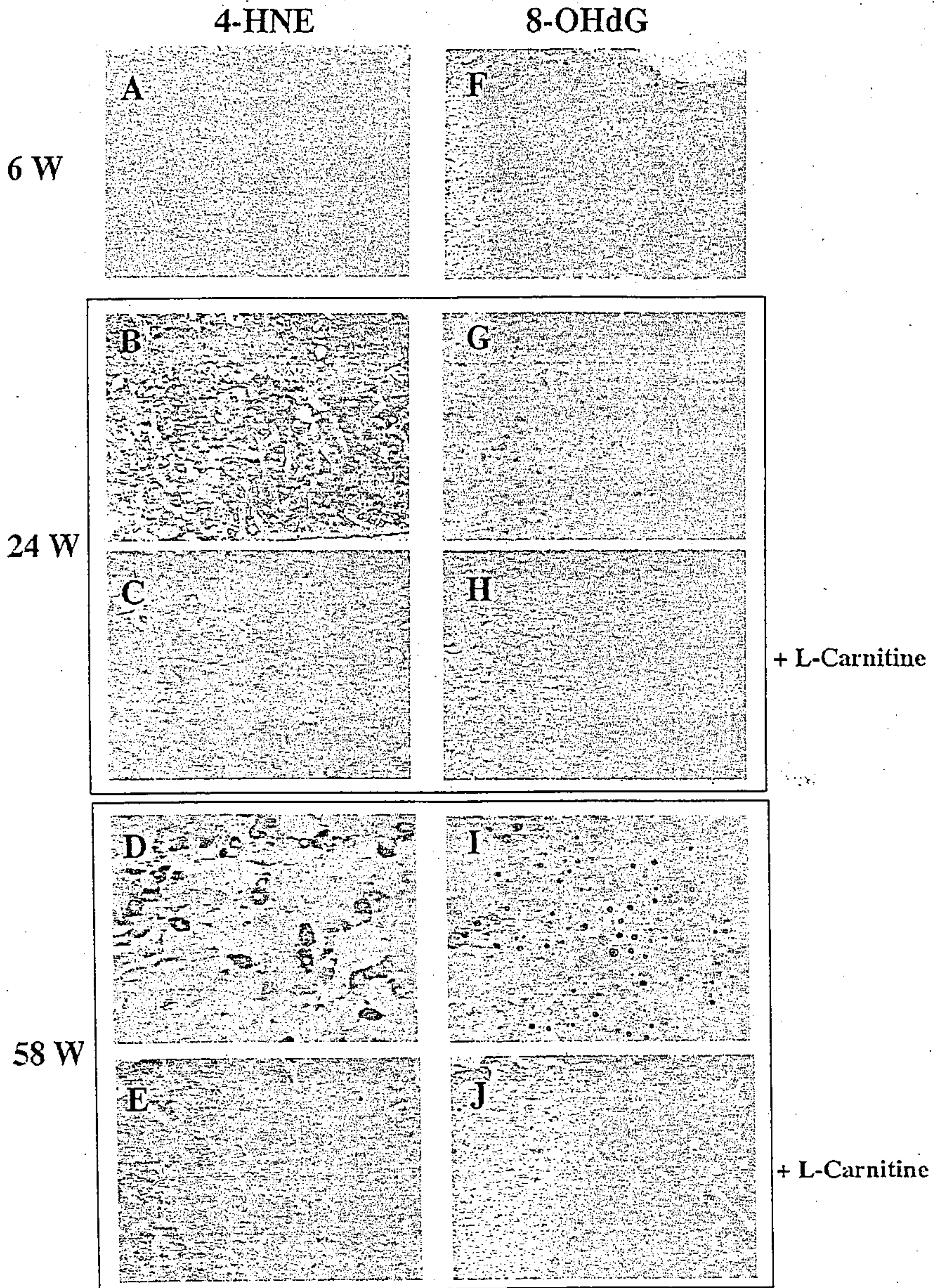

FIGURE 8 – Effects of carnitine on hepatic formation of 4-HNE adducts and 8-OHdG. The degrees of lipid peroxidation and oxidative DNA damage were assessed by immunohistochemical staining for 4-HNE (*a–e*) adducts and 8-OHdG (*f–j*) at 6 (*a* and *f*), 24 (*b*, *c*, *g* and *h*) and 58 weeks (*d*, *e*, *i* and *j*). (*a*, *b*, *d*, *f*, *g* and *i*), untreated groups; (*c*, *e*, *h* and *j*), carnitine-treated groups. Data show 1 typical result of 4 separate experiments. Magnification: 200×.

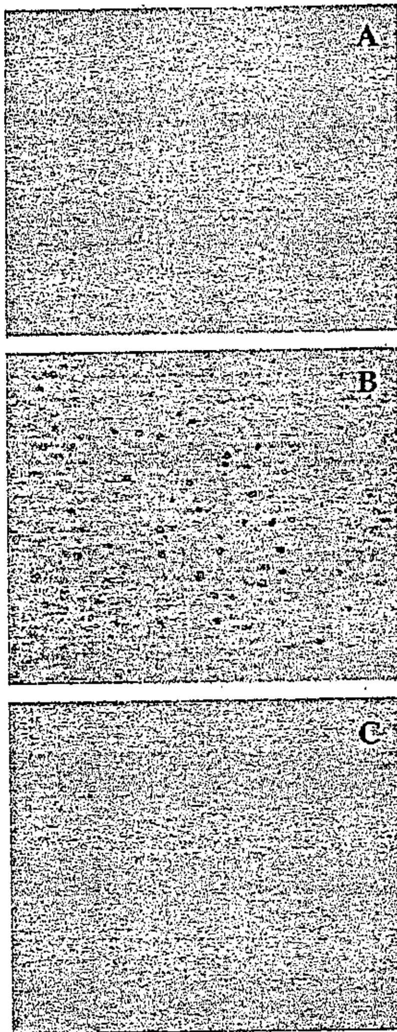

FIGURE 9 – Effects of carnitine on the occurrence of TUNEL-positive cells in acute hepatitis. Effects of carnitine on the occurrence of apoptosis in the liver were examined at 6 (*a*) and 24 weeks (*b* and *c*). (*a* and *b*), untreated groups; (*c*), carnitine-treated group. Data show 1 typical result of 4 separate experiments. Magnification: 200×.

Analysis of lipid peroxidation and oxidative DNA damage

To analyze lipid peroxidation and oxidative DNA damage, immunohistochemical staining for 4-hydroxy-2-nonenal (4-HNE) adducts and 8-hydroxydeoxyguanosine (8-OHdG) was performed using the avidin-biotin-peroxidase complex (ABC) method as previously described[33] with a Vectastain elite ABC kit (Vector Laboratories, Burlingame, CA). Briefly, liver sections 5 μm thick were deparaffinized with xylene and alcohol series. After inactivation of endogenous peroxidase with a methanol solution containing 0.3% H_2O_2 and blocking with normal blocking serum, the sections were incubated with monoclonal mouse antirat 4-HNE and 8-OHdG antibodies (100 × and 50 × dilution, respectively; Japanese Aging Control Institute, Shizuoka, Japan) overnight at 4°C. After washing 3 times with PBS solution (pH 7.4), the sections were incubated with the biotinylated second antibody for 1 hr at room temperature, followed by treatment with the ABC reagent for 30 min. Peroxidase activity was developed with 0.025% 3,3′-diaminobenzidine tetrahydrochloride in the presence of 0.015% H_2O_2 in PBS solution (pH 7.4).

In situ *detection of apoptosis*

Apoptosis of hepatocytes was assessed by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) using an *in situ* apoptosis detection kit. Briefly, after deparaffinization of formalin-fixed and paraffin-embedded liver specimens (5 μm sections) in xylene and dehydration through graded ethanol, specimens were incubated with 20 μg/ml proteinase K for 15 min at room temperature to decrease background contamination, and with 3% H_2O_2 for 5 min at room temperature to quench endogenous peroxidase activity. After incubation with the TdT enzyme and a labeling safe buffer in a humidified chamber for 90 min at 37°C, specimens were incubated with anti-FITC HRP conjugate in a humidified chamber for 30 min at 37°C, followed by thorough washing with PBS (pH 7.4) at each step above. The final staining was developed using diaminobenzidine/peroxidase substrate (H_2O_2) for 8–10 min under microscopic control and stopped with distilled water. After mounting slides using glass coverslips, DNA fragmentation in the liver specimens was detected under a light microscope.

Analysis of preneoplastic liver lesions

To analyze the preneoplastic lesions, immunohistochemical staining for the glutathione S-transferase placental (GST-P) form was performed using the ABC method as described above with a polyclonal rabbit antirat GST-P antibody (200 × dilution; MBL). GST-P-positive foci larger than 0.002 mm^2 were counted. The numbers per square centimeter and the size (mm^2 per cm^2 of liver specimen) were measured using a personal Image Analysis System LA-555 (Pias, Osaka, Japan).

Analysis of number of tumors

When LEC rats were sacrificed at 58 weeks of age, visible liver cancers large than 1 mm^3 were counted.

Statistical analysis

Values are expressed as mean ± SD. Statistical analysis was performed by the unpaired student's *t*-test and significance was set at $p < 0.05$ or $p < 0.01$.

Results

Effects of carnitine on hepatic disorder

To study the effect of carnitine on hepatic dysfunction in LEC rats in the stage of acute and chronic hepatitis, changes in plasma levels of ALT, AST and T-Bil were determined at 6, 24 and 58 weeks, respectively (Fig. 1). Plasma levels of ALT, AST and T-Bil in the untreated group increased significantly at 24-week stage in the acute hepatitis rats and decreased thereafter to slightly higher levels than 6 weeks at 58 weeks in chronic hepatitis rats. However, treatment of animals with carnitine significantly inhibited the increase in plasma levels of ALT, AST and T-Bil at the 24-week stage. Thus, acute hepatic dysfunction in LEC rats was inhibited significantly by carnitine.

F1

Effects of carnitine on hepatic structure

Histologic examination of the livers from untreated LEC rats showed no significant changes at 6 weeks (Fig. 2*a*), massive necrosis at 24 weeks and spotty necrosis and megalocytic changes in the hepatocytes with giant nuclei at 58 weeks (Fig. 2*b* and *d*). The livers from the carnitine-treated group showed a much lower degree of hepatic damage at both 24 and 58 weeks (Fig. 2*c* and *e*).

F2

Serum and organic levels of total carnitine

We analyzed the serum and hepatic levels of total carnitine in the untreated and carnitine-treated groups. Serum levels in both groups showed a transient rise at the 24-week stage in acute hepatitis. The plasma level of carnitine in the carnitine-treated group was higher than that in the untreated group over the whole period (Fig. 3*a*). There was a slight increase in the livers

F3

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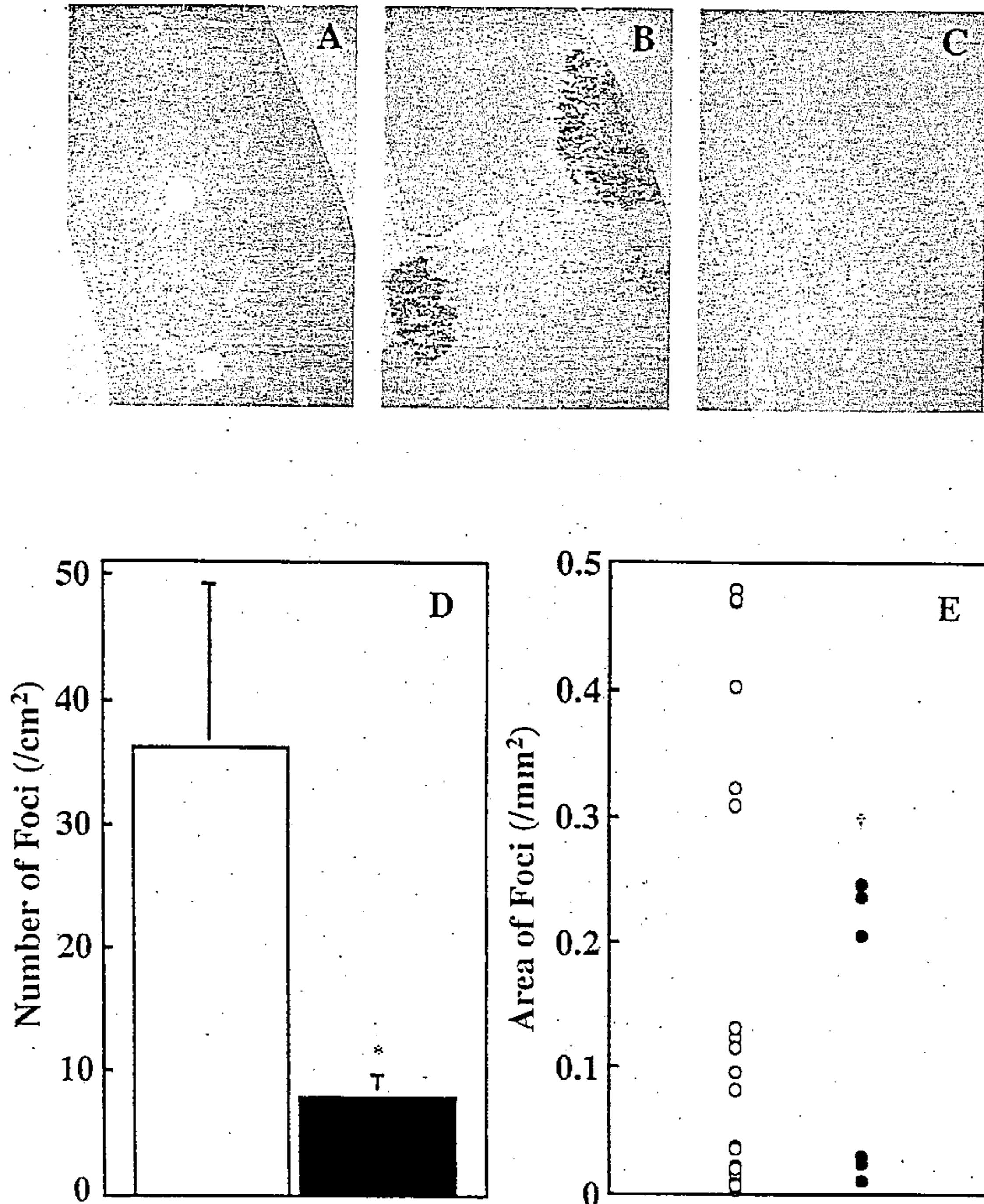

FIGURE 10 – Effects of carnitine on preneoplastic lesions. Preneoplastic liver lesions were assessed by immunohistochemical staining of GST-P at 6 (*a*) and 58 weeks (*b* and *c*). GST-P-positive foci larger than 0.002 mm^2 were counted. The number (*d*) and the size (*e*) per square centimeter of liver specimen were measured. (*a* and *b*), untreated groups; (*c*), carnitine-treated group. Data show 1 typical result of 4 separate experiments. Magnification: 20× (*a–c*). Open circles and columns, untreated group; closed circles and columns, carnitine-treated group. Asterisk, $p < 0.01$ *vs.* untreated group; dagger, $p < 0.05$ *vs.* untreated group.

of the carnitine-treated group compared to the untreated group (Fig. 3*c*).

Western blotting analysis revealed that the expression of OCTN-2, which is a carnitine transporter, in the livers of 24-week-old untreated rats decreased, while that of 58-week-old untreated and carnitine-treated rats recovered (Fig. 3*b*).

Effect of carnitine on serum and hepatic levels of FFA

To test the possible involvement of FFA in the mechanism by which carnitine inhibited hepatitis, its effect on serum and hepatic levels of FFA was examined. As shown in Figure 4, there was a significant increase of hepatic FFA levels at 24 and 58 weeks, an increase of serum FFA levels at 24 weeks. However, treatment

F4