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Comparison of hepatic oxidative DNA damage in patients with chronic hepatitis B and C

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SUMMARY. 8-Hydroxydeoxyguanosine (8-OHdG) is a promutagenic DNA lesion produced by hydroxyl radicals and is recognized as a useful marker in estimating DNA damage induced by oxidative stress. The aim of this study was to clarify the clinical significance of hepatic 8-OHdG levels in patients with chronic viral hepatitis. Hepatic 8-OHdG accumulation was investigated in patients with chronic hepatitis C (CH-C) (n = 77) and chronic hepatitis B (CH-B) (n = 34) by immunohistochemical staining of liver biopsy samples. 8-OHdG positive hepatocytes were significantly higher in patients with CH-C compared to CH-B (median 55.0 *vs* 18.8 cells/10^5 μm^2, $P < 0.0001$). The number of positive hepatocytes significantly increased with the elevation of serum aminotransferase levels, especially in CH-C patients (8-OHdG *vs* alanine aminotransferase (ALT)/aspartate aminotrasferase (AST) were r = 0.738/0.720 in CH-C and 0.506/0.515 in CH-B). 8-OHdG reactivity was strongly correlated with body and hepatic iron storage markers in CH-C (*vs* serum ferritin, r = 0.615; *vs* hepatic total iron score, r = 0.520; *vs* hepatic hepcidin mRNA levels, r = 0.571), although it was related to serum HBV-DNA titers (r = 0.540) and age of patients (r = −0.559) in CH-B. These results indicate that hepatic oxidative DNA damage is common in chronic viral hepatitis, in particular chronic HCV-infected patients, suggesting a possible link between chronic hepatic inflammation and hepatocarcinogenesis. The strong positive correlation between hepatic DNA damage and iron overload suggests that iron content is one of the most likely mediators of hepatic oxidative stress and iron reduction may be beneficial to reduce the incidence of hepatic cancer in CH-C patients.

Keywords: alanine aminotransferase/aspartate aminotrasferase; hepatitis B virus, hepatitis C virus, hepatocellular carcinoma, iron, oxidative stress.

INTRODUCTION

Reactive oxygen species (ROS) in living cells have been implicated in a number of pathologies, including aging, inflammatory diseases and the development of cancer, because they cause oxidative damage to nucleic acids, proteins, and lipids [1]. ROS include oxygen-centred radicals and non-radical compounds. Among the many radicals, the hydroxyl radical is the most reactive and is also responsible for the formation of 8-hydroxydeoxyguanosine (8-OHdG) [2,3]. 8-OHdG is known to induce G-C to T-A transversions during DNA replication [4]. Therefore, 8-OHdG is considered a useful marker for oxidatively generated DNA damage, leading to carcinogenesis [3].

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, and the death rate due to this tumour has been increasing over the past

Abbreviations: 8-OHdG, 8-hydroxydeoxyguanosine; CH-B, chronic hepatitis B; CH-C, chronic hepatitis C; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HIS, hepatic iron score; mRNA, messenger RNA; NO, nitric oxide; NOS2, nitric oxide synthase 2; NSAIDs, nonsteroidal anti-inflammatory drugs; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PIS, portal iron score; ROS, reactive oxygen species; SIS, sinusoidal iron score; TfR2, transferrin receptor 2; TIS, total iron score; TMA, transcription-mediated amplification.

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20–30 years in the Unites States [5] and in Japan [6]. It has been shown that chronic hepatitis C virus (HCV) and hepatitis B virus (HBV) infections are strong and independent risk factors associated with the likelihood of HCC [7,8], but the precise mechanism(s) of hepatocarcinogenesis during chronic viral hepatitis in humans are still largely unknown. Recently, there has been an increasing body of evidence suggesting that oxidative stress may play a pathogenic role in chronic hepatitis, especially in patients with chronic hepatitis C (CH-C). It has been demonstrated that plasma samples from HCV-infected patients have increased lipid peroxidation products [9], superoxide dismutase activity [10], and 8-OHdG level in circulating leukocyte DNA [11]. Immunohistochemistry has also documented the presence of oxidative stress formation in the livers of CH-C patients [9,12]. Involvement of oxidative stress in pathogenesis during chronic HCV infection is also supported by the fact that antioxidant therapy improved liver injury caused by HCV infection [11,13]. Despite these evidences, little is understood about the mechanisms of oxidative stress formation during chronic HCV infection.

In the case of chronic HBV infection, mechanisms of hepatocarcinogensis have been proposed by using several theoretical roles. Early studies suggested that the direct integration of the HBV genome into human chromosomes might cause inactivation of tumour suppressor/proto-oncogenes [14]. The HBV encoded X protein has been shown to initiate transactivation as well as induction of signal transduction pathways such as Ras/Raf-1 [15], and the large surface HBV protein has been shown to induce HCC in a transgenic mouse model [16]. But the pathogenic role(s) and clinical significance of oxidative stress formation in the livers of patients with chronic hepatitis B (CH-B) has not been sufficiently investigated.

To examine the potential role of hepatic oxidative stress formation for the pathogenesis of liver injury caused by chronic HCV and HBV infections, we compared 8-OHdG-positive hepatocyte count in liver biopsy specimens of CH-C and CH-B patients, and evaluated the association between hepatic oxidative DNA damage and demographic, biochemical and histological findings.

PATIENTS AND METHODS

Patients with CH-C and CH-B

A total of 111 consecutive non-selected patients with CH-C and CH-B who underwent needle liver biopsy at Mie University Hospital between March 1998 and September 2005 were enrolled in this study. About 77 of them had CH-C and included 45 males and 32 females, with a median age of 55.0 (range, 25–81) years. Diagnosis of chronic HCV infection was based on the consistent detection of serum anti-HCV antibody [the third-generation enzyme-linked immunosorbent assay (ELISA); Ortho Diagnostic Systems, Raritan, NJ, USA] and HCV-RNA (Amplicor-HCV assay; Roche Molecular Diag. Co., Tokyo, Japan). Serum HCV-RNA titre was quantified by the Amplicor monitor assay (Roche Molecular Diag. Co.). About 34 patients with CH-B [24 males and 10 females, with a median age of 54.0 (range, 24–75) years] who underwent liver biopsy during the same period were also recruited. All CH-B patients were positive for both HBsAg and anti-HBc [commercial enzyme immunoassay kits (EIA); Abbott Laboratories, North Chicago, IL, USA], and 22 patients (64.7%) were HBeAg (EIA; Abbott Laboratories) positive. Serum HBV-DNA titre was measured using the transcription-mediated amplification (TMA) assay (Mitsubishi Kagaku BML, Tokyo, Japan). Patients with other liver diseases (drug-induced, autoimmune and metabolic) were excluded by appropriate serological testing and clinical history. None of the patients were co-infected with both HBV and HCV, or received any antiviral or immunomodulatory treatment in the preceding 6 months of the study. Patients with concurrent diseases or those taking medications capable of interfering with free radical production, such as nonsteroidal anti-inflammatory drugs (NSAIDs), vitamins and iron-containing drugs were also excluded.

The following parameters were obtained form each patient at the time of liver biopsy: age; sex; body mass index; alcohol intake; biochemical, haematological, iron-related [serum iron, transferrin saturation (calculated and expressed as a percentage: serum iron/total iron binding capacity × 100%), and ferritin levels], and virological serum markers; liver histological findings; presence of hepatic 8-OHdG; and hepatic messenger RNA (mRNA) levels of transferrin receptor 2 (TfR2) and hepcidin. Informed consent was obtained from each patient included in the study and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a *priori* approval by the Ethical Committee of Mie University.

Histological evaluation

Liver biopsy specimens were immediately divided in to two parts, and one portion was fixed in 10% buffered formalin and embedded in paraffin for routine histological examination and the other was frozen and stored at −80 °C for RNA extraction. The former samples were stained with haematoxylin–eosin and Masson's trichrome, and were graded for the degree of necroinflammatory activity and staged for the extent of fibrosis using the criteria of Desmet *et al.* [17]. The histological quantification of hepatic iron using liver samples stained with Perls' Prussian blue was carried out according to Deugnier *et al.* [18] by scoring iron separately within hepatocytes [Hepatic Iron Score (HIS), 0–36], sinusoidal cells [Sinusoidal Iron Score (SIS), 0–12], and portal tracts [Portal Iron Score (PIS), 0–12]. The Total Iron Score (TIS, 0–60) represented the sum of these scores. This score has been

shown to correlate highly with the biochemical hepatic iron index and hepatic iron concentration measured by atomic absorption spectrophotometry in patients with chronic liver disease [19–21].

Immunohistochemical detection of 8-OHdG adducts in liver biopsy samples

Immunohistochemical staining of 8-OHdG was performed as previously described [22]. A mouse monoclonal antibody against 8-OHdG (Japanese Aging Control Institute, Shizuoka, Japan) and Alexa 488-labelled goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA) were used. The degree of immunoreactivity was estimated by counting the number of stained hepatocyte nuclei using Adobe Photoshop version 5.5 and NIH Image free software (Vers. 1.62, National Institute of Health, image program) [22]. The specificity of this anti-8-OHdG monoclonal antibody was confirmed by (i) comparison with adjacent sections in which the primary antibody was omitted, or (ii) using normal mouse serum instead of the primary antibody, or (iii) absorption with purified 8-OHdG (Sigma, Tokyo, Japan) or guanosine (Sigma), or (iv) RNA digestion. The primary antibody was incubated for 5 h at room temperature with serial dilutions of purified 8-OHdG or guanosine in phosphate buffered saline (PBS) ranging from 2.5 mg/mL through 2.5 ng/mL and applied to the sections. RNA digestion was performed before the immunostaining procedures in PBS containing DNase-free RNase (5 $\mu g/\mu L$) for 1 h at 37 °C.

TfR2 and hepcidin mRNA quantification in liver biopsy samples

Hepatic mRNAs of TfR2 and hepcidin were measured using the TaqMan real-time detection-polymerase chain reaction (PCR) assay (Applied Biosystems, Tokyo, Japan), as previously described [23,24]. mRNA was extracted from liver biopsy specimens using the SV-RNA Isolation System (Promega corporation, Madison, USA). Primer and probe sequences are shown in Table 1. The results for TfR2 and hepcidin mRNA were expressed as the amount relative to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA quantified simultaneously in each liver sample.

Statistical analysis

Results were expressed as median with range. Comparisons between groups were performed using the Mann–Whitney *U*-test or the Kruskal–Wallis test for continuous variables and the chi-square or Fisher's exact test for categorical data. Correlation coefficients between numerical variables were calculated as Spearman's rank test. All tests were two-tailed and P values < 0.05 were considered as statistically significant. Statistical analysis was performed using the commercially available software SPSS 11.5 software (SPSS Inc., Chicago, IL, USA).

Table 1 List of primers and probes used for real-time detection-PCR Assay

Primers and probes	Sequence
TfR2	
Forward primer	5' -TGGTGACTTTGGAAGCGTG-3'
Reverse primer	5'-CTGGTCTTGGCATGAAACTTG-3'
Probe	5-FAM-TAGTGTACGTGAGCCTGGA-CAACGCAGT-TAMRA-3'
Hepcidin	
Forward primer	5'-TTCCCCATCTGCATTTTCTG-3'
Reverse primer	5'-TCTACGTCTTGCAGCACATCC-3'
Probe	5'-FAM-TGCGGCTGCTGTCATCGAT-CAA-TAMRA-3'
GAPDH	
Forward primer	5'-GAAGGTGAAGGTCGGAGTC-3'
Reverse primer	5'-GAAGATGGTGATGGGATTC-3'
Probe	5'-FAM-CAAGCTTCCCGTTCTCAG-CC-TAMRA-3'

PCR, polymerase chain reaction; TfR2, transferrin receptor 2; FAM, 6-carboxyfluorecein; TAMRA, 6-carboxytetramethyl-rhodamine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

RESULTS

Comparison of clinical profiles of patients with CH-C and CH-B

Demographic and laboratory data and histological features of patients with CH-C and CH-B are shown in Table 2. There was no significant difference in the median age and gender distribution between the HCV and HBV groups. Laboratory data, except for serum ferritin, were not significantly different between the two groups. CH-C patients had significantly higher ferritin levels compared to CH-B [211.6 (range 13.2–792) *vs* 101.9 (9.1–322) ng/mL, $P = 0.0032$]. Among the 77 CH-C patients, serum ferritin levels were elevated above the normal range (> 220 ng/mL for male and > 100 ng/mL for female) in 45 cases (58.4%) in contrast to 11 cases (32.4%) from 34 CH-B patients, and this reached statistical significance ($P = 0.0198$). Liver histology showed no significant difference in grading and staging score between the CH-C and CH-B patients. Iron deposition in the liver was more prominent in CH-C patients; TIS was significantly higher in CH-C compared to CH-B patients [7.0 (0–22) *vs* 3.0 (0–16), $P = 0.0033$]. Hepatic TfR2 mRNA levels were significantly higher in CH-C patients than in CH-B [3740 (237–30 700) *vs* 2095 (66.7–72 100), $P = 0.0055$].

Table 2 Clinical characteristics of patients with chronic hepatitis C and B

Characteristics	Chronic hepatitis C (n = 77)	Chronic hepatitis B (n = 34)	P-Value
Age (years)	55 (25–81)	54 (24–75)	NS
Gender (M/F)	45/32	24/10	NS
Body mass index (kg/m^2)	24.0 (16.5–31.3)	24.0 (18.3–28.2)	NS
Laboratory data			
ALT (IU/L)	56 (19–411)	49 (11–561)	NS
AST (IU/L)	60 (19–565)	58.5 (18–702)	NS
Hyaluronic acid (ng/mL)	58.7 (9.0–649)	49.0 (11.0–324)	NS
Platelet count (× 10^4/mm^3)	14.9 (4.9–29.8)	14.3 (3.6–25.3)	NS
Red blood cell count (× 10^4/mm^3)	425 (234–565)	447.5 (280–566)	NS
Haemoglobin (g/dL)	13.7 (7.9–16.8)	13.8 (9.2–17.1)	NS
Serum iron (μg/dL)	128 (32–228)	128.5 (20–240)	NS
Transferrin saturation (%)	39.1 (8.5–85.4)	41.5 (6.1–85.4)	NS
Serum ferritin (ng/mL)	211.6 (13.2–792)	101.9 (9.1–322)	0.0032
Viral titre			
HCV-RNA (KIU/mL)	682 (10.4–5100)	–	
HBV-DNA (LGE/mL)	–	5.4 (<3.7–8.7)	
Liver histology			
Inflammatory activity (0/1/2/3)*	1/28/42/6	1/12/14/7	NS
Fibrosis staging (0/1/2/3/4)†	2/25/21/15/14	0/6/10/9/9	NS
Total iron score‡	7 (0–22)	3 (0–16)	0.0033
TfR2 mRNA (/GAPDH)	3740 (237–30 700)	2095 (66.7–72 100)	0.0055
Hepcidin mRNA (/GAPDH)	4500 (230–35 200)	3290 (250–41 000)	NS

Data are expressed as median (range).

*Inflammatory activity was graded according to the intensity of necroinflammatory lesions: 0, no histological activity; 1, mild activity; 2, moderate activity; 3, severe activity.

†Fibrosis staging was scored: 0, no fibrosis; 1, portal fibrosis without septa; 2, portal fibrosis with few septa; 3, numerous septa without cirrhosis; 4, cirrhosis.

‡Histological quantification of iron was assessed by total iron score proposed by Deugnier *et al* (1992).

ALT, alanine aminotransferase; AST, aspartate aminotransferase; TfR2, transferrin receptor 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

although the hepcidin levels were not significantly different between the two groups, as reported previously [23,24].

In situ detection of 8-OHdG positive hepatocytes using liver biopsy samples

Figures 1a,b show the 8-OHdG immunohistochemical staining in liver biopsy samples in patients with CH-C and CH-B. 8-OHdG immunoreactivity was strongly observed in the nuclei (and weekly in the cytoplasm) of hepatocytes, Kupffer cells and infiltrated inflammatory cells in CH-C liver biopsies (Fig. 1a). The hepatocyte nuclei were differentiated from the nuclei of other cells using computed analyses based on nuclear shape and size. 8-OHdG-immunoreactive hepatocytes were distributed throughout the whole acinus in the livers of patients. Using the liver samples of patients with CH-B, relatively faint immunoreactivity of 8-OHdG was observed in the nuclei of hepatocytes and was rarely in the cytoplasm (Fig. 1b). As a whole, 8-OHdG-positive hepatocyte counts were significantly higher in CH-C patients than in CH-B [55.0 (range 12–126) *vs* 18.8 (6.3–138) cells/10^5 μm^2, $P < 0.0001$] (Fig. 1c). Stratifying the patients according to the histological inflammatory grade, in the mild and moderate hepatitis subgroup, CH-C patients had significantly higher hepatic 8-OHdG levels than CH-B [in grade 0/1, 47.3 (12–126) *vs* 16.3 (6.3–36.7) cells/10^5 μm^2, $P < 0.0001$; in grade 2, 54.7 (21.3–121) *vs* 18.2 (6.7–39.3) cells/10^5 μm^2, $P < 0.0001$] (Fig. 1d).

The specificity of the anti-8-OHdG antibody used in this study was confirmed by several parallel experiments. Sections in which the primary antibody was omitted or those treated with normal control serum instead of the primary 8-OHdG antibody consistently yielded negative staining. Localization of 8-OHdG was considered specific because the recognition of hepatocytes was completely blocked by previous incubation with 25 ng/mL of 8-OHdG, but not by

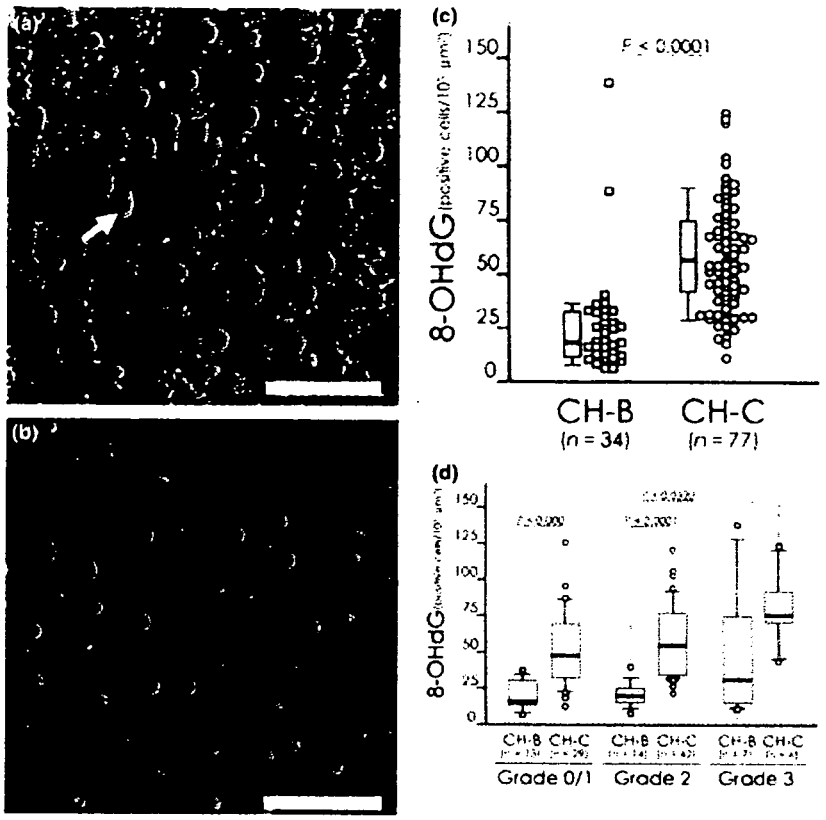

Fig. 1 Representative 8-hydroxydeoxyguanosine (8-OHdG) immunohistochemical staining in liver tissues from patients with chronic hepatitis C (CH-C) (a) and chronic hepatitis B (CH-B) (b). In the liver of CH-C patient, 8-OHdG immunoreactivity was strongly observed throughout the whole acinus and mainly in the nuclei of hepatocytes and Kupffer cells [arrow in (a)]. In the liver of CH-B patient, the relatively faint immunoreactivity of 8-OHdG was observed in the nuclei of hepatocytes and rarely in the cytoplasm. Scale bar 100 μm. (c) Comparison between 8-OHdG-positive hepatocytic nuclear count in patients with CH-C and CH-B. Positive cells were significantly higher in CH-C patients than in CH-B. Box and whisker graphs depict the median (line within the box), 25–75 percentiles (upper and lower border of the box), and 10–90 percentiles (whiskers). Open circles and squares refer to patients with CH-C and CH-B respectively. (d) 8-OHdG-positive hepatocytic counts were stratified according to histological grading score in patients with CH-C and CH-B. Box and whisker graphs depict the median (line within the box), 25–75 percentiles (upper and lower border of the box), and 10–90 percentiles (whiskers), with outliners plotted individually.

over a thousand-fold greater concentration of guanosine. Further, enzymatic treatment with RNase did not affect the immunoreaction of oxidized DNA.

Clinical variables that correlate with hepatic oxidative DNA damage in patients with CH-C

To estimate the source of oxidative-generated DNA damage frequently occurring in the livers of patients with CH-C, the correlation of clinical and histological findings with the degree of hepatic damaged DNA was evaluated, and the results are summarized in Table 3. The age of patients was not related to the degree of hepatic oxidative DNA damage in CH-C patients. Serum transaminase levels were significantly correlated with hepatic 8-OHdG levels in patients with CH-C (8-OHdG *vs* ALT, $r = 0.738$, $P < 0.0001$; *vs* AST, $r = 0.720$, $P < 0.0001$) (Table 3; Figs 2a,b). Hepatic 8-OHdG was significantly higher in a subgroup of CH-C patients with histologically advanced to severe hepatitis (grade 3) than in those with mild hepatitis (grade 0/1) [74.5 (43–123) *vs* 47.3 (12–126) cells/10^5 μm^2, $P = 0.0320$] (Fig. 1d). It is noteworthy that the hepatic 8-OHdG levels were strongly and positively correlated with body and hepatic iron deposition markers in patients with CH-C; serum ferritin levels and the hepatic iron deposit grade, i.e. TIS, were strongly correlated with 8-OHdG-positive hepatocyte nuclei (8-OHdG *vs* ferritin, $r = 0.615$, $P < 0.0001$; *vs* TIS, $r = 0.520$, $P < 0.0001$) (Table 3; Figs 2c,d). Hepcidin, that is exclusively synthesized in the liver, was recently identified as a key regulatory hormone of iron homeostasis, and is reported to be up-regulated in response to iron overload [24–26]. Therefore, we evaluated the relation of 8-OHdG levels with

Table 3 Correlations between clinical findings and hepatic 8-OHdG expression levels in patients with chronic hepatitis C and B

	Chronic hepatitis C (n = 77)		Chronic hepatitis B (n = 34)	
Characteristics	r	P Values	r	P Values
Age (years)	0.140	0.2212	**-0.559**	**0.0013**
Body mass index (kg/m^2)	0.265	0.0209	−0.291	0.0944
Laboratory data				
ALT (IU/L)	**0.738**	**<0.0001**	**0.506**	**0.0037**
AST (IU/L)	**0.720**	**<0.0001**	**0.515**	**0.0031**
T-Bilirubin (mg/dL)	0.351	0.0022	0.050	0.7717
T-Cholesterol (mg/dL)	−0.029	0.7994	0.227	0.1924
Hyaluronic acid (ng/mL)	0.226	0.0487	−0.253	0.1453
Platelet count (× 10^4/mm^3)	−0.266	0.0205	0.302	0.0825
Red blood cell count (× 10^4/mm^3)	0.027	0.8147	−0.032	0.8544
Haemoglobin (g/dL)	0.125	0.2768	0.033	0.8516
Serum iron (μg/dL)	0.270	0.0185	−0.078	0.6549
Transferrin saturation (%)	0.318	0.0056	−0.075	0.6664
Serum ferritin (ng/mL)	**0.615**	**<0.0001**	0.064	0.6928
Viral litre				
HCV-RNA (KIU/mL)	−0.024	0.8324	-	
HBV-DNA (LGE/mL)	-		**0.540**	**0.0019**
Liver histology				
Total iron score*	**0.520**	**<0.0001**	−0.126	0.4079
mRNA (/GAPDH)				
TfR2	−0.258	0.0243	−0.167	0.3457
Hepcidin	**0.571**	**<0.0001**	0.070	0.6883

*Histological quantification of iron was assessed by total iron score proposed by Deugnier *et al* (1992).
8-OHdG, 8-hydroxyguanosine; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TfR2, transferrin receptor 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
Bold values indicate that correlations between these two variables are very close ($r > 0.5$).

hepatic hepcidin mRNA expression levels, as a marker for iron overload. A statistically significant positive correlation was also observed between 8-OHdG levels and hepcidin mRNA levels in the liver of patients with CH-C ($r = 0.571$, $P < 0.0001$) (Table 3; Fig. 2e).

Clinical variables that correlate with hepatic oxidative DNA damage in patients with CH-B

The correlation of clinical and histological findings with the degree of hepatic oxidative DNA damage was also evaluated in CH-B patients. Serum transaminase levels were significantly correlated with hepatic 8-OHdG count in CH-B patients (*vs* ALT, $r = 0.506$, $P = 0.0037$; *vs* AST, $r = 0.515$, $P = 0.0031$) (Table 3; Figs 3a,b), although the statistical coefficients of Spearman were smaller compared to those of CH-C. Hepatic 8-OHdG was increased in accordance with the progression of hepatic histological inflammatory grading in CH-B patients, but these differences did not reach statistical significance (Fig. 1d). Unlike cases of HCV infection, hepatic 8-OHdG levels were not related to body and hepatic iron deposition markers (serum ferritin, TIS, hepcidin mRNA levels), but were significantly correlated with individual serum HBV-DNA titres and age of the patients (Table 3; Fig. 3c,d).

DISCUSSION

Especially in the case of HCV infection, underlining mechanisms by which hepatitis viruses cause liver cell injury are largely unknown. Oxidative stress is one of the most probable mediators, because patients with chronic HCV infection show an increase in serum or liver content of oxidative stress markers, such as lipid peroxidation products [20], superoxide dismutase [10], and 8-isoprostane [27]. In this study, using specific and sensitive immunohistochemical staining with anti-8-OHdG antibodies, 8-OHdG positive signals in the liver tissue were detected in all patients examined, indicating that oxidatively generated DNA damage occurs frequently in the liver of patients with chronic HCV and HBV infections. The relatively high 8-OHdG expression levels in our study compared to previous reports [28,29] are most likely related to differences in methodology. Variations in 8-OHdG levels have been

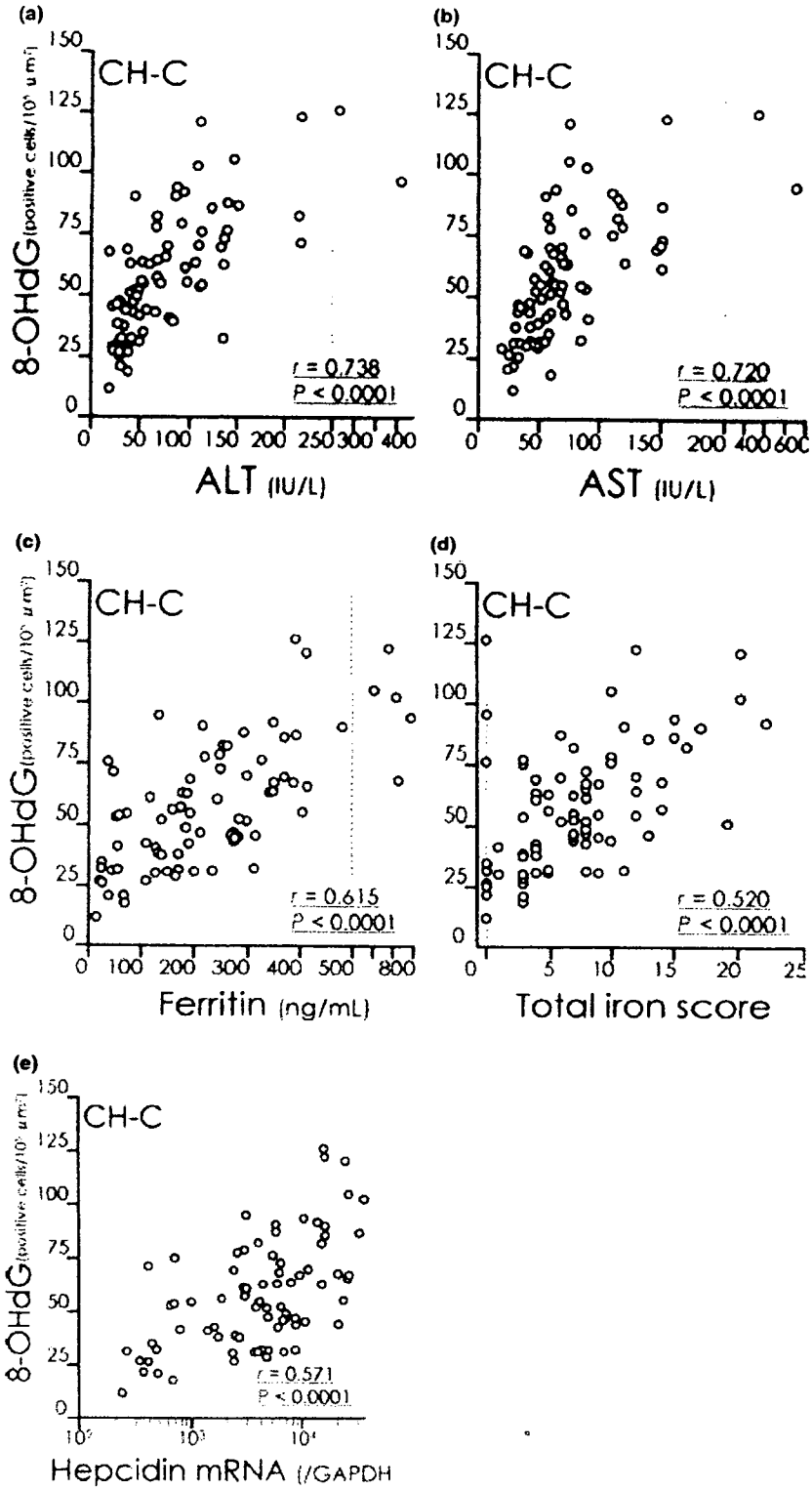

Fig. 2 Correlation between the 8-hydroxydeoxyguanosine (8-OHdG)-positive hepatocytic nuclear count and clinical variables in 77 patients with chronic hepatitis C. (a) 8-OHdG count and serum ALT levels. (b) 8-OHdG count and serum AST levels. (c) 8-OHdG count and serum ferritin levels. (d) 8-OHdG count and total iron score in hepatic tissue. (e) 8-OHdG count and hepcidin messenger RNA levels in hepatic tissue.

reported depending on DNA extraction procedures and detection methods used [30]. Therefore, a comparison of absolute 8-OHdG values between different studies can be done with caution.

The first conclusion of our study is that quantitative hepatocytic 8-OHdG immunoreactivity was significantly correlated with serum transaminase levels, the representative serum marker of hepatic inflammation, both in CH-C

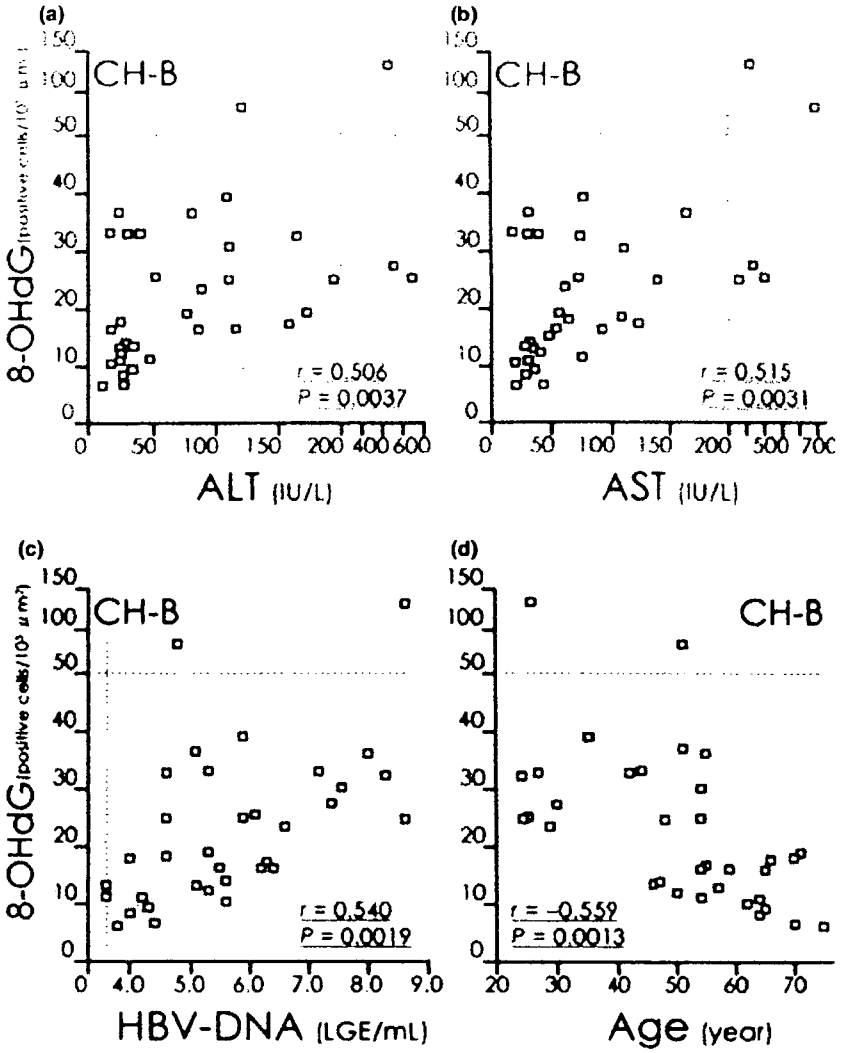

Fig. 3 Correlation between 8-hydroxydeoxyguanosine (8-OHdG)-positive hepatocytic nuclear count and clinical variables in 34 patients with chronic hepatitis B. (a) 8-OHdG count and serum ALT levels. (b) 8-OHdG count and serum AST levels. (c) 8-OHdG count and serum HBV-DNA titers. (d) 8-OHdG count and age of patients.

and CH-B. In the case of HCV, these results are in accordance with previous reports showing that plasma levels of lipid peroxidation products are correlated with aminotransferase levels in CH-C patients [31]. Shimoda *et al.* [28] also established a positive correlation between 8-OHdG concentration of extracted DNA from CH-C liver biopsy specimens and serum ALT activity. A possible link between the degree of hepatic oxidative DNA damage and hepatic inflammation may indicate the direct involvement of hepatic oxidative stress in the pathogenesis and progression of liver cell injury in chronic viral hepatitis. Certainly, it is unclear whether the oxidative stress is the cause or the consequence of liver cell injury from our results, but whichever is correct, hepatic DNA damage could be the cause of hepatocarcinogenesis which is frequently observed during chronic HCV and HBV infection, as 8-OHdG is a promutagenic lesion as it mispairs during DNA replication [4]. Therefore, it is conceivable that the treatment of chronic liver disease to suppress hepatic inflammation may have a preventive benefit for HCC development in chronic hepatitis patients. In fact, clinical studies have suggested that continuous hepatitis with high transaminase activity after curable resection of HCC is an independent risk factor for new development of HCC [32], and that treatment of chronic active hepatitis C with interferon may diminish the incidence of HCC in patients who normalized transaminases, even when viraemia is persistent [33].

Our second major conclusion is that the 8-OHdG-positive hepatocytic count in HCV-infected livers was significantly higher than in HBV-infected ones, suggesting that HCV infection may cause a more advanced oxidative stress in the liver during chronic infection. Because the baseline characteristics including serum aminotransferase levels and histological grading of inflammation were not significantly different between CH-C and CH-B, the difference in the hepatic inflammation status does not seem to be responsible for the abundant hepatic oxidative stress in CH-C patients. Stratifying the patients with histological grade, CH-C

patients in the subgroup with mild to moderate inflammatory activities (grading scores 0/1 and 2) also had significantly higher 8-OHdG levels as compared to CH-B with the same inflammatory activities, indicating that HCV infection itself has a more direct influence on hepatic oxidative stress formation. Most plausible candidate for the source of hepatic oxidative stress formation in CH-C is excessive deposition of iron in the liver, that is frequently implicated in chronic HCV infection [20,21,34], because the serum ferritin levels, that reflect the iron burden in the body of chronic liver disease patients [35], and TIS, the marker of hepatic iron deposition, were significantly higher in CH-C patients than in CH-B. The hepatic 8-OHdG levels were significantly correlated with the ferritin levels and TIS scores, also indicating a strong relationship between the oxidative DNA damage and iron overload status in the liver of patients with CH-C. It is known that free iron promotes generation of oxygen radicals by catalysing the Fenton reaction in which Fe^{2+} reacts with H_2O_2 to generate highly reactive hydroxyl radicals. Therefore, it is plausible that ROS production during chronic HCV infection is the effect of high iron levels in hepatic tissues in CH-C patients, which lead to progressive liver inflammation, and increased risk for developing liver cancer. The mechanisms involved in such a modification of iron metabolism associated with chronic HCV infection remain unclear. We have reported previously that the hepatic TfR2 mRNA expression levels were remarkably increased in CH-C patients [23] and the results were confirmed in this report using another study population. Therefore, hepatic iron overload may be caused by this upregulation of hepatic TfR2 expression in the liver of HCV infected patients. Recently, hepcidin, that is exclusively synthesized in the liver, was identified as a key regulator of body iron balance [25,26]. It has been clearly demonstrated that hepatic hepcidin expression is immediately and strictly regulated in response to hepatic iron levels [25]. In this study, a significant correlation was observed between hepatic 8-OHdG count and hepatic hepcidin expression levels in CH-C patients, also suggesting direct involvement of iron overload in hepatic oxidative stress formation, although the hepcidin expression levels were not significantly different between HCV- and HBV-infected patients. Further studies are necessary to identify the molecular mechanism responsible for iron overload that is frequently seen in CH-C patients.

Hepatic oxidative stress also seems to be concerned with hepatic inflammation in the case of chronic HBV infection, because the serum transaminase levels were significantly correlated with the hepatic 8-OHdG count among the CH-B patients. However, serum ferritin and TIS were not correlated to hepatic 8-OHdG levels in CH-B, therefore the involvement of iron overload for hepatic oxidative stress formation is relatively weak or none in chronic HBV infection. On the other hand, hepatic 8-OHdG levels were positively correlated with serum HBV-DNA titers. Majano *et al.* [36] demonstrated that the HBV genome could directly upregulate nitric oxide synthase 2 (NOS2) gene expression in cultured human hepatocyte-derived cells. The NOS2 gene generated long-term over-production of nitric oxide (NO), resulting in an increase in NO-related DNA damage [37]. Therefore, such a mechanism by which HBV directly induces hepatocarcinogenesis may be involved in the formation of hepatocytic DNA damage in chronic HBV-infected patients. The correlation of hepatic 8-OHdG count with age may be on account of the correlation between age and HBV-DNA titres in these patients ($r = -0.551$, $P = 0.0015$).

In summary, we have demonstrated that oxidative DNA damage widely occurs in the livers of patients with chronic viral hepatitis especially in CH-C, and its relation to serum trasaminase levels indicates that hepatic oxidative stress is one of the mechanisms fuelling necro-inflammatory changes in chronic viral hepatitis. As a matter of fact, it should be noted that 8-OHdG represents an ongoing mutagenic phenomenon even in liver cirrhosis where high transaminase levels are unusual. Thus, hepatic 8-OHdG levels may be the most potent predictive marker for hepatocarcinogenesis during chronic HCV and HBV infection. The strong positive correlations between hepatic oxidative DNA damage and iron overload in CH-C suggest that hepatic iron content is one of the most probable mediators of hepatic oxidative stress in HCV infection, and iron reduction therapy may be beneficial in reducing HCC incidence in CH-C patients.

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