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Original Article

Analysis of hepatic genes involved in the metabolism of fatty acids and iron in nonalcoholic fatty liver disease

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Aims: Hepatic steatosis and iron cause oxidative stress, thereby progressing steatosis to steatohepatitis. We quantified the expression of genes involved in the metabolism of fatty acids and iron in patients with nonalcoholic fatty liver disease (NAFLD).

Methods: The levels of transcripts for the following genes were quantified from biopsy specimens of 74 patients with NAFLD: thioredoxin (Trx), fatty acid transport protein 5 (FATP5), sterol regulatory element-binding protein 1c (SREBP1c), fatty acid synthase (FASN), acetyl-coenzyme A carboxylase (ACAC), peroxisome proliferative activated receptor α (PPARα), cytochrome P-450 2E1 (CYP2E1), acyl-coenzyme A dehydrogenase (ACADM), acyl-coenzyme A oxidase (ACOX), microsomal triglyceride transfer protein (MTP), transferrin receptor 1 (TfR1), transferrin receptor 2 (TfR2) and hepcidin. Twelve samples of human liver RNA were used as controls. Histological evaluation followed the methods of Brunt.

Results: The levels of all genes were significantly higher in the NAFLD patients than in controls. The Trx level increased as the stage progressed. The levels of FATP5, SREBP1c, ACAC, PPARα, CYP2E1, ACADM and MTP significantly decreased as the stage and grade progressed ($P < 0.05$). Hepatic iron score (HIS) increased as the stage progressed. The TfR1 level significantly increased as the stage progressed ($P < 0.05$), whereas TfR2 level significantly decreased ($P < 0.05$). The ratio of hepcidin mRNA/ferritin ($P < 0.001$) or hepcidin mRNA/HIS ($P < 0.01$) was significantly lower in NASH patients than simple steatosis patients.

Conclusions: Steatosis-related metabolism is attenuated as NAFLD progresses, whereas iron-related metabolism is exacerbated. Appropriate therapies should be considered on the basis of metabolic changes.

Key words: fatty acids, iron, NAFLD, oxidative stress

Abbreviations
Trx, thioredoxin; FATP5, fatty acid transport protein 5; SREBP1c, sterol regulatory element-binding protein 1c; FASN, fatty acid synthase; ACAC, acetyl-coenzyme A carboxylase; PPARα, peroxisome proliferative activated receptor α; CYP2E1, cytochrome P-450 2E1; ACADM, acyl-coenzyme A dehydrogenase; ACOX, acyl-coenzyme A oxidase; MTP, microsomal triglyceride transfer protein; TfR1, transferrin receptor 1; TfR2, transferrin receptor 2.

INTRODUCTION

NON ALCOHOLIC FATTY liver disease (NAFLD) is a wide-spectrum liver disease, ranging from simple steatosis to steatohepatitis.[1] Owing to the obesity epidemic, NAFLD is now recognized as a leading health problem worldwide.[1] Since NAFLD has been documented to progress to liver failure[2] and/or hepatocellular carcinoma,[3] various therapeutic studies for NAFLD or nonalcoholic steatohepatitis (NASH) have been conducted to date.[4–8] These studies included weight reduction,[4] use of insulin sensitizers,[5] antioxidants,[6] phlebotomy[7] and hepato-protective drugs,[8] albeit with limited success. Although these treatments are aimed at addressing the pathogenesis of NAFLD, they would not always be efficient at every stage of this "wide spectrum" disease.

NASH is thought to develop through a "two-hit theory".[9] The first hit includes insulin resistance, mostly due to obesity.[9] The second hits include oxidative stress, inflammatory cytokines, and bacterial endotoxin.[9] In particular, the accumulation of fatty acids in the liver results in oxidative stress through oxidation of fatty

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acids.[10] In addition, hepatic iron load, which also induces oxidative stress, has been reported in some groups of patients with NAFLD.[11] Therefore, hepatic metabolism of fatty acids and iron should be the therapeutic target for NAFLD. However, their roles in the development of NAFLD have not yet been studied

In this study, we quantified the expression of genes involved in hepatic metabolism of fatty acids and iron using liver biopsy specimens from patients with NAFLD, and compared them with liver histology. Based on the results, we explored the role of the metabolism of fatty acids and iron in NAFLD. Our study should improve out understanding of the pathogenesis of NAFLD and contribute to the identification of putative therapeutic pathways.

PATIENTS AND METHODS

Patients

NAFLD PATIENTS WHO underwent liver biopsies in our institute between April 2000 and March 2007 were retrospectively selected according to the following criteria: no excessive alcohol intake (more than 20 g/day), as assessed by interview (on at least three occasions); no history of treatment with steatosis-inducing drugs within the 12 months prior to the study; negative serum hepatitis C virus (HCV) antibody; negative for hepatitis B surface antigen or antibodies to human immunodeficiency virus; and an absence of other forms of chronic liver disease, such as autoimmune liver diseases. Anthropometry and laboratory data were collected from all patients at the time of the liver biopsy. All patients had given written informed consent for the analysis of metabolic genes and liver biopsies before the study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of the Kyoto Prefectural University of Medicine.

Laboratory determinations

After a 12-h overnight fast, venous blood samples were drawn to determine asparatate aminotransferase (AST), alanine aminotransferase (ALT), albumin, total cholesterol, triglyceride, fasting plasma glucose (FPG), glycosylated haemoglobin (HbA_{1c}), insulin and ferritin levels. These parameters were measured using standard techniques from clinical chemistry laboratories. The index of insulin resistance was calculated only in patients without overt diabetes (fasting plasma glucose >126 mg/dL), according to the homeostasis model assessment (HOMA).

Histological evaluation

Formalin-fixed and paraffin-embedded liver biopsy specimens were stained with hematoxylin–eosin, Masson's trichrome, and Perl's Prussian blue. The stage of hepatic fibrosis was scored according to Brunt[12]: 1, zone 3 fibrosis; 2, zone 3 fibrosis with periportal fibrosis; 3, bridging fibrosis; and 4, cirrhosis. The grade of inflammation was scored as follows[12]: 1, mild; 2, moderate; and 3, severe. We considered the scores of stage and grade of simple steatosis as "0". Steatosis was assessed according to the percentage of hepatocytes containing fat droplets. The degree of iron loading was graded using a Perl's score of 0–4, as described previously.[13]

Quantification of the expression of hepatic genes

Liver specimens were immediately frozen after the biopsy and were stored at −80°C until use. Total RNA was isolated from biopsy specimens using the RNeasy kit (Qiagen, Hilden, Germany). First-strand cDNA was obtained from total RNA using the QuantiTect Reverse Transcription kit (Qiagen). PCR was performed using the Light Cycler 2.0 System (Roche, Mannheim, Germany), and the mRNA levels were normalized to those of β-actin. Comprehensive target genes were as follows: thioredoxin (Trx), fatty acid transport protein 5 (FATP5), sterol regulatory element-binding protein 1c (SREBP1c), fatty acid synthase (FASN), acetyl-coenzyme A carboxylase (ACAC), peroxisome proliferative activated receptor α (PPARα), cytochrome P-450 2E1 (CYP2E1), acyl-coenzyme A dehydrogenase, C4 to C12 straight chain (ACADM), acyl-coenzyme A oxidase (ACOX), microsomal triglyceride transfer protein (MTP), transferrin receptor 1 (TfR1), transferrin receptor 2 (TfR2) and hepcidin. Table 1 summarizes the specific primers for these target genes. Twelve samples of human total liver RNA were obtained from commercial sources (Stratagene, CA, USA; Clontech Laboratories, CA, USA; Ambion, TX, USA; Becton, Dickinson, NJ, USA; Cell Applications, CA, USA), and used as controls.

Statistical analysis

Associations between variables were analyzed using the Spearman's correlation coefficient by rank. Differences between variables were analyzed using the Mann–Whitney U-test or Kruskal–Wallis test. All analyses were performed using SPSS software for Windows, version

Table 1 The specific primers used for the target genes

	Sense primers	Antisense primers
Trx	5′-CTGCTTTTCAGGAAGCCTTG-3′	5′-ACCCACCTTTTGTCCCTTCT-3′
FATP5	5′-ACACACTCGGTGTCCCTTTC-3′	5′-CTACAGGGCCCACTGTCATT-3′
SREBP1c	5′-TGCATTTTCTGACACGCTTC-3′	5′-CCAAGCTGTACAGGCTCTCC-3′
FASN	5′-TTCCGAGATTCCATCCTACG-3′	5′-TGTCATCAAAGGTGCTCTCG-3′
ACAC	5′-GAGAACTGCCCTTTCTGCAC-3′	5′-CCAAGCTCCAGGCTTCATAG-3′
PPARα	5′-GGAAAGCCCACTCTGCCCCCT-3′	5′-AGTCACCGAGGAGGGGCTCGA-3′
CYP2E1	5′-CCCAAAGGATATCGACCTCA-3′	5′-AGGGTGTCCTCCACACACTC-3′
ACADM	5′-TTGAGTTCACCGAACAGCAG-3′	5′-AGGGGGACTGGATATTCACC-3′
ACOX	5′-TGATGCGAATGAGTTTCTGC-3′	5′-AGTGCCACAGCTGAGAGGTT-3′
MTP	5′-CATCTGGCGACCCTATCAGT-3′	5′-GGCCAGCTTTCACAAAAGAG-3′
TfR1	5′-ATGCATTTTGCAGCAGTGAG-3′	5′-TCCAAAAGGCCCTACTCCTT-3′
TfR2	5′-GACCCTGCAGTGGGTGTACT-3′	5′-CAGTCGCTCGTCTCTCTCCT-3′
hepcidin	5′-ACCAGAGCAAGCTCAAGACC-3′	5′-AAACAGAGCCACTGGTCAGG-3′

Note: The role of genes analyzed in lipid and iron metabolisms is as follows: oxidative stress-induced, Trx; uptake of fatty acid, FATP5; synthesis of fatty acid, SREBP1c, FASN, ACAC; oxidation of fatty acid, PPARα, CYP2E1, ACADM, ACOX; secretion of triglyceride, MTP; uptake of transferrin-bound iron, TfR1, TfR2; regulation of iron metabolism, hepcidin.
Trx, thioredoxin; FATP5, fatty acid transport protein 5; SREBP1c, sterol regulatory element-binding protein 1c; FASN, fatty acid synthase; ACAC, acetyl-coenzyme A carboxylase; PPARα, peroxisome proliferative activated receptor α; CYP2E1, cytochrome P-450 2E1; ACADM, acyl-coenzyme A dehydrogenase; ACOX, acyl-coenzyme A oxidase; MTP, microsomal triglyceride transfer protein; TfR1, transferrin receptor 1; TfR2, transferrin receptor 2.

14.0 (SPSS, Chicago, IL, USA). A P value of less than 0.05 was considered significant.

RESULTS

The characteristics of patients

TABLES 2 AND 3 summarize the characteristics of patients and the results of liver histology, respectively. Of the 16 diabetic patients, 3 had been treated with metformin, 2 with pioglitazone, 2 with sulfonylurea, and the others had been followed with diet restriction. Serum triglyceride levels were greater in the simple steatosis patients than in the NASH patients. Although the values of HbA_{1c} were comparable in the two groups, those of HOMA-IR [index of insulin resistance (IR)] were significantly higher in the NASH

Table 2 Patients characteristics

	Simple steatosis ($n = 33$)	NASH ($n = 41$)	P value
Age	55.4 ± 15.0	61.2 ± 12.7	0.051
BMI (kg /m^2)	27.5 ± 2.4	26.5 ± 4.4	0.748
Sex (male/female)	24/9	25/16	0.208
Diabetes (yes/no)	7/26	9/32	0.584
Plt	21.6 ± 3.9	19.1 ± 6.3	0.006
AST	43.0 ± 21.4	72.9 ± 30.5	0.0002
ALT	62.3 ± 30.8	89.8 ± 50.3	0.006
Alb	4.7 ± 0.3	4.6 ± 0.3	0.023
T-Cho	231.1 ± 50.5	199.9 ± 44.0	0.006
TG	205.0 ± 105.8	140.9 ± 103.2	0.015
FPG	145.1 ± 68.4	116.7 ± 21.5	0.356
HbA_{1c}	6.6 ± 1.8	6.0 ± 0.6	0.533
HOMA-IR	2.9 ± 1.2	4.6 ± 1.8	0.012
ferritin	223.1 ± 106.0	197.7 ± 160.7	0.227

Note: The value is expressed as either mean ± S.D. or the number of patients.
ALT, alanine aminotransferase; AST, asparatate aminotransferase; Alb, albumin; BMI, body mass index; FPG, fasting plasma glucose; HbA_{1c}, glycosylated haemoglobin; HOMA-IR, homeostasis model assessment-index of insulin resistance; T-Cho, total cholesterol; TG, triglyceride.

Table 3 Results of liver biopsy

	Simple steatosis	NASH
Stage: 1/2/3/4		13/13/13/2
Grade: 1/2/3		27/10/4
Iron: 0/1/2/3	11/12/3/1	14/8/6/6
Steatosis:		
<30%	14	18
30%–60%	7	13
60% <	2	10

NASH, nonalcoholic steatohepatitis.

patients than in the simple steatosis patients. Neither significant fibrosis nor inflammation was observed in the biopsy specimens from patients with simple steatosis. Six specimens from simple steatosis patients and seven specimens from NASH patients were not available for iron staining.

Hepatic oxidative stress

We evaluated hepatic oxidative stress by the level of hepatic Trx, since Trx is known to be a redox-sensitive molecule.[14] We have previously reported that serum Trx levels are a marker of NASH.[15] We measured hepatic thioredoxin mRNA, because it would reflect the redox status of the liver more precisely than serum thioredoxin levels. Hepatic thioredoxin consists of both reduced and oxidized forms, whereas serum thioredoxin is an oxidized form. Therefore, hepatic thioredoxin levels do not correlate with serum thioredoxin levels. The Trx level increased in the order of controls, then simple steatosis patients with the highest levels in NASH patients (Table 4). The differences among the groups were significant (Table 4). The Trx level tended to increase as the stage progressed; however, it did not show any association with the grade (Table 5).

Fatty acid metabolism

The levels of transcripts for the genes involved in fatty acid metabolism were increased in the order of controls, then NASH patients with the highest levels in simple steatosis patients (Table 4). The differences among the groups were significant (Table 4). When values were compared between simple steatosis and NASH patients by the Mann–Whitney's test, the difference was significant in FATP5 ($P < 0.01$), ACAC ($P < 0.05$), PPARα ($P < 0.05$), CYP2E1 ($P < 0.05$), ACADM ($P < 0.05$), ACOX ($P < 0.05$), MTP ($P < 0.05$). Levels of all these genes were significantly higher in the simple steatosis patients than the NASH patients. When compared with the liver histology, the levels of FATP5, SREBP1c, ACAC, PPARα, CYP2E1, ACADM and MTP significantly decreased as the stage and grade progressed (Table 5). The level of ACOX tended to decrease as the stage and grade progressed (Table 5). The level of FASN was similarly decreased, although the difference between groups

Table 4 The levels of hepatic gene involved in lipid and iron metabolism

	Control	Simple steatosis	NASH	*P* value
Trx	1.0 ± 1.1	2.3 ± 0.9	2.5 ± 1.0	$P < 0.00001$
FATP5	1.0 ± 0.4	6.1 ± 3.6	4.3 ± 2.5	$P < 0.00001$
SREBP1c	1.0 ± 0.6	73.9 ± 74.3	56.0 ± 85.4	$P < 0.00001$
FASN	1.0 ± 1.0	28.2 ± 26.8	17.8 ± 15.1	$P < 0.00001$
ACAC	1.0 ± 0.8	12.2 ± 5.9	8.7 ± 3.4	$P < 0.00001$
PPARα	1.0 ± 0.8	21.1 ± 11.3	15.5 ± 8.1	$P < 0.00001$
CYP2E1	1.0 ± 0.4	8.0 ± 4.2	6.2 ± 3.2	$P < 0.00001$
ACADM	1.0 ± 0.9	17.8 ± 9.7	13.1 ± 6.1	$P < 0.00001$
ACOX	1.0 ± 0.9	16.6 ± 9.2	12.0 ± 5.7	$P < 0.00001$
MTP	1.0 ± 1.0	10.8 ± 3.8	8.8 ± 3.3	$P < 0.00001$
TfR1	1.0 ± 1.1	10.8 ± 11.3	11.8 ± 10.3	$P < 0.00001$
TfR2	1.0 ± 0.4	7.6 ± 3.6	5.6 ± 2.8	$P < 0.00001$
hepcidin	1.0 ± 0.9	11.2 ± 9.6	5.7 ± 3.9	$P < 0.00001$

Note: The value is expressed as folds to mean control values (mean ± S.D.). The deference between the groups was determined using the Kruskal–Wallis test.

Trx, thioredoxin; FATP5, fatty acid transport protein 5; SREBP1c, sterol regulatory element-binding protein 1c; FASN, fatty acid synthase; ACAC, acetyl-coenzyme A carboxylase; PPARα, peroxisome proliferative activated receptor α; CYP2E1, cytochrome P-450 2E1; ACADM, acyl-coenzyme A dehydrogenase; ACOX, acyl-coenzyme A oxidase; MTP, microsomal triglyceride transfer protein; TfR1, transferrin receptor 1; TfR2, transferrin receptor 2.

Table 5 Correlation of the gene levels with liver histology*

	Stage		Grade	
	r	*P* value	*r*	*P* value
Trx	0.209	0.074	0.132	0.266
FATP5	−0.334	0.004	−0.339	0.003
SREBP1c	−0.264	0.024	−0.283	0.015
FASN	−0.158	0.178	−0.182	0.124
ACAC	−0.264	0.024	−0.313	0.007
PPARα	−0.253	0.031	−0.244	0.038
CYP2E1	−0.264	0.024	−0.293	0.012
ACADM	−0.241	0.040	−0.246	0.036
ACOX	−0.213	0.070	−0.213	0.071
MTP	−0.262	0.025	−0.271	0.020
TfR1	0.227	0.037	0.182	0.089
TfR2	−0.307	0.008	−0.318	0.006
hepcidin	−0.251	0.032	−0.221	0.060

*Using Spearman's test. Trx, thioredoxin; FATP5, fatty acid transport protein 5; SREBP1c, sterol regulatory element-binding protein 1c; FASN, fatty acid synthase; ACAC, acetyl-coenzyme A carboxylase; PPARα, peroxisome proliferative activated receptor α; CYP2E1, cytochrome P-450 2E1; ACADM, acyl-coenzyme A dehydrogenase; ACOX, acyl-coenzyme A oxidase; MTP, microsomal triglyceride transfer protein; TfR1, transferrin receptor 1; TfR2, transferrin receptor 2.

did not reach statistical significance (Table 5). In parallel with these findings, the level of hepatic steatosis decreased as the stage and grade progressed (Fig. 1). None of these genes was independently correlated with hepatic steatosis (not shown).

TfR1 and TfR2

The hepatic iron score (HIS) tended to increase as the stage progressed (Table 6). We examined the levels of TfR1 and TfR2, since the uptake of serum iron by hepatocytes is largely through a transferrin-bound form.[16] The levels of both of these genes were significantly higher in the NAFLD patients than in the controls (Table 4). When values were compared between simple steatosis and NASH using the Mann–Whitney's test, the TfR2 level was significantly ($P < 0.01$) higher in the simple steatosis patients than the NASH patients. The TfR1 level significantly increased as the stage progressed, whereas that of TfR2 significantly decreased as the stage and grade progressed (Table 5). Neither TfR1 nor TfR2 were independently correlated with HIS (not shown).

Table 6 Hepatic iron score and the stage

	Hepatic iron score				
	0	1	2	3	4
Stage 0	11	11	3	0	1
Stage 1	7	1	1	1	0
Stage 2	3	4	3	2	0
Stage 3	4	4	2	2	0
Stage 4	0	0	0	0	1

Note: The value represents the number of patients. Simple steatosis was considered as stage "0". $r = 0.213$, $P = 0.099$, iron score *vs* stage: Spearman's test.

Hepcidin

Hepcidin is known to be secreted from hepatocytes and regulates systemic iron transport.[16] The hepcidin level was significantly different among the controls, the simple steatosis patients and the NASH patients. The value was higher in the simple steatosis patients than in the NASH patients (Table 4). Hepcidin level decreased significantly as the stage progressed (Table 5). Since the ratio of hepcidin to iron load has been reported to evaluate the appropriateness of the hepcidin response to iron overload,[17] we divided hepcidin mRNA levels by serum ferritin levels or HIS. The ratios of hepcidin mRNA/ferritin and hepcidin mNA/HIS were signifi-

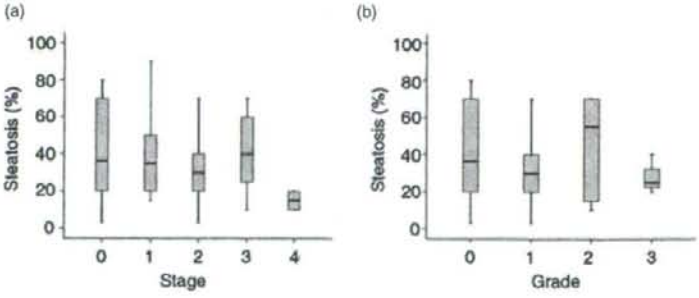

Figure 1 Distributions of the level of hepatic steatosis in association with the stage (a) and grade (b). The level of steatosis decreased as the stage and grade progressed.

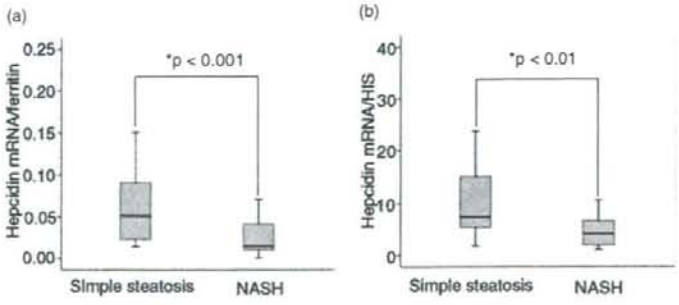

Figure 2 The ratio of hepcidin mRNA levels to serum ferritin levels (a) and that of hepcidin mRNA levels to hepatic iron score (HIS) (b). Hepcidin mRNA levels corrected for iron overload were significantly lower in NASH patients than in simple steatosis patients. *Mann-Whitney U-test.

cantly lower in NASH patients than simple steatosis patients (Fig. 2). The ratio of hepcidin mRNA/ferritin was significantly correlated with stage ($r = -0.523$, $P < 0.00005$) and grade ($r = -0.436$, $P < 0.0005$). The same results were obtained from the ratio of hepcidin mRNA/HIS ($r = -0.424$, $P < 0.01$ vs stage; $r = -0.373$, $P < 0.05$ vs grade). We compared hepcidin mRNA levels with metabolic variables and found that the level of hepcidin was significantly correlated with both total cholesterol ($r = 0.323$, $P < 0.01$) and triglyceride ($r = 0.323$, $P < 0.01$). The ratio of hepcidin mRNA/ferritin was also significantly correlated with total cholesterol ($r = 0.365$, $P < 0.005$).

DISCUSSION

IN THIS STUDY, we investigated the expression levels of hepatic genes that play significant roles in the metabolism of fatty acids and iron. Their roles in hepatocytes include the uptake, synthesis, oxidation, storage and excretion of fatty acids,[10,18,19] the uptake of iron and the regulation of systemic iron transport.[16] We found that the levels of these genes were significantly higher in NAFLD patients than controls. In addition, we found some novel findings. However, none of the individual genes was independently correlated with hepatic steatosis. These results indicated that neither the lack of nor increase in the expression levels of any of these genes plays an independent role in the development of fatty liver.

Insulin resistance is the "first hit" in the development of NASH,[9] which is characterized by an increase in the uptake and synthesis of fatty acids in hepatocytes.[19] Nevertheless, our results showed that the levels of fatty acid-related genes decreased in the later stages despite the presence of insulin resistance. In parallel with these findings, the level of hepatic steatosis also decreased. Considering that fat is the fuel involved in progressive liver injuries,[20] these findings might be associated with "burnout" NASH.[21] Although the underlying reason for this is unclear, some possibilities should be considered. Because hepatic adenosine 5'-triphosphate (ATP) levels tend to be decreased in fatty liver,[22] hepatic adenosine monophosphate-activated protein kinase (AMPK) should be activated.[23] AMPK is known to activate catabolic pathways and switch off protein, carbohydrate and lipid synthesis, such that cellular energy levels remain unchanged.[23] Thus, activated AMPK in hepatocytes might contribute to the decrease in the expression levels of fatty acid-related genes. Anti-diabetic drugs, which ameliorate liver injuries in patients with NASH, have been reported to activate AMPK.[24] Interestingly, the levels of all the genes involved in fatty acid metabolism were lower in the patients treated with insulin sensitizers than in those treated with other agents or followed with diet restriction. Statistical significance was achieved only in FATP5 ($P < 0.05$, Mann–Whitney's test). However, these results may be difficult to evaluate or apply generally, because the numbers of patients were small.

Hepatic iron load has been documented to be another key player in the progression from steatosis to steatohepatitis.[11] Hepatic iron load has been attributed to the Cys282Tyr mutation in the hemochromatosis gene.[11] This mutation decreases hepatic synthesis of hepcidin, resulting in the facilitation of iron absorption from the duodenum.[16] Our results showed that hepatic iron scores tended to correlate with the histological stage of NAFLD. Furthermore, the ratios of hepcidin mRNA/ferritin and hepcidin mRNA/HIS were significantly lower in NASH patients than in simple steatosis patients. This insufficient production of hepcidin may not be attributed to the genetic mutation, since known mutations of hemochromatosis-associated genes have been reported to be rare among Japanese patients.[25]

Interestingly, the hepcidin level was significantly correlated with the levels of total cholesterol and triglycerides. These findings coincide with those recently reported by Barisani *et al.*,[17] who reported that the hepcidin mRNA/ferritin ratio and the hepcidin mRNA/tissue iron score ratio were significantly lower in the NAFLD group with hepatic iron overload than in the NAFLD group without iron overload,[17] and that the level of hepatic hepcidin mRNA was significantly correlated with lipid parameters.[17] Our findings, in concert with those of Barisani *et al.*, suggest that more severe forms of NAFLD are associated with insufficient hepcidin production, and that lipid metabolism might be involved in hepcidin synthesis. Alternatively, the hepatic levels of TfR1 and TfR2 were significantly higher in NAFLD patients than controls. Therefore, TfR1 and TfR2 would be expected to promote hepatic iron load irrespective of iron absorption from the duodenum.

TfR1 is ubiquitously expressed in the human body,[16] while TfR2 is dominantly expressed in specific organs including the liver.[26] TfR1 has a high affinity with transferrin[27] and its expression is regulated by the iron-responsive element (IRE) in the 3′-untranslated regions of mRNAs.[16] In the NAFLD patients, the TfR1 level increased significantly as the stage progressed. Since ROS stabilize TfR1 mRNA via activation of iron regulatory proteins that interact with IRE,[16] hepatic oxidative stress should upregulate TfR1 in NAFLD.

TfR2 was recently identified as a novel transferrin receptor,[28] although the expression mechanisms have not been fully determined.[28] Similarly, neither the physiological nor pathological role of TfR2 in the liver has been documented. The expression level of TfR2 was higher in NAFLD patients than controls. At present, the association between the level of TfR2 and the pathogenesis of NAFLD remains unknown. Regardless of the role of TfR2, we have reported that the TfR2 level is significantly correlated with that of PPARα.[29] It is of much interest to speculate that PPARα might contribute to the regulation of TfR2, since PPARα may be upregulated in NAFLD by intrinsic PPARα ligands. This hypothesis is under investigation in our institute.

In summary, we investigated the metabolism of fatty acids and iron in the livers of NAFLD patients. Steatosis-related metabolism is attenuated as the disease progresses, whereas iron load-related metabolism is exacerbated. Based on these findings, we hypothesize that anti-lipid synthesis should be considered in the early stages and that iron reduction should be considered in the later stages. The former therapies may thus include body weight reduction and insulin-sensitizing drugs, and the latter therapies may include phlebotomy, iron-restriction diets and/or antioxidants.

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Iron overload and cofactors with special reference to alcohol, hepatitis C virus infection and steatosis/insulin resistance

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Abstract

There are several cofactors which affect body iron metabolism and accelerate iron overload. Alcohol and hepatic viral infections are the most typical examples for clarifying the role of cofactors in iron overload. In these conditions, iron is deposited in hepatocytes and Kupffer cells and reactive oxygen species (ROS) produced through Fenton reaction have key role to facilitate cellular uptake of transferrin-bound iron. Furthermore, hepcidin, antimicrobial peptide produced mainly in the liver is also responsible for intestinal iron absorption and reticuloendothelial iron release. In patients with ceruloplasmin deficiency, anemia and secondary iron overload in liver and neurodegeneration are reported. Furthermore, there is accumulating evidence that fatty acid accumulation without alcohol and obesity itself modifies iron overload states. Ineffective erythropoiesis is also an important factor to accelerate iron overload, which is associated with diseases such as thalassemia and myelodysplastic syndrome. When this condition persists, the dietary iron absorption is increased due to the increment of bone marrow erythropoiesis and tissue iron overload will thereafter occurs. In porphyria cutanea tarda, iron is secondarily accumulated in the liver.

Key words: Iron overload; Cofactors; Alcohol; Chronic hepatic C; Non-alcoholic steatohepatitis; Insulin resistance; Hepatocellular carcinoma

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INTRODUCTION

In hereditary hemochromatosis, patients having HFE trait are more susceptible to iron overload when cofactors such as alcohol, hepatitis viruses, and abnormal porphyrin metabolism are present. Even in the absence of hereditary hemochromatosis, there are several conditions associated with secondary iron overload in which iron deposition is rather mild[1]. For example, in alcoholics and patients with chronic hepatitis C, intrahepatic iron is increased and liver injury is accelerated, followed by development of fibrosis, cirrhosis and hepatocellular carcinoma (HCC). In addition, abnormal copper metabolism and several causes for iron-loaded anemia are also important cofactors which influence the background iron overload. Furthermore, there is accumulating evidences that fatty acid accumulation without alcohol and obesity itself modifies insulin resistance through iron[2] and fibrogenesis of the liver[3]. In this review, the role of cofactors on iron overload will be discussed in three categories such as alcohol, hepatitis C virus infection and steatosis with obesity, the most common cofactors in liver iron overload.

COFACTORS AFFECTING BODY IRON METABOLISM AND IRON OVERLOAD

There are several factors which affect body iron metabolism and accelerates iron overload. Table 1 lists cofactors and disease conditions which are known to accelerate hepatic iron accumulation independent from responsible genes for hereditary hemochromatosis. Alcoholic and hepatic viral infections are the most typical examples for clarifying the role of cofactors in iron overload. In addition, abnormal copper metabolism and several causes for iron-loaded anemia such as thalassemia and myelodysplastic syndrome are also important factors which influence the background iron overload. When this condition persists, the dietary iron absorption is increased due to the increment of bone marrow erythropoiesis[4] and tissue iron overload will occur thereafter. These patients are usually anemic in spite of increased body iron stores (iron-

Table 1 Cofactors of iron overload

1	Alcohol (Alcoholic liver disease)
2	Infection (Hepatitis C virus infection, *etc*)
3	Obesity and insulin resistance (Nonalcoholic steatohepatitis)
4	Copper (Ceruloplasmin deficiency)
5	Porphyrin (Porphyria)
6	Ineffective erythropoiesis (Thalassemia, myelodysplastic syndrome)
7	Others

loaded anemia), and require frequent blood transfusions, which further exaggerate secondary iron overload, in which conditions of new oral iron chelators are effective[5]. In patients with ceruloplasmin deficiency, anemia and secondary iron overload in liver and neurodegeneration are reported[6]. Furthermore, there are accumulating evidences that fatty acid accumulation without alcohol and obesity itself modifies iron overload states. Ineffective erythropoiesis is also an important factor to accelerate iron overload. This condition is associated with diseases such as thalassemia, aplastic anemia, and myelodysplastic syndrome. In porphyria cutanea tarda, iron is secondarily accumulated in the liver and phlebotomy and oral iron chelators are effective as well as in hemochromatosis.

ALCOHOL

Alcohol is one of the most important cofactors to modify or enhance iron accumulation in the liver. Excess intake of alcohol induces alcoholic liver diseases (ALD) such as fatty liver, fibrosis, hepatitis, and cirrhosis, in which iron overload is frequently associated[7]. By Perls' iron stain, excess iron accumulation was found in hepatic tissues with ALD, but not in any normal hepatic tissues[8]. In ALD, iron is deposited in both hepatocytes and reticuloendothelial (Kupffer) cells. In advanced cases of ALD, which is also called as "alcoholic siderosis", the reticuloendothelial iron deposition is dominant. In earlier stages of ALD such as fatty liver and fibrosis, iron deposition is mild and is preferentially present in hepatocytes rather than in Kupffer cells, which finding is more frequently observed in Japanese patients who have mild clinical phenotype comparing with those in US[9].

The reactive oxygen species (ROS) produced play an important role in the development of ALD[10]. The expression of 4-hydroxy-2-nonenal (HNE)-protein adducts, which is a lipid peroxidative product is increased in oxidized hepatocytes[11]. Chronic alcohol ingestion in experimental animals is associated with oxidative stress as reflected by increased hepatic levels of lipid peroxidation products such as malondialdehyde and HNE, both of which have been implicated in hepatic fibrogenesis in the intragastric ethanol infusion model[12]. Furthermore, lipid peroxidation products induce gene expression of procollagen α-1 (I) and increase collagen production by several folds in cultured hepatic stellate cell[13]. In human ALD, there is a positive correlation between iron deposition and histological intensity of HNE-protein adduct[14]. As shown in Figure 1, the distribution of HNE-protein adducts and iron granules appeared to be identical, suggesting that iron may be associated with the production of HNE-protein adduct. As hepatic iron is visualized by Perls' reaction as an insoluble protein-bound iron such as hemosiderin, this form of iron may be inactive for the production of ROS. But, the free iron responsible for Fenton reaction should be present close to the protein-bound iron, and may be involved in the production of HNE-protein adducts. There are two pathways to generate ROS through ethanol metabolism. Oxidation of ethanol by alcohol dehydrogenase to form acetaldehyde, which is subsequently oxidized to acetate and ultimately carbon dioxide and water. During the oxidation process of acetaldehyde involving aldehyde oxidase and xanthine oxidase, superoxide (O_2^-) is produced[15]. In addition, cytochrome P450 is involved in the metabolism of ethanol, in which ROS are also generated in microsomes[16]. Among ROS, hydroxy radical (OH) is most potent, which is produced via Fenton reaction in the presence of free iron and the resulted OH can easily cause cell damage by oxidizing lipid, proteins, and nucleic acids. In an intragastric infusion mouse model of ALD, supplementation of carbonyl iron advanced peri-venular fibrosis to bridging fibrosis and cirrhosis[17]. Oxidative stress arising from hepatocytes and macrophage activates hepatic stellate cells by increasing the production of cytokines such as transforming growth factor-β (TGFβ), directly or indirectly. The dietary iron supplementation was associated with increased NF-κB activation[18], and the up

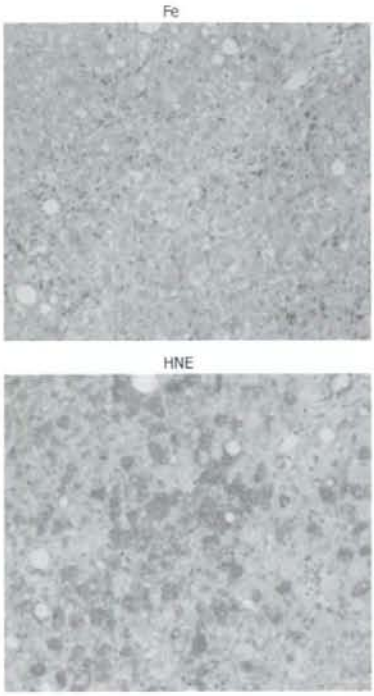

Figure 1 Iron staining and immunohistochemical staining of 4-hydroxy-2-nonenal-modified protein (HNE-protein) adducts in human alcoholic liver disease. The localization of HNE-protein adducts and iron in hepatocytes appeared to be identical (from ref. 14 with some modifications).

regulation of NF-κB responsive proinflammatory genes such as IL-1β, TNFα, and MIP-1[19].

In advanced cases of ALD, iron is accumulated more prominently in Kupffer cells than in hepatocytes, mainly due to repeated endotoxemia and hyper-cytokinemia of TNFα and IL-1β[20]. These cytokines induced hepatic uptake of transferrin iron *in vitro*[21] and *in vivo*[22]. In mild cases of ALD, iron is preferentially stained in hepatocytes, rather than in Kupffer cells, suggesting that hepatocyte is the main site of early iron storage in the liver. However, it is not clear why iron is accumulated in liver parenchymal cells of alcoholics in such conditions. Two possibilities can be drawn: one is the increased uptake of iron in hepatocytes, and another is the increased iron absorption through hepcidin, which is a newly found antimicrobial peptide, and is a negative regulator of iron absorption and reticuloendothelial iron releases[23]. Hepatocytes have several pathways for iron uptake. Iron in serum is usually bound to transferrin and iron-bound transferrin is taken up via transferrin receptor (TfR) with high affinity or via other unknown mechanism with greater capacity, but low affinity independent of high affinity receptor[24]. There are two molecules of transferrin receptor: transferrin receptor 1 (TfR1) and transferrin receptor 2 (TfR2). TfR1 has a high affinity to serum transferrin and considered to be functional, while the function of TfR2 is not clear yet, even though the TfR2 gene is responsible for genetic hemochromatosis[25]. In normal hepatocytes, TfR2 is constitutively expressed. But, TfR1 is down-regulated, suggesting that TfR1 does not contribute to the steady state hepatic iron uptake. Recently, Wallace *et al* reported that homozygous TfR2 knockout mice had no TfR2 associated with typical iron overload, and there was no upregulation of hepcidin mRNA, suggesting that TfR2 is required to iron regulated expression and is involved in a pathway to HFE and hemojuvelin[26]. In addition, DMT1 may be involved when serum iron concentration exceeds transferrin iron binding capacity[27]. It is noteworthy that TfR1 is regulated by cellular iron levels or oxidative stresses post-transcriptionally and it is possible that ethanol may augment TfR1 expression by producing oxidative stresses. According to immunohistochemical investigation, TfR1 expression was increased in hepatocytes in 80% of hepatic tissues with ALD, but was not detected in any normal hepatic tissues[28]. It is noteworthy that the mean duration of abstinence of patients who demonstrated positive TfR1 expression in hepatocytes was significantly shorter than that of patients who demonstrated negative TfR1 expression.

Ethanol exposure in the presence of iron to the primary cultured-hepatocytes demonstrated an increase of TfR expression, and this augmentation was suppressed by the inhibitor of alcohol dehydrogenase, 4-methyo pyrazole, but enhanced by a inhibitor of acetaldehyde dehydrogenase, cyanamide, suggesting that ethanol metabolite acetaldehyde itself is involved for the induction of TfR1 by ethanol[29]. By functional uptake assay using ^{59}Fe-transferrin, the additional ethanol exposure increased transferrin-iron uptake into hepatocytes, while non-transferrin-bound iron (NTBI) uptake[30] was not increased. It has been reported that TfR1 expression was up-regulated both transcriptionally[31] and posttranscriptionally[32]. This regulation is induced either by iron deficiency state or oxidative stress such as H_2O_2 and nitric oxide via iron regulatory protein, IRP[33]. In addition to the direct cell toxicity, acetaldehyde produces free radicals[34] and free radicals modify IRP activity[35,36].

Table 2 Speculated effects of iron on HCV

1	Immunological modification (Immunological escape of HCV)
	Decrease of Th1 activity
	Impaired function of macrophage and Kupffer cells
	Decrease of innate immunity (Natural resistance macrophage protein 2)
2	Increase of liver toxicity by iron-mediated radical formation
	Reactive oxygen production through fenton reaction
	Induction of apoptosis
	Acceleration of fibrinogenesis
	DNA damage and carcinogenesis
3	Effect on cell signalling
	Decrease of interferon responsiveness by NFκB activation
4	HCV proliferation
	Activation of translation initiation factor 3 (eIF3)
	Suppression of HCV RNA polymerase (NS5B) activity

Body iron homeostasis is strictly regulated by a balance between the processes such as dietary iron absorption in enterocytes, iron transport by transferrin in circulation, iron utilization and storage in bone marrow and liver. The increase of intestinal iron absorption was one of the mechanisms of the hepatic iron deposition in alcoholics[37]. In patients with hereditary hemochromatosis, serum pro-hepcidin is lower than that of normal controls, suggesting that iron absorption is increased in spite of high iron storage[38]. It is speculated that down-regulation of hepcidin might be one of important factors for pathogenesis of iron overload in ALD[39]. Serum pro-hepcidin concentration in ALD was significantly lower than that in healthy subjects, and pro-hepcidin/ferritin ratios in ALD were lower than healthy subjects[40]. In the ethanol-loaded mouse model which has a mild steatotic change, the hepcidin mRNA and protein expression were significantly lower than that of control. In addition, alcohol-loading might disrupt the sensing signal of inflammatory cytokines, and then down-regulate hepcidin expression, following the increased iron absorption from small intestine. Recently, the mechanism of hepcidin downregulation by alcohol has been elucidated: a decreased hepcidin expression in mouse liver is accompanied with an increase of DMT1 and ferroportin1, and a decrease of hepcidin promoter activity and DNA-binding activity of CCAAT/enhancer-binding, protein α (C/EBPα)[41].

HEPATITIS C VIRUS INFECTION

Hepatitis C virus infection is one of the most common disorders in liver diseases involving chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC). Table 2 summarizes the effect of iron on hepatitis C virus infection. In the Third National Health and Nutrition Examination Survey, HCV infection is significantly associated with higher serum levels of ferritin and iron in the US population[42]. The mean serum levels of

ferritin and iron were significantly higher among subjects with HCV infection than among subjects without liver disease[43]. In addition, serum ferritin levels were directly and significantly correlated with serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and γ-glutamyl transpeptidase, whereas platelet counts were inversely correlated with serum ferritin. It is also found that lipid peroxidative products such as malondialdehyde are increased in hepatic tissues with CH-C[44]. In 1994, an initial report was published that phlebotomy was effective in improving the serum ALT level in patients with CH-C[45] and a national prospective study confirmed the results[46]. Since then, it was reported that hepatic iron accumulation in CH-C predict a response to interferon (IFN) therapy[47], and phlebotomy before and during IFN therapy improved virological and histological response to short-term IFN therapy evaluated at the end-of-treatment[48]. This observation is reasonable considering the finding that oxidative stress impairs interferon alpha signal by blocking JAK-STAT pathway[49]. The standard therapy for hepatitis C is now a combined therapy of interferon-α and ribavirin, in which patients with viral response to treatment seemed to develop higher soluble transferrin receptor levels[50] with decline in serum iron and ferritin than non-responders, revealing intracellular reduction of iron store depending on the result of treatment including hemolytic reaction by ribavirin[51]. This is an interesting observation that decrease of iron status may be an additional effect of the combination therapy with interferon and ribavirin. Moreover, HFE mutations are also associated with increased sustained virologic responses by antiviral long-term treatment, while it is well known that HFE mutations are associated with increased iron loading[52]. However, some reports suggest that iron depletion was unable to trigger interferon response, so that there are conflicting data. It should be further investigated whether hepatic iron content modify the response to interferon[53,54]

From these observations, iron and related molecules seem to be key factors in the hepatocytes to influence the disease condition of CH-C, and also development of cirrhosis and maybe hepatocellular carcinoma. Clinical data on phlebotomy on CH-C generally indicates that phlebotomy does not influence the viral load in vivo. On the other hand, in vitro study on HCV replication is controversial: iron promotes HCV translation by up-regulating expression of the translation initiation factor eIF3 by reporter assay[55], whereas iron suppresses HCV replication by inactivating the RNA polymerase NS5B[56].

As previously described, hepatocytes have two iron uptake systems, transferrin-mediated and nontransferrin-bound iron-mediated pathway. Transferrin and TfR1 are molecules involved in the classical pathway of cellular iron uptake, but are faintly expressed in normal hepatocytes, and is down-regulated in iron-loaded hepatic tissues with hemochromatosis. Concerning the post-transcriptional regulation of TfR1, two mechanisms are postulated through the activity change of IRP which is already mentioned. In CH-C, TfR1 expression was up-regulated and DMT1 expression was down-regulated in the condition of hepatic excess iron accumulation, suggesting that regulation of DMT1 expression is iron-dependent, but that of TfR1 expression is iron-independent in CH-C[57]. In patients with CH-C, serum values of inflammatory cytokines such as IL-1β, IL-6, and TNFα have been reported to be high in comparison with those in normal controls. In addition, TfR1 was up-regulated by IL-1β, IL-6, and TNFα in HepG2. Administration of IL-6 augments hepatic uptake of transferrin-bound iron (^{59}Fe), and this is mainly mediated through hepatocytes, but not through Kupffer cells. These results suggest that the up-regulation of TfR1 expression in CH-C might be caused by increase of inflammatory cytokines that proceeded from HCV infection, although there is a possibility that the components of HCV themselves may induce TfR1 expression directly or indirectly.

Like wise, the up-regulated TfR1 might act as a key molecule for hepatic excess iron accumulation in CH-C; however, there are several candidate molecules which cause this condition. For instance, each mutant of HFE, TfR2, hepcidin, hemojuvelin and ferroportin1 (also known as Ireg1 or MTP1) with substitution of amino acid causes the similar phenotype of hemochromatosis. That is, these facts indicate that at least 5 molecules are involved in the familiar hemochromatosis[58]. In hepatocytes, TfR2 predominantly expresses in the normal condition[59] and the disruption of TfR2 gene caused the hepatic iron overload, a phenotype of hemochromatosis, suggesting that TfR2 should also have important role in hepatic iron metabolism[60]. This receptor might act as a sensor of iron status because hepatic TfR2 protein level was increased in iron loaded rats and was decreased in iron deficient rats. Recently, Takeo et al reported that in CH-C TfR2 protein expression is increased parallel with ferroportin1[61], although the meaning of this TfR2 elevation is still to be elucidated[62].

In addition, there was a significant correlation of hepcidin mRNA expression in the liver with hepatic iron concentration and serum ferritin, but did not correlate with ALT, AST, HAI, or viral load. In inflammatory conditions, hepcidin is regulated transcriptionally by IL-6[63] and IL-1β[64] independent of liver iron content. It is noteworthy that, in contrast to other inflammatory states, hepcidin mRNA expression in the liver was independent of markers of inflammation in hepatitis C, suggesting that iron stores in patients with hepatitis C regulate hepcidin expression, and that iron loading in chronic hepatitis C is not due to inappropriate hepcidin expression[65]. However, there is still a controversial result concerning the hepcidin metabolism in chronic hepatitis C that serum pro-hepcidin is down-regulated[66]. The role of hepcidin in chronic hepatits C seems to need further consideration.

The role of iron on the hepatocellular carcinoma (HCC) development in patients with chronic hepatitis C is another major concern. In primary hemochromatosis, iron could be involved in the development of HCC in associated with cirrhosis, suggesting a strong link between heavy iron overload and HCC development. In cases of chronic hepatitis C, it is also known that HCC are developed 20 to 30 years after the infection of hepatitis C virus through the progression of the disease from chronic hepatitis and cirrhosis. In Long-Evans Cinnamon (LEC) rat, an animal model of human

Wilson disease which spontaneously developed hepatitis and liver fibrosis, HCC is frequently developed after the rats have recovered from initial fulminant hepatitis and subsequent liver fibrosis. This is considered to relate to progressive iron accumulation in the animal[67], and iron depletion prevents their development of hepatic cancer[68]. Even though the iron deposition in chronic hepatitis C is mild compared with that in hemochromatosis, iron may be an independent factor on the risk of HCC. It is reported that liver fibrosis is a favorable environment of proliferation of cancer cells by releasing transforming growth factor β, and there is a strong link between liver fibrosis and liver iron deposition. In clinical trials of phlebotomy, the hepatic content of 8-OH deoxyguanosine is decreased and fibrotic score is improved. An important issue in hepatocaricinogenesis in chronic hepatitis C is the closely related sustained production of ROS during inflammation and fibrosis. Moriya *et al* reported that HCC developed in HCV core transgenic mice after the age of 16 mo, and showed high hepatic lipid peroxidation levels in old (more than 16 mo) core transgenic mice, than in control[69]. However, the association of HCV transgenic mice, and HCC development disappeared with advanced passaging of animals, suggesting that HCC development in HCV transgenic mice cannot be simply explained by HCV infection, but requires additional cofactors. A recent study by Furutani *et al* clearly showed that hepatic iron overload induces HCC in transgenic mice expressing HCV polyprotein[70]. Transgenic animal carrying full length polyprotein-coding region (core to NS5B, nts 342-9378) by using pAlb promoter/enhancer was fed with excess iron diet. After 6 mo feeding, the transgenic mice showed marked steatosis and increased 8 hydroxy-2'deoxyguanosine content in association with the hepatic iron accumulation. Twelve months after feeding, 45% of transgenic mice developed hepatic tumors including HCC. It is noteworthy that the steatosis does not accompany with inflammation but a remarkable ultrastructural alteration of mitochondria associated with decreased degradation activity of fatty acids.

STEATOSIS AND INSULIN RESISTANCE

Nonalcoholic steatohepatitis (NASH) is a clinical entity characterized by the development of histopathological changes in the liver that are nearly identical to those induced by excessive alcohol intake, but in the absence of alcohol abuse; the presence of macrovesicular steatosis and mixes inflammatory infiltrate associate with varying amounts of Mallory's hyaline, glycogenated nuclei, and focal hepatocyte ballooning degeneration. Clinical features of NASH include obesity, hyperlipidemia, diabetes mellitus, and hypertension. In US population, approximately 25% is obese, and at least 20% of the obese individuals have hepatic steatosis. Thus, non-alcoholic liver disease (NAFLD) is the most common cause of liver dysfunction, and it is believed that NASH becomes a cause of cryptogenic cirrhosis and hepatocellular carcinoma (HCC). In patients with homozygote of HFE-related hemochromatosis, obesity and steatosis affect liver disease progression, and will be cofactors for iron overload. There is one study of Australia that showed that the prevalence of abnormal genotype of HFE in NASH is 31% compared to a normal prevalence of 13% in the general population, sugget that excess iron might be important. A study on North American subjects showed similar results that the prevalence of the *HFE* gene mutation associated with hereditary hemochromatosis are increasing in patients with NASH[71]. In the study dealing Japanese NASH patients, who had no *HFE* gene mutations, a significant staining of liver iron and increased level of thioredoxin, a marker of oxidative stress in addition to the increase of serum ferritin, was observed.

As diabetes and obesity were background conditions of NAFLD, and is thought to be a initial triggering factor, insulin resistance is now considered the fundamental operative mechanism. Insulin resistance is probably the "first step" in NASH, and a close correlation between insulin resistance and iron is speculated. Even though it is not still clear whether secondary iron accumulation increases insulin resistance, or vice versa, oxidative stress may be the elusive "second" hit of possibly multiple steps in the progression of steatosis to fibrosing steatohepatitis[72]. This may be due to the activation of stellate cells[73].

Because hepatic iron promotes oxidative stress, it seems that iron is a contributory cofactor in NASH. This proposal is strengthened by an association with hepatic fibrosis with NASH[74] and was confirmed by measuring serum markers of oxidative stress[75-77]. Excess hepatic iron also occur in insulin resistance-associated iron overload (IRHIO), characterized by hyperferritinemia with normal to mild increases in transferrin saturation. There is an interesting clinical study that venesections and restricted diet are effective in patients with IRHIO[78]. As in IRHIO, restriction of dietary calories, fat and iron improved NAFLD in addition the decrease of levels of serum aminotransferases and ferritin[79]. It seems that the simultaneous disorder of iron and glucose and/or lipid metabolism, in most cases associated with insulin resistance, is responsible for persistent hyperferritinemia and identifies patients at risk for NASH[80]. However, it is still unclear why iron is deposited in IRHIO and NAFLD. There is an interesting report by Bekri *et al* that there is an increase of hepcidin in adipose tissue of the severely obese but of liver, suggesting that severe obesity itself cause hypoferremia due to the overproduction of hepcidin in the adipocytes[81]. This finding may explain the hypoferremia in severe obese patients, but does not show the mechanism of hepatic iron deposition in IRHIO and NASH. Further studies are needed to clarify this issue, including an increase of transferrin iron influx into hepatocytes in NAFLD.

In patients with NASH, increased transferrin saturation correlated positively with the severity of fibrosis in univariate analysis, although it became insignificant when age, obesity, diabetes, and AST/ALT ratio were controlled. A recent study showed improvement in insulin sensitivity with the use of venesection in 11 patients with NASH. Biweekly phlebotomy until serum ferritin concentration became lower than or equal to 30 ng/mL reduced mean serum ALT activity without a significant change of

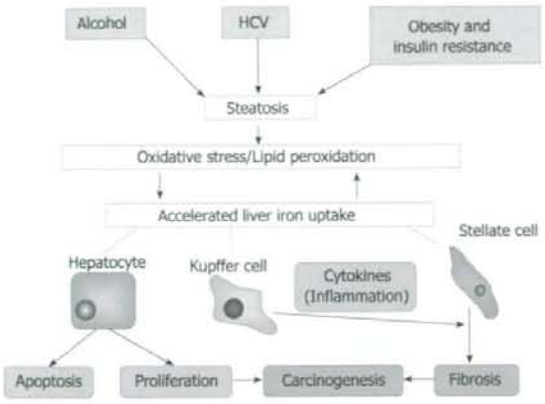

Figure 2 Postulated schema of liver damage occurred by alcohol, HCV infection, obesity and insulin resistant. A common pathway through steatosis/oxidative stress may be responsible for the development of liver fibrosis and carcinogenesis by iron.

body weight, suggesting that iron reduction therapy by phlebotomy will be one of the promising therapies for NASH[82], although this approach cannot be implemented without extensive review.

The natural history of NASH is still unclear, but some patients follow advanced liver fibrosis progressing to cirrhosis and sometimes HCC[83]. It is also known that diabetes increases the risk of hepatocellular carcinoma in US[84]. Further studies are needed to clarify this issue, especially the relation between hepatocarcinogenesis from mild iron accumulation in NASH.

As shown in Figure 2, a common pathway through steatosis/oxidative stress may be present for the development of liver fibrosis and carcinogenesis by iron.

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Lipopolysaccharide induces adipose differentiation-related protein expression and lipid accumulation in the liver through inhibition of fatty acid oxidation in mice

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Background. In the present study, we examined the effect of lipopolysaccharide (LPS) on liver histopathology with special reference to lipid metabolism in mice. ***Methods.*** Mice were injected with LPS intraperitoneally, and its effect on the liver was investigated pathologically and biochemically. ***Results.*** Oil-red O staining and adipose differentiation-related protein (ADRP) immunohistochemistry demonstrated that injection of LPS transiently induced lipid accumulation and ADRP expression in hepatocytes, especially around the portal vein. Microscopic observation revealed that lipid accumulation started 12 h after LPS injection. Time-course studies showed that LPS rapidly, within 2 h, decreased hepatic expression of nuclear hormone receptors, including peroxisome proliferator-activated receptor (PPAR) α. LPS inhibited the expression of PPARα-target genes involved in fatty acid oxidation in the liver such as those coding for enoyl-CoA hydratase, acyl-CoA dehydrogenase, and carnitine palmitoyl transferase-1, whereas LPS also suppressed the expression of genes related to fatty acid synthesis such as those for fatty acid synthase, stearoyl-CoA desaturase, and acetyl-CoA carboxylase α. ***Conclusions.*** LPS induces transient lipid accumulation and expression of ADRP in the liver through inhibition of fatty acid oxidation by down-regulation of the PPARα-related transcriptional mechanism.

Key words: LPS, adipose differentiation-related protein, liver steatosis, PPARα, fatty acid oxidation

Introduction

Hyperlipidemia frequently accompanies infectious and inflammatory diseases.[1] The administration of endotoxin or lipopolysaccharide (LPS) has been used to mimic infection, and studies have demonstrated that a single administration of LPS is sufficient to produce hypertriglyceridemia,[2–4] suggesting that LPS is involved in the hyperlipidemia induced by infection and inflammation. The doses of LPS that produce hypertriglyceridemia in rodents are similar to those that produce fever, anorexia, and changes in acute-phase protein synthesis, suggesting that hypertriglyceridemia is a very sensitive, physiological part of the host response to infection rather than a manifestation of toxicity.[5] Although a number of lines of evidence obtained from biochemical and molecular biological studies have shown that LPS is capable to changing lipid and lipoprotein metabolism in the liver,[1] little is known about LPS-induced histopathological changes in the liver with particular reference to lipid metabolism. In the present study, we examined the effect of LPS on liver pathology associated with changes in lipid metabolism caused by LPS and the possible mechanism of that effect.

Materials and methods

Animal studies

Ten-week-old male C57Bl/6Ncrj mice (Charles River Japan, Tokyo, Japan) were housed under a 12 h light/dark cycle (light on at 7 a.m.) at 22°C and given food and water ad libitum. Mice were fed a normal diet in which 13.2% of calories were from fat (MF, Oriental Yeast, Tokyo, Japan). Mice were injected with LPS (Sigma-Aldrich, St. Louis, MO, USA) intraperitoneally, and each mouse was anesthetized with diethyl ether and weighed at several time points. To test the possibility

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that reduced food intake by itself could induce lipid accumulation in the liver, we also examined the effect of fasting for 24 h on the liver histology. Mice deprived of food but with free access to water were killed after fasting for 24 h and their livers were dissected out for later histological analysis. Blood was collected by cardiac puncture and subsequently assayed for biochemical parameters. Livers and epididymal fat pads were dissected out, weighed, and frozen in liquid nitrogen. Samples of the resected liver were used for later histological and polymerase chain reaction (PCR) analysis. All experiments were carried out in accordance with rules and guidelines of the Animal Experiment Committee of Asahikawa Medical College.

Biochemical analyses

The serum biochemical markers alanine aminotransferase (ALT) and triglycerides were measured with an Automatic Analyzer 7180 (Hitachi High-Technologies, Tokyo, Japan).

Histopathologic evaluation

Samples of the liver tissue were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and the sections stained with hematoxylin and eosin (HE). To detect fat deposition in the liver, frozen sections were rinsed with distilled water, stained with 0.18% oil red O (Sigma-Aldrich) with 60% 2-propanol (Sigma-Aldrich) for 20 min at 37°C, and then rinsed with distilled water as in our previous study.[6] Immunohistochemistry for adipose differentiation-related protein (ADRP) was performed as described in our previous study.[7]

RNA isolation and first-strand cDNA synthesis

Total hepatic RNA was isolated from small pieces of mouse tissue (80–100 μg) by using Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA (1 μg) was reverse-transcribed with RETROscript (Ambion, Austin, Texas, USA). From each mouse, 1 μg of total RNA was mixed with 2 μl of random decamers and nuclease-free water in a total volume of 12 μl and heated at 80°C for 3 min. The mixture was then chilled on ice and incubated with 2 μl 10× reverse transcriptase buffer, 4 μl dNTP mix, 1 μl RNase inhibitor, and 1 μl reverse transcriptase at 44°C for 1 h. The reaction mixtures were further incubated for 10 min at 92°C. The cDNA was stored at –30°C until used for real-time PCR.

Real-time PCR

Real-time PCR were performed in a 7500 Real Time PCR system (Applied Biosystems, Foster City, CA, USA) with a Taqman probe. Primers sets for tumor necrosis factor (TNF) α (Mm00443258_m1), peroxisome proliferator-activated receptor γ (PPARγ) (Mm00440945_m1), PPARα (Mm00440939_m1), retinoid X receptor (RXR) α (Mm00441182_m1), sterol regulatory element binding protein (SREBP) 1 (Mm00550338_m1), fatty acid synthetase (Mm00662319_m1), stearoyl-CoA desaturase (Mm00772290_m1), acetyl-CoA carboxylase (Mm01304283_g1), enoyl-CoA hydratase (Mm00470091_s1), very long acyl CoA dehydrogenase (Mm00444296_m1), carnitine palmitoyl transferase (Mm00487202_m1), and ADRP (Mm00475794_m1) were purchased from Applied Biosystems.

Statistical analysis

The results are expressed as means ± SEM. Statistical analysis was performed by repeated measures analysis of variance and subsequent Fisher's LSD test. $P < 0.05$ was considered statistically significant.

Results

LPS caused changes in body, liver, and fat weight in mice (Fig. 1). LPS at a dose of 30 mg/kg gradually caused a reduction in body weight, which reached a minimum 24 h after LPS administration (Fig. 1, left). LPS also caused reductions in the weights of liver and fat, at least during the first 12 h. The inhibitory effects of LPS on each parameter were dose dependent (Fig. 1, right).

Biochemical markers in serum of mice administered 30 mg/kg LPS also changed over time (Fig. 2). Serum ALT and triglycerides did not change during the first 6 h but significantly increased by 12 h after LPS administration. Thus, high doses of LPS, which induced liver steatosis in the present study (see below), also increased serum triglyceride levels, supporting previous evidence that a single dose of LPS is sufficient to produce hypertriglyceridemia.[5]

Histologically, a number of small lipid droplets were clearly seen in the liver 24 h after LPS administration (Fig. 3A, B). The lipid droplets were observed predominantly in hepatocytes around the portal vein rather than around the central veins. Inflammatory cells infiltrating the liver were rarely observed. Although accumulation of small lipid droplets was clearly seen after 24 h, droplets were not observed 48 or 72 h after LPS administration. We also evaluated the dose–response effect of LPS on the induction of lipid droplets in the liver 24 h after LPS injection and observed the accumulation of lipid droplets in mice administered LPS at doses of 0.3 mg/kg or larger (data not shown). To test the possibility that reduced food intake by itself could induce lipid accumu-

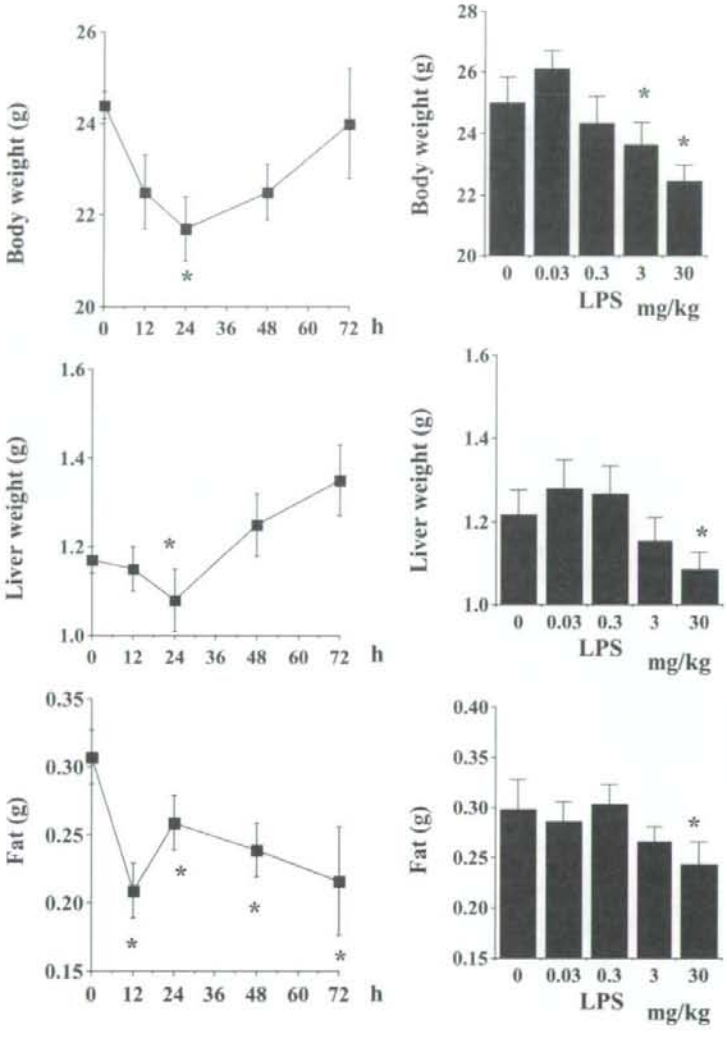

Fig. 1. Body, fat, and liver weight in mice injected intraperitoneally with lipopolysaccharide (*LPS*) and then killed at several time points. *Left panels* shows the time-course change in these parameters in mice administered 30 mg/kg of LPS. *Right panels* illustrates the dose–response effect of LPS on the parameters in mice 24 h after several injections of LPS. Each data point represents the mean ± SEM of five animals. *$P < 0.01$, compared with control

lation in the liver, we examined the effect of fasting for 24 h on the liver histology. Histological observation (Fig. 4) indicated that 24 h of fasting by itself did not induce lipid accumulation in the liver, strongly suggesting that reduced food intake was not a major factor causing liver steatosis. Oil red O staining confirmed that the lipid droplet-like structures seen in HE staining were indeed lipid droplets containing triglycerides (Fig. 3C, D). The time-course results with oil-red O staining showed that fat accumulation started within 12 h after injection of LPS, lipid deposition reached a maximum in the liver 24 h after LPS administration, and then the lipid content in the liver gradually decreased. Thus, a single intraperitoneal injection of LPS induced liver steatosis within 12 h, and fat accumulation disappeared by 48 h after LPS injection, establishing a novel transient liver steatosis model in mice by LPS.

To further clarify whether LPS transiently induced liver steatosis, expression of ADRP, a protein that coats lipid droplets, was evaluated. ADRP was expressed in the liver 12 and 24 h after 30 mg/kg LPS administration (Fig. 5A). High-power observation of a liver specimen stained with ADRP antibody showed that ADRP coated the lipid droplets in the hepatocytes (Fig. 5B). Moreover, ADRP protein was expressed transiently, in good agreement with the HE and oil red O staining results. LPS induced ADRP expression in a dose-dependent manner (Fig. 5C). Obvious induction of ADRP expres-