to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in *a priori* approval by the Ethical Committee of Mie University.

Quantitative assay for serum hepcidin using the SELDI-TOF MS system

We used SELDI-TOF MS system to measure the serum hepcidin levels, as reported previously.[17,19] Serially diluted synthetic hepcidin-25 (Peptide Institute, Osaka, Japan) was used for external mass calibration. The data from the peak intensities were normalized with the total ion current using Biomarker Wizard, v. 3.1.1 (Ciphergen ProteinChip; Chiphergen Biosystems, Fremont, CA, USA) to compensate for the variations in sample concentrations loaded onto a spot; the data were represented as arbitrary units (AU) calculated using this software.

Statistical analysis

Data are expressed as the mean ± standard deviation (SD). The baseline characteristics were compared using the unpaired Student *t*-test or Mann–Whitney *U*-test for continuous variables and χ^2-test for categorical variables. Correlations between two variables were assessed by the Spearman rank correlation test. To analyze the changes of laboratory data before and after the iron reduction, paired Student's *t*-test was used. Two-sided *P* values of <0.05 were considered as statistically significant. All statistical analyses were performed using the commercially available software SPSS 11.5 software (SPSS, Chicago, IL, USA).

RESULTS

CLINICAL CHARACTERISTICS OF patients are shown in Table 1. Patients with C-HCV had significantly higher serum iron, transferrin saturation, and ferritin levels compared to healthy subjects, indicating that iron overload condition is relatively common in chronic HCV-infected patients, as reported previously. Serum hepcidin was measurable in all 9 C-HCV patients and 10 healthy controls. The mean serum hepcidin was 91.2 ± 50.0 AU in C-HCV before the iron reduction, and this value was significantly higher than that of healthy controls (34.2 ± 15.6 AU) (Fig. 1a). When the correlation between serum hepcidin and clinical characteristics was investigated, serum ferritin levels were strongly related to serum hepcidin, indicating that hepcidin is expressed in response to iron burden (Fig. 1b). Hepcidin expression is also known to increase in response to inflammatory stimuli,[20] but there were no significant correlations between serum hepcidin and serum transaminase levels and histological inflammatory grading in C-HCV patients.

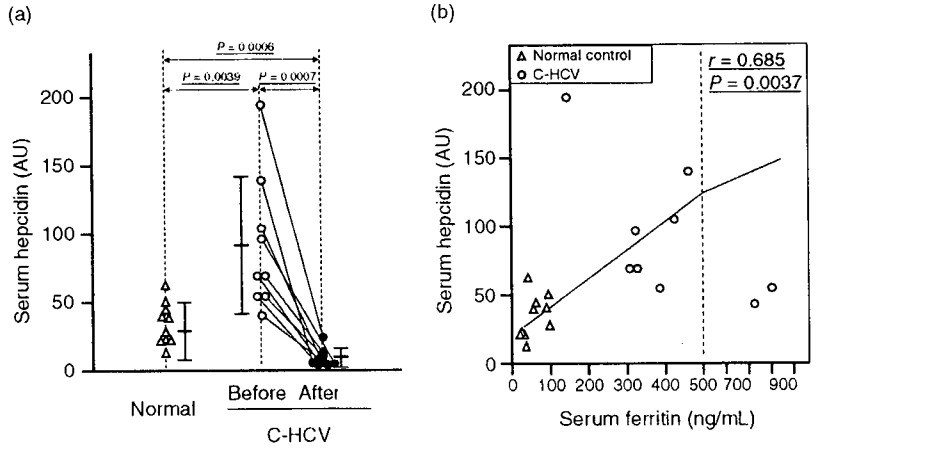

Figure 1 (a) Serum hepcidin concentrations in normal subjects (open triangles) ($n = 10$) and in patients with chronic hepatitis C virus (C-HCV) before (open circles) and after (closed circles) the phlebotomy ($n = 9$). Serum hepcidin was measured by surface-enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF-MS) system. Before the phlebotomy, C-HCV patients had significantly higher serum hepcidin concentrations than those of normal subjects. Serum hepcidin levels were drastically decreased in all C-HCV patients after the phlebotomy, and its mean value was significantly lower as compared to that of healthy subjects. Bar shows mean ± SD. (b) Correlation between serum hepcidin concentrations and serum ferritin levels in normal subjects (open triangles) ($n = 10$) and in patients with C-HCV (open circles) ($n = 9$). Regression line is shown.

A mean blood volume of 2910 ± 920 mL was removed by 9.0 ± 2.89 venesection times performed over a period of 12.0 ± 2.8 months in 9 C-HCV patients (Table 2). Red blood cell counts, hemoglobin concentrations, serum iron, and ferritin levels were reduced in all patients after phlebotomy, except for 1 case of serum iron. Serum transaminase levels were also decreased in all treated patients, indicating that liver damage was improved after the iron reduction in C-HCV. Serum hepcidin levels were drastically decreased in all C-HCV patients after the therapy, and its mean value (9.0 ± 6.0 AU) was significantly lower as compared to the level of healthy subjects (Fig. 1a). Phlebotomy stimulates erythropoietic activity, as the result of iron deficiency. Indeed, mean reticulocyte count, a marker of erythropoieses, was significantly increased after phlebotomy (Table 2), but pretreatment reticulocytes were not correlated with serum hepcidin levels in CH-C patients ($r = 0.118$, $P = 0.7393$; Spearman rank correlation).

Our findings of strong positive correlations between serum hepcidin and ferritin, and significant reduction of serum hepcidin after phlebotomy also suggest the feedback mechanism of hepcidin expression against iron signal. Then, to evaluate the appropriateness of hepcidin expression relative to iron overload,[21] the ratio of hepcidin to ferritin levels was calculated in each patient. Before the phlebotomy, the ratio of C-HCV was significantly lower compared to healthy subjects (0.33 ± 0.41 vs. 0.73 ± 0.36, $P = 0.0068$) (Fig. 2). Hepcidin/ferritin ratio was not significantly changed after phlebotomy, and its mean was still significantly lower than that of controls (0.32 ± 0.14, $P = 0.0338$). These results indicate that the hepcidin expression is impaired in chronic HCV-infected patients when adjusted for serum concentrations of ferritin, and this relative impairment is not improved when the iron overload condition is cured.

DISCUSSION

HEPCIDIN MAINTAINS BODY iron balance by acting as a regulatory feedback mechanism between body iron needs and intestinal iron absorption, and measurement of serum hepcidin is useful for understanding the mechanism of iron dysregulation in patients suffering from iron regulatory disorders.[11] Hepcidin is recognized as the hormone that almost exclusively synthesized in hepatocytes[22] and we previously confirmed that serum hepcidin levels were strongly correlated with hepatic mRNA expression levels of hepcidin in C-HCV patients.[19] In this study,

Table 2 Profile and changes in individual data after phlebotomy in patients with chronic hepatitis C virus

Patients no.	Age/ Gender	Phlebotomy Period/ Volume	RBC ($\times 10^4$/mm^3)		Hemoglobin (g/dL)		Serum iron (μg/dL)		Ferritin (ng/mL)		Reticulocytes (%)		ALT (IU/L)		AST (IU/L)	
			Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
1	65/M	16 M/4200 mL	421	389	14.1	12.7	159	120	805	35	0.72	3.11	112	47	158	48
2	56/M	14 M/2800 mL	383	348	15.3	13.5	199	114	387	19	0.95	7.29	128	79	120	59
3	56/F	12 M/1800 mL	342	303	12.9	12.0	160	66	141	33	1.52	2.68	66	35	40	32
4	43/M	12 M/4000 mL	387	352	14.8	13.6	112	87	305	21	1.20	4.24	94	38	68	35
5	27/M	13 M/3600 mL	381	365	15.1	13.3	106	110	327	28	1.16	4.66	102	68	89	49
6	65/M	6 M/2000 mL	339	295	14.2	13.0	203	58	724	49	1.37	3.01	115	39	162	40
7	64/M	15 M/3400 mL	432	403	13.9	12.9	139	89	423	25	0.77	2.30	78	51	79	68
8	59/F	12 M/2000 mL	342	310	13.7	12.4	170	79	462	20	1.13	1.98	59	47	38	34
9	57/M	12 M/2400 mL	420	367	14.5	12.9	91	68	321	24	0.94	4.63	145	87	82	62
Mean			383*	348*	14.3**	12.9**	149***	88***	433****	28****	1.08*****	3.77*****	100******	55******	93*******	47*******

*Statistically significant difference at $P = 0.0439$ (paired *t*-test).
**Statistically significant difference at $P = 0.0072$ (paired *t*-test).
***Statistically significant difference at $P = 0.0043$ (paired *t*-test).
****Statistically significant difference at $P = 0.0003$ (paired *t*-test).
*****Statistically significant difference at $P = 0.0015$ (paired *t*-test).
******Statistically significant difference at $P = 0.0002$ (paired *t*-test).
*******Statistically significant difference at $P = 0.0145$ (paired *t*-test).
ALT, alanine aminotransferase; AST, aspertate aminotransferase; RBC, red blood cell counts.

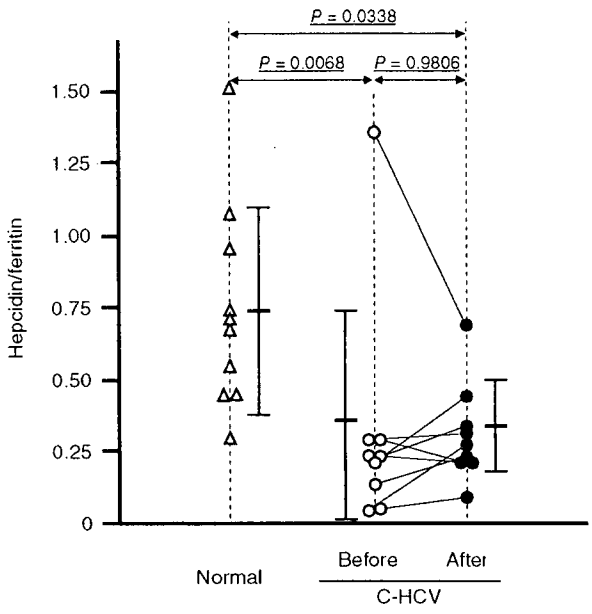

Figure 2 Serum hepcidin/ferritin ratio in normal subjects (open triangles) ($n = 10$) and in patients with chronic hepatitis C virus (C-HCV) before (open circles) and after (closed circles) the phlebotomy ($n = 9$). Serum hepcidin was measured by surface-enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF-MS) system. Before the phlebotomy, C-HCV patients had significantly lower hepcidin/ferritin ratio than those of normal subjects, and this relative low ratio was not significantly changed during phlebotomy in C-HCV patients. Bar shows mean ± SD.

serum hepcidin levels of C-HCV were significantly higher than those of healthy controls. Serum iron, transferrin saturation, and ferritin were also significantly higher in C-HCV than in control, suggesting that the many C-HCV patients are in iron overload condition, and recollecting the hepcidin expression in response to iron overload. Drastically decrease of serum hepcidin by phlebotomy in C-HCV also indicates that the hepcidin is responded to iron overload signal, even in the chronically HCV-infected liver. But hepcidin to ferritin ratio was significantly lower in C-HCV patients than in controls, indicating that up-regulation of hepcidin by increased body-stored iron may be impaired in the HCV-infected liver. Serum hepcidin, consistent with ferritin, was significantly decreased after phlebotomy, but the hepcidin/ferritin ratio was not significantly changed during the treatment. From these results, relative imparment of hepcidin production in C-HCV seems to be directly related to HCV infection, not to iron overload condition. Recently, Nishina *et al.*[23] clearly demonstrated that transgenic mice expressing HCV ploprotein had decreased hepcidin expression and increased ferritin expression in the liver. They also showed the down-regulation of hepcidin promoter activity and increased hepatic reactive oxygen species. Because it is well known that hepatic oxidative stress is elevated in patients with C-HCV,[24] HCV-induced oxidative stress formation may involve the down-regulation of hepcidin, causing iron accumulation during chronic HCV infection.

Besides hepcidin, numerous iron-related proteins [hemojuvelin, ferroportin, transferrin receptors (TfR) 1 & 2, ferritin, etc.] involve the keeping of iron balance in the body. We have previously examined these hepatic mRNA expression levels in several phlebotomized C-HCV patients at preliminary.[25] Although ferroportin and TfR2 mRNA levels were not significantly changed, hepatic TfR1 levels were significantly increased by phlebotomy. Large number and more detailed analysis would be performed in the future.

It is reported that increased iron storage plays a role as a cofactor for liver disease progression during chronic HCV infection. Indeed, a beneficial effect of phlebotomy on serum transaminase levels and histological inflammation has already been reported in C-HCV.[5–9] In this study, all C-HCV patients also reduced serum transaminase levels after phlebotomy. But in this subclinical iron deficiency condition by phlebotomy, depleted stores iron further decreased serum hepcidin concentrations, and may lead to a vicious circle of frequent maintenance phlebotomies to counteract an upregulated iron absorption from the gut in C-HCV. Efficacy of iron restricted diet judging from serum trasamianse levels was also documented in C-HCV,[26] and combination with phlebotomy and iron restricted diet may be more beneficial for the amelioration of liver injury in C-HCV patients. Further clinical prospective studies should be necessary.

In conclusion, we measured serum hepcidin levels in patients with C-HCV and its strict response to iron signaling was observed. But, its response was relatively diminished and it was irreversible after iron reduction by phlebotomy in C-HCV patients. The consequences of this hepcidin dysregulation may be an important mechanism underlying the iron overload seen in many patients, and may have significant implications for the management of C-HCV infection. Improvement for the regulation or supplementation of hepcidin may be beneficial for iron-overloaded patients with C-HCV.

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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

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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 (×10^4/mm^3)	18.0 (4.9-37.0)	23.0 (13.1-45.2)	0.0146
RBC count (×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

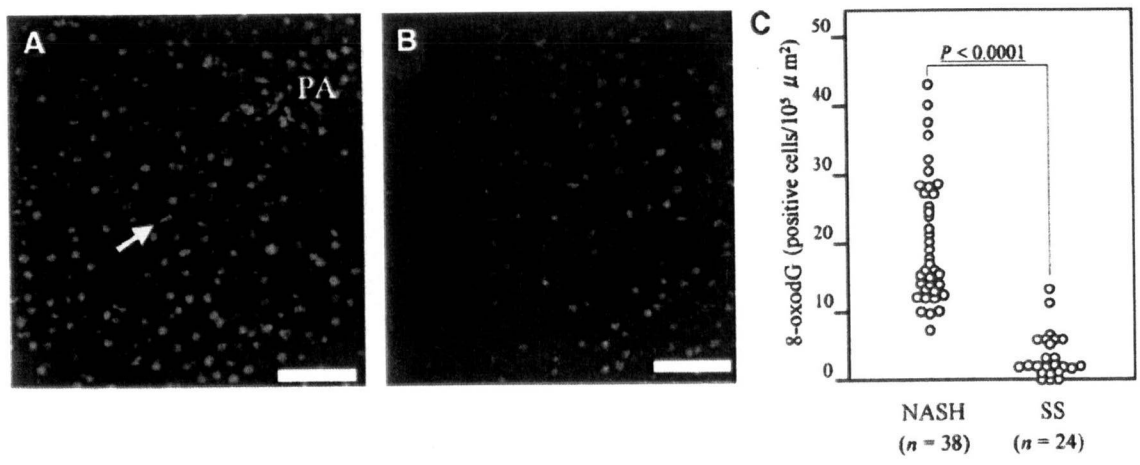

Figure 1. A and **B.** Representative 8-oxodG immunohistochemical staining in liver tissues from patients with NASH (**A**) and simple steatosis (**B**). In the liver of NASH, 8-oxodG immunoreactivity was strongly observed at the nuclei of many hepatocytes and several Kupffer cells (*arrow*) throughout 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. O, 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 (positive cells/10^5 μm^2)	Statistics	
		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.

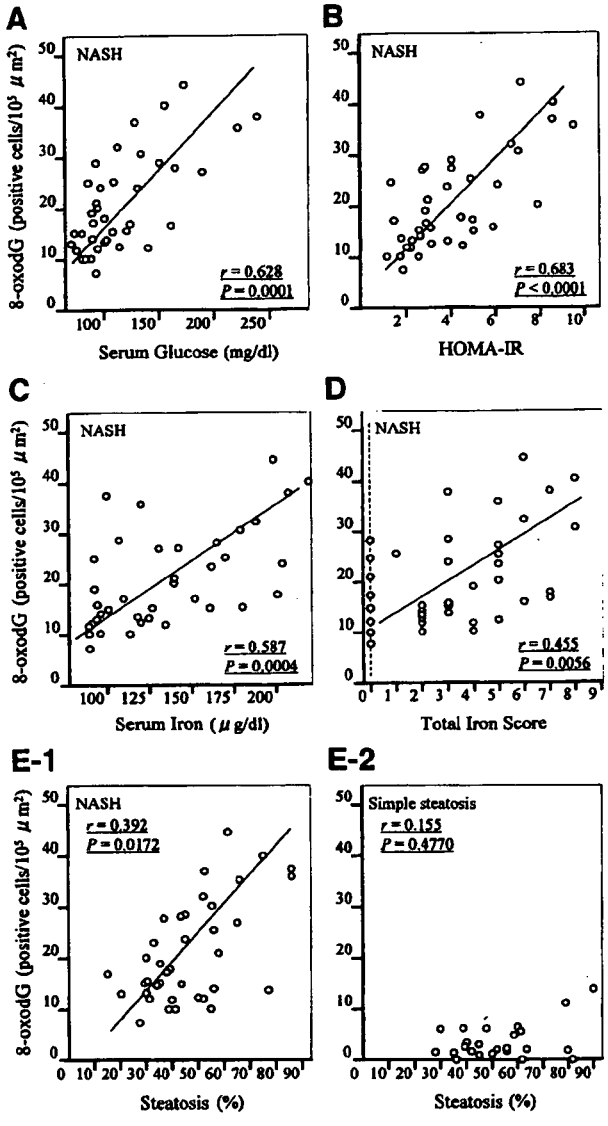

Figure 2. Correlations between 8-oxodG–positive hepatocytic nuclear counts and clinical variables in 38 NASH or 24 simple steatosis patients. **A.** 8-oxodG counts and serum glucose levels in NASH. **B.** 8-oxodG counts and HOMA-IR in NASH. **C.** 8-oxodG counts and serum iron levels in NASH. **D.** 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.

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

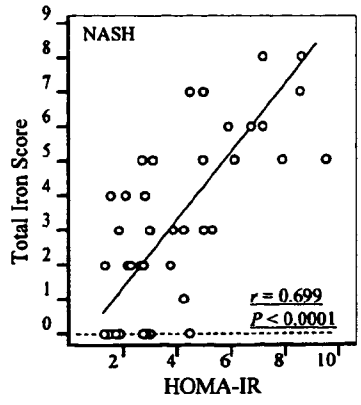

Figure 3. Correlation between TIS in hepatic tissues and HOMA-IR in NASH patients. Dotted horizontal line indicates that the TIS is 0.

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/m^2)		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/mm^3)		−0.265*	NS*
RBC count (×10^4/mm^3)		−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/m^2)		ALT (IU/L)		Hemoglobin (g/dL)		Serum iron (μg/dL)		Ferritin (ng/mL)		TIS		8-oxodG ($/10^5$ μm^2)	
			Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	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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特集II NASH起因発癌のエビデンス

NASH肝発癌における肝内酸化的DNA障害の関与とその発生機序*

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Key Words：8-OHdG, chronic hepatitis C, iron overload, insulin resistance, iron restricted therapy

はじめに

「飽食の時代」と言われる今日の高エネルギー・高脂肪の欧米型食生活への変化に加え，自動車社会の発達や喫煙・飲酒などの生活習慣の変化によって，近年本邦においても肥満人口の急激な増加が大きな社会的問題となっている．非アルコール性脂肪性肝炎(nonalcoholic steatohepatitis：NASH)は1980年にLudwig[1]が「明らかな飲酒歴がないにもかかわらず，肝病理学上アルコール性肝障害に類似した組織像を呈する疾患」として提唱して以来すでに四半世紀が経過したが，肥満や糖尿病(インスリン抵抗性)との深い関連性や少なからず肝硬変や肝癌への進展例が存在することも明らかとなり[2]，今後その臨床的重要性は増すばかりである．

NASH発症にはまず肝に脂肪沈着が起こり(first hit)，これになんらかのsecond hitが加わることによって発症するといったtwo hit theoryが現在のところもっとも有力視されている[3]．Second hit本体の解明はその病因論も含めてまだまだ研究途上であるが，その有力な候補として酸化ストレスがあげられている[4)~6)]．とくに過剰鉄の存在下でFenton反応により発生するヒドロキシラジカル(·OH)は(図1)もっとも細胞毒性の強い活性酸素種(reactive oxygen species：ROS)の一種で，これが核酸のグアニンの8位にアダクトすることにより8-hydroxy-2'-deoxyguanosine(8-OHdG)が産生される(図1)．8-OHdGはその立体構造の変化により本来の相手であるシトシンのみならずアデニンとも対合するようになり，その結果として癌遺伝子や癌抑制遺伝子に変異を生じさせやすくすると考えられている[7]．実際，多くの発癌モデルや癌臨床検体においてDNA中の8-OHdGの過剰蓄積が証明されている[8]．

われわれは以前よりさまざまな肝障害における酸化ストレスの関与を検討しており[9)~13)]，ここではそのデータを供覧し，NASH肝発癌における酸化ストレスの関与について言及したい．

ウイルス性慢性肝炎における肝内酸化的DNA障害の肝発癌への関与

われわれは以前にウイルス性慢性肝炎患者における肝内酸化的DNA障害の肝発癌への関与を検討した[14]．1995～2001年の間に当科にて肝生検が施行されたB型およびC型慢性肝炎152例を対象とし(表1)，肝内8-OHdG量を測定，以後の発癌率との関係を検討した．肝内8-OHdG量の測定は，既報のごとく[15)16)]肝組織に抗8-OHdG抗体(日本老化制御研究所，静岡)による蛍光免疫染

* Hepatic oxidative DNA damage and hepatocarcinogenesis in patients with nonalcoholic steatohepatitis.

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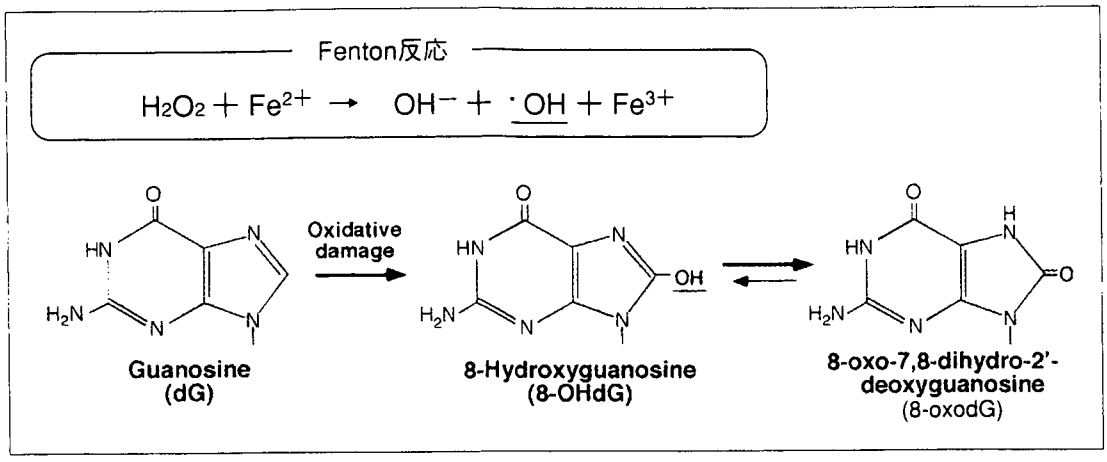

図1　鉄によるヒドロキシラジカルの産生および8-OHdGの産生経路
過剰な自由2価鉄はFenton反応によって3価鉄に変換される．この際，強力な活性酸素種であるヒドロキシラジカル(·OH)が発生する．このヒドロキシラジカルがグアニンの8位にアダクトすることで8-hydroxy-2'-deoxyguanosine(8-OHdG)が産生される．

表1　B型およびC型慢性肝炎患者

Characteristics	C型慢性肝炎 (N＝118)	B型慢性肝炎 (N＝34)	*P* values
年齢（歳）	57.5 (18-82)	54.0 (24-75)	NS
性別（男/女）	66/52	24/10	NS
BMI (kg/m²)	24.3 (16.3-35.2)	24.0 (18.3-28.2)	NS
血液データー			
ALT (IU/L)	58.0 (11-225)	49.0 (11-561)	NS
AST (IU/L)	61.5 (17-183)	58.5 (18-702)	NS
ヒアルロン酸 (ng/ml)	101.8 (5.4-736)	49.0 (11.0-324)	0.0024
血小板 (× 10⁴/mm³)	14.6 (2.9-34.5)	14.3 (3.6-25.3)	NS
赤血球 (× 10⁴/mm³)	429.5 (283-520)	447.5 (280-566)	NS
ヘモグロビン (g/dl)	13.5 (8.3-16.5)	13.8 (9.2-17.1)	NS
血清鉄 (μg/dl)	135.5 (17-334)	128.5 (20-240)	NS
トランスフェリン飽和度（%）	37.2 (3.2-86.2)	41.5 (6.1-85.4)	NS
血清フェリチン (ng/ml)	139.8 (6.0-884)	101.9 (9.1-322)	0.0032
ウイルス量			
HCV-RNA (KIU/ml)	1220 (23.6-5000)	−	
HBV-DNA (LGE/ml)	−	5.4 (<3.7-8.7)	
肝組織所見			
Inflammatory activity (0/1/2/3)	1/41/49/27	1/12/14/7	NS
Fibrosis staging (0/1/2/3/4)	1/29/26/27/35	0/6/10/9/9	NS
肝内鉄スコア	7 (0-22)	3 (0-16)	0.0033

Data are expressed as median(range). HCV：hepatitis C virus, HBV：hepatitis B virus

色を施し，image analyzerを用い陽性肝細胞数をカウントした．なお，全例interferonや核酸アナログなどの抗ウイルス療法は経過中施行されていない．その結果，C型肝炎においては平均6.7±3.3年の経過観察中36例に肝癌の発生を認め，その年率発癌率は3.89%であった．発癌群では肝生検時，すでにより多くの肝内8-OHdGの蓄積を認め(図2)，肝内8-OHdG量を25(cells/10^5 μm²)ごとの4群の層別化したところ，以後の発癌率は有意に異なっていた(図3-A)．さらにC型肝炎において肝癌発生に寄与する因子につき多変量解析を行ったところ，肝線維化に加え肝内8-OHdG量が以後の肝発癌に寄与する独立した因子であった(表2)．B型肝炎においては平均7.7±2.2年の間に8例発癌し(年率発癌率3.42%)，B型においても同様に発癌群では肝生検時すでにより多くの8-OHdGが肝内に蓄積しており(図2)，やはり肝内8-OHdG量ごとに以後の発癌率に差異

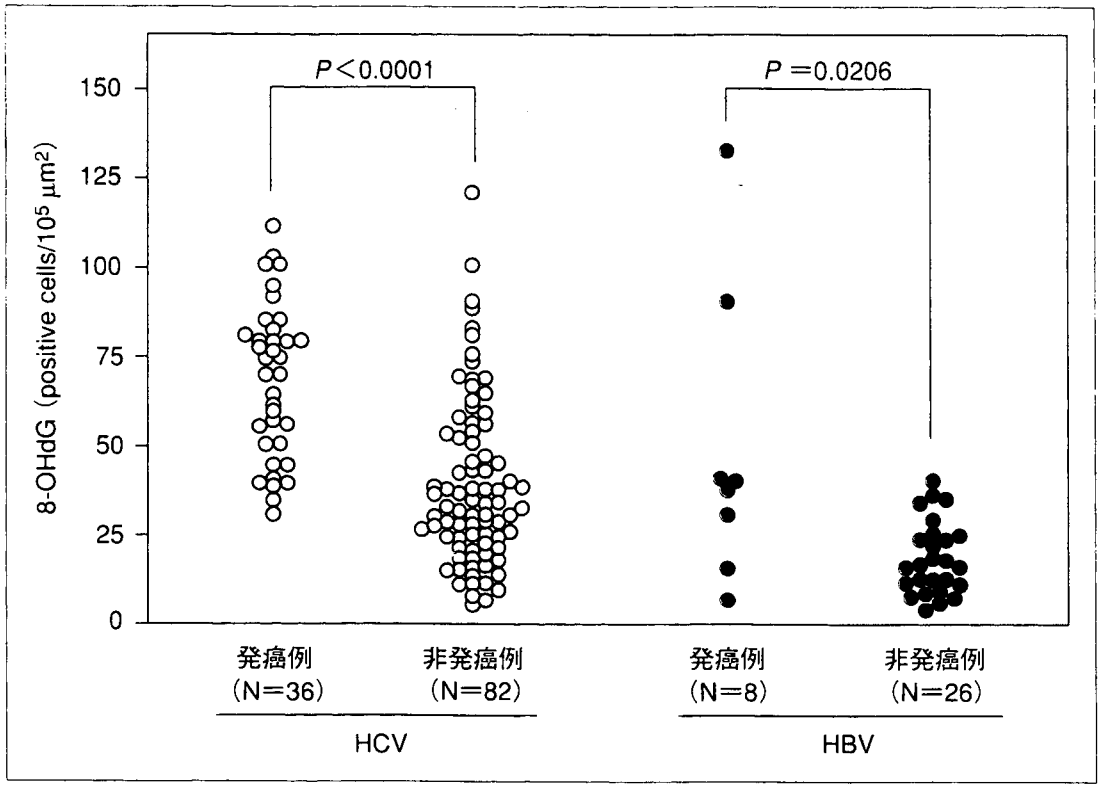

図 2　C 型および B 型慢性肝炎患者における発癌群と非発癌群における肝内8-OHdG量の比較
C 型，B 型慢性肝炎ともに肝生検施行時の肝内8-OHdG量が高値である症例より，以後有意に多くの症例に肝癌が発生している．HCV：hepatitis C virus, HBV：hepatitis B virus

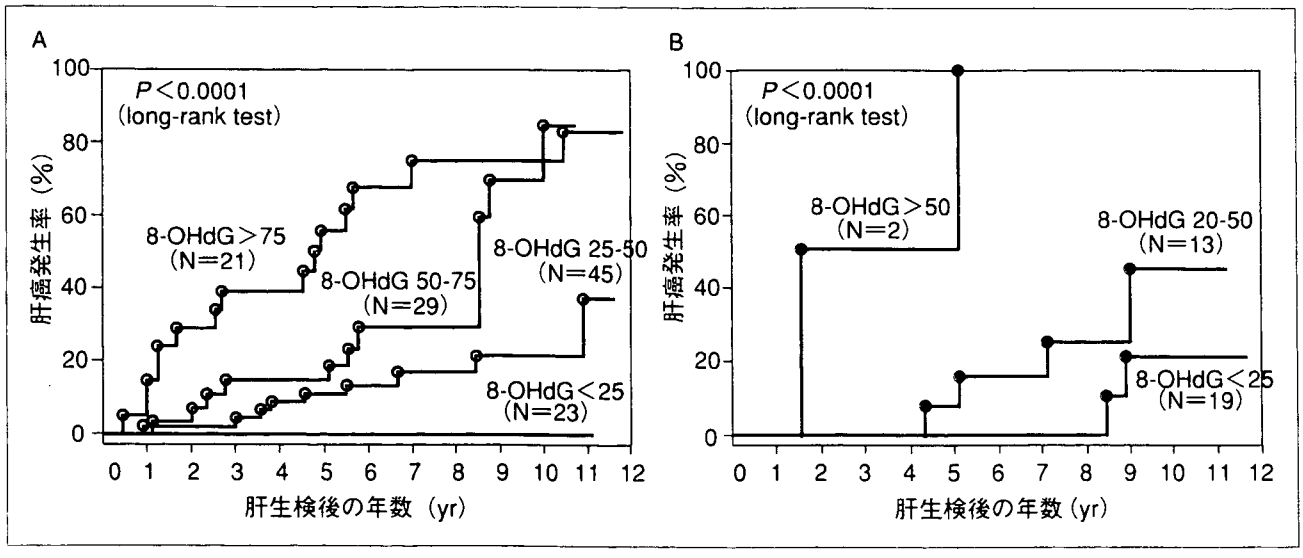

図 3　C 型および B 型慢性肝炎患者における肝内8-OHdG量ごとの以後の肝癌発生率の推移(Keplan-Meier)
C 型(A)および B 型(B)慢性肝炎患者における肝生検施行時の肝内8-OHdG量ごとの以後の肝癌発生率の推移をKeplan-Meier法にて示した．C 型，B 型肝炎とも肝内8-OHdGの多い症例から有意に早期に，かつ高頻度に肝癌の発生を認めた．

を認めた(図3-B)．

以上の結果は，原因ウイルスにかかわらず慢性肝炎においては，肝内酸化ストレスが肝発癌における重要な発癌起因物質として作用している可能性を示している．

NASHにおける肝内酸化的DNA障害

NASHは肥満，とくに内臓肥満を合併していることが多く，基礎病態としてインスリン抵抗性を認めることが多い．インスリン抵抗性は末梢

表2　C型慢性肝炎における肝発癌に寄与する因子(Coxの比例Hazard modelによる多変量解析)

Factor	Odds ratio	95%CI	*P* values
8-OHdG陽性肝細胞数(10 cells/10^5 μm^{2}上昇ごと)	1.487	1.12-1.97	0.0058
Fibrosis staging(stage 1上昇ごと)	4.090	1.27-13.15	0.0181

CI : confidence interval

表3　NASH患者と単純性脂肪肝患者

Characteristics	NASH (N=38)	単純性脂肪肝 (N=24)	*P* values
年齢(歳)	59.0 (29-78)	51.0 (19-73)	NS
性別(男/女)	22/16	11/13	NS
BMI (kg/m^2)	25.6 (22.5-36.7)	24.7 (16.3-35.1)	NS
糖尿病〔N (%)〕	18 (47.4%)	8 (33.3%)	NS
高血圧症〔N (%)〕	14 (36.8%)	9 (37.5%)	NS
高脂血症〔N (%)〕	25 (65.8%)	15 (62.5%)	NS
血液データー			
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
総コレステロール (mg/d*l*)	201 (151-358)	216 (155-276)	NS
中性脂肪 (mg/d*l*)	155 (63-443)	125 (73-261)	NS
空腹時血糖 (mg/d*l*)	102 (71-241)	99 (73-427)	NS
血清インスリン (μU/m*l*)	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
ヒアルロン酸 (ng/m*l*)	66.5 (5-365)	19.6 (6-258)	0.0004
血小板 (×10^4/mm^3)	18.0 (4.9-37.0)	23.0 (13.1-45.2)	0.0146
赤血球 (×10^4/mm^3)	448 (274-633)	461 (367-558)	NS
ヘモグロビン (g/d*l*)	14.3 (8.3-18.9)	14.7 (11.5-18.9)	NS
血清鉄 (μg/d*l*)	126 (88-220)	93 (25-188)	0.0059
トランスフェリン飽和度 (%)	38.0 (22.3-87.6)	32.4 (9.4-43.8)	0.0152
血清フェリチン (ng/m*l*)	283 (69-847)	139 (18-640)	<0.0001
肝組織所見			
Inflammatory activity (1/2/3)	14/21/3	−	−
Fibrosis staging (1/2/3/4)	8/17/11/2	−	−
脂肪化 (%)	43 (15-86)	51 (28-90)	NS
肝内鉄スコア	3 (0-8)	0 (0-7)	<0.0001

HOMA-IR : Homeostasis of model assessment-insulin resistance. Results are presented as numbers (percentages) for qualitative data and as medians (ranges) for quantitative data.

の脂肪組織の中性脂肪の加水分解を亢進させ遊離脂肪酸の増加を起こす．また，高インスリン血症によりグルコースからも肝細胞へ取り込まれる脂肪酸が増加し，肝臓内の脂肪酸プールは過剰となる．この過剰な脂肪酸がミトコンドリアやペルオキシゾーム，ミクロソームにてβ酸化やω酸化をうける際に，電子伝達系を活発にさせROSを発生させる[17]．また，この過剰な脂肪酸がCYP2E1の活性化を誘導しROS産生が亢進することもわかっている[18]．また，NASHでは血中のTNFαやエンドトキシンがROSの産生を亢進する可能性もいわれている[19]．このようにNASHの病態にも酸化ストレスが深く関与しており[4)~6)]，われわれはウイルス性肝炎と同様，NASH症例においても肝内8-OHdG量の測定を行うこととした．

1．NASHにおける肝内8-OHdG量の病態への関与

2003年3月より2006年12月の間に当科において肝機能異常の精査のために入院し肝生検が行われ，NASHと診断された38例を対象とした(表3)．なお，同時期に肝生検が行われるも肝炎の所見なく単純性脂肪肝とされた24例を比較

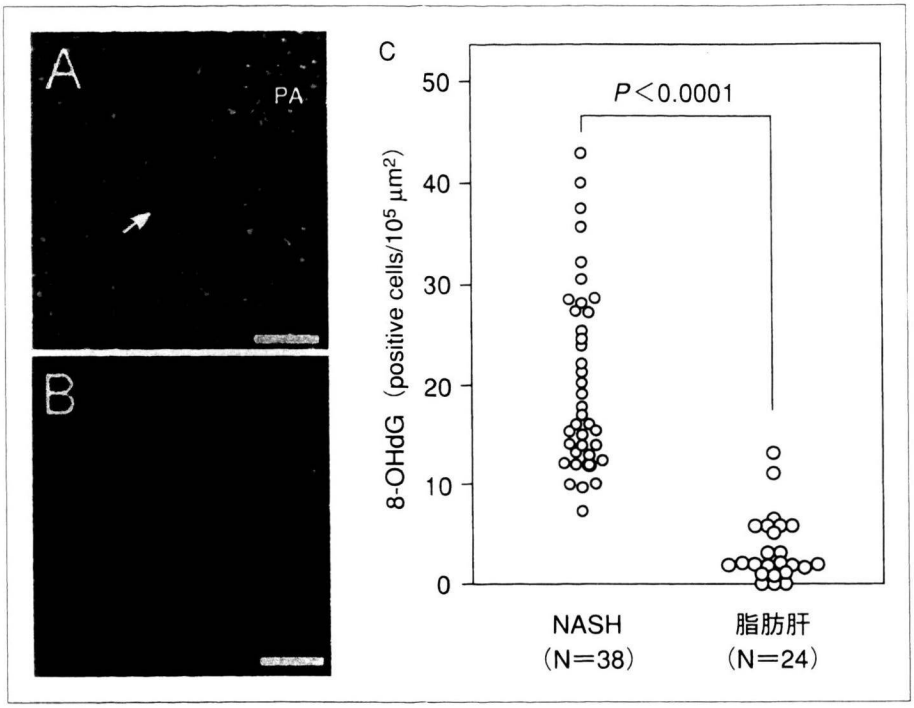

図4　NASHおよび単純性脂肪肝における免疫染色法による肝組織内8-OHdGの同定

マウス抗ヒト8-OHdGモノクローナル抗体を一次抗体に，Alexa488でラベルしたヤギ抗マウスIgG抗体を二次抗体に用い免疫染色を施し，蛍光顕微鏡にて観察している．A：NASHでは比較的多くの肝細胞やKupffer細胞(白矢印)の核内に8-OHdG陽性所見を認める(PA＝potal area)．B：一方単純性脂肪肝では多くの症例でその染色性は弱い．Scale bar＝100μm．C：一定の視野あたりの8-OHdG陽性肝細胞数はNASH群で単純性脂肪肝群に比し有意に高値であった．

対象とした．なお，全例HCV抗体・HBs抗原陰性，また飲酒量は20g/日以下であった．

NASH群と脂肪肝群では年齢・性別・体重・合併症の頻度に有意差を認めなかったが，NASH群ではAST値やヒアルロン酸・血小板数など，より肝病変の進展を示唆する血液検査所見であった．また，血中インスリン値やHomeostasis of model assessment-insulin resistance(HOMA-IR)によるインスリン抵抗性も，よりNASH群に強く，さらには各種血液鉄関連マーカーや肝内鉄量もNASH群でより高値であった．つまり，NASH群ではより鉄過剰状態にあると思われた．

図4に典型的なNASHと脂肪肝における肝内8-OHdG染色を示す．NASH症例では比較的強く肝細胞核内に8-OHdG陽性シグナルを認めるのに対し，脂肪肝症例においては，比較的その染色性は弱く，全例の比較で8-OHdG陽性肝細胞数に有意差を認めた(median＝17.5 vs. 2.0 cells/10^5 μm^2)(図4-C)．そこでNASH群において肝内8-OHdG量と各種臨床データーとの相関をみてみると，8-OHdGと血清鉄値や肝内鉄量とは有意な正の相関関係にあった(図5-A, B)．さらに8-OHdG量は血糖値やHOMA-IR, 肝内脂肪量とも正の相関関係にあった(図5-C, D, E)．そしてNASH群ではインスリン抵抗性と肝内鉄量も正の相関関係にあった(図5-F)．つまり，NASHにおいては肝脂肪化－インスリン抵抗性－鉄過剰が，酸化ストレスの発生を介して，それぞれがそれぞれに作用しあい，より肝病変を進展させている可能性が想定される(図6)．そして，NASHにおいてもこのような酸化ストレスを介した発癌機構の存在の可能性が考えられる．しかし，今回のNASH対象症例からは観察期間が短いためか(2.9±1.3年)，現在のところいまだ発癌例は認めていない．

2．NASHにおける除鉄療法の効果

上記の肝脂肪化－インスリン抵抗性－鉄過剰のcrosstalkによる肝病態への関与はC型慢性肝炎にみられるそれと同様である[22)~24)]．C型慢性肝炎においては瀉血[25)26)]や鉄制限食[27)]といった除

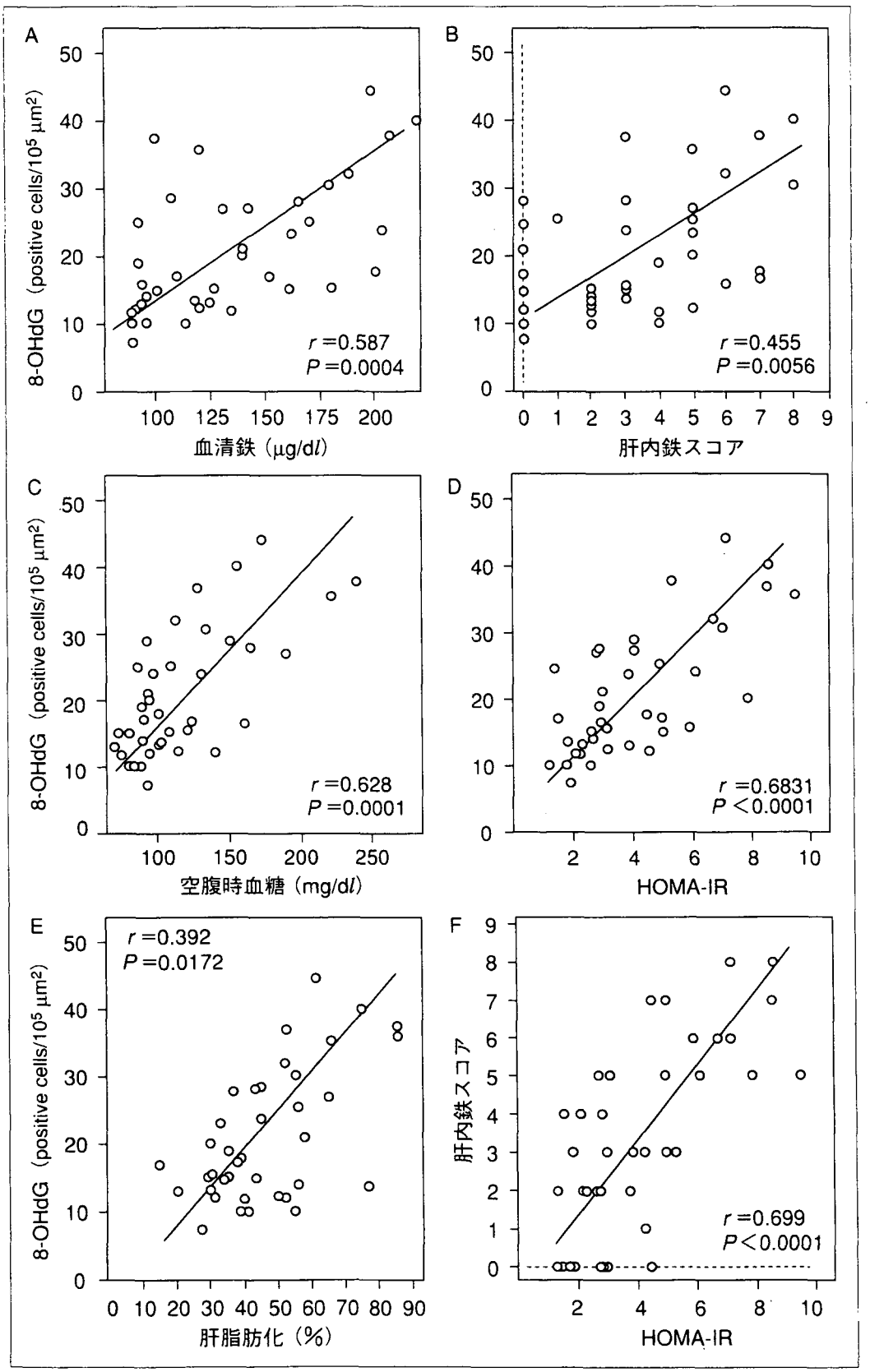

図5　NASHにおける肝内8-OHdG量と各種検査値との相関関係
NASH患者38例において免疫染色による肝内8-OHdG量は血清鉄(A)，肝内鉄量(B)，空腹時血糖(C)，インスリン抵抗性(HOMA-IR)(D)，肝内脂肪化の程度(E)と正の相関関係にあった．さらにNASHにおいてはインスリン抵抗性と肝内鉄量も有意な相関関係にあった(F)．

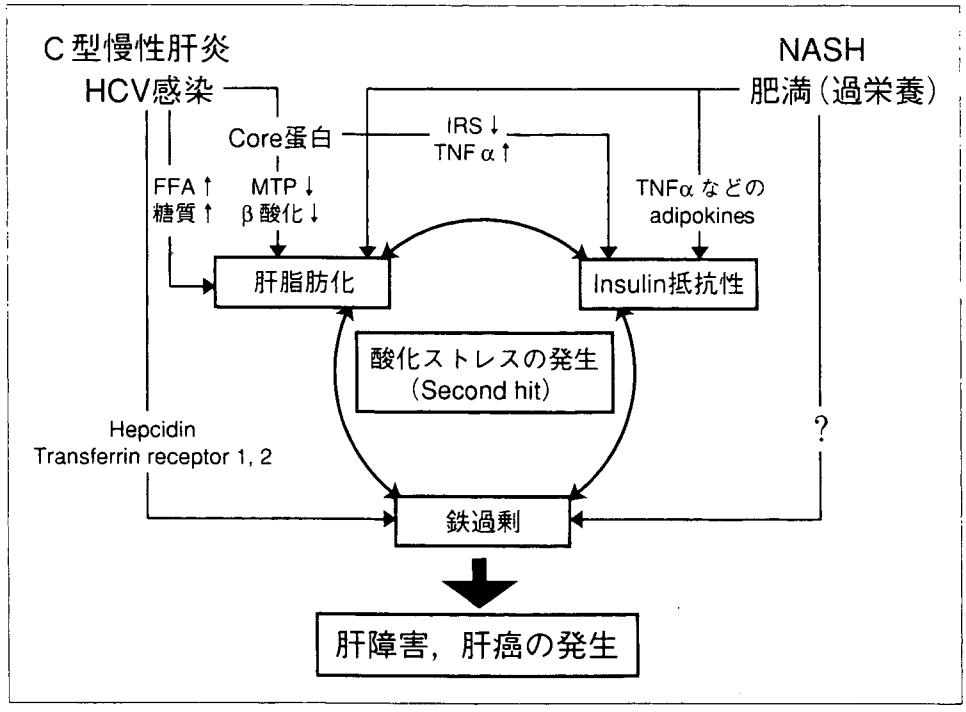

図6　C型慢性肝炎とNASHにおける肝内酸化ストレスの発生を介した肝病態進展のメカニズム

C型慢性肝炎においては過栄養による肝脂肪化の発生に加えて，core蛋白などによる直接的な脂肪化やインスリン抵抗性の発生が指摘されている．さらに，C型肝炎においてはさまざまな鉄調整関連分子の発現異常によって鉄過剰が発生することがわかっている[20)21)]．そしてこれらが互いの増悪因子となるmalignant cycleを形成しながら，酸化ストレスの発生を介して肝病態を進行させ，肝発癌に至る．NASHにおいても同様の病態形成機構が働いているものと想定されるが，鉄過剰発生のメカニズムは不明である．

鉄療法がALT値や肝組織所見を改善することが知られているが，さらには肝内8-OHdG量が低下するといった報告もみられる[16)28)]．そこで上記のNASH群の中でダイエット療法の反応性に乏しい11例に対し，瀉血＋鉄制限食の除鉄療法を施し，その前後にて肝内8-OHdG量を評価した．虚血性心疾患などのリスクを考慮し，全例70歳未満，血清フェリチン値高値，また明らかな心疾患のないものとした(表4)．方法は既報のごとく[29)]フェリチン値50ng/mlもしくはヘモグロビン値12g/dlを目標に，1回200mlを1～2週ごとに瀉血を行った．その結果，約1年の経過にてALT値は1例を除いて低下し，その平均値は有意に低下した．また，肝内8-OHdG量も10例に低下を認め，やはりその平均は有意に低下した．なお，とくに重篤な合併症などは認めなかった．

考　案

NASHにおいては鉄過剰蓄積を高頻度に認め，これが肝脂肪化やインスリン抵抗性とのcrosstalkを介して肝内に酸化ストレスを誘導し，肝発癌に関与している可能性を示した．近年，さまざまな鉄吸収関連分子やその調整機構が解明されつつあり，NASHにおける鉄過剰蓄積の原因機序の解明が待たれる．現在NASHに対しては，食事や運動療法以外の有効な薬物療法は確立されておらず，その対策が急務である．今後，鉄過剰を含めたNASH発症の根本的な病態および病因の解明が進み，機序に基づいた，より特異的で有効な治療法の開発が進むことを期待したい．

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