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Immunohistochemical Evaluation of Oxidative Stress Markers in Chronic Hepatitis C

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ABSTRACT

Oxidative stress (OS) plays a major role in chronic hepatitis C. Various OS markers have been found to be elevated in hepatitis C virus (HCV)-related liver disease. This study detected the presence of OS in serum and liver biopsy specimens of HCV patients. Reactive oxygen molecules (ROM) in sera of 54 HCV patients were compared with 23 controls. OS markers 8-hydroxydeoxyguanosine (8-OHdG), 4-hydroxy-2-nonenal, malondialdehyde, and thioredoxin were measured in liver biopsy specimens of 18 HCV patients with fibrosis staging F1 (six); F2 (two), F3 (four), and F4 (six). The interferon (IFN) response and hepatocellular carcinoma (HCC) occurrence in the presence of OS markers were also evaluated. The level of ROM in HCV patients was 318 ± 56.7 Carr compared with 248 ± 40.8 Carr in controls ($p = 0.032$). Multivariate analysis found age ($p = 0.0236$) to be the only independent variable associated with increase in ROM in sera. In liver biopsy specimens, OS markers were found mainly around the area of piecemeal necrosis or the periportal area. The presence of OS markers seemed to increase with fibrosis staging, although not significantly. The OS DNA damage marker 8-OHdG was detected in the nucleus of hepatocytes. Thirteen patients received IFN therapy. During the 4-year follow-up period, HCC developed in four nonresponders to IFN and in one untreated patient. OS markers were stained in both HCC cells and non-HCC cells in HCC patients. OS markers were found in serum and liver specimens of HCV-associated liver disease and in HCC tissue. Detection of OS markers may be important for monitoring disease progression in HCV patients. Antioxidant therapy in combination with antiviral therapy may minimize liver damage and aid in the prevention and subsequent development of HCC.
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INTRODUCTION

OXIDATIVE STRESS (OS) is considered to be involved in many pathological conditions, such as aging, atherosclerosis, cancer, inflammatory diseases, and acquired immune deficiency syndrome. Imbalance in the oxidant–antioxidant status is also suggested to play a major role in viral hepatitis (3). Hepatitis C virus (HCV) infects almost 170 million worldwide, and chronic liver disease is said to develop in 70% of the infected cases (15). HCV infection produces acute and chronic hepatitis, liver steatosis, cirrhosis, and ultimately hepatocellular carcinoma (HCC). The pathogenic mechanisms for liver injury and fibrosis in chronic hepatitis C (CHC) are still unclear, but are reported to include immunological liver damage, direct cytotoxicity by various viral products, and induction of oxidative stress (13). The risk of developing HCC is significantly increased in patients with chronic viral hepatitis. Hepatocarcinogenesis is considered a multistep process, and HCC may be attributed to genetic alterations accumulating in hepatocytes during the course of chronic liver disease (6, 8, 20, 24). HCV viral structural proteins, particularly the core protein, have been shown

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TABLE 1. CLINICAL BACKGROUND OF 18 PATIENTS WITH HCV-ASSOCIATED LIVER DISEASE

No. of patients	*Genotype*	*ALT (IU/L)*	*Serum ferritin*	*Fibrosis staging*	*Activity grading*	*S markers*			*Fat deposition*	*IFN response*	*Long prognosis*
						TRX	*HNE*	*MDA*			*HCC*
1	2b	170	76.4	1	2	±	±	±	—	SVR	—
2	1b	51	260	1	1	1+	±	1+	—	—	—
3	2b	31	—	1	1	1+	1+	1+	—	—	—
4	1b	17	91.7	1	1	2+	1+	1–2+	—	—	
5	1b	76	395	1	2	2+	1+	1–2+	—	NR	—
6	2b	59	120	1	1	2+	2+	1+	±	NR	—
7	2b	46	150	2	1	2+	±	1+	2+	SVR	—
8	1b	135	839	2	1	2+	2+	1+	2+	NR	+
9	2a	47	211	3	2	2+	±	2+	1+	NR	—
10	1b	35	215	3	2	2+	2+	2+	—	NR	—
11	1b	81	180	3	3	2+	2+	2+	1+	NR	+
12	1b	78	198	3–4	2	2+	2+	2+	±	NR	+
13	2a	103	—	4	2–3	2+	2+	1+	—	SVR	—
14	1b	177	364	4	3	2+	1–2+	2+	1+	NR	+
15	2b	61	25.1	4	3	2+	1+	2+	±	—	+
16	2b	127	207	4	3	2+	2+	1–2+	1–2+	SVR	—
17	1b	115	10.2	4	3	2+	2+	2+	—	NR	—
18	1b	49	78	4	3	2+	2+	2+	—	—	—

Data are means.

to alter the oxidant–antioxidant status in the liver, in the absence of inflammation and contribute to oxidative stress and eventually facilitate HCC formation in HCV infection (19). Free radicals have been shown to play a role in CHC liver damage. Antioxidants, enzymatic and nonenzymatic, scavenge free radicals and prevent liver tissue injury (27). There are many OS markers. Among them are the following: (a) oxygen radicals such as reactive oxygen molecules (ROM); (b) 8-hydroxydeoxyguanosine (8-OHdG), a DNA base-modified product generated by reactive oxygen species (ROS) produced by activated macrophages, and an indicator of DNA damage (10); (c) reactive aldehydes arising as a consequence of lipid peroxidation, such as 4-hydroxy-2-nonenal (HNE) and malondialdehyde (MDA), which are shown to be increased in damage due to oxidative stress in HCV (2, 23); and (d) antioxidant molecules such as thioredoxin (TRX), a stress-inducible thiol-containing protein, also reported to be increased in serum of HCV patients with the progression of fibrosis. Individual studies have reported increased levels of 8-OHdG, HNE, MDA, and TRX in HCV infection (4, 9, 12, 21, 25). In this study, we evaluated the expression of all the above OS markers in biopsy specimens and sera in a group of HCV-infected patients.

MATERIALS AND METHODS

Study groups

This study was carried out in three steps. (a) The presence of oxidative stress markers, 8-OHdG, HNE, MDA, and TRX, was evaluated in biopsy specimens of 18 HCV patients, with

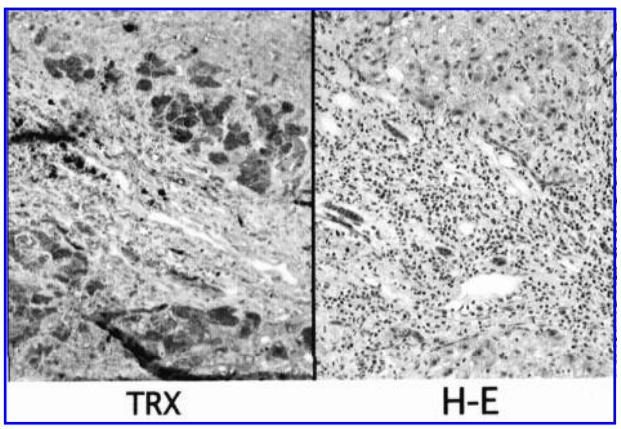

FIG. 1. HE staining of hepatic tissue and TRX staining in serial sections of liver tissue in HCV-associated liver disease.

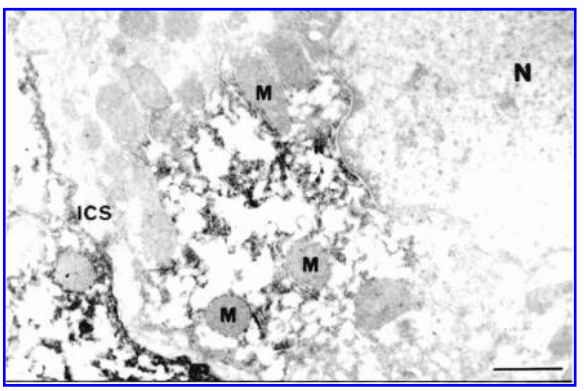

FIG. 2. The immunoelectron microscopic observation of hepatocytes with focal and diffuse TRX staining. M, mitochondria; n, nucleus; ICS, intercellular space. Bar = 1 μm.

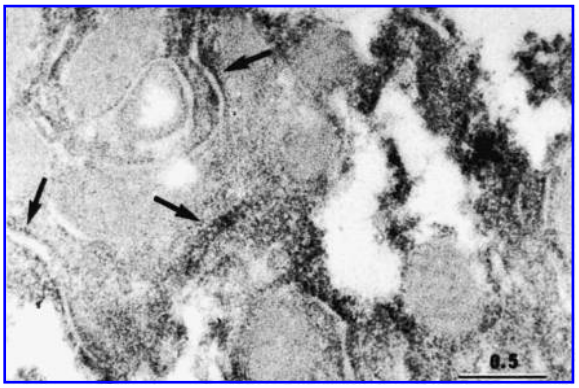

FIG. 3. Higher magnification immunoelectron microscopic observation of hepatocytes with TRX staining. Arrows indicate presence of TRX in ribosomes of rough endoplasmic reticulum. Bar = 0.5 μm.

fibrosis staging F1 (six), F2 (two), F3 (four), and F4 (six), (b) Interferon (IFN) was administered to 13 patients, and the IFN response and HCC occurrence, in relation to OS markers, were observed. (c) ROM in sera of 54 HCV patients and 23 controls were compared.

Liver samples

A total of 18 liver biopsy samples were used in this study. Each liver sample was divided into two sections: one for routine light microscopy and the other for immunohistochemical study. Liver specimens were obtained by needle biopsy for diagnostic purposes before treatment. Liver tissue obtained from HCC patients during operation were also used, for the presence of OS markers in HCC and non-HCC tissues. Informed consent was obtained from all patients for this study.

Histological evaluation

Hematoxylin–eosin (HE) staining and Azan–Mallory staining were performed for histological diagnosis of liver tissue. For cases of chronic hepatitis, liver specimens were scored for the stage of liver fibrosis and grade of inflammatory activity according to the Inuyama classification (7).

TABLE 2. DISTRIBUTION OF OS MARKERS IN NON-HCC AND HCC AREAS OF LIVER TISSUES OF HCV PATIENTS

	Non-HCC area			*HCC area*		
n = 4	*TRX*	*HNE*	*MDA*	*TRX*	*HNE*	*MDA*
Case 1	2+	1+	2+	2+	—	—
Case 2	2+	1+	1+	2+	1+	2+
Case 3	2+	2+	2+	2+	2+	2+
Case 4	2+	1+	1+	2+	1+	2+

Immunohistochemical detection of 8-OHdG/HNE/TRX/MDA

Tissue parts were fixed in a periodate lysine 2% paraformaldehyde (PLP) fixative for immunohistochemical studies (28). Cryostat sections were used for the detection of 8-OHdG, HNE, MDA, and TRX. The specimens were then reacted with purified mouse monoclonal antibody against 8-OHdG (Japanese Aging Control Institute, Shizuoka, Japan); rabbit anti-HNE antiserum (Alpha Diagnostics, Japan), rabbit anti-MDA antiserum (Alpha Diagnostics, Japan), and mouse anti-TRX antiserum (kindly supplied by Dr. Nakamura of Kyoto University, Japan), respectively, overnight. For the second antibody, we used goat anti-rabbit horseradish peroxidase (HRP) (dilution 1:400, from Dako) for HNE and MDA and goat anti-mouse HRP labeled Fab′ anti-mouse immunoglobulins (MBL) for 8-OHdG and TRX. The specificity of the monoclonal antibodies to 8-OHdG, HNE, MDA, and TRX, was confirmed by comparison with adjacent sections.

For immunoelectron microscopy, the sections were reacted with the antibodies in the same way as for light microscopy. They were fixed with 2% glutaraldehyde and reacted sequentially with diaminobenzidine containing hydrogen peroxide for 2 minutes and fixed with 1% osmium tetroxide for 30 min. They were then dehydrated in graded ethanols and embedded in epoxy resin and araldite resin. Ultrathin sections were examined without counterstaining under an electron microscope at magnifications of 5,000–30,000. Control sections were incubated with diluted normal, rabbit, or mouse serum or phosphate-buffered saline, instead of the rabbit antisera or mouse monoclonal antibodies.

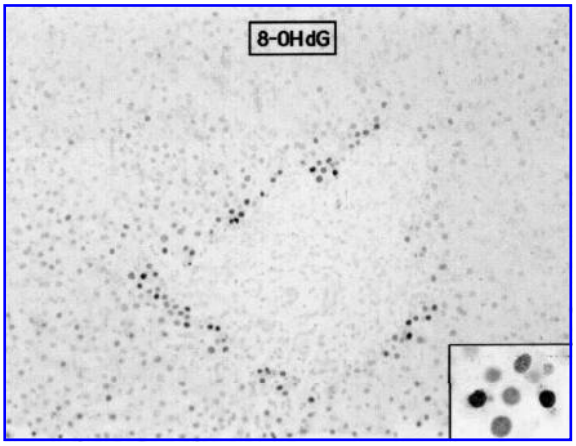

FIG. 4. The expression of 8-OHdG in CHC.

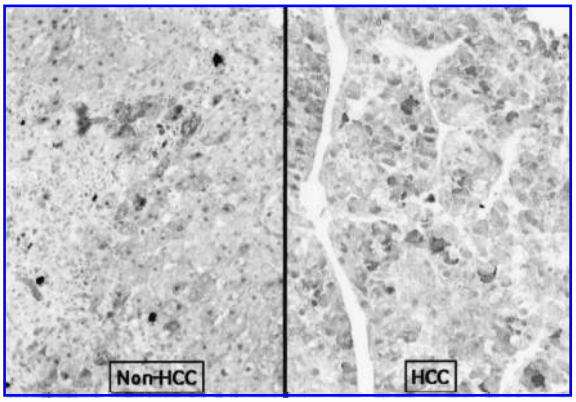

FIG. 5. Presence of TRX in HCC and non-HCC tissue sections of patients with HCC.

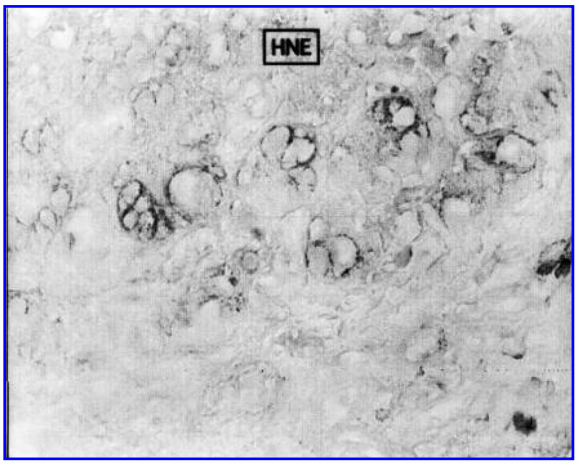

FIG. 6. Presence of OS marker (HNE) in liver tissue of HCV-infected patient with fat deposition.

Labeling index for 8-OHdG/HNE/TRX/MDA

The distributions of HNE-, TRX-, and MDA-positive hepatocytes and 8-OHdG-positive hepatocyte nuclei were determined in the following manner.

Liver sections in which most lobules had diffusely or partly positive staining were designated as 2+; sections where less than half of the lobules were positively stained were designated as 1+; and sections where throughout the specimen some hepatocytes were stained but were not diffuse or localized were designated as ±. Based upon the above detecting system, the extent of OS in liver biopsy specimens was evaluated.

Detection of ROM in sera

Serum samples of 54 HCV patients and 23 healthy controls were subjected to the d-ROMs test, to detect ROM in sera by a Free Radical Analytical System (Iram, Italy). The ROM concentration is measured in U Carr units.

TABLE 3. MULTIVARIATE ANLAYSIS SHOWING THE BEST PREDICTOR OF ROM INCREASE IN SERA OF HCV-ASSOCIATED LIVER DISEASE

Variable	*Odds ratio*	*95% CI*	*p value*
Age	2.75	0.4482–5.1214	0.0236*
Sex	0.32	0.6635–2.8657	0.091
ALT	0.98	0.0116–1.0917	0.296

CI, confidence interval.
*Statistically significant.

Biochemical determinations

The serum levels of alanine aminotransferase (ALT) and ferritin were measured in all patients prior to IFN therapy.

RESULTS

The clinical background of 18 HCV patients is given in Table 1. During the 4-year follow-up period, five patients developed HCC over an average period of 1.8 years. Four out of five HCC patients were found to be HCV genotype 1b, with average serum ALT levels of >80 IU/l and high serum ferritin levels, and nonresponder (NR) to IFN therapy. There were no HCC cases among sustained viral responder (SVR) patients. OS markers (TRX, HNE, MDA) were found in liver biopsy specimens of most patients, and their expression appeared to increase with the staging of fibrosis, although not significantly. Figure 1 shows the HE staining of hepatic tissue and TRX staining in serial sections. TRX was found to be clustered around portal areas with piecemeal necrosis. The immunoelectron mi-

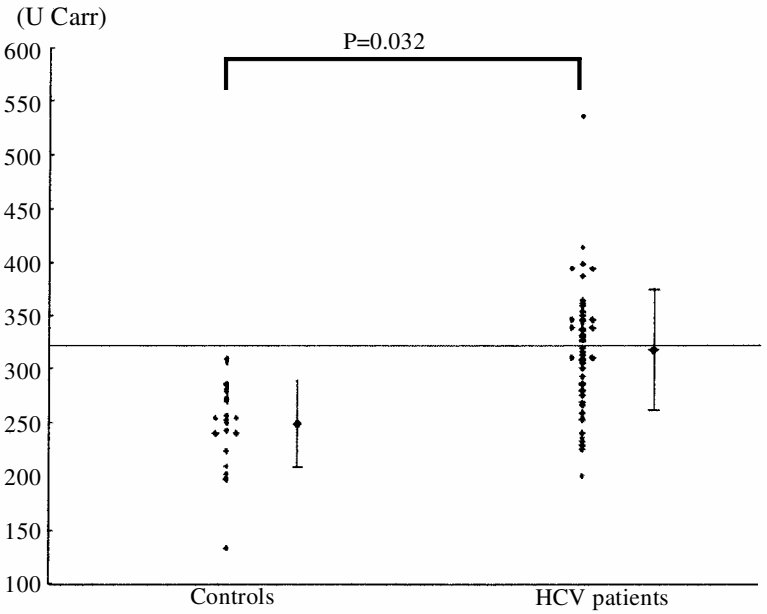

FIG. 7. Comparison of the presence of ROM in HCV patients and controls.

croscopic observation of hepatocytes with TRX expression is shown in Figs. 2 and 3. The reaction products of TRX were distributed focally in some hepatocytes and diffusely in others (Fig. 2) and localized in the cytosol and ribosomes of rough endoplasmic reticulum (Fig. 3). The expression of 8-OHdG was studied in eight patients with CHC. 8-OHdG was observed in the nuclei of hepatocytes in all cases, around the portal area (Fig. 4). Table 2 shows the extent of OS in liver tissue of four HCC cases. OS stress markers were present in both HCC and non-HCC tissues, but slightly strongly positive in HCC tissues. Figure 5 shows the presence of TRX in HCC and non-HCC tissue sections of HCC patients. Both HCC cells and non-HCC cells were positively stained. OS markers were found to be scattered in the cytoplasm of HCC cells and around the portal tracts in non-HCC cells. Figure 6 shows the localization of HNE in hepatocytes with fat deposition particularly near the periportal area. Figure 7 compares the presence of ROM in HCV patients and controls. The average ROM of HCV patients was 318 ± 56.7 Carr compared with 248 ± 40.8 Carr in controls (p = 0.032). Multivariate analysis of three variables significant on univariate analysis, with respect to increase in ROM (Table 3), found age to be the only independent predictor (p = 0.0236).

DISCUSSION

In all types of liver damage, there has been evidence of enhanced production of free radicals and/or significant decrease of antioxidant defense. The HCV core protein is said to cause mitochondrial injury leading to OS. OS disturbs lipid metabolism and causes other damage that leads to steatosis and sometimes apoptosis, the latter of which is a standard feature of viral hepatitis (14). ROS at the submicromolar level act as novel intra- and intercellular secondary messengers and modulate various aspects of cellular function, including proliferation, apoptosis, and gene expression (1). Reactive aldehydes arising as a consequence of lipid peroxidation have been reported to directly activate hepatic stellate cells (16, 26), transforming them into myoblasts, leading to hepatic fibrosis and cirrhosis. Lipid peroxidation products such as MDA and HNE in liver sections of HCV-associated patients indicate the presence of OS (22). TRX has been found to increase with the progression of fibrosis in HCV patients, and elevated TRX in sera and biopsy specimens of HCC patients has been documented (11, 18). In periportal areas with prominent lymphocytic infiltration, the presence of 8-OHdG damages hepatocytes leading to OS (21). Liver iron accumulation has been previously associated with HCV infection (5), and liver cell injury by ROM has also been reported.

In the present study, we used an immunohistochemical approach using monoclonal antibodies against various OS markers, to find a relationship between OS and HCV related liver damage. In our study, ROM were found to be significantly higher in HCV patients compared with controls and increased with age. An increase in ROM with age could indicate subsequent development of HCC in older patients, as endogenous OS occurs in mitochondria of hepatocytes and development of cancer increases in old cells, along with DNA damage. Older patients with HCV-associated liver disease need further attention regarding the presence of OS markers, for the prevention of HCC development. TRX immunostaining was found to be the strongest among all other OS markers studied, present in most lobules of hepatic tissue. MDA was found mainly in hepatocytes and very weakly stained, and not mainly in perisinusoidal cells as reported earlier. HNE was stained positively but weakly in periportal areas, not lobules. 8-OHdG stained the nucleus of hepatocytes surrounding the portal areas. The expression of OS markers in HCC was somewhat stronger compared with that in the non-HCC group, although not significant. The presence of OS markers in HCC tissue as well as in non-HCC tissue indicates that OS is closely related to the development of HCC and that non-HCC tissue under OS may become carcinomatous in the future. The importance of detection of OS markers and antioxidant therapy in HCV-associated liver disease is to slow down disease progression and HCC occurrence. Successful IFN therapy has been found to reverse enhanced hepatic iron accumulation and lipid peroxidation in CHC (19). Other antioxidant therapies, such as vitamin E therapy, has been found to reduce serum TRX levels and ALT levels in CHC patients (17). Early detection, continuous monitoring, and subsequent treatment of OS in HCV-related liver disease may prevent further liver damage and slow down or prevent HCC development.

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ABBREVIATIONS

ALT, alanine aminotransferase; CHC, chronic hepatitis C; F1–F4, fibrosis staging 1, 2, 3, 4; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HE, hematoxylin–eosin; HNE, 4-hydroxy-2-nonenal; HRP, horseradish peroxidase; IFN, interferon; MDA, malondialdehyde; NR, nonresponder; 8-OHdG, 8 hydroxydeoxyguanosine; OS, oxidative stress; ROM, reactive oxygen molecule; ROS, reactive oxygen species; SVR, sustained viral responder; TRX, thioredoxin.

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