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J Gastroenterol (2010) 45:426–434
DOI 10.1007/s00535-009-0154-3

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Hepatic senescence marker protein-30 is involved in the progression of nonalcoholic fatty liver disease

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Received: 3 July 2009 / Accepted: 10 October 2009 / Published online: 28 November 2009

Abstract
Background Both insulin resistance and increased oxidative stress in the liver are associated with the pathogenesis of nonalcoholic fatty liver disease (NAFLD). Senescence marker protein-30 (SMP30) was initially identified as a novel protein in the rat liver, and acts as an antioxidant and antiapoptotic protein. Our aim was to determine whether hepatic SMP30 levels are associated with the development and progression of NAFLD.
Methods Liver biopsies and blood samples were obtained from patients with an NAFLD activity score (NAS) ≤ 2 ($n = 18$), NAS of 3–4 ($n = 14$), and NAS ≥ 5 ($n = 66$).
Results Patients with NAS ≥ 5 had significantly lower hepatic SMP30 levels (12.5 ± 8.4 ng/mg protein) than patients with NAS ≤ 2 (30.5 ± 14.2 ng/mg protein) and patients with NAS = 3–4 (24.6 ± 12.2 ng/mg protein). Hepatic SMP30 decreased in a fibrosis stage-dependent manner. Hepatic SMP30 levels were correlated positively with the platelet count ($r = 0.291$) and negatively with the homeostasis model assessment of insulin resistance ($r = -0.298$), the net electronegative charge modified-low-density lipoprotein ($r = -0.442$), and type IV collagen 7S ($r = -0.350$). The immunostaining intensity levels of 4-hydroxynonenal in the liver were significantly and inversely correlated with hepatic SMP30 levels. Both serum large very low-density lipoprotein (VLDL) and very small low-density lipoprotein (LDL) levels in patients with NAS ≥ 5 were significantly higher than those seen in patients with NAS ≤ 2, and these lipoprotein fractions were significantly and inversely correlated with hepatic SMP30.
Conclusion These results suggest that hepatic SMP30 is closely associated with the pathogenesis of NAFLD, although it is not known whether decreased hepatic SMP30 is a result or a cause of cirrhosis.

Keywords SMP30 · NAFLD · NASH · Insulin resistance · Oxidative stress

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is one of the most common causes of chronic liver injury throughout the

world [1–3]. It represents a spectrum of conditions characterized histologically by macrovesicular hepatic steatosis, and the diagnosis is made in patients who have not consumed alcohol in amounts sufficient to be considered harmful to the liver.

NAFLD encompasses varying microscopic features that range from simple steatosis, which has a good prognosis, to nonalcoholic steatohepatitis (NASH), which has a poor prognosis. Liver biopsy is recommended as the gold standard for both the diagnosis and staging of fibrosis in patients with NASH [1, 4–6]. Hyperlipidemia, insulin resistance, and oxidative stress can contribute heavily to the initiation and progression of NAFLD [7–9]. However, the exact intricacies of the molecular and cellular mechanisms responsible for the progression from simple steatosis to NASH have not been fully elucidated.

Senescence marker protein-30 (SMP30), a 34-kDa protein originally identified in the rat liver, is a novel molecule that decreases in concentration with aging in an androgen-independent manner [10, 11]. SMP30 transcripts have been detected in a multitude of tissues, and its amino acid alignment reveals a highly conserved structure among humans, rats, and mice [11]. We have reported previously that SMP30 participates in Ca^{2+} efflux by activating the calmodulin-dependent Ca^{2+}-pump in HepG2 cells and renal tubular cells, conferring on these cells resistance to injury caused by high intracellular Ca^{2+} concentrations [12, 13]. Recently, we identified SMP30 as gluconolactonase (GNL), which is involved in L-ascorbic acid biosynthesis in mammals, although human beings are unable to synthesize vitamin C because there are many mutations in the gulonolactone oxidase gene, which catalyzes the conversion from L-gulono-γ-lactone to L-ascorbic acid [14]. To clarify whether a causal relationship exists between a decrease in SMP30/GNL levels and age-associated organ disorders, we established SMP30/GNL knockout (KO) mice [15]. The livers of SMP30/GNL KO mice are highly susceptible to tumor necrosis factor-α (TNF α) and Fas-mediated apoptosis [15]. In addition, they showed mitochondrial damage and abnormal accumulations of triglycerides, cholesterol, and phospholipids [16]. Furthermore, SMP30/GNL in brain and lung tissue appeared to have protective properties against oxidative stress associated with aging [17–20]. Because the SMP30/GNL KO mice showed changes in the liver that mimic the processes of NAFLD, we hypothesized that decreased levels of SMP30 may be linked to the pathogenesis of NAFLD. The purpose of this study was to investigate the role of SMP30 in the pathogenesis of NAFLD.

Patients and methods

Patients

The study protocol was approved by the ethics committee of Saiseikai Suita Hospital and Kyoto Prefectural University of Medicine, and informed consent was obtained from all subjects prior to their enrollment in the study. A total of 98 patients histologically diagnosed as having NAFLD at Saiseikai Suita Hospital or Kyoto Prefectural University Hospital between 2006 and 2008 were enrolled in this study.

All liver biopsy specimens were stained with hematoxylin–eosin and Masson's trichrome stains and examined by two experienced pathologists blinded to the patients' clinical or laboratory data or liver biopsy sequence. Patients with NAFLD were divided into the following groups: simple steatosis and mild NASH (stages 0–1), moderate NASH (stage 2), and advanced NASH (stage 3–4) according to the classification proposed by Brunt et al. [6]. Several liver tissues samples from these groups were embedded in Tissue-Tek OCT (Sakura Finetech, Tokyo, Japan) compound and stained with Oil Red O. The fibrosis staging system was classified as follows: stage 0, no fibrosis; stage 1, zone 3 predominant pericellular fibrosis; stage 2, zone 3 fibrosis plus periportal fibrosis; stage 3, bridging fibrosis; stage 4, cirrhosis. The grade of steatosis was defined as mild ($\leq$33%), moderate (34–65%), or advanced ($\geq$66%). In addition, the NAFLD activity score (NAS) system was used to classify NAFLD into "not NASH" (NAS $\leq$ 2), "borderline NASH" (NAS = 3–4), and "definite NASH" (NAS $\geq$ 5), as shown in Table 1, because the NAS system has been reported as a reliable scoring system for diagnosing NASH [5]. We excluded patients with alcohol intake exceeding 20 g/day and those who reported signs, symptoms, and/or a history of known liver disease including viral, genetic, autoimmune, and drug-induced liver disease, before evaluation of liver histology.

Immunohistochemistry

Liver biopsy specimens were preserved in 10% formalin and embedded in paraffin. Specimens were serially sectioned onto microscope slides at a thickness of 4 μm and then deparaffinized. After removal of paraffin, the liver sections were heated by microwaving in 0.1 M citrate buffer (pH 7.0), followed by inactivation of endogenous peroxidases by incubation with 1% hydrogen peroxide (H_2O_2) in methanol. The primary antibodies used were monoclonal antibody raised against recombinant human SMP30 (1:2000 dilution) [14] and anti-4-hydroxynoneral

Table 1 Clinical features and laboratory data of three patient groups classified according to NAS scores

	Group A (n = 18) NAS ≤ 2	Group B (n = 14) NAS 3–4	Group C (n = 66) NAS ≥ 5
Male/female	9/9	6/8	34/32
Age (years)	60.9 ± 13.1	53.3 ± 16.8	60.4 ± 12.2
Body mass index (kg/m^2)	26.4 ± 4.8	27.5 ± 4.4	27.5 ± 4.8
Systolic blood pressure (mmHg)	133 ± 18	137 ± 8	140 ± 18
Diastolic blood pressure (mmHg)	77 ± 9	84 ± 12	82 ± 12
HbA1c (%)	6.2 ± 1.6	6.4 ± 1.8	6.3 ± 1.3
Fasting glucose (mg/dL)	116 ± 32	125 ± 41	124 ± 44
Fasting insulin (μU/mL)	8.5 ± 4.9	10.9 ± 4.7	12.7 ± 7.6
HOMA-R	2.9 ± 1.7	3.7 ± 2.1	3.9 ± 2.8*
AST (U/L)	39 ± 12	43 ± 12	44 ± 23
ALT (U/L)	37 ± 11	41 ± 10	53 ± 25
Triglyceride (mg/dL)	160 ± 67	149 ± 72	165 ± 94
Total cholesterol (mg/dL)	221 ± 42	208 ± 36	202 ± 34
HDL cholesterol (mg/dL)	55 ± 22	50 ± 7	49 ± 12
LDL cholesterol (mg/dL)	134 ± 25	119 ± 28	129 ± 30
Oxidized LDL (U/ml)	13.3 ± 2.6	13.8 ± 1.1	14.8 ± 2.2
Electronegative charge modified-LDL (ecd)	3.1 ± 3.0	3.1 ± 3.2	6.4 ± 3.5*
Type IV collagen 7S (ng/dL)	3.9 ± 0.5	4.0 ± 1.2	5.7 ± 1.9*
Platelet count (× 10^4/μL)	21.9 ± 2.9	21.9 ± 4.5	17.1 ± 4.7*
SMP30 in liver tissue (ng/mg protein)	30.5 ± 14.2	24.6 ± 12.2	12.5 ± 8.4**·***
75 g OGTT (NGT/IGT/DM)	4/7/7	3/6/5	13/29/24

Data are expressed as mean ± SD

* $P < 0.05$ versus group A versus group B. ** $P < 0.01$ versus group B. *** $P < 0.001$ versus group A

NAS nonalcoholic fatty liver disease (NAFLD) activity score, *HOMA-R* homeostasis model assessment of insulin resistance, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *HDL* high-density lipoprotein, *LDL* low-density lipoprotein, *ecd* electronegative-charge density, *SMP30* senescence marker protein-30, *OGTT* oral glucose tolerance test, *NGT* normal glucose tolerance, *IGT* impaired glucose tolerance, *DM* diabetes mellitus

(4-HNE) monoclonal antibody (1:100 dilution; Nihon Yushi, Tokyo, Japan). SMP30 and 4-HNE were detected by indirect immunoperoxidase staining using corresponding Histofine Simple Stain MAX-PO kits (Nichirei Biosciences, Tokyo, Japan) and 3, 3-diaminobenzidine (DAB) as a chromogenic substrate. After DAB staining, nuclei were counterstained with Mayer's hematoxylin. Two independent observers evaluated the intensity of immunostaining for 4-HNE as 0, 1, 2, or 3 (negative, weak, moderate, or strong, respectively).

Quantification of hepatic SMP30 content by enzyme-linked immunosorbent assay (ELISA)

A portion of each liver biopsy specimen was immediately frozen and stored at −80°C for hepatic SMP30 measurement. Frozen liver biopsy specimens were suspended in ice-cold phosphate-buffered saline (PBS; pH 7.4). After disruption by homogenization and sonication, samples were centrifuged (15,000 g, 15 min, 4°C) and supernatants were stored at −80°C until assay. SMP30 in supernatant fractions was determined by a sandwich ELISA using a polyclonal anti-SMP30 antibody (Cosmo Bio, Tokyo, Japan) and a monoclonal anti-SMP30 antibody. In brief, microtiter plates were coated with affinity-purified anti-SMP30 rabbit IgG (2.0 μg/ml) diluted with 10 mM carbonate buffer (pH 9.3) for 2 h at room temperature. After washing, nonspecific binding sites in each well were blocked with 10 mM carbonate buffer containing 0.5% bovine serum albumin (BSA). Standard solution (0–2,000 pg/ml recombinant SMP30) and supernatant samples diluted (1:10) with sample buffer (50 mM Tris–HCl buffer, pH 7.0, containing 200 mM NaCl, 10 mM $CaCl_2$, 0.1% Triton X-100, and 1% BSA) were added to the wells, and the plate was incubated for 2 h at room temperature. After a washing with BSA-free sample buffer, biotinylated anti-monoclonal SMP30 antibody was added to each well. The plate was incubated for 2 h at room temperature, washed, and then incubated for an additional 2 h at room temperature with streptavidin-horseradish peroxidase (HRP) diluted 1:10,000 (Vector Laboratories, Burlingame, CA, USA). After a final washing, the plate was treated for 20 min with

a substrate solution of 3,3′,5,5′-tetramethylbenzidine and H_2O_2 was added to each well and allowed to react for 15 min at room temperature. The reaction was stopped by the addition of 1 M phosphoric acid, after which optical density (OD) values at 450 nm were read with an ELISA plate reader. The detection limit of the assay was 20 pg/ml and the intra- and interassay coefficients of variation were 6.4 and 8.2% at 50 pg/ml and 4.6 and 7.0% at 500 pg/ml, respectively. The concentration of SMP30 in liver tissue was expressed based on milligrams of total protein. The protein concentration was determined using a Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA, USA) with human serum albumin as a standard.

Laboratory investigations

Blood samples were obtained in the morning after an overnight fast. Plasma glucose was measured by the glucose oxidase method and HbA1c was determined by high-performance liquid chromatography (HPLC; Arkray, Kyoto, Japan). Serum insulin (immunoreactive insulin; IRI) concentrations were measured by an immunoradiometric assay (Insulin-RIAbead II, Abbott Japan, Tokyo, Japan). The homeostasis model assessment of insulin resistance (HOMA-R) was calculated from fasting insulin and glucose levels by the following equation: HOMA-R = fasting IRI (mU/ml) × fasting plasma glucose (PG) (mg/dl)/405. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol (T-Ch), high-density lipoprotein cholesterol (HDL-Ch), low-density lipoprotein cholesterol (LDL-Ch), and triglyceride (TG) were measured by enzymatic methods using a chemical autoanalyzer (Hitachi, Tokyo, Japan). Serum type IV collagen 7S was measured with a radioimmunoassay kit (Mitsubishi Chemical Group, Tokyo, Japan). Serum oxidized LDL (oxLDL) was measured with an ELISA kit (Kyowa Medex, Tokyo, Japan).

The net electronegative charge modified-LDL (emLDL) was analyzed using an agarose gel electrophoresis lipoprotein fraction system, according to the manufacturer's instructions (Chol/Trig Combo System; Helena Labs, Saitama, Japan). The percentage frequency of emLDL was calculated on a computer from the migration distance (b) of the LDL fraction in the test samples and the migration distance (a) of normal control sera, according to the following formula: emLDL density = $[b - a/a] \times 100\%$. The intraassay coefficient of variation in this method was <1%. In our preliminary study, the value of emLDL in normal healthy subjects ($n = 45$, mean age 46.5 ± SD 3.9 years) was 0.3 ± 2.6% (unpublished data). Serum lipoproteins were also analyzed by an HPLC system according to the procedure described by Okazaki et al. [21], while lipoprotein particle size was determined based on individual elution times that corresponded to peaks on the chromatographic pattern of cholesterol fractions. In this study, we defined very low-density lipoprotein (VLDL), LDL, and HDL subclasses according to lipoprotein particle size, expressed as diameter [22].

Statistical analysis

All statistical analyses were performed with Statview version 5.0 (Abacus Concepts, Berkeley, CA, USA), with data expressed as mean ± SD. When the data were not normally distributed, logarithmic transformation was performed. Differences between the groups were determined by Student's t test or one-way analysis of variance (ANOVA) with Scheffé's multiple comparison test. Categorical data were assessed by the χ^2 test. The degree of correlation between selected variables was determined by Pearson's correlation analysis or Spearman's correlation analysis. The relationship between hepatic SMP30 levels and other clinical parameters was also analyzed by stepwise multiple regression analysis using forward direction, with the F value for entry set at 4.0. A P value of <0.05 was considered statistically significant.

Results

The clinical, biochemical, and laboratory data of the three patient groups classified by NAS score are summarized in Table 1. Patients with NAS ≥ 5 had significantly lower hepatic SMP30 levels than patients with NAS ≤ 2 ($P < 0.001$) and patients with NAS of 3–4 ($P < 0.01$). Patients with NAS ≥ 5 had significantly higher HOMA-R ($P < 0.05$), serum emLDL ($P < 0.05$), and serum type IV collagen 7S ($P < 0.05$) and had lower platelet counts ($P < 0.05$) than patients with NAS ≤ 2 or patients with NAS of 3–4. There was no significant difference in the other clinical and laboratory data among the three patient groups.

Hepatic SMP30 levels were significantly and positively correlated with platelet count ($P < 0.05$), and were significantly and inversely correlated with HOMA-R ($P < 0.05$), serum emLDL ($P < 0.01$), and serum type IV collagen 7S ($P < 0.01$; Table 2). Stepwise multiple regression analysis also showed that hepatic SMP30 levels were associated with HOMA-R ($F = 4.08$) emLDL ($F = 11.19$), and type IV collagen 7S ($F = 5.23$; Table 2).

As shown in Fig. 1A, immunohistochemical staining showed strong expression of SMP30 protein in parenchymal cells in liver tissue from patients with simple steatosis (Fig. 1A-a) compared with liver tissue from patients with mild NASH (stages 0–1; Fig. 1A-b), moderate NASH (stage 2; Fig. 1A-c), and advanced NASH (stages 3–4; Fig. 1A-d). The level of hepatic SMP30 was significantly higher in patients with simple steatosis (28.5 ± 9.5 ng/mg

Table 2 Pearson's correlation and stepwise multiple regression analysis of the relationship between hepatic SMP-30 and 18 clinical variables

	Pearson's correlation	Stepwise multiple regression	
	r	*β*	*F*
Age	−0.111	–	–
Body mass index	−0.116	–	–
Systolic blood pressure	−0.034	–	–
Diastolic blood pressure	−0.040	–	–
HbA1c (%)	0.026	–	–
Fasting glucose	−0.201	–	–
Fasting insulin	−0.158	–	–
HOMA-R	−0.298*	−0.243	4.08*
AST	−0.127	–	–
ALT	−0.190	–	–
Triglyceride	−0.175	–	–
Total cholesterol	0.026	–	–
HDL cholesterol	0.031	–	–
LDL cholesterol	−0.158	–	–
Oxidized LDL (U/ml)	−0.241	–	–
Electronegative charge modified-LDL	−0.442**	−0.380	11.19**
Type IV collagen 7S	−0.350**	−0.260	5.23*
Platelet count	0.291*	–	–

* $P < 0.05$, ** $P < 0.01$

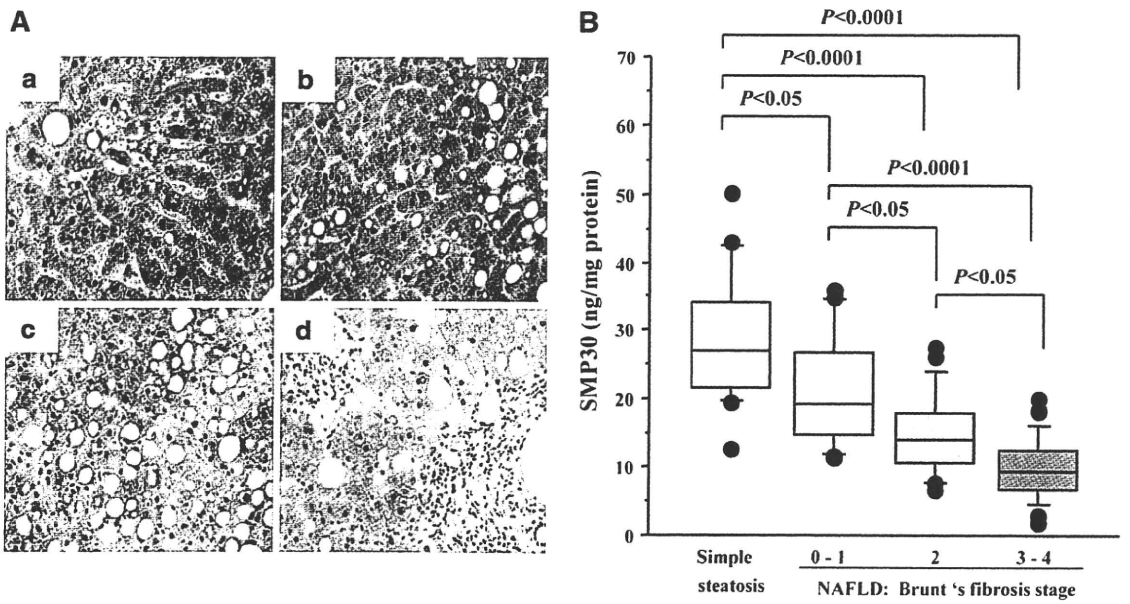

Fig. 1 **A** Immunostaining of senescence marker protein-30 (*SMP30*) in liver tissue from patients with *a* simple steatosis, *b* mild nonalcoholic steatohepatitis (NASH; stages 0–1), *c* moderate NASH (stage 2), and *d* advanced NASH (stage 3–4), ×400. **B** Hepatic SMP30 levels in patients with simple steatosis, mild NASH, moderate NASH, or advanced NASH. The *box plots* include the medians (*horizontal lines*) and interquartile ranges (*boxes*), whereas the *whiskers* represent the 10–90th percentiles, and the *dots* represent the 5–95th percentiles and 1–99th percentiles, respectively. *NAFLD* nonalcoholic fatty liver disease

protein) compared with that in patients with NASH (vs. mild; 21.2 ± 7.9, $P < 0.05$, vs. moderate; 14.9 ± 5.9, $P < 0.001$, and vs. advanced; 9.6 ± 4.6, $P < 0.001$), and was observed as decreasing in a stage-dependent manner (Fig. 1B).

The grade of fatty change evaluated with hematoxylin-eosin staining (Fig. 2a) was correlated well with the intensity of Oil Red O staining (Fig. 2b).

In this study, to estimate oxidative stress in liver tissue, we investigated the expression of 4-HNE, a marker of

lipid peroxidation products. As shown in Fig. 3a, b, the immunostaining intensity of 4-HNE in tissues from patients with NAS ≥ 5 (Fig. 3A-c and -d) was significantly greater than that seen in patients with NAS ≤ 2 (Fig. 3A-a) and patients with NAS of 3–4 (Fig. 3A-b). Spearman's correlation analysis showed that the immunostaining intensity levels of 4-HNE had a significant negative correlation with hepatic SMP30 levels ($\rho = -0.649$, $P < 0.01$; Fig. 3c).

We investigated serum lipoprotein profiles in the patients with NAS ≤ 2 ($n = 15$) and those with NAS ≥ 5 ($n = 20$). As shown in Fig. 4, levels of large VLDL and very small LDL in patients with NAS ≥ 5 were significantly higher than those in patients with NAS ≤ 2 (5.6 ± 1.3 vs. 4.5 ± 1.2 mg/dl, $P = 0.008$ and 20.4 ± 5.4 vs. 17.0 ± 4.0 mg/dl, $P = 0.047$, respectively). There was no significant difference in the other lipoprotein subclass levels between patients with mild and advanced NASH. Both large VLDL and very small LDL levels were correlated negatively with hepatic SMP30 levels ($r = -0.379$, $P = 0.024$ and $r = -0.357$, $P = 0.035$, respectively; Fig. 5).

Discussion

In the present study, we have demonstrated for the first time, by both ELISA and immunohistochemical studies, the significant reduction of hepatic SMP30 levels in a stage-dependent manner in patients with NAFLD. An additional noteworthy finding of this study is that the hepatic SMP30 level was strongly and inversely correlated with the grade of immunohistochemical staining of 4-HNE, which is an aldehydic end product of lipid peroxidation in hepatocytes. Furthermore, we also demonstrated that hepatic SMP30 levels were significantly and positively correlated with the platelet count, and were significantly and inversely correlated with HOMA-R, serum emLDL, and serum type IV collagen 7S. It is well known that increased lipotoxicity and oxidative stress in the liver play a critical role in the progression of NAFLD. Accumulated reactive oxygen species (ROS) activate nonparenchymal cells, including Kupffer cells and hepatic stellate cells [23]. By way of a paracrine mechanism, activated Kupffer cells release transforming growth factor (TGF)-β, a known profibrotic factor that has been implicated in the activation

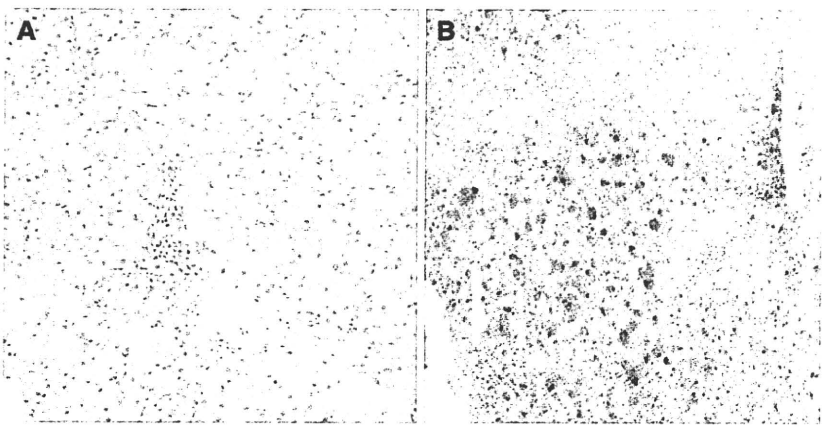

Fig. 2 **a** H&E staining in liver tissue from a patient with mild NASH. Many large fat droplets are noted around the central vein. **b** Oil Red O staining in liver tissue from a patient with large fat droplets. **a**, **b** ×200

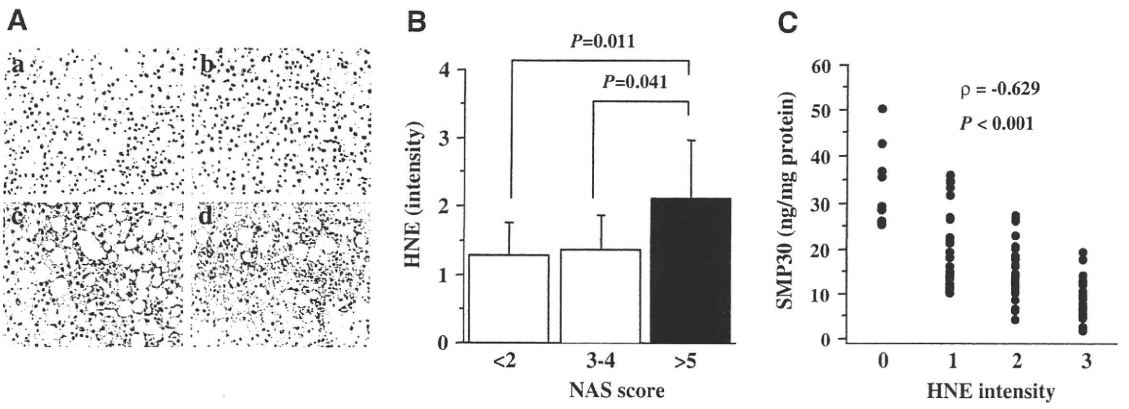

Fig. 3 **A** Immunostaining of 4-hydroxynoneral (*4-HNE*) in liver tissues from patients with *a* NAFLD activity score (*NAS*) ≤ 2, *b* NAS = 3–4, and *c*, *d* NAS ≥ 5, ×200. **B** Hepatic 4-HNE intensity in the three patient groups classified according to the NAS scores. *c* Spearman's correlation between hepatic SMP-30 and 4-HNE intensity

of other neighboring hepatic cells, including hepatic stellate cells [24]. Recently, Tomita et al. [25] reported that hepatic expression of TGF-β1 was enhanced at both early and late fibrotic stages of NASH, and that elimination of hepatic ROS accumulation in the liver decreased hepatic TGF-β1, whereas enhancement of ROS in the liver increased TGF-β1 mRNA levels. It has also been reported that 4-HNE stimulates procollagen type I synthesis in human hepatic stellate cells [26, 27]. SMP30 maintains calcium homeostasis by activating the calmodulin-dependent Ca^{2+}-pump, and has gluconolactonase activity. Several functional studies of SMP30 have demonstrated that SMP30 plays a role as an antiapoptotic protein and antioxidant [15, 17–20, 28, 29]. Therefore, our findings strongly suggest that the reduction of hepatic SMP30 is associated with the progression of hepatic fibrosis through increased oxidative stress in the liver. In our preliminary study, hepatic SMP30 was significantly decreased in hepatitis C virus (HCV)-infected patients with cirrhosis (data not shown). It is suggested that hepatic SMP30 is decreased in cirrhotic liver by other etiologies as well as NAFLD. However, it is not known whether decreased hepatic SMP30 is a result or a cause of cirrhosis. Further studies are needed to clarify this point.

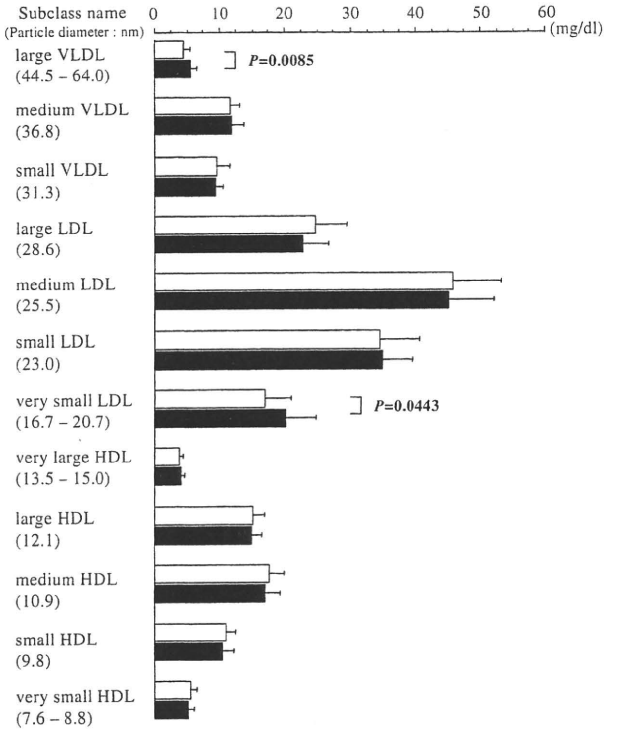

Fig. 4 Comparison of the levels of each lipoprotein subclass grouped according to particle size. The *open columns* represent data from 15 patients with NAS $\leq$ 2 and the *closed columns* represent data from 20 patients with NAS $\geq$ 5. *VLDL*, Very low-density lipoprotein; *LDL*, low-density lipoprotein; *HDL*, high-density lipoprotein

In the present study, we found that emLDL was significantly increased in an NAS-dependent fashion. Similarly, using lipoprotein profile analysis by HPLC, we also found that in patients with NAS $\geq$ 5, serum levels of both large VLDL, which corresponds to VLDL1 (Svedberg flotation, Sf 60–400), and very small LDL were significantly higher than those in patients with NAS $\leq$ 2. A noteworthy finding is that these lipoproteins were significantly and inversely correlated with hepatic SMP30. Griffin and Packard [30] and Packard [31] have demonstrated that large VLDL1 is a precursor of small dense LDL, and VLDL1 is preferentially produced in the liver during the development of insulin resistance. Adiels et al. [32, 33] have reported that hyperglycemia stimulates VLDL1 production in type 2 diabetes, and that overproduction of VLDL1 is significantly correlated with increased liver fat and plasma glucose in patients with type 2 diabetes. Previously, we reported that accumulation of cholesterol in the livers of SMP30-deficient (SMP30Y/−) mice was markedly higher than that in age-matched wild-type mice [16]. In the SMP30Y/− mice, mitochondrial damage and many fat droplets were observed in hepatocytes by electron microscopy [16]. Our present findings, taken together with these findings in the SMP30Y/− mice, lead us to speculate

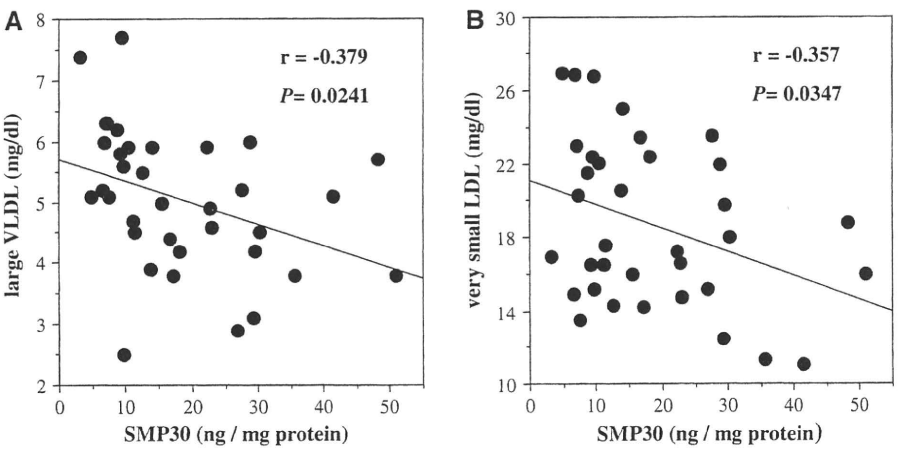

Fig. 5 Pearson's correlations between **a** hepatic SMP-30 and serum large VLDL (particle size 44.5–64.0 nm) and **b** between hepatic SMP-30 and serum very small LDL (particle size 16.7–20.7 nm)

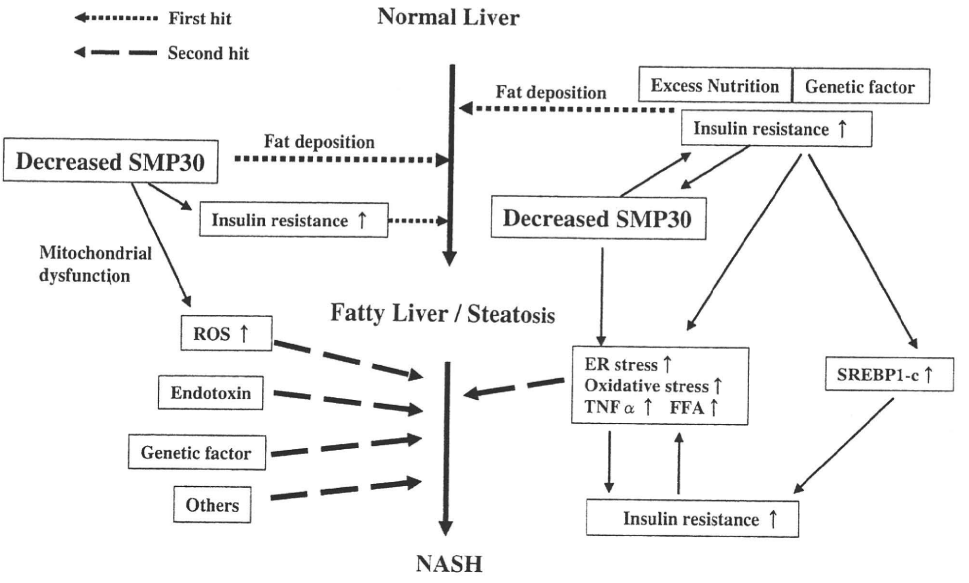

Fig. 6 Schematic diagram depicting disease progression of NAFLD according to the two-hit hypothesis, showing involvement of hepatic SMP30. *FFA*, free fatty acid; *SREBP*, sterol regulatory element-binding proteins; *TNF*, tumor necrosis factor

that steadily decreasing hepatic SMP30 levels are associated with the progression of hepatic insulin resistance.

In conclusion, the two-hit theory proposed by Day and James [9], in which the initial trigger is the hepatic accumulation of excessive fat, followed by the second hit of the development of oxidative stress, is widely advocated as a pathogenic mechanism for NASH. Therefore, our findings in the present study strongly suggest that SMP30 plays an important role in the pathogenesis of NAFLD (Fig. 6), and that increasing levels of SMP30 in the liver will serve as a promising target in the treatment of NASH.

Acknowledgments This study was supported by a Grant-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science (Goji Hasegawa), and a Grant-in-Aid from the Ministry of Health, Labour and Welfare (Takeshi Okanoue).

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Ⅲ. 臨床的メカニズムの解明

3. NASH患者と酸化ストレス

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[Summary]

非アルコール性脂肪肝炎(NASH)の発症機序は1st hitとしての肝細胞への脂肪の蓄積に加えて，酸化ストレスを中心とした2nd hitが加わることによって発症すると推察されている．酸化ストレスの要因として遊離脂肪酸，炎症性サイトカイン，エンドトキシン，アディポカイン，鉄などがあげられるがその詳細については不明な点も多い．NASHにおいて血清や肝組織での酸化ストレスマーカーが高値を示すとの報告が散見されるが確立されたものは存在しない．NASHの治療として抗酸化療法の有用性が期待され，ビタミンEや瀉血療法は多くのデータが集積され始めているが，今後さらなる大規模臨床試験が必要である．

Key Words:

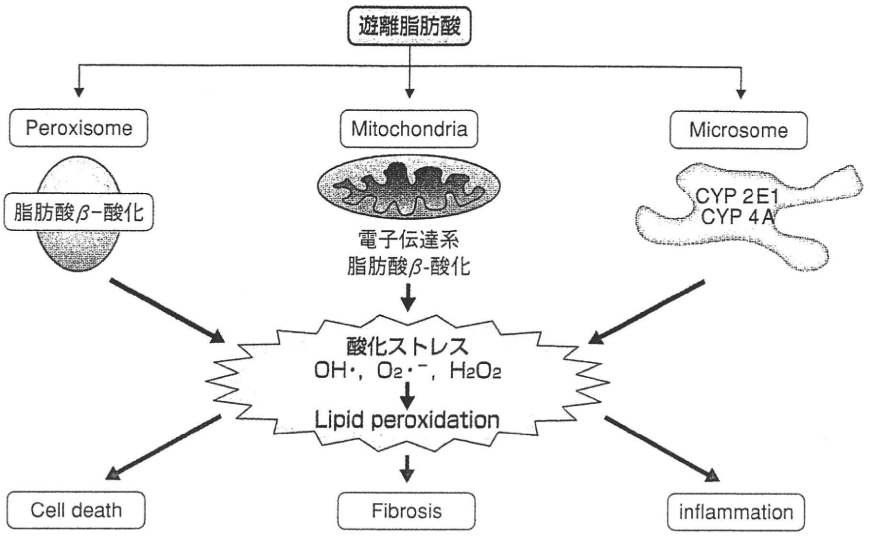
遊離脂肪酸□アディポカイン□ビタミンE□鉄□酸化ストレス

はじめに

近年食生活の欧米化，運動不足により肥満人口は増加の一途をたどり内臓肥満を基盤に発症するメタボリックシンドロームが問題視されている．メタボリックシンドロームの肝臓での表現型とされる非アルコール性脂肪肝疾患(nonalcoholic fatty liver disease; NAFLD)には，良好な経過をたどる単純性脂肪肝(simple steatosis; SS)と，肝硬変，肝癌へと進展する可能性のある非アルコール性脂肪肝炎(nonalcoholic steatohepatitis; NASH)が含まれる[1]．NASHの病理学的機序は明らかではないが，Dayらが提唱した"Two hit theory"が広く受け入れられている[2]．1st hitとして肝細胞への脂肪沈着，2nd hitとして脂肪酸代謝異常，インスリン抵抗性(insulin resistance; IR)，アディポカイン，エンドトキシン，炎症性サイトカインなどといった酸化ストレスが関与し，NASHが発症するというものである．

NASHの病態と酸化ストレス

脂質過酸化物は好中球の遊走を促進し，肝星細胞(hepatic stellate cells; HSC)を活性化し，コラーゲン産生を促進させる血小板由来増殖因子(platelet-derived growth



図❶　遊離脂肪酸による酸化ストレスとその作用

（文献3より引用）

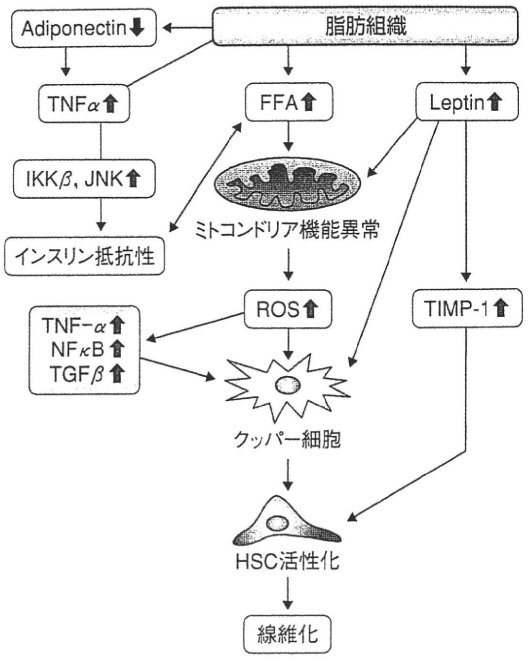
factor；PDGF）や形質転換増殖因子（transforming growth factor；TGF）-βといったサイトカイン産生を亢進し，線維化を誘導する．NASHにおける酸化ストレスの要因はいまだ十分に解明されていないが，以下のようなメカニズムが推定されている．

1．遊離脂肪酸（free fatty acid；FFA）（図❶）[3]

肥満や糖尿病に伴ってIR状態になると，食事中の中性脂肪由来の脂肪酸や末梢脂肪組織由来の脂肪酸の肝への流入量が増加する．中性脂肪合成に利用されないFFAはミトコンドリア，ペルオキシソーム，ミクロソームで代謝を受ける．ミトコンドリアでFFAが代謝される際にミトコンドリア内膜の呼吸鎖複合体による電子伝達系が活発になり，superoxideなどのラジカルが生じる．NASHではmegamitochondriaやcrystalline inclusionsなどの形態異常を示すミトコンドリアが高頻度に観察され，このミトコンドリア異常が電子伝達系の異常をきたし，活性酸素種（ROS）の過剰産生に関連する．また過剰に産生されたROSによりミトコンドリア膜の脂質が過酸化反応を受け，さらなるミトコンドリア障害が惹起され，ROS産生が増加する．過剰なFFAにより，ミトコンドリアでのβ酸化が飽和状態になると，ペルオキシソームでのβ酸化が亢進し，その代謝過程で過酸化水素などのラジカルを生じる．ミクロソームのcytochrome P450（CYP）によってω酸化が亢進し，その副産物として過酸化水素が発生し，細胞障害を惹起する．NASH患者ではアルコール性肝障害と同様にCYP2E1の発現が亢進していることが証明されている[4]．またω酸化の代謝産物であるジカルボン酸はミトコンドリアのβ酸化を抑制すると同時にペルオキシソームでのβ酸化の基質となるので，さらに上記の機序でROS産生を助長する．

2．炎症性サイトカイン

空回腸バイパス術後の患者でNASHが多くみられること



図❷ NASH発症におけるアディポカインの役割

FFA: free fatty acids, HSC: hepatic stellate cells; IKKβ: inhibitor kappa kinase beta, JNK: Jun N-terminal kinase, NF-κβ: nuclear kappa beta transcriptional factor, ROS: reactive oxygen species, TGF-β: transforming growth factor beta, TIMP-1: tissue inhibitor of metalloprotease-1, TNF-α: tumor necrosis factor alpha

から，腸内細菌由来のエンドトキシンがTNF-α，IL-6，IL-8などの炎症性サイトカインを誘導し，酸化ストレスの一因となる．TNF-αはc-Jun N-terminal kinase (JNK) やinhibitor kappa kinase beta (IKKβ) などを活性化させてインスリン受容体に結合する基質 (insulin receptor substrate; IRS) をセリンリン酸化させる．その結果インスリンによるチロシンリン酸化が阻害されIRを惹起するのみならず，ミトコンドリアの膜透過性を亢進させ，ミトコンドリア障害からROS産生を促進する．血清TNF-αはNASHで高値を示し，NAFLD activity scoreと良好な相関を示すといった報告があるが，血清TNF-αは両者で差がないとする報告もあり，一定しない．Tokushigeらによりの一塩基多型 (TNF-α position-1031Cもしくは TNF-α position-863A) キャリアはNASH患者において高頻度で，可溶性TNF受容体 (sTNFR)-2濃度が高いと報告している[5]．またIL-6の血中，肝組織レベルはNASHで高値を示し，IRと相関する[6]．

3．アディポサイトカイン(図❷)

脂肪組織は単なる余剰エネルギーの貯蔵庫と考えられてきたが，近年この臓器は種々の生理活性物質“アディポサイトカイン”を分泌する巨大な内分泌臓器であることが明らかとなってきた．アディポサイトカインには炎症性サイトカインであるTNF-α，IL-6，食欲抑制作用をもつレプチン，IR惹起作用のあるレジスチンなどの悪玉サイトカインに対して，善玉サイトカインであるアディポネクチンは肝臓へのFFAの流入を抑制し，インスリン作用を促進し，脂肪酸酸化を促進するのみならず，TNF-αの産生を抑制する作用を有する．NASH患者ではアディポネクチンが低下することが報告されているが，SSと差がないとの報告もあり，さらに多数例での解析が必要である．アディポネクチンノックアウトマウスではCYP2E1の発現を亢進して酸化ストレス状態を惹起することや，低アディポネクチン状態の患者で尿中の8-epi-prostaglandin F2が増加していることから，アディポネクチンは抗酸化作用を有すると考えられている[7]．

4．鉄

鉄はフェントン反応を介して，反応性の高いヒドロキシラジカル (・OH) を生成することから，NASHにおける酸化ストレスの一因となる．NASHでは血清ferritin値が高値を示し，鉄蓄積が線維化と相関するとの報告がある一方で，関係ないとする報告もあり，一定しない[8]．鉄蓄積の機序は明らかでなく，欧米ではヘモクロマトーシスの責任遺伝子であるHEF変異が示唆されたが，少なくともわが国の症例ではその関与は否定的である．Mitsuyoshiらは鉄量の増加に伴い，肝で産生され鉄吸収調節ペプチドであるhepcidinの発現が低下していることに起因するとしてい

表❶ NASHにおける酸化ストレスマーカーの文献的報告

報告者(年)	対象(n)	サンプル	測定項目	NASH vs. control	NASH vs. SS
Loguercio (2001)[16]	NAFLD (n=81) NASH/SS=34 : 14	血 清 赤血球	HNE, MDA	↑	↑
Seki (2002)[19]	NASH (n=17) SS (n=23) control (n=7)	肝	8-OHdG HNE	↑ ↑ ↑	→ →～↑
Sumida Y (2003)[22]	NASH (n=25) SS (n=15) control (n=17)	血 清	TRX	↑	↑
Koruk (2004)[25]	NASH (n=18) control (n=16)	血 清	GSH, MDA, NO SOD GSH-Px, GR	↑ ↓ →	
Chalassani (2004)[18]	NASH (n=21) control (n=19)	血 清	酸化LDL, TBARS	↑	
Horoz (2005)[26]	NASH (n=22) control (n=22)	血 清	Total antioxidant response (TAR) Total plasma peroxide	↓ ↑	
Yesilova (2005)[15]	NAFLD (n=51) control (n=30) Viral hepatitis (n=30)	血 清	MDA CuZn-SOD, カタラーゼ, コエンザイムQ	↑ ↓	
Machado (2008)[21]	NASH (n=43) control (n=33)	血 清 赤血球	8-OHdG 還元型GSH, 還元型GSH/酸化型GSH比 total antioxidant status (TAS), ビタミンE GSH-Px, GR	→ ↓ ↑ →	
Fujita (2009)[20]	NASH (n=38) SS (n=24) control (n=10)	肝	8-OHdG	↑	↑

SS: simple steatosis, HNE: 4-hydroxy-2-nonenal, MDA: malondialdehyde, 8-OHdG: 8-hydroxy deoxyguanosine, TRX: thioredoxin, GSH: glutathione, NO: nitric oxide, TBARS: thiobarbituric acid-reactive substances, SOD: superoxide dismutase, GSH-Px: glutathione peroxidase, GR: glutathione reductase

る[9]. Tsuchiyaらによるレチノイン酸シグナルの減弱が鉄蓄積に寄与するとの興味ある報告[10]もあり，今後の展開が期待される.

5. 好中球由来ミエロペルオキシダーゼ(MPO)

NASHでは肝臓内の好中球が増加し，MPO活性陽性のクッパー細胞が増加し，MPO活性化により生じた酸化修飾物がCXCケモカインを誘導するとの報告がなされた[11]. Ikuraらは酸化フォスファチジルコリン(oxPC)の免疫染色において，oxPCの発現強度がNASHの病態の進行度やMPO陽性好中球数と相関していることを明らかにしている[12].

6. その他

NASH患者では飽和脂肪酸やコレステロールの摂取量が多く，不飽和脂肪酸や抗酸化ビタミンであるビタミンE，Cの摂取量が少ないと報告されており，酸化ストレスに曝露されやすい[13]. また，肥満患者では腸内細菌叢が産生するエタノールによって呼気中のエタノール濃度が増加し，NASHではアルコール性肝障害と同様の組織所見を呈するとの推察もある[14]. このようにNASHの発生に酸化ストレスが深くかかわっているとする考え方には異論はないが，

表❷　NAFLD/NASHに対するビタミンEに関する文献的報告

報告者(年)	試験形式	対　象	治療内容	期　間	効　果	組織学的効果
Lavine (2000)[27]	パイロット	小児NAFLD (n=11)	ビタミンE	4～10ヵ月	ALT↓	評価なし
Hasegawa (2001)[28]	パイロット	NASH (n=12) SS (n=10)	ビタミンE	12ヵ月	ALT↓(NASHのみ) TGF-β1↓	NASH9例において 6例が脂肪化↓ 5例が炎症→，線維化↓
Harrison (2003)[29]	無作為対照比較試験	NASH (n=45)	ビタミンE＋C (n=23) プラセボ (n=22)	6ヵ月	ALT↓ (プラセボ群)	線維化↓ (ビタミンE＋C群) (特に糖尿病合併例) 炎症→
Kugelmas (2003)[30]	パイロット	NASH (n=16)	食事療法/ビタミンE (n=9) 食事療法のみ (n=7)	12週	全体としてALT↓ (2群間に有意差なし) HA↓，IL-6↓ TNF→，IL-8→	評価なし
Vajro (2004)[31]	無作為対照比較試験	小児NAFLD (n=28)	食事療法/ビタミンE (n=14) 食事療法/プラセボ (n=14)	5ヵ月	全体としてALT↓ (2群間に有意差なし)	評価なし
Kawanaka (2004)[32]	パイロット	NASH (n=10)	ビタミンE	6ヵ月	ALT↓，TRX↓， TBARS↓	評価なし
Dufour (2006)[33]	無作為対照比較試験	NASH (n=48)	ビタミンE/UDCA (n=15) プラセボ/UDCA (n=18) プラセボ/プラセボ (n=15)	2年	AST↓，ALT↓ (ビタミンE/UDCA群)	脂肪化↓ (ビタミンE/UDCA群)
Yakaryilmaz (2007)[34]	パイロット	NASH (n=9)	ビタミンE	6ヵ月	AST↓，ALT↓， HOMA-IR↓	脂肪化↓ 炎症→，線維化→

SS：simple steatosis，TGF-β1：transforming growth factor β1，HA：hyaluronic acid，TRX：thioredoxin，TBARS：thiobarbituric acid-reactive substances，UDCA：ursodeoxycholic acid

酸化ストレス発生機序の詳細は今後さらに明確にされるべき課題である．

NASH患者における酸化ストレスマーカー(表❶)

血清の脂質過酸化物であるmalondialdehyde (MDA)，4-hydroxy-2-nonenal (HNE)は，NASHでは健常者やSSより高値との報告がある一方で[15,16]，MDAに関してはNASHでは健常者に比較して高値を示すがむしろSSより低く，組織の進行度とも無関係との報告[17]もあり一定しない．NASH患者では年齢，性別，BMIをマッチさせた対照群と比較して酸化LDLが高値を示す[18]．DNAの酸化ストレス障害を指標である8-hydroxy-2'-deoxyguanosine (8-OHdG)に関してSekiらはNASHではSSや健常者と比較して肝臓内の8-OHdGが上昇し，炎症と関連すると報告し[19]，Fujitaらは8-OHdGが鉄蓄積やIRと関連し，瀉血により8-OHdGが低下したと報告している[20]．一方，NASHとSSでは血清の8-OHdGに差がないとの報告もある[21]．

一方，生体内の代表的還元物質であるglutathione (GSH)に関しては，血清の還元型GSHや還元型GSH/酸化型GSH比が低下しているとの報告がある[21]．GSHと同様に細胞内の酸化/還元(redox)制御を行う蛋白としてチオレドキシン(thioredoxin；TRX)があげられる．筆者ら

は京都大学ウイルス研究所の淀井淳司博士らとの共同研究によりSSと比べてNASHではTRXが高値を示し，肝組織内の鉄の増加にともなってTRXが上昇することを報告した[22]．抗酸化系酵素や抗酸化物質についてはほかにも男性NAFLD患者51例（うち45例がNASH）と，年齢とBMIをマッチさせた対照群とを比較して，coenzyme Q10, CuZn-superoxide dismutase（CuZn-SOD），catalase activityなど抗酸化系酵素が低下していると報告されている[15]．さらに，ヘム蛋白（heme）を分解し，生体から酸化ストレスを防御する役割を果たしているヘムオキシゲナーゼ-1（heme oxygenase；HO-1）はNASHではSSと比べて活性上昇を認め，血清ferritin，肝組織中の鉄量や脂質過酸化物と相関し，特にGSHの低下を伴う症例でHO-1の発現亢進があり，生体防御反応のひとつと考えられる[23]．このようにNASHにおける酸化ストレスマーカーについては少数例での報告が多く，確立されたものは存在しない．今後，簡便に測定でき，かつ肝内の酸化ストレス状態を鋭敏に反映するマーカーの開発が期待される．

NASHに対する抗酸化療法

脂溶性ビタミンの一種で代表的な抗酸化物質であるビタミンEはNASHに対する有用性が期待される（**表❷**）．Nonalcoholic Steatohepatitis Clinical Research Network Research Groupは糖尿病を有しない成人NASH患者に対するビタミンE 800mg/day，ピオグリタゾン30mg/day，プラセボの第Ⅲ相試験（96週）や小児NAFLDに対するメトホルミン500mg/day，ビタミンE 400mg/day，プラセボの第Ⅲ相試験（TONIC trial）を開始しており，今後これらの結果が期待される．ほかにもGSHの前駆体であるN-アセチルシステイン，コリンの代謝物であるベタイン，抗酸化作用を有する脂質異常症治療薬として知られているプロブコールなどが抗酸化療法として期待されている．さらに細胞質内からミトコンドリアへのアシルCoAの取り込みに重要なL-carnitineの補充がALT値のみでなく，TNF-αやHOMA-IRを低下させ，組織学的改善効果があると最近のRCTで明らかにされた[24]．前述のように鉄はNASHにおける酸化ストレスの一因となりうることから，瀉血療法も広義の抗酸化療法として期待されている[8]．ALTの低下のみならず，IRの改善まで期待できるとの報告もあるが，現時点ではNASHに対する瀉血療法は保険適用がなく，今後大規模なRCTが必要である．

おわりに

NASHの病態には多種多様の要因が関与しているがなかでも酸化ストレスの果たす役割は大きい．抗酸化療法として抗酸化物質，除鉄療法などの有効性に関する報告が相次いでいるが，現段階で確立されたものはなく今後の大規模臨床試験の結果に期待したい．そしてこれらの抗酸化療法によりNASHからの肝不全や肝発癌をいかに減らすことができるかが今後の最重要課題である．

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カレントテラピー

別刷

月刊カレントテラピー [別刷] 2010 Vol.28 No.12 12月号