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Iron Overload Is Associated with Hepatic Oxidative Damage to DNA in Nonalcoholic Steatohepatitis

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Abstract

Several lines of evidence have suggested that oxidative stress plays an important role for the pathogenesis of nonalcoholic steatohepatitis (NASH). Therefore, by using immunohistochemical staining of liver biopsy samples, we measured hepatic 7,8-dihydro-8-oxo-2′ deoxyguanosine (8-oxodG), a DNA base-modified product generated by hydroxyl radicals, of 38 NASH patients and compared with 24 simple steatosis and 10 healthy subjects. Relation of hepatic 8-oxodG with clinical, biochemical, and histologic variables and changes after iron reduction therapy (phlebotomy plus iron–restricted diet) were also examined. Hepatic 8-oxodG levels were significantly higher in NASH compared with simple steatosis (17.5 versus 2.0 8-oxodG–positive cells/10^5 μm^2; $P < 0.0001$). 8-oxodG was significantly related to iron overload condition, glucose-insulin metabolic abnormality, and severities of hepatic steatosis in NASH patients. Logistic regression analysis also showed that hepatic iron deposit and insulin resistance were independent variables associated with elevated hepatic 8-oxodG. After the iron reduction therapy, hepatic 8-oxodG levels were significantly decreased (from 20.7 to 13.8 positive cells/10^5 μm^2; $P < 0.01$) with concomitant reductions of serum transaminase levels in NASH patients. In conclusion, iron overload may play an important role in the pathogenesis of NASH by generating oxidative DNA damage and iron reduction therapy may reduce hepatocellular carcinoma incidence in patients with NASH. (Cancer Epidemiol Biomarkers Prev 2009;18(2):424–32)

Introduction

Nonalcoholic fatty liver disease, the leading cause of liver disease in Western countries, includes a spectrum of clinical entities ranging from pure fatty liver to nonalcoholic steatohepatitis (NASH; ref. 1). Simple steatosis is usually considered benign, but the development of NASH is recognized as a precursor to more severe liver disease and sometimes evolves into cryptogenic cirrhosis and hepatocellular carcinoma (2). A commonly accepted model for the pathogenesis of NASH is the so-called "two-hit" hypothesis, wherein the "first hit" leads to accumulation of hepatic free fatty acids resulting in a histologic picture of macrovesicular steatosis (3). Several lines of evidence have suggested that oxidative stress may play an important role for the pathogenesis of NASH as the "second hit" (4-6), but little is understood about the molecular mechanisms of its formation in the liver of NASH and involvement of hepatocarcinogenesis. One convincing candidate for the source of oxidative stress is excessive accumulated iron in the liver of patients with NASH because mild to moderate iron overload in the liver is common in NASH (7-9). It is known that ferrous iron in the presence of hydrogen peroxide generates hydroxyl radical through the Fenton reaction (10). In the representative iron-related liver injury disorder, genetic hemochromatosis, it is clearly shown that hepatic iron is responsible for liver damage through reactive oxygen species formation leading to lipid peroxidation and accumulated oxidative stress, which causes hepatic cancer (11). It is therefore plausible that hepatic iron overload may contribute to oxidative stress formation among patients with NASH.

7,8-Dihydro-8-oxo-2′ deoxyguanosine (8-oxodG) is a modification of guanine that induces a point mutation in the daughter DNA strands (12) and it is used as a marker of oxidatively generated DNA damage in several diseases (13). Therefore, we examined 8-oxodG levels in the liver of NASH patients, compared with those of simple steatosis, and evaluated its relation with clinical, biochemical, and histologic findings. Changes of hepatic 8-oxodG levels after iron reduction therapy were also investigated in NASH patients with hyperferritinemia.

Materials and Methods

Patients. A total of 38 NASH and 24 simple steatosis patients who underwent needle liver biopsy at Mie University Hospital between March 2003 and December 2006 were enrolled in the study (Table 1). In addition, 10 liver specimens from HBV/HCV-negative and normal liver function patients (6 males and 4 females; median age, 59 y; range, 41-70 y) were obtained during hepatobiliary surgery for either resection of hemangioma or benign tumors, and their histologically

Table 1. Clinical characteristics of patients with NASH and simple steatosis

Characteristics	NASH ($n = 38$)	Simple steatosis ($n = 24$)	P
Age (y)	59.0 (29-78)	51.0 (19-73)	NS
Gender (M/F)	22/16	11/13	NS
BMI (kg/m^2)	25.6 (22.5-36.7)	24.7 (16.3-35.1)	NS
Obesity	23 (60.5%)	11 (45.8%)	NS
Type II diabetes, n (%)	18 (47.4%)	8 (33.3%)	NS
Hypertension, n (%)	14 (36.8%)	9 (37.5%)	NS
Hyperlipidemia, n (%)	25 (65.8%)	15 (62.5%)	NS
Laboratory data			
ALT (IU/L)	63.0 (23-171)	59.0 (12-863)	NS
AST (IU/L)	58.0 (27-134)	37.0 (17-443)	0.0047
Total cholesterol (mg/dL)	201 (151-358)	216 (155-276)	NS
Triglyceride (mg/dL)	155 (63-443)	125 (73-261)	NS
Glucose (mg/dL)	102 (71-241)	99 (73-427)	NS
Serum insulin (microunits/mL)	12.1 (2.4-34)	9.2 (1.0-18)	0.0083
HOMA-IR	3.48 (1.51-9.56)	2.21 (1.05-9.24)	0.0010
Hyaluronic acid (ng/mL)	66.5 (5-365)	19.6 (6-258)	0.0004
Platelet count ($\times 10^4$/mm^3)	18.0 (4.9-37.0)	23.0 (13.1-45.2)	0.0146
RBC count ($\times 10^4$/mm^3)	448 (274-633)	461 (367-558)	NS
Hemoglobin (g/dL)	14.3 (8.3-18.9)	14.7 (11.5-18.9)	NS
Serum iron (μg/dL)	126 (88-220)	93 (25-188)	0.0059
Transferrin saturation (%)	38.0 (22.3-87.6)	32.4 (9.4-43.8)	0.0152
Serum ferritin (ng/mL)	283 (69-847)	139 (18-640)	<0.0001
Liver histology			
Inflammatory activity (1/2/3)*	14/21/3	—	—
Fibrosis staging (1/2/3/4)*	8/17/11/2	—	—
Steatosis (%) [†]	43 (15-86)	51 (28-90)	NS
TIS [‡]	3 (0-8)	0 (0-7)	<0.0001

NOTE: Results are presented as numbers (percentages) for qualitative data and as medians (ranges) for quantitative data.
Abbreviation: NS, not significant.
*Inflammatory activity and fibrosis staging in NASH was scored according to Brunt classification (16).
[†] Hepatic steatosis degree was assessed based on the percentage of affected hepatocytes.
[‡] The histologic quantification of iron was assessed by TIS proposed by Deugnier et al. (17).

normal liver tissue surrounding the resected lesion was used as a control. A diagnosis of NASH was established if a combination of the following clinical and histopathologic features was present: (*a*) a persistent abnormal liver biochemistry for >3 mo; (*b*) a liver biopsy showing steatosis (>10%) in the presence of lobular and/or portal inflammation, with or without Mallory bodies or fibrosis; and (*c*) the exclusion of other liver diseases, such as viral hepatitis, autoimmune hepatitis, drug-induced liver disease, primary biliary cirrhosis, biliary obstruction, hemochromatosis, Wilson's disease, and α-1-antitrypsin deficiency–associated liver disease. Patients consuming >20 g of alcohol per day were excluded from the study. None of the patients had ingested drugs known to produce hepatic steatosis (including corticosteroids, high-dose estrogens, methotrexate, tetracycline, calcium channel blockers, or amiodarone) or those capable of interfering with free radical production (nonsteroidal anti-inflammatory drugs, vitamins, and iron-containing drugs) in the previous 6 mo. One patient with NASH had a history of gastrointestinal surgery. Simple steatosis was also diagnosed by liver biopsy. Obesity was defined as a body mass index (BMI) of >25 kg/m^2, according to the criteria of the Japan Society for the Study of Obesity (14). Patients were assigned a diagnosis of diabetes mellitus if a documented use of oral hypoglycemic medication or insulin, a random glucose level in excess of 200 mg/dL, or a fasting glucose of >126 mg/dL on at least two occasions was present (15). Hyperlipidemia was diagnosed if the cholesterol level was higher than

220 mg/dL and/or the triglyceride level was over 160 mg/dL. Hypertension was diagnosed if the patients were on antihypertensive medication and/or had a resting recumbent blood pressure of ≥140/90 mmHg on at least two occasions. Serum biochemical, hematologic, and iron-related markers were obtained from medical and laboratory records closest to the dates of liver biopsies. Informed consent was obtained from each patient and the study was approved by the Ethical Committee of Mie University. The study was carried out according to the ethical guidelines of the 1975 Declaration of Helsinki.

Histologic Evaluation. Biopsy specimens were fixed in buffered formalin and embedded in paraffin. Sections were stained with H&E for morphologic evaluation, Masson's trichrome for assessment of fibrosis, and Perls' Prussian blue stain for assessment of iron loading. The histologic findings of NASH were interpreted and scored according to the classification proposed by Brunt et al. (16). The activity of hepatitis (necroinflammatory grade) was determined by the presence of hepatocellular steatosis, ballooning, and inflammation (acinar and portal) features as follows: grade 1, mild; grade 2, moderate; and grade 3, severe. The severity of hepatic fibrosis (stage) was defined as follows: stage 1, zone 3 perisinusoidal fibrosis; stage 2, zone 3 perisinusoidal fibrosis with portal fibrosis; stage 3, zone 3 perisinusoidal fibrosis and portal fibrosis with bridging fibrosis; and stage 4, cirrhosis. Macrovesicular steatosis was quantified as the percentage of hepatocytes that

contained fat droplets. The histologic quantification of hepatic iron was done according to Deugnier et al. (17) by scoring iron separately within hepatocytes (hepatic iron score, 0-36), sinusoidal cells (sinusoidal iron score, 0-12), and portal tracts or fibrotic tissue (portal iron score, 0-12). The total iron score (TIS, 0-60) was defined by the sum of these scores. This score has been shown to highly correlate with the biochemical hepatic iron index and hepatic iron concentration as measured by the atomic absorption spectrophotometry in patients with chronic liver diseases (18-20). All histologic grading and staging were done by a single pathologist without knowledge of the patients' clinical and laboratory data.

Immunohistochemical Detection of 8-oxodG Adducts in Liver Biopsy Samples. Immunohistochemical staining of 8-oxodG was done as previously described (21). Mouse monoclonal antibody against 8-oxodG (Japan Institute for the Control of Aging, Shizuoka, Japan) and Alexa Fluor 488–labeled goat antibody against mouse IgG (Molecular Probes) 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 (version 1.62, NIH, Image program; ref. 21).

The specificity of the anti-8-oxodG 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-oxodG antibody consistently yielded negative staining. Localization of 8-oxodG was considered specific because the recognition of hepatocytes was completely blocked by previous incubation with 25 ng/mL of 8-oxodG but not by over a thousand-fold greater concentration of guanosine. When the primary antibody was preincubated with graded

8-oxodG competitively, a similar blocking of immunolabeling was obtained. Further, enzymatic treatment with RNase did not affect the immunoreactivity toward oxidized DNA.

Iron Reduction Therapy for NASH. To evaluate the clinical effects of iron reduction for NASH, 11 NASH patients with iron overload [serum ferritin levels were elevated above the reference range (>300 ng/mL for male and >200 ng/mL for female)] underwent iron reduction therapy and the changes of serum and histologic features were analyzed. We selected patients that fulfilled the following criteria for iron reduction: no complication with hypertension and/or cardiovascular disorder, <70 y, and their histology showed without cirrhosis and TIS is not score 0. Iron depletion was accomplished by doing intermittent phlebotomies in combination with regulation of dietary iron intake as described previously (22). In brief, at the initial phase of iron depletion, all patients underwent weekly or biweekly phlebotomy of 200 g until a state of mild iron deficiency was achieved (defined by a serum ferritin levels <50 ng/mL and/or a blood hemoglobin concentration of 12 g/dL). The mild iron deficiency state was maintained by additional phlebotomies during the study period: patients were followed up every 1 to 2 mo for the duration, and a phlebotomy was done if the serum ferritin level exceeded 80 ng/mL. In addition, those subjects were instructed both orally and in writing by a registered dietitian to reduce their intake of iron-rich foods during the intervention. The subjects were not required to alter their total caloric intake but were expected to replace iron-rich foods with appropriate substitutes.

Statistical Analysis. Results are presented as the medians and ranges for quantitative data or as numbers with percentages in parentheses for qualitative data. Demographic and baseline data were compared
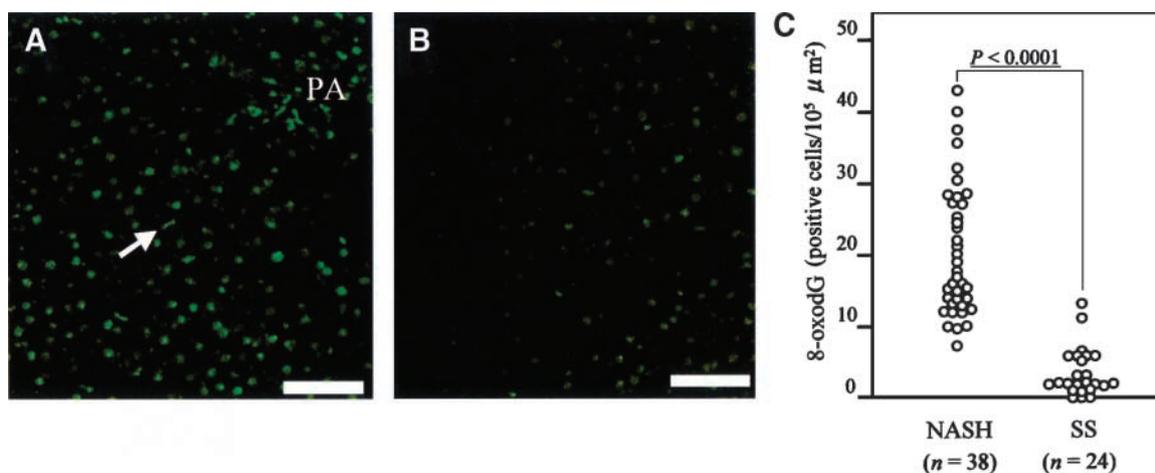

Figure 1. **A** and **B**. Representative 8-oxodG immunohistochemical staining in liver tissues from patients with NASH (**A**) and simple steatosis (**B**). In the liver of NASH, 8-oxodG immunoreactivity was strongly observed at the nuclei of many hepatocytes and several Kupffer cells (*arrow*) throughout the whole acinus. *PA,* portal area. In the liver of simple steatosis, relatively faint immunoreactivity of 8-oxodG was observed in the nuclei of hepatocytes and rarely in the cytoplasm. *Scale bar,* 100 μm. **C.** Comparison between 8-oxodG–positive hepatocytic nuclear counts of patients with NASH and those of simple steatosis (*SS*). Positive cells were significantly higher in NASH patients than in simple steatosis. ○, individual data of patients.

Table 2. Correlations between clinical findings and 8-oxodG levels in the liver of patients with NASH (n = 38)

Characteristics	8-oxodG	Statistics	
	(positive cells/10^5 μm^2)	r	P
Age (y)		0.048*	NS*
Gender			
Male (n = 22)	20.7 (10.0-43.3) [†]		NS [‡]
Female (n = 16)	15.4 (7.3-35.7) [†]		
BMI (kg/m^2)		−0.057*	NS*
Laboratory data			
ALT (IU/L)		−0.012*	NS*
AST (IU/L)		0.068*	NS*
Total cholesterol (mg/dL)		−0.258*	NS*
Triglyceride (mg/dL)		−0.050*	NS*
Glucose (mg/dL)		0.628*	0.0001*
Serum insulin (microunits/mL)		0.359*	0.0294*
HOMA-IR		0.683*	<0.0001*
Hyaluronic acid (ng/mL)		0.307*	NS*
Platelet count (×10^4/mm^3)		−0.491*	0.0028*
RBC count (×10^4/mm^3)		−0.119*	NS*
Hemoglobin (g/dL)		0.009*	NS*
Serum iron (μg/dL)		0.587*	0.0004*
Transferrin saturation (%)		0.364*	0.0267*
Serum ferritin (ng/mL)		0.325*	0.0481*
Liver histology			
Inflammatory activity[§]			
A1 (n = 14)	18.9 (10.0-40.0) [†]		
A2 (n = 21)	19.0 (7.3-43.3) [†]		NS[‖]
A3 (n = 3)	14.7 (12.0-17.6) [†]		
Fibrosis staging[§]			
F1 (n = 8)	14.9 (10.0-43.3) [†]		
F2 (n = 17)	15.0 (7.3-37.7) [†]		NS[‖]
F3/4 (n = 13)	21.0 (12.0-40.0) [†]		
Steatosis[¶]		0.392*	0.0172*
TIS**		0.455*	0.0056*

*Spearman rank correlation test.
[†] Data are expressed as median (range).
[‡] Unpaired Student's t test.
[§] Inflammatory activity and fibrosis staging in NASH was scored according to Brunt classification (16).
[‖] One-way factorial ANOVA and multiple comparison test.
[¶] Hepatic steatosis degree was assessed based on the percentage of affected hepatocytes.
**The histologic quantification of iron was assessed by TIS proposed by Deugnier et al. (17).

by use of Kruskal-Wallis ANOVA, which is independent of the distribution of the data. Distribution of variables was first evaluated to determine the most appropriate statistical method across group comparisons. Normally distributed data were compared using one-way ANOVA. Data that were not normally distributed were analyzed using Kruskal-Wallis ANOVA. The mean values of two groups of normally distributed data were compared by a t test, and the median values of two groups of data that were not normally distributed were compared using the Mann-Whitney U test. Spearman rank correlation was used to quantify the association between continuous or ordered categorical variables. To analyze the changes of BMI, serum, and histologic variables after the iron reduction therapy, paired Student's t test was used. Logistic regression analysis was used to identify significant factors that influence elevated hepatic 8-oxodG expression in NASH and simple steatosis patients. Categorical variables with more than two levels were coded as dummy variables. All tests were two tailed, and P values <0.05 were considered as statistically significant. Statistical analysis was done using the commercially available software Statistical Package for the Social Sciences 11.5 (SPSS, Inc.).

Results

Clinical Characteristics of the Patients with NASH and Simple Steatosis. The main demographic and clinical laboratory features of the patients with NASH and simple steatosis are compared in Table 1. Patients with NASH were older, and more male and obese subjects than in simple steatosis, but they did not reach the statistical significance. The prevalence of type II diabetes, hypertension, and hyperlipidemia, and serum total cholesterol, triglyceride, and glucose levels were not significantly different between the two groups. Serum aspartate aminotransferase (AST), fasting insulin levels, insulin resistance [assessed by homeostasis model assessment of insulin resistance (HOMA-IR)], and hyaluronic acid were significantly higher in NASH than in simple steatosis. Iron-related serum markers (i.e., serum iron, transferrin saturation, and ferritin) were found to be significantly elevated in NASH compared with those of simple steatosis. Although liver histology showed no significant difference in steatosis degree between the NASH and simple steatosis, hepatic iron deposition was more prominent in NASH; TIS was significantly higher in NASH compared with simple steatosis [3 (0-8) versus 0 (0-5); P < 0.0001].

Hepatic 8-oxodG Levels in NASH and Simple Steatosis Patients. Figure 1A and B showed the 8-oxodG immunohistochemical staining in liver biopsy samples in patients with NASH and simple steatosis, as representative. 8-oxodG immunoreactivity was strongly observed in the nuclei (and weakly in the cytoplasm) of hepatocytes, Kupffer cells, and infiltrated inflammatory cells in NASH patients' liver biopsy specimen (Fig. 1A). The hepatocyte nuclei were differentiated from the nuclei of other cells using computed analyses at the point of nuclear shape and size. 8-oxodG–immunoreactive hepatocytes were distributed throughout the whole acinus in liver of patients. Using the liver samples of patients with simple steatosis, relatively faint immunoreactivity of 8-oxodG was observed in the nuclei of hepatocytes and was rarely in the cytoplasm (Fig. 1B). As a whole, 8-oxodG–positive hepatocyte counts were significantly higher in NASH patients than in simple steatosis [17.5 (range, 7.3-43.3) versus 2.0 (range, 0.0-13.3) cells/10^5 μm^2; $P < 0.0001$; Fig. 1C]. In the liver of 10 healthy controls, immunoreactivities of 8-oxodG were rarely detected in the nuclei of hepatocytes.

Clinical Variables That Correlate with Hepatic 8-oxodG Levels in NASH Patients. To estimate the source of oxidant-generated DNA damage that frequently occurred in the livers of patients with NASH, the correlations of clinical and histologic findings with the degree of hepatic damaged DNA were evaluated, and the results are summarized in Table 2. Patients' age, gender, and BMI were not related to hepatic 8-oxodG counts in NASH patients. Although the 8-oxodG–positive hepatocytic counts were not correlated with serum transaminases, cholesterol, and triglyceride levels, hepatic 8-oxodG levels were elevated in parallel with increase of fasting glucose, serum insulin, and HOMA-IR in patients with NASH [8-oxodG versus glucose ($r = 0.628$, $P = 0.0001$) versus serum insulin ($r = 0.359$, $P = 0.0294$) versus HOMA-IR ($r = 0.683$, $P < 0.0001$); Table 2; Fig. 2A and B]. It is noteworthy that the hepatic 8-oxodG levels were also positively correlated with body and hepatic iron deposition markers; serum iron, transferrin saturation, ferritin, and the hepatic iron deposit grade (i.e., TIS) were significantly correlated with 8-oxodG–positive hepatocyte nucleus counts [8-oxodG versus iron ($r = 0.587$, $P = 0.0004$) versus transferrin saturation ($r = 0.364$, $P = 0.0267$) versus ferritin ($r = 0.325$, $P = 0.0481$) versus TIS ($r = 0.455$, $P = 0.0056$); Table 2; Fig. 2C and D]. Platelet count was also correlated with hepatic 8-oxodG levels, but histologic features (inflammatory activity and fibrosis staging) were not related to hepatic oxidative damage to DNA in patients with NASH. Moreover, elevated hepatocytic 8-oxodG levels were significantly correlated with the extent of hepatic steatosis in patients with NASH (8-oxodG versus steatosis, $r = 0.392$, $P = 0.0172$; Fig. 2E-1), but these two variables were not related in patients with simple steatosis (Fig. 2E-2). The degree of hepatic iron deposition (TIS) and insulin resistance (HOMA-IR) was also correlated mutually in patients with NASH (Fig. 3).

Clinical Variables That Correlate with Hepatic 8-oxodG Levels in Simple Steatosis Patients. The correlations of clinical and histologic findings with the hepatic 8-oxodG levels were also investigated in simple steatosis patients (Table 3). Patients' age and serum ferritin levels were significantly related to hepatic 8-oxodG levels in simple steatosis, but other variables, including HOMA-IR, serum iron levels, and TIS, were not correlated.

Factors Independently Associated with Elevated Hepatic 8-oxodG Levels. To identify the variables independently associated with elevated hepatic 8-oxodG
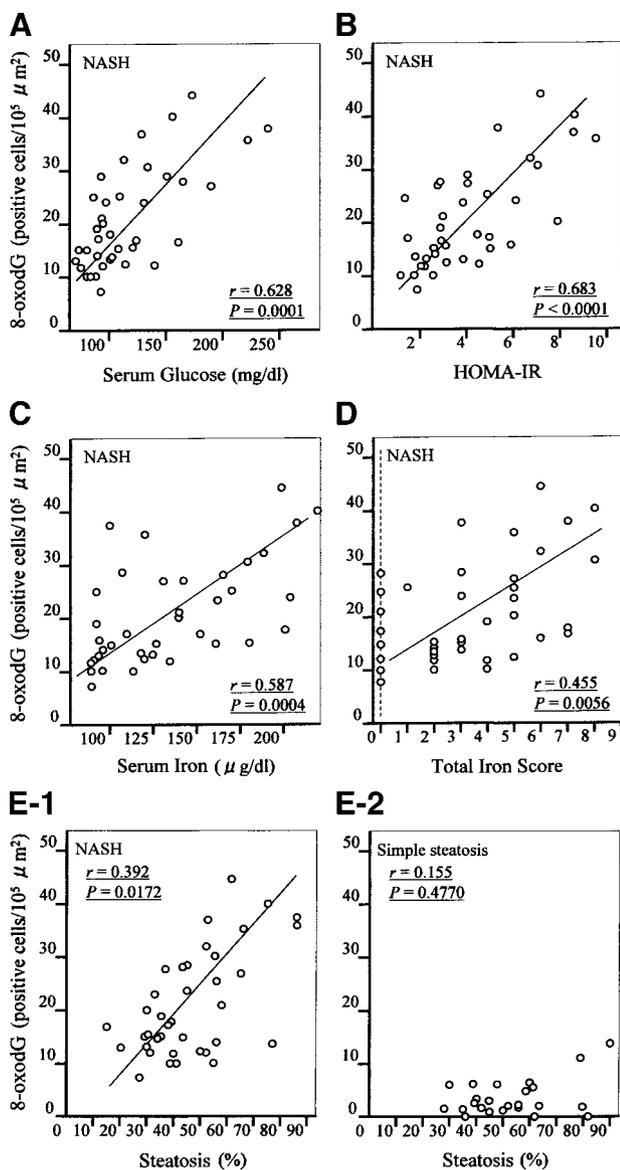

Figure 2. Correlations between 8-oxodG–positive hepatocytic nuclear counts and clinical variables in 38 NASH or 24 simple steatosis patients. **A.** 8-oxodG counts and serum glucose levels in NASH. **B.** 8-oxodG counts and HOMA-IR in NASH. **C.** 8-oxodG counts and serum iron levels in NASH. **D.** 8-oxodG counts and TIS in hepatic tissues in NASH. Dotted vertical line indicates that the TIS is 0. **E-1.** 8-oxodG counts and extent of hepatic steatosis in NASH. **E-2.** 8-oxodG counts and extent of hepatic steatosis in simple steatosis.
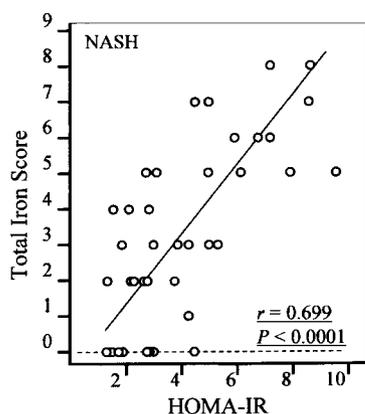
Figure 3. Correlation between TIS in hepatic tissues and HOMA-IR in NASH patients. Dotted horizontal line indicates that the TIS is 0.

levels in NASH and simple steatosis patients, logistic regression analysis was done using the variables recorded in Tables 2 and 3. When the analysis was done in combination NASH and simple steatosis, positive for hepatic iron deposit (i.e., TIS > 0) and insulin resistance (i.e., HOMA-IR > 2) were independent variables contributing to elevated (>10 positive cells/10^5 μm^2) hepatic 8-oxodG (Table 4).

Changes of Serum and Hepatic Histologic Features by Iron Reduction in NASH Patients. To directly evaluate the effect of iron overload to oxidatively

generated damage to DNA in the liver of patients with NASH, iron reduction therapy (phlebotomy plus iron–restricted diet) was done in 11 hyperferritinemic NASH patients (7 males and 4 females; range, 39-67 years) and changes of serum and histologic variables were examined (Table 5). A mean blood volume of 1,700 ± 630 mL was removed by 8.5 ± 3.1 venesection times done over a period of 10.8 ± 1.9 months. Serum hemoglobin, iron, and ferritin levels were decreased in all treated patients at the end of iron reduction. Serum alanine aminotransferase (ALT), TIS score, and hepatic 8-oxodG levels were also decreased in most treated patients, and mean values were significantly decreased after the treatment. Serum cholesterol, triglyceride, fasting glucose, and insulin levels were not significantly changed by iron reduction therapy.

Discussion

In this study, we used immunohistochemical approaches using a monoclonal antibody against 8-oxodG in formalin-fixed, paraffin-embedded liver sections for assessment of oxidatively generated damage to DNA in the liver of nonalcoholic fatty liver disease. Using this approach, 8-oxodG–positive signals in liver tissue were detected in all patients with NASH, suggesting that oxidative stress is a frequent event in the liver of NASH patients. At present, a commonly accepted model for the pathogenesis of NASH is the so-called two-hit hypothesis; first hit leads to accumulation of hepatic free fatty acids resulting in a histologic picture of macrovesicular steatosis, and a subsequent second hit may result in liver

Table 3. Correlations between clinical findings and 8-oxodG levels in the liver of patients with simple steatosis (*n* = 24)

Characteristics	8-oxodG (positive cells/10^5 μm^2)	Statistics	
		r	P
Age (y)		0.485*	0.0251*
Gender			
Male (*n* = 11)	2.0 (0.7-13.3) [†]		NS [‡]
Female (*n* = 13)	2.0 (0.0-6.3) [†]		
BMI (kg/m²)		0.221*	NS*
Laboratory data			
ALT (IU/L)		0.276*	NS*
AST (IU/L)		0.310*	NS*
Total cholesterol (mg/dL)		0.009*	NS*
Triglyceride (mg/dL)		−0.070*	NS*
Glucose (mg/dL)		0.321*	NS*
Serum insulin (microunits/mL)		−0.225*	NS*
HOMA-IR		0.001*	NS*
Hyaluronic acid (ng/mL)		0.360*	NS*
Platelet count (×10^4/mm³)		−0.265*	NS*
RBC count (×10^4/mm³)		−0.265*	NS*
Hemoglobin (g/dL)		−0.237*	NS*
Serum iron (μg/dL)		0.094*	NS*
Transferrin saturation (%)		0.141*	NS*
Serum ferritin (ng/mL)		0.577*	0.0082*
Liver histology			
Steatosis[§]		0.155*	NS*
TIS[‖]		0.282*	NS*

*Spearman rank correlation test.
[†] Data are expressed as median (range).
[‡] Unpaired Student's *t* test.
[§] Hepatic steatosis degree was assessed based on the percentage of affected hepatocytes.
[‖] The histologic quantification of iron was assessed by TIS proposed by Deugnier et al. (17).

Table 4. Factors associated with the elevated hepatic 8-oxodG in NASH and simple steatosis patients by regression analysis

Factors	RR (95% CI)	P
TIS > 0	3.69 (2.18-13.97)	0.0088
HOMA-IR > 2	2.61 (1.50-6.46)	0.0273

Abbreviations: RR, relative risk; 95% CI, confidence interval.

injury (3). Although the precise mechanism of how the second hit occurs and concerns in liver disease progression remains unclear, oxidative stress is recognized as the most convincing mediator of second hit in NASH (4-6). Significantly elevated hepatic 8-oxodG in NASH compared with simple steatosis supports the hypothesis that oxidative stress may contribute to the pathogenesis of NASH. Because the hepatocytic 8-oxodG counts were significantly correlated with platelet count, oxidative stress may be related to disease progression in NASH, especially fibrogenesis. Seki et al. (4) also reported that hepatic oxidative stress formation as assessed by the level of 4-hydroxy-2′-2 nonenal was significantly increased with the progression of histologic fibrosis staging in NASH. The degree of hepatic fat deposit seems to be relevant to hepatic oxidative stress formation in NASH because hepatic 8-oxodG levels were positively correlated to the extent of steatosis in NASH. But steatosis alone could not cause the hepatic oxidative stress because the degree of hepatic steatosis was not significantly different between the NASH and simple steatosis, and steatosis and 8-oxodG levels were not correlated in simple steatosis patients. These results clearly indicate that second hit is necessary for the development from simple steatosis to NASH.

Some authors believe that iron may be the substrate of oxidative stress and could be responsible for the second hit in patients with NASH (23, 24). In steatotic livers, the saturation of β-oxidation by excess free fatty acids will ultimately lead to the generation of hydrogen peroxide, which in turn can be converted to highly reactive hydroxyl radicals in the presence of free iron via Fenton reaction (10). Indeed, there is strong evidence, from *in vitro* and *in vivo* studies, that iron overload enhances oxidative stress (25-27). Consistent with several previous findings (7-9), the present data showing that serum iron, transferrin saturation, and ferritin levels and the grade of hepatic iron staining (TIS) are significantly higher in NASH compared with simple steatosis also suggest that iron overload may be responsible for the second hit and pathogenesis of NASH. Quantitative analysis revealed that hepatocytic 8-oxodG levels were significantly correlated with these iron-related markers in NASH, strongly indicating that the increase in the body stored iron is specifically related to increased hepatocytic oxidatively generated damage to DNA in NASH patients.

Because serum insulin and HOMA-IR were significantly higher in NASH than in simple steatosis, and fasting glucose levels and HOMA-IR were significantly correlated with hepatic oxidative damage to DNA in NASH patients, another important factor for hepatic oxidative stress formation in NASH may be insulin resistance, as same as the iron overload. A strong association between iron overload and insulin resistance has been proposed. In fact, Mendler et al. (28) defined a syndrome of "insulin resistance-associated iron overload" in the presence of unexplained hepatic iron overload and at least one component of the insulin resistance. Insulin resistance also seemed to be closely linked to total body iron stores in the general population. Body iron stores are positively associated with the development of glucose intolerance and type 2 diabetes (29, 30). Iron overload and insulin resistance relationship also confirms the fact that iron depletion can improve insulin sensitivity (31-33). Iron overload can interfere with insulin signaling through the induction of reactive oxygen species, the latter impairing insulin uptake through a direct effect on insulin receptor function, by inhibiting the translocation of glucose

Table 5. Profile, phlebotomy, and changes in individual data after iron reduction therapy in patients with NASH

Patient no.	Age/gender	Phlebotomy period (mo)/volume (mL)	BMI (kg/m²) Before	BMI After	ALT (IU/L) Before	ALT After	Hemoglobin (g/dL) Before	Hemoglobin After	Serum iron (µg/dL) Before	Serum iron After	Ferritin (ng/mL) Before	Ferritin After	TIS Before	TIS After	8-oxodG (/10⁵ µm²) Before	8-oxodG After
1	47/M	9/2,200	31.0	29.7	59	32	15.6	14.7	220	172	718	77	8	4	40.0	21.7
2	66/M	9/1,000	26.2	25.9	171	110	14.5	14.2	188	155	431	382	6	8	32.0	39.0
3	67/M	12/1,800	27.3	26.5	98	56	14.2	13.6	141	115	539	123	5	4	27.0	22.3
4	41/F	14/2,400	29.0	27.8	136	97	14.5	13.2	170	122	481	53	5	2	25.3	12.3
5	59/F	13/1,400	35.0	32.2	46	38	14.9	13.6	138	101	223	75	5	1	20.0	12.7
6	41/M	12/2,800	25.1	25.3	122	89	15.3	13.9	92	72	374	68	4	4	19.0	5.7
7	59/F	10/800	23.5	22.2	133	72	12.2	11.5	202	154	847	272	7	5	17.6	6.7
8	54/F	8/1,200	25.2	25.3	82	49	15.7	15.2	124	107	300	109	2	2	13.3	12.0
9	42/M	12/1,800	28.1	28.3	94	42	16.4	14.7	120	77	537	39	5	2	12.3	5.0
10	39/M	9/2,000	28.1	24.5	118	77	16.2	14.3	134	100	306	34	2	0	11.7	3.7
11	64/M	11/1,200	30.4	30.1	37	49	15.0	14.2	96	94	339	46	4	2	10.0	10.7
Mean			28.1*	27.1*	99.6[†]	64.6[†]	15.0[‡]	13.9[‡]	148[§]	115[§]	463[∥]	116[∥]	4.8[¶]	3.1[¶]	20.7**	13.8**

*Statistically significant difference at $P = 0.0222$ (paired t test).
[†] Statistically significant difference at $P = 0.0003$ (paired t test).
[‡] Statistically significant difference at $P < 0.0001$ (paired t test).
[§] Statistically significant difference at $P < 0.0001$ (paired t test).
[∥] Statistically significant difference at $P < 0.0001$ (paired t test).
[¶] Statistically significant difference at $P = 0.0113$ (paired t test).
**Statistically significant difference at $P = 0.0092$ (paired t test).

transporter GLUT4 to the plasma membrane (34, 35). The relation of insulin resistance and iron overload is also important in reverse, as insulin stimulates cellular iron uptake through increased transferrin receptor externalization (36, 37). It is also known that the glycation of transferrin decreases its ability to bind ferrous iron (38) and, by increasing the pool of free iron, stimulates ferritin synthesis. Glycated holotransferrin is additionally known to facilitate the production of free oxygen radicals, which further amplify the oxidative effects of iron (38). Reciprocally, the oxidative stress also induces both insulin resistance [by decreasing internalization of insulin (34)] and increased ferritin synthesis. Therefore, iron overload, insulin resistance, and oxidative stress may amplify each other and may compose the vicious cycle to progress liver injury in NASH.

The above-mentioned results prompted us to investigate the possibility of iron reduction for improvement of hepatic oxidative damage to DNA in NASH. Iron reduction (phlebotomy plus iron–restricted diet) therapy for NASH significantly reduced the serum ALT and hepatic 8-oxodG levels, suggesting the possibility of iron reduction for treatment option for NASH. Recently, Valenti et al. (33) reported that iron reduction also improved insulin resistance in 64 phlebotomized nonalcoholic fatty liver disease patients with hyperferritinemia. A randomized study also suggests that iron reduction may recover insulin action in type 2 diabetic patients (39). But in our treated NASH patients, iron reduction did not significantly affect insulin resistance state. Large randomized controlled studies, considering histology as final outcomes, are nonetheless required to determine the clinical effect of iron reduction therapy in patients with NASH before this therapy can be proposed.

In conclusion, iron overload, insulin resistance, and hepatic oxidatively generated damage to DNA tightly correlate each other in NASH patients, suggesting that these three factors may play an important role in the pathogenesis of NASH. Simple and inexpensive therapies, such as phlebotomy and iron–restricted diet, may be emerging as effective treatment options, which may lead to reduction of hepatocellular carcinoma incidence in NASH patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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