Table 5 Reasons for discontinuation of combination therapy

Patients aged < 60 years			Patients aged ≥ 60 years		
Reason	n	Weeks after starting treatment	Reason	n	Weeks after starting treatment
Cerebral hemorrhage	1	**4**	**Infarction in the retina**	1	**14**
Rash	5	1,1,5,22,25	Fatigue	4	4,12,12.14
Fatigue	5	6,12,20,20,21	Anemia	3	10,16, 22
Depression	2	4,10	Anorexia	2	1,19
Anorexia	2	21,23	Nervousness	1	2
Vomiting	1	2	Dizziness	1	6
Anemia	1	4	Vomiting	1	16
Worsening diabetes	1	16	Depression	1	18
Spontaneous pneumothorax	1	17			
Hypothyroidism	1	18			
Uterine cancer	1	20			
Thyroiditis	1	24			
Pancytopenia	1	37			

Bold, serious adverse effect.
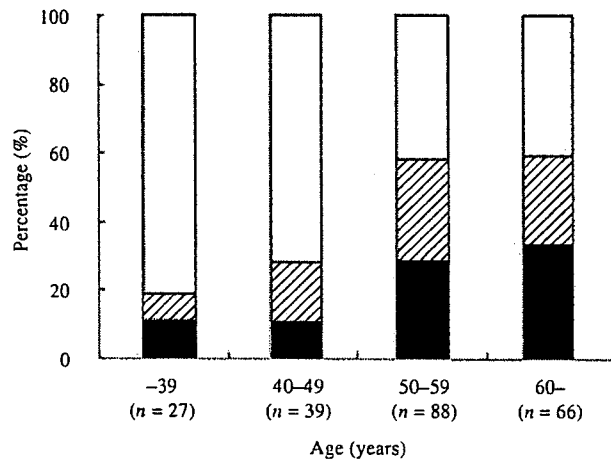
Figure 4 Ribavirin dose reduction and discontinuation rates according to age of patients (n = 220). (□) Completion: SVR 44.7% (51/114); (▨) dose reduction: SVR 36.5% (19/52); (■) treatment discontinuation: SVR 18.5% (10/54). SVR, sustained virologic response.

viral load by per-protocol analysis, 27.5% vs 6.7%, P = 0.032; in patients with genotype 2 and a high viral load by per-protocol analysis, 83.3% vs 23.1%, P = 0.0048; Figs 2,3). Moreover, the SVR rate among patients aged ≥60 years with HCV genotype 1 did not decrease with age. Neither did the SVR rate change with age for patients ≥60 years with genotype 2. Patients with genotype 2 achieved a high SVR rate of approximately 80% in all age categories. Adverse effects are thought to increase in elderly patients, but adverse effects necessitating discontinuation of IFN and ribavirin did not differ significantly between the older and younger patients (21.2% vs 14.9%). In addition, the severe adverse effects were not associated with age. These findings were similar to previously reported findings that there was no difference between young and elderly patients with respect to adverse

effects.[11,12] The treatment discontinuation rate tended to be higher in combination therapy (14/66) than in monotherapy (4/47) among patients aged ≥60 years, but there was no significantly difference between the two groups. (Table 4). This is because there was a small number of patients in the monotherapy group. The reason for discontinuation of combination therapy in seven of 14 patients was ribavirin-related adverse effects such as general fatigue or anemia.

The ribavirin dose reduction and discontinuation rates increased with age, but the SVR rate did not differ significantly between patients with and without dose reduction who completed the treatment schedule (36.5% vs 44.7%). These findings are consistent with previously reported findings.[19] In patients aged ≥60 years with HCV genotype 1 and a high viral load, the SVR rate did not differ significantly between combination therapy and IFN monotherapy by intention-to-treat analysis, but it did differ significantly by per-protocol analysis. These findings indicate that rather than discontinuing treatment, we should continue as permitted by dose reduction of ribavirin. In groups 50–59 years and >60 years of age the rate of dose reduction and treatment discontinuation was similarly high. In contrast, in groups <50 years of age the rate was low. In the present study we focused on patients aged ≥60 years because 60 years is often used as a cut-off for older patients; if we had focused on patients ≥65 years the number of study patients would have decreased and the comparison would have been difficult. There were high dose-reduction and discontinuation rates in the patients aged ≥50 years, so we should consider dose modification for these patients in advance.

Careful monitoring and appropriate reduction of the ribavirin dose is required to circumvent the need for discontinuation in elderly patients.[20,21] Also, it will be necessary to be careful when treating elderly patients with other diseases commonly observed in this age group, such as diabetes or hypertension. The present study, however, was limited due to being a retrospective analysis and using of historical controls, therefore further prospective studies are needed.

In conclusion, combination therapy was shown to be of comparable efficacy for chronic hepatitis C between patients aged <60 years and those aged ≥60 years, although the rate of ribavirin

discontinuation was shown to be higher among the older patients than among the younger patients. The efficacy of combination therapy was shown to be greater than that of IFN monotherapy in older patients.

Acknowledgments

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TNF-α Induces Hepatic Steatosis in Mice by Enhancing Gene Expression of Sterol Regulatory Element Binding Protein-1c (SREBP-1c)

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We investigated the effect of tumor necrosis factor-α (TNF-α), a member of the proinflammatory cytokine family, on steatosis of the mouse liver by analyzing morphological changes and hepatic triglyceride content in response to TNF-α. We also examined expression of the sterol regulatory element binding protein-1c gene. Intraperitoneal injection of TNF-α acutely and dramatically accelerated the accumulation of fat in the liver, as evidenced by histological analysis and hepatic triglyceride content. This treatment increased liver weight, increased serum levels of free fatty acids, and increased fatty acid synthase and sterol regulatory element binding protein-1c mRNA expression. Furthermore, intraperitoneal injection of lipopolysaccaride (LPS) to induce TNF-α expression also accelerated hepatic fat accumulation. Pretreatment with anti-TNF-α antibody attenuated the development of LPS-induced fatty change in the liver. Antibody pretreatment not only decreased sterol regulatory element binding protein-1c expression in LPS-treated mice but also attenuated the expression of suppressors of cytokine signaling-3 mRNA. This study suggests that TNF-α, acting downstream of LPS, increases intrahepatic fat deposition by affecting hepatic lipogenetic metabolism involving sterol regulatory element binding protein-1c. Exp Biol Med 232:614–621, 2007

Key words: TNF-α; LPS; liver; SREBP-1c

Introduction

Proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), have been suggested to cause obesity-related metabolic disorders, including insulin resistance (1). In addition to direct impairment of insulin signaling (2–4), lipotoxicity induced by fat accumulation in the liver also leads to hepatic insulin resistance (5–7). TNF-α may thus affect hepatic lipid metabolism. TNF-α treatment has been shown to accelerate hepatic triglyceride production and hyperlipidemia (8–11). However, the molecular mechanisms underlying TNF-α–induced fatty liver disease are not clear. A previous study suggested that TNF increases triglyceride (TG) production in the liver by providing an increased amount of fatty acids as substrate, since TNF did not increase the activity of triglyceride synthesis enzymes (10).

Fat accumulation in the liver is regulated by a number of lipogenetic and lipolytic factors. In particular, fatty acid synthase (FAS) and sterol regulatory element binding protein-1c (SREBP-1c) play important roles in lipogenetic processes in the liver (12–14). FAS is an enzyme necessary for *de novo* synthesis of fatty acids, which is regulated both transcriptionally and post-transcriptionally in response to nutrients and hormones (14). SREBP-1c, a transcription factor integral to maintaining lipid homeostasis, regulates gene expression related to fatty acid metabolism, including expression of FAS (12, 13, 15). Several studies have demonstrated that these lipogenetic processes are influenced by obesity and insulin-resistant conditions (16–18). Recently, suppressors of cytokine signalling proteins (SOCS) have been suggested to play pathogenetic roles in obesity-related metabolic disorders, including insulin resistance and fatty liver disease, by affecting cytokine signaling (19, 20). Overexpression of SOCS-1 and SOCS-3 in the liver has been shown to induce insulin resistance accompanied by elevation of SREBP-1c (19). This study suggests that proinflammatory cytokines, including TNF-α, may affect steatosis of the liver by modulating SOCS and SREBP-1c.

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However, it is still not clear how TNF-α actually affects the development of fatty liver *in vivo*. To address this issue, we examined the acute effects of TNF-α on hepatic lipid metabolism by analyzing lipogenetic and lipolytic markers in the liver. In addition, we examined the effect of TNF-α inhibition on lipopolysaccaride (LPS)-induced fatty changes in the liver.

Materials and Methods

Animals. Mice (C57BL/6, Seac Yoshitomi Ltd, Fukuoka, Japan) weighing 20–27 g at 10–11 weeks of age, were housed in a room illuminated daily from 0700–1900 hrs (12:12-hr light:dark cycle) at constant temperature (21°C ± 1°C) and humidity (55% ± 5%). The mice were allowed free access to standard mice chow pellets (CLEA Japan Ltd., Tokyo, Japan) and tap water. The animals were treated humanely in accordance with the Oita Medical University Guidelines for the Care and Use of Laboratory Animals.

Reagents. Recombinant human TNF-α (Dainippon Pharmacy Co. Ltd., Tokyo, Japan) was dissolved in saline to a final concentration of 50 μg/ml, anti-TNF-α antibody (Merck & Co., Whitehouse Station, NJ) was dissolved in saline to a final concentration of 1 mg/ml, and the LPS (Sigma Chemical Co., St. Louis, MO) was dissolved in saline to a final concentration of 1 mg/ml. The pH of each solution was adjusted to 6.8–7.4, and each solution was freshly prepared on the day of use.

TNF-α and Anti-TNF-α Antibody Treatment. After being matched according to body mass and food intake during a pre-experimental adaptation period, the mice were divided into two groups. One group was treated with recombinant human TNF-α, and the other group (control) was treated with saline. TNF-α was injected ip at 0.166 mg/ kg, and the controls received the same volume of saline at the same time of day (1500 hrs). This TNF-α dose, determined based on a preliminary dosage study, causes necroinflammatory changes in the liver and is relevant to clinical conditions such as sepsis. Peak serum TNF-α levels were reached 3 hrs following treatment during our experimental period (3, 6, 9, 24 hrs after treatment). To avoid any influence of food consumption on lipid metabolism, food was withdrawn after the injection. Mice were decapitated after their body weights were measured at 6 hrs (2100 hrs) or 24 hrs (1500 hrs, the next day) following TNF-α injection.

Another set of mice was divided into four groups. The animals were pretreated with either 3.3 mg/kg anti-TNF-α antibody or the same volume of physiological saline by ip injection 30 mins before (1430 hrs) the administration of either 3.3 mg/kg LPS or the same volume of saline (1500 hrs). Food was withdrawn after the second injection. Mice were decapitated after their body weights were measured 6 hrs (2100 hrs) or 24 hrs (1500 hrs, the next day) following LPS injection. As with TNF-α, the doses of LPS and anti-

TNF-α antibody were based on our preliminary dosage study (data not shown).

Tissue and Blood Sampling. Blood samples were obtained following decapitation at 3 hrs, 6 hrs, 9 hrs, and 24 hrs post-TNF-α treatment. Plasma was isolated, immediately frozen at −20°C, and stored until analysis. The livers were surgically removed and weighed, and the samples were immediately frozen in liquid nitrogen and stored at −80°C until RNA extraction. Levels of serum glucose, insulin, TG, and free fatty acids were measured by using commercially available assay kits (WAKO Chemical, Tokyo, Japan).

Histological Analysis. Liver samples were fixed with 10% formalin and embedded in paraffin. Sections measuring 5 μm were cut and stained with hematoxylin/ eosin (HE) and Sudan III. Liver histology was examined using the analysis system (Olympus, Tokyo, Japan).

Liver Triglycerides. Liver (100 mg) was homogenized in 2 ml of solution containing 150 mM NaCl, 0.1% Triton X-100, and 10 mM Tris using a polytron homogenizer (NS-310E; Micro Tech Nichion, Chiba, Japan) for 1 min. The TG content of this 100-μl solution was determined by using a commercially available kit (WAKO). To normalize the samples by protein content, the protein concentration of each sample was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction. Liver FAS, SREBP-1c, PPAR-α, and SOCS3 mRNAs were detected by polymerase chain reaction (PCR) amplification and quantified by real-time quantitative PCR. Total cellular RNA was prepared from mouse tissue using TRIzol (Lifetech, Tokyo, Japan) according to the manufacturer's protocol. Total RNA (20 μg) was separated by electrophoresis on 1.2% formaldehyde-agarose gels. RNA quality and quantity were assessed by EtBr staining and by measuring the relative absorbance at 260 nm versus 280 nm. cDNA was synthesized from 150 ng of total RNA in a volume of 20 μl with the ReverTra Dash reverse transcriptase kit (Toyobo, Tokyo, Japan) using random hexamer primers. Reactions were diluted to 50 μl with sterile distilled H$_2$O and stored at −20°C. Primers for genes were designed, synthesized, optimized, and provided as preoptimized kits: FAS (Cat No. Mm00662319m1), SREBP1-c (primers generated by Nihon Gene Research Laboratory, Sedai, Miyagi, Japan), PPAR-α (Catalog No. Mm00440939m1), SOCS3 (Catalog No. Mm00545913-s1). Primers for ribosomal RNA as internal controls were also provided as a preoptimized kit (Catalog No. Hs99999901). Using an ABI PRISM 7000 sequence detector, PCR amplifications were performed in 50-μl volumes containing 100 ng cDNA template in 2× PCR Master Mix (Roche, Branchburg, NJ) according to the following program: 50°C for 2 mins; 95°C for 10 mins; 40 cycles at 95°C for 15 secs, and 60°C for 1 min. Duplicate samples were processed. Results were analyzed with Sequence Detection Software (ABI), and expression levels of FAS, SREBP-1c, and SOCS3 mRNAs

A

N.S.

B

*
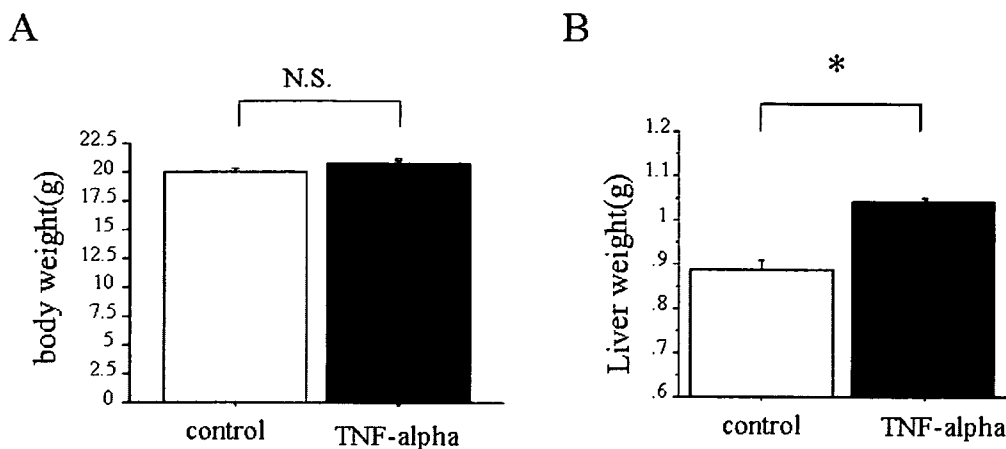
Figure 1. Body weight (A) and liver weight (B) 24 hrs after intraperitoneal injection of TNF-α at a dose of 0.166 mg/kg (TNF-α) or injection with the same volume of saline (control). Values and vertical bars: mean ± SEM ($n = 4$–8 for each). * $P < 0.05$ versus the corresponding controls.

were normalized to ribosomal RNA, as outlined in Perkin-Elmer's User Bulletin No. 2.

Statistical Analysis. All data are expressed as mean ± SEM. We used ANOVA to analyze differences for multiple comparisons (StatView 4.0, Abacus Concepts, Berkeley, CA); when appropriate, we used the Mann-Whitney U test.

Results

Effect of TNF-α on Body and Liver Weight. As shown in Figure 1A, administration of TNF-α at a dose of 0.166 mg/kg did not induce changes in body weight compared with the controls (Fig. 1A, $P > 0.1$). In contrast, this dose of TNF-α acutely increased liver weight, even under fasting conditions ($P < 0.05$) (Fig. 1B).

Effect of TNF-α on Serum Levels of Blood Glucose (BG), Insulin, TG, and Free Fatty Acids (FFA). We investigated the effect of TNF-α on serum levels of BG, insulin, TG, and FFA at 3 hrs, 6 hrs, 9 hrs, and 24 hrs after treatment. TNF-α treatment increased serum FFA levels compared with controls 6 hrs after treatment ($P < 0.05$) (Fig. 2D). However, serum levels of BG, insulin, and TG showed no remarkable change 6 hrs after TNF-α treatment (Fig. 2A, B, and C). In addition, no significant change in serum levels of BG, insulin, TG, and FFA were found at 3 hrs, 9 hrs, and 24 hrs after treatment.

Effect of TNF-α on Intrahepatic TG Content and Liver Histology. TNF-α treatment significantly increased liver TG content compared with controls ($P < 0.05$) (Fig. 3B). Histological analysis using HE and SUDAN-III staining also showed fat accumulation in the liver induced by TNF-α administration (Fig. 3A).

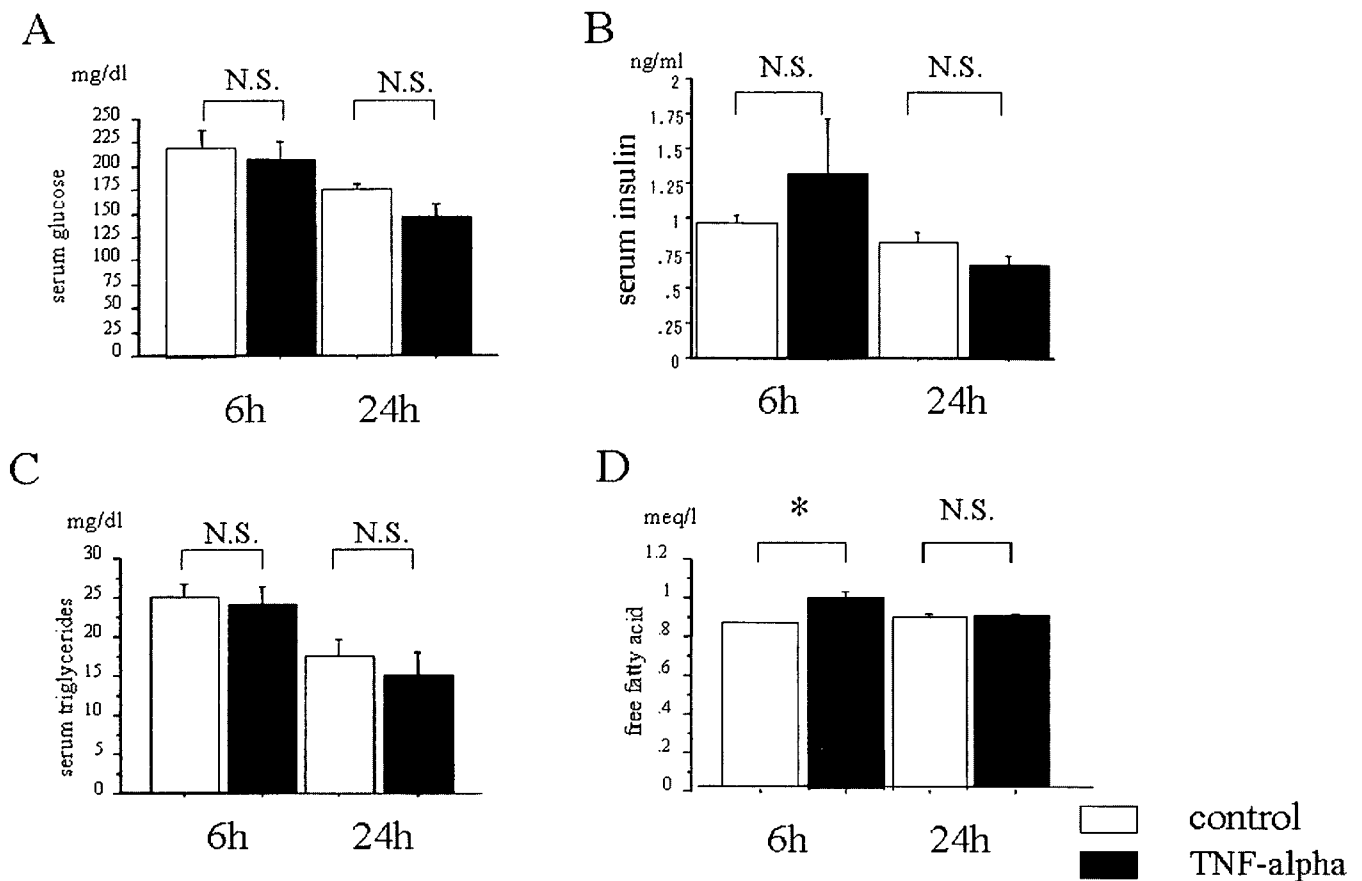**Effect of TNF-α on Hepatic FAS, PPAR-α, and SREBP-1c mRNA Expression.** After TNF-α treatment, liver SREBP-1c, and FAS mRNA expression increased 1.4-fold and 1.8-fold, respectively, compared with controls ($P < 0.05$ for each) (Fig. 4). Peroxisome proliferator–activated receptor mRNA-α expression was not significantly changed

after TNF-α treatment (control, 100.0% ± 4.1%; TNF-α, 109% ± 5.9%) ($P > 0.1$).

Effect of Anti-TNF-α Antibody on LPS-Induced Fatty Change in the Liver. Administration of LPS, similar to administration of TNF-α, accelerated fat accumulation in the liver as assessed by liver TG content ($P < 0.05$). This LPS-induced change in the liver was attenuated by pretreatment with anti-TNF-α antibody (control, 9.6 mg/dl ± 1.8 mg/dl; LPS, 29.5 mg/dl ± 6.9 mg/dl; LPS–anti-TNF-α, 12 mg/dl ± 4.0 mg/dl [control vs. LPS, $P < 0.05$; LPS vs. LPS–anti-TNF-α, $P < 0.05$]) (Fig. 5B). The attenuating effect of anti-TNF-α antibody was confirmed by histological analysis (Fig. 5A). SREBP1-c mRNA expression in LPS-treated mice decreased 0.8-fold after pretreatment with anti-TNF-α antibody (control, 100.0% ± 8.0%; LPS, 102.0% ± 8.1%; LPS–anti-TNF-α, 76.8% ± 1.8% [LPS vs. LPS–anti-TNF-α, $P < 0.05$]). FAS mRNA expression was not significantly changed. In addition, SOCS3 mRNA expression was attenuated by pretreatment with anti-TNF-α antibody (control, 100.0% ± 9.8%; LPS, 139% ± 11.7%; LPS–anti-TNF-α, 101% ± 8.6% [control vs. LPS, $P < 0.05$; LPS vs. LPS–anti-TNF-α, $P < 0.05$]) (Fig. 6).

Discussion

The present study demonstrates that TNF-α treatment in mice increases liver weight and hepatic TG levels. Histological analysis of TNF-α–treated mice by SUDAN-III staining also showed fatty changes in the liver tissue. These findings indicate that TNF-α treatment acutely accelerates the development of fatty liver in mice. Hypertriglyceridemia is often observed in patients or animals with fatty livers, reflecting the increased secretion of TG-rich lipoprotein from the liver (21–23). In fact, a previous study showed that TNF-α induced an increase in hepatic TG production, causing hyperlipidemia (24). However, this TNF-α–induced response was not observed in fasted animals, indicating that TNF-α effects are influenced by

A

mg/dl

N.S.   N.S.

250
225
200
175
150
125
100
75
50
25
0

serum glucose

6h   24h

B

ng/ml   N.S.   N.S.

2
1.75
1.5
1.25
1
.75
.5
.25
0

serum insulin

6h   24h

C

mg/dl

N.S.   N.S.

30

25
20
15
10
5
0

serum triglycerides

6h   24h

D

meq/l   *   N.S.

1.2

1
.8
.6
.4
.2
0

free fatty acid

6h   24h

☐ control
■ TNF-alpha

Figure 2. Serum glucose (A), serum insulin (B), serum triglycerides (C), and free fatty acid (D) levels at 6 hrs and 24 hrs after ip injection of TNF-α at a dose of 0.166 mg/kg (TNF-α) or injection with the same volume of saline (control). Values and vertical bars: mean ± SEM ($n = 4$ for each). * $P < 0.05$ versus the corresponding controls.

dietary factors (9). In the present study, serum TG content showed no response to acute TNF-α treatment. It is difficult to explain the difference in the serum TG responses. We speculate that the acute effect of the TNF-α treatment in our study may be restricted to fatty acid synthesis and TG production and may not influence very-low-density-lipoprotein secretion or insulin resistance.

We observed that serum FFA levels increased after TNF-α treatment. This result is consistent with a previous study showing that TNF-α treatment increases serum FFA level by activation of lipolysis in adipose tissue (25–27). It was also demonstrated that pretreatment with an antilipolytic drug prevented the TNF-induced increase in serum FFA (10). Taken together, these data indicate that TNF-α treatment induces lipolysis in adipose tissue and lipogenesis in the liver. However, in the present study, only large doses of TNF-α were used, and the results were similar to severe sepsis. Thus, further studies are needed to investigate the involvement of plasma and intrahepatic TNF-α in nonalcoholic fatty liver disease and nonalcoholic steatohepatitis (NASH). Previous studies demonstrated that obese patients with nonalcoholic steatohepatitis presented with increased expression of TNF-α mRNA in the liver (28). In addition, plasma TNF-α concentration was also elevated in patients

with NASH (29). Furthermore, genetically obese mice with NASH enhanced intestinal permeability leading to increased portal endotoxemia (30). These findings suggest that the LPS and TNF-α system may be involved in the pathogenesis of NASH in human and rodent.

The next important question we addressed was how TNF-α accelerates the development of fatty liver. A previous study showed that TG production was increased by the delivery of fatty acids from adipose tissue to the liver but not by activation of TG synthetic enzymes, such as diacylglycerol acyltransferase (10). In the study using sucrose-fed rats, TNF-α treatment was shown to stimulate hepatic de novo FFA synthesis, which provides FFA. Thus, the mechanism by which TNF-α stimulates hepatic triglyceride production seems to depend on dietary conditions (9, 10). However, the present study demonstrated that TNF-α treatment caused an upregulation of SREBP-1c in vivo, a key regulator of fatty acid synthesis in the liver, and FAS, an enzyme that catalyzes long-chain fatty acid biosynthesis through the condensation of acetyl-CoA and malonyl-CoA (14). The SREBP family is a family of transcription factors that activate genes encoding enzymes that regulate cholesterol and fatty acid biosynthesis. In particular, SREBP-1c preferentially enhances transcription
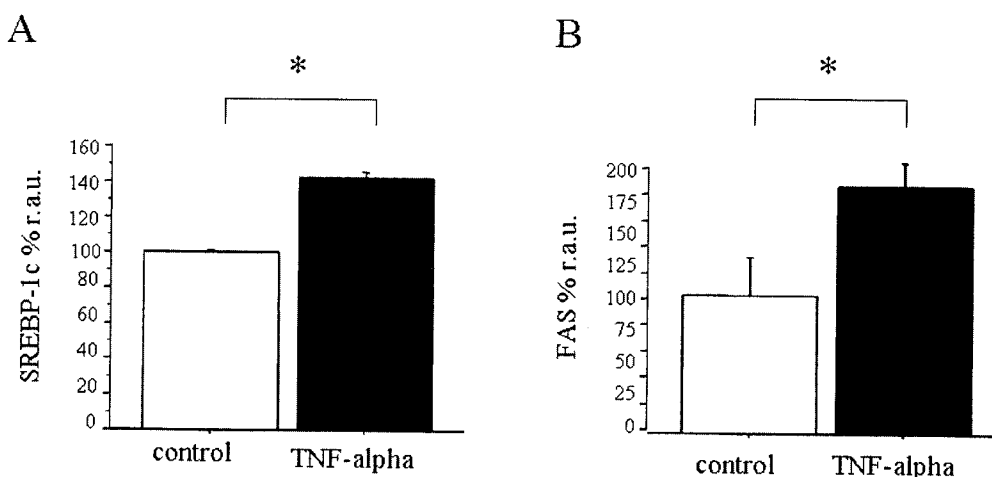
A   **B**
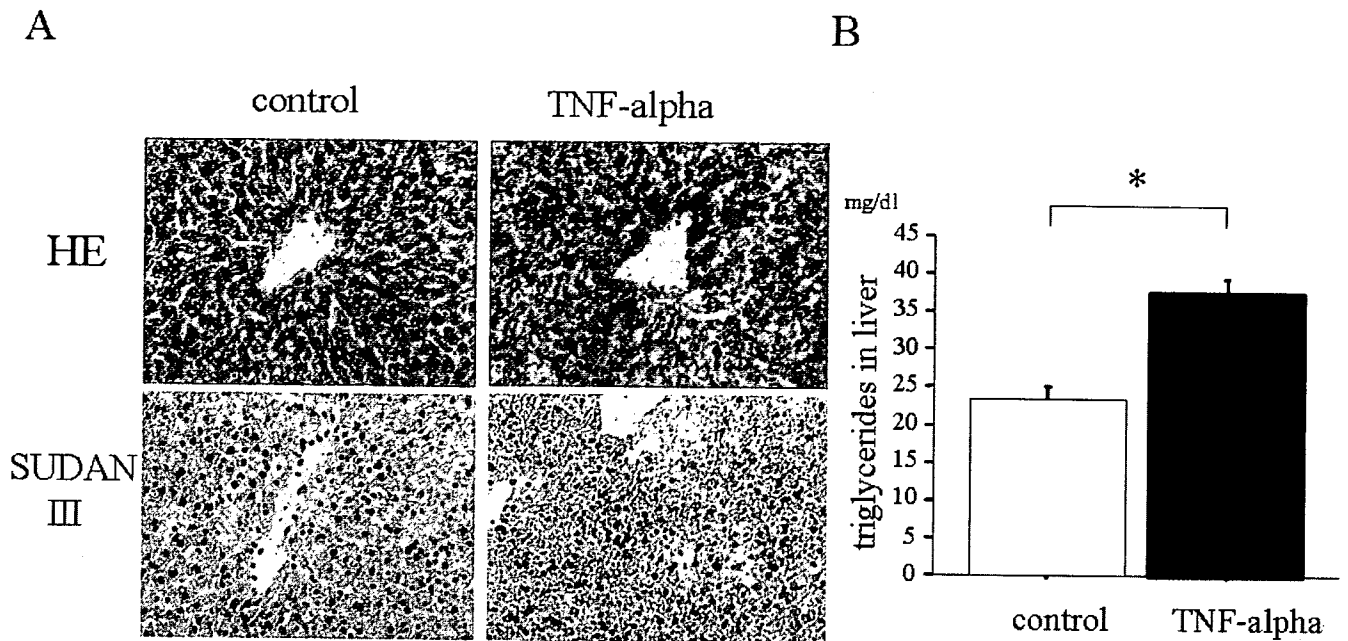
Figure 3. Histological changes (A) assessed by HE and SUDAN III (×200 magnification), and liver triglyceride levels (B) in the liver 24 hrs after ip injection of TNF-α at a dose of 0.166 mg/kg (TNF-α) or injection with the same volume of saline (control). Values and vertical bars: mean ± SEM ($n = 4$ for each). * $P < 0.05$ versus the corresponding controls.

of genes associated with fatty acid synthesis, including FAS (12, 13). TNF-α–induced activation of FAS has also been investigated in a previous study using a binding-activity assay (31).

Considering these data, a picture of the mechanism by which TNF-α induces fatty liver emerges. First, TNF-α treatment up-regulates SREBP-1c and FAS; second, these lipogenetic factors increase synthesis of fatty acids; and third, the synthesized fatty acids may be used as substrates for TG production in the liver.

How does cytokine signaling contribute to the functional linkage between TNF-α and SREBP-1c expression? Recently, suppressors of cytokine signaling (SOCS) pro-

teins have been shown to play an important role in cytokine signaling in the regulation of insulin resistance and the development of fatty change of liver (32). First, increased expression of SOCS-1 and SOCS-3 was observed in livers of obese, insulin-resistant animals (33). Second, overexpression of SOCS-1 or SOCS-3 in the liver caused insulin resistance. Third, the inhibition of SOCS-1 and SOCS-3 in livers of obese mice by antisense treatment normalizes the increased expression of SREBP-1 (33). Finally, proinflammatory cytokines have been shown to stimulate production of SOCS family proteins (19, 32). With these considerations, TNF-α treatment in our study may be increasing SREBP-1c expression and fatty acid

A   **B**

Figure 4. Expression of SREBP-1c mRNA (A) and FAS mRNA (B) in the liver 24 hrs after ip injection of TNF-α at a dose of 0.166 mg/kg (TNF-α) or injection with the same volume of saline (control). Real-time PCR was performed with total RNA from the liver samples. Values and vertical bars: mean ± SEM ($n = 4$ for each). * $P < 0.05$ versus the corresponding controls.
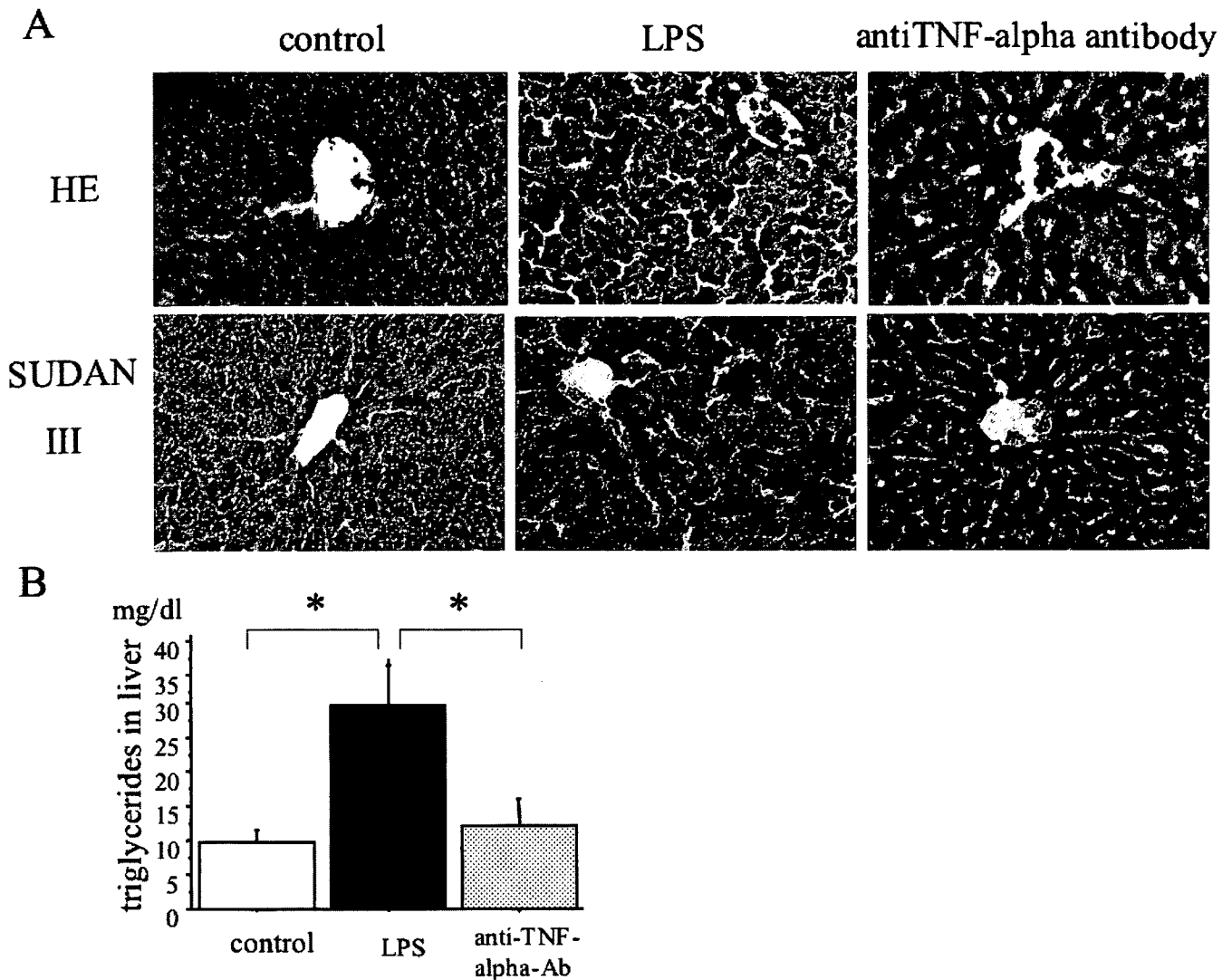
Figure 5. Effect of anti-TNF-α antibody on LPS-induced development of fatty liver 24 hrs after treatment. (A) Histological changes in the liver assessed by HE and SUDAN III staining (×200 magnification). (B) Liver triglyceride levels. Mice were injected with ip anti-TNF-α antibody at a dose of 3.3 mg/kg (anti-TNF-α antibody) or saline (LPS) before the administration of LPS at a dose of 3.3 mg/kg. Alternatively, both injections contained saline (control). Values and vertical bars: mean ± SEM ($n = 4$ for each). * $P < 0.05$ versus the corresponding controls.

synthesis in the liver through the action of SOCS proteins. We used LPS and anti-TNF-α antibody to examine the contribution of this cytokine signaling system and the specificity of TNF-α treatment in the development of cytokine-induced fatty change of liver. Similar to TNF-α, LPS induced fatty changes in the liver as assessed by TG content and histological observation of fat accumulation. The LPS-induced increase in liver TG content was partially attenuated by pretreatment with anti-TNF antibody. Histological analysis also demonstrated attenuation of hepatic fat accumulation by pretreatment with anti-TNF-α antibody. In addition, as expected by the functional role of SOCS proteins, expression of SREBP-1c and SOCS3 mRNA in LPS-treated mice was also attenuated by pretreatment with anti-TNF-α antibody. These results indicate that TNF-α may partially mediate LPS action to induce the development of fatty liver disease, and that SOCS3 and SREBP-1c may be

involved in this signaling mechanism. LPS is known to activate not only TNF-α but also other cytokines (34). A previous study showed that LPS induced fatty liver through cytokines such as TNF-α and interleukin-1 (11, 35). Thus, it is highly probable that TNF-α is one of the mediators of LPS-induced acceleration of fatty liver development as well as increased SREBP-1c expression.

The present study has some limitations. In the present study, hepatic SREBP-1c expression was not increased by LPS treatment compared with vehicle-treated controls although the anti-TNF-α antibody did down-regulate hepatic SREBP-1c expression. It may be due to the sensitivity or timing of our analysis system. In addition, it is also possible that other lipogenetic markers besides SREBP-1c in the liver may be involved in LPS and TNF-α–induced fatty liver. In anyway, further study would be necessary to clarify that point.

A   B   C
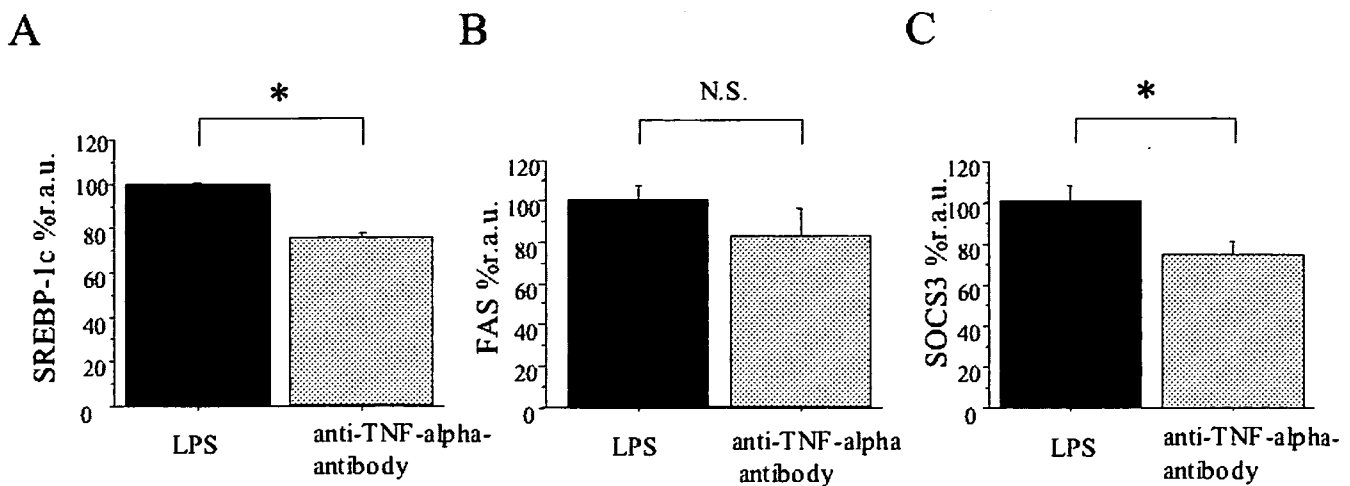
Figure 6. Expression of SREBP-1c mRNA (A), FAS mRNA (B), and SOCS3 (C) in the liver 24 hrs after treatment with anti-TNF-α antibody at a dose of 3.3 mg/kg (anti-TNF-α antibody) or saline (LPS) 30 mins before the administration of LPS at a dose of 3.3 mg/kg. Real-time PCR was performed with total RNA from the liver samples. Values and vertical bars: mean ± SEM ($n=4$ for each). * $P < 0.05$ versus the corresponding controls.

In summary, the present study indicates that TNF-α enhances the expression of SREBP-1c and FAS *in vivo* can acutely increase intrahepatic fat deposition. It can be expected that further studies of interactions among cytokine signaling and hepatic lipid metabolism involving SREBP-1c may provide a novel strategy for the treatment of inflammation-related disorders.

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Original

RFAにおける人工腹水注入液の選択

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Effective artifical ascites for RFA : whether we should use saline or 5% glucose solution?

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Abstract

The aim of this study is to evaluate the merits of various solutions when we perform artificial acites as an aid to percutaneous RFA therapy. We made an experimental model of RFA using a special plastic container. A piece of swine liver as ablation sample was placed in this container full of 1,500 mL saline or 5% glucose solution as artificial ascites. RFA was performed using 17 G cooled electrode with 2 cm exposed tip. The duration of ablation was 5 minutes and generated power was 20 W. We assessed necrotic area, tissue temperature and impedance. Ablation area was 28 mm and 22 mm using saline and 5% glucose solution respectively. Temperature of ablation tissue was 84°C and 79°C for each. Our data suggest that we had more effective RFA by using saline than 5% glucose solution in artificial ascites. We found saline to be recommended for artificial ascites technique.

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緒言

ラジオ波焼灼療法（RFA）を中心とした局所治療の進歩により，肝細胞癌の長期の治療成績は向上し，長期生存例が増加している．そのため再々発，再々々発の腫瘍を治療する機会も多くなり，腫瘍は肝の様々な部位に存在するため，腫瘍の穿刺は難易度が増し，熟練を要するようになってきた．更に良好な長期治療成績を残すためには，様々な肝癌に対応できる治療環境や技術の獲得が必要となってきた．

腹部超音波検査で死角にある腫瘍，隣接臓器に近接している腫瘍に対する治療機会は多い．このような場合，経皮的アプローチの他，腹腔鏡的なアプローチや外科的切除により対応するという戦略もあるが，肝細胞癌患者は高齢化による肝機能低下例が多いことから，より低侵襲的なアプローチが望まれる．そこで，局所麻酔下の経皮的な局所療法による治療対象の拡大を進めていく戦略が必要となってくる．

画像上死角になり描出できないにもかかわらず治療機会の多いドーム直下の腫瘍に対して，従来は人工胸水法が行われてきた[1]-[3]が問題点も多い．一方，人工腹水法はドーム直下の腫瘍に加え，腸管近接部の腫瘍を治療する際にも臓器の熱損傷を予防できる利点がある[4]．そのため，人工腹水法は経皮的アプローチでは必要不可欠の手技である[5]．

人工腹水法は，既にウサギ肝を用いて腹水法の安全性と効果の報告[6]がされている他，肝細胞癌症例において

も有用性が報告されている[2]．注入液については生理食塩水（生食水）もしくは5％ブドウ糖液が用いられているが，どちらが有用であるかという検討はない．経験的にどちらかの注入液が各施設で用いられているに過ぎない．そこでブタ肝を用い，RFAの際，どちらが効果的で有用であるかを検討したので報告する．

I. 方法

プラスチック容器の底を開けて金属トレイに固定し，トレイの下面に対極板を貼り，図1に示す容器を作製した．容器に320～400gのブタ肝を入れ，生食水（大塚製薬株式会社），5％ブドウ糖液（テルモ株式会社）それぞれ1,500mLで満たした．2cm針（2020）のCool-tip®針（Radionics社）を用いて20W・5分間焼灼した．容器内の使用した生食水と5％ブドウ糖液の水温は24～25℃であった．

①Cool-tip®針の非絶縁部をブタ肝内に穿刺した場合，②対極板の接触面を狭くした場合（より生理的条件に近づけるため），③針の非絶縁部が肝表面から出ている場合，④針先が肝臓の外に出た場合，以上の穿刺条件で各々焼灼範囲，焼灼後の組織内温度を測定した．

II. 成績
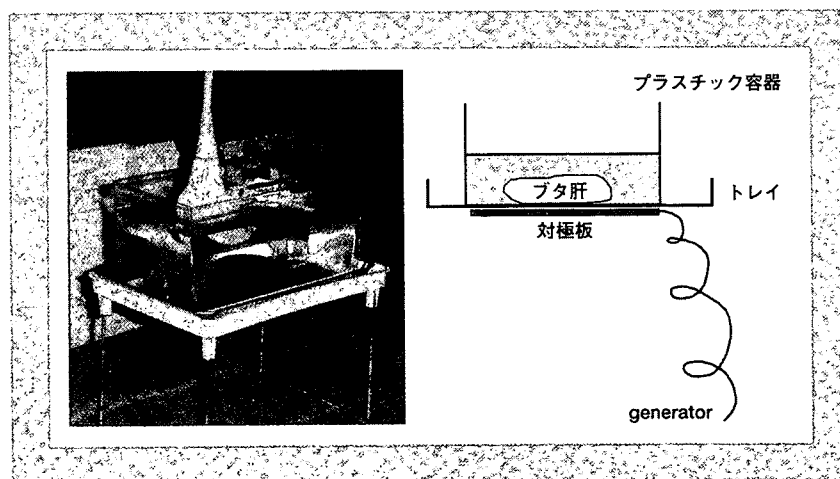
通常のRFAを想定し，肝にCool-tip®針を刺入した．同様の実験を3回行った．焼灼範囲（長径×短径，mm）

図1　実験装置
生理食塩水，5％ブドウ糖液1,500mLを容器にそれぞれ注入し，人工腹水環境を作製した．

は生食水で35×23，30×22，32×22，焼灼後の組織内温度は平均83（81～84）℃であった．一方，5％ブドウ糖液を用いた場合，焼灼範囲（長径×短径，mm）は27×22，29×22，27×22，平均組織内温度は78℃（77～79）であり，生食水を用いた方が高かった．一方，インピーダンスは生食水が低かった（図2）．

対極板の接する面積を狭くし，同様の実験を2回行った．これは，肝の直下に対極板を敷く（①）の通電環境は現実的でないため，より生理的条件に近づけるために行ったものである．生食水では焼灼範囲（長径×短径，mm）は24×20，24×18，組織内温度は90℃，88℃であった．5％ブドウ糖液を用いた場合では，焼灼範囲（長径×短径，mm）は17×10，10×8で，組織内温度は54℃，45℃であった．生食水を用いた方が焼灼範囲は広く，組織内温度も高かった（図3）．

Cool-tip®針の非絶縁部が肝外にある場合，同様の実験を行った．この場合は生食水では十分な焼灼が得られなかった（図4）．

針先が肝外へ出た場合，③と同様な結果であった．

III. 考察

経皮的ラジオ波焼灼療法（RFA）において，人工腹水法は死角への対応の他，横隔膜直下の腫瘍に対する治療時の疼痛軽減，また隣接臓器へ熱損傷を防ぐ観点から経皮的アプローチを行う場合，必要不可欠な手技である．

当科では，生食水を用いて既に100例を超える症例に行ってきた．一部に肝切除後など腹部の手術歴のある例で，人工腹水の作製が困難な例が存在する．隣接臓器への熱損傷を防ぐ目的では，78％で臓器と肝の分離が可能であったとの報告がある[4]．

人工腹水注入液についての検討はない．生食水や5％ブドウ糖液が使用されているが，施設により異なる．当科においても生食水で焼灼できず，5％ブドウ糖液を用いてRFAを行い成功した症例を経験した（図5）．注入液の選択は重要であると考えられるが，注入液の有用性についての検討はない．

そこで，実際の治療環境に適応する特殊容器を作製し，生食水，5％ブドウ糖液を人工腹水として用い，その有用性を検討した．Cool-tip®針を用いて20W・5分間の焼灼を行った．通常のRFAを想定した場合，生食水の方が焼灼範囲は広く，組織内温度も高かった．しかし，針の非絶縁部が肝外にある場合はブドウ糖液の方が焼灼範囲，組織内温度ともに高かった．これは，生食水が電解質で通電するのに対し，5％ブドウ糖液は通電しないためである．そのため，針の非絶縁部が肝外にあると，組織で高周波電流が生じないため熱が発生せず，凝固が起きないものと考えられる．
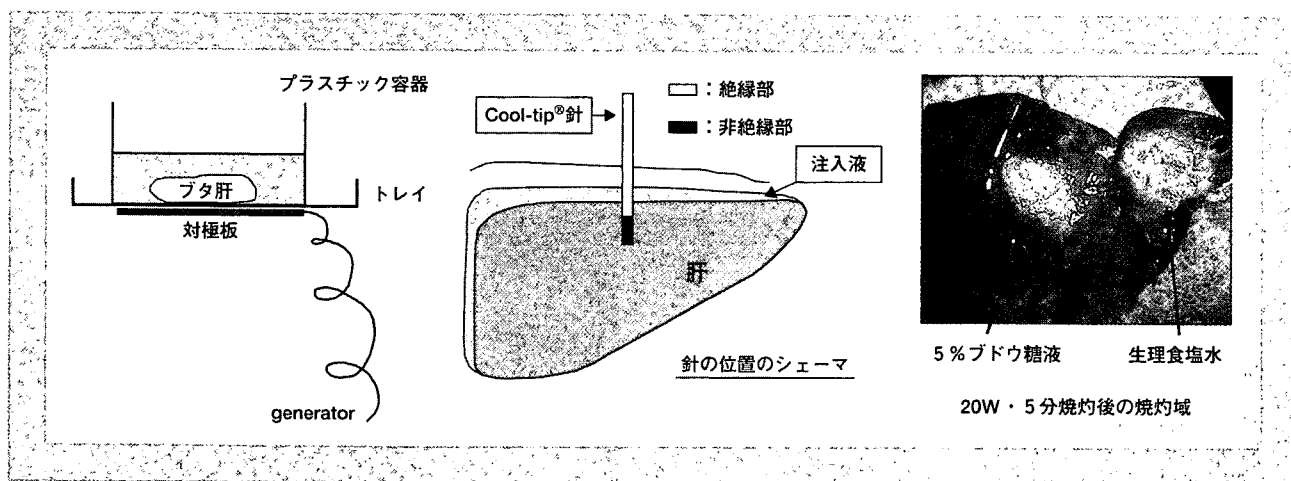
針先が肝外へ出ることや，針の非絶縁部が肝外にあ

図2　非絶縁部が肝内にある場合
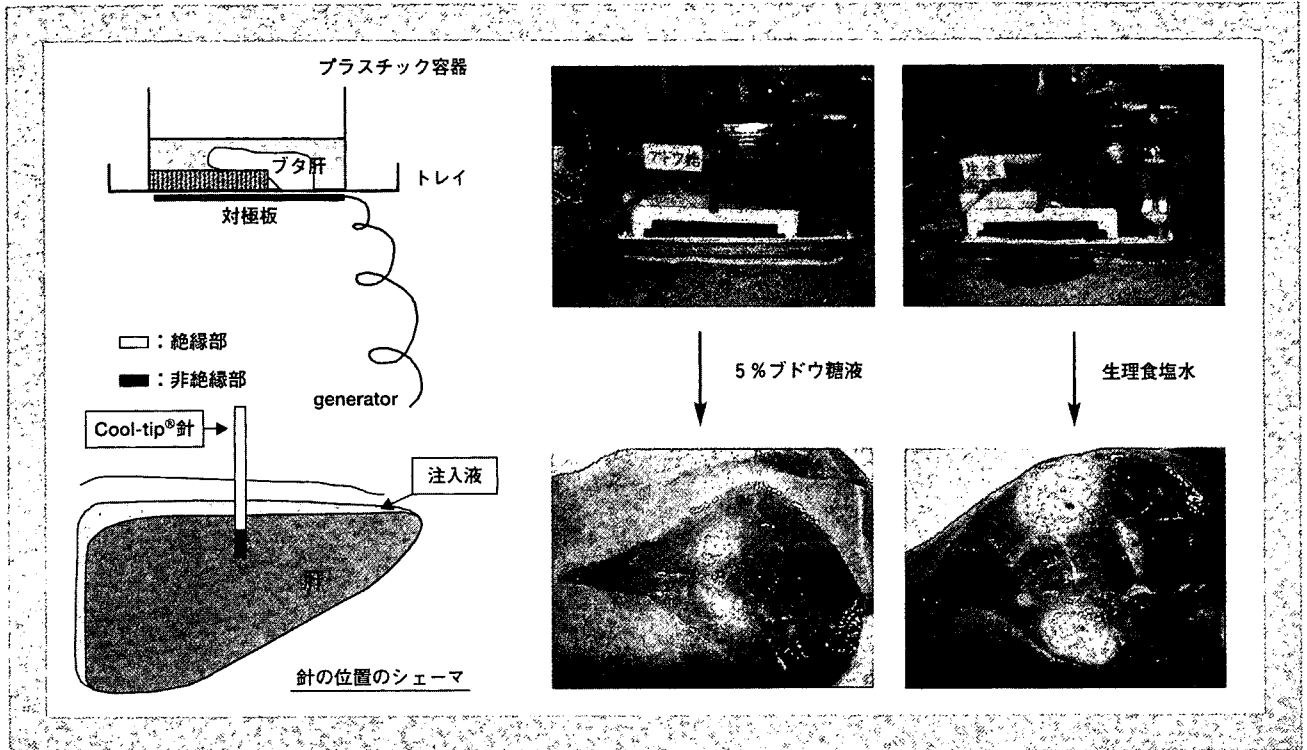実験装置に生理食塩水，5％ブドウ糖液1,500mLを注入し，人工腹水環境を作製した．穿刺の非絶縁部は肝内にある．通常のRFAを想定している．

図3 対極板と肝の接触面積を減少させた場合

より生理的条件に近づけるため，肝を浮かせて対極板に接触する面積を少なくした状態で，20W・5分間焼灼した．ブドウ糖液では十分な焼灼範囲が得られなかった．

るようなことは実際の臨床ではあってならないことである．つまり針先が肝外にある場合，針先が隣接臓器に接するとその熱損傷が生じる．また，非絶縁部が皮下や横隔膜内にあれば同部位の熱傷となる[7]．そのため，注入液は通常，生理食塩水で行い，肝のS6，S2 tipでは肝内に電極の非絶縁部2 cm，3 cmが確保できない場合のみブドウ糖液を用い，針先に注意してRFAを行うことが推奨される．

結　語

経皮的RFAに人工腹水法は，死角の克服，近接臓器の熱損傷予防，疼痛の軽減において必要な手技である．また，人工腹水注入液の適正な選択が重要である．通常は生理食塩水で安全かつ効果的に十分な焼灼範囲が得られる．

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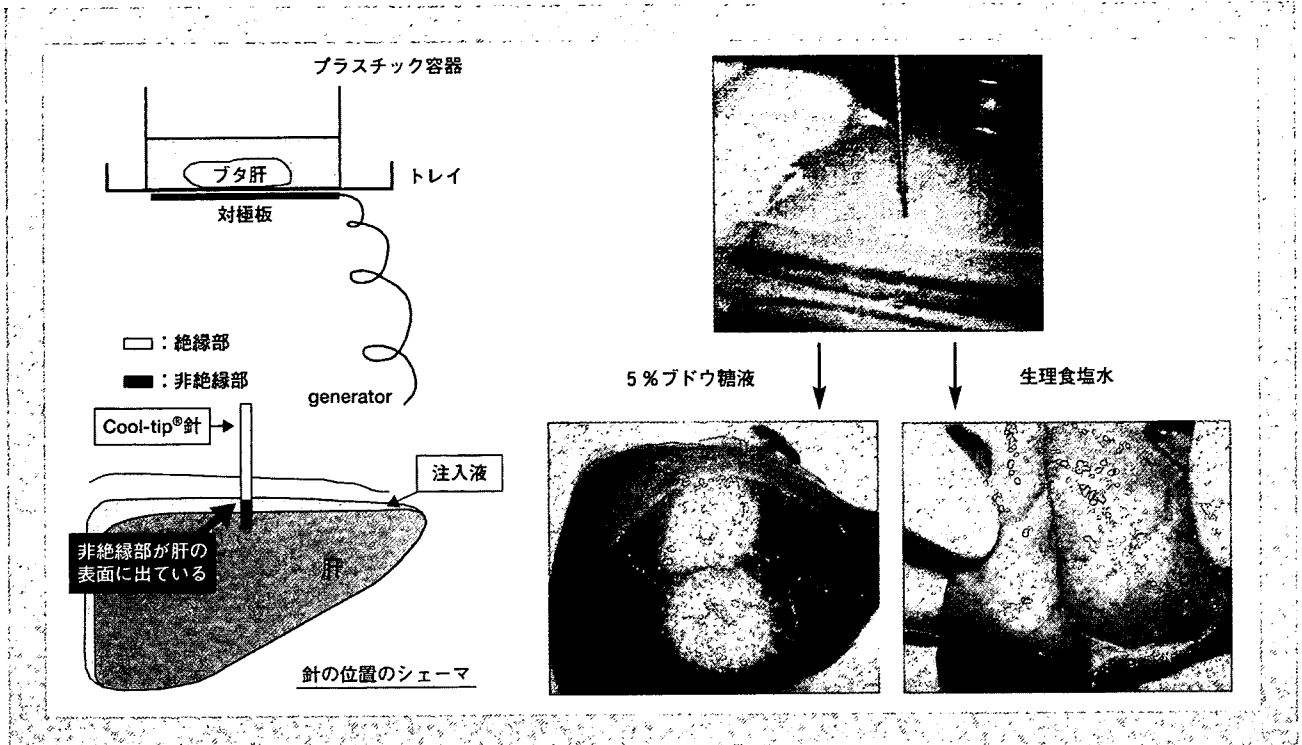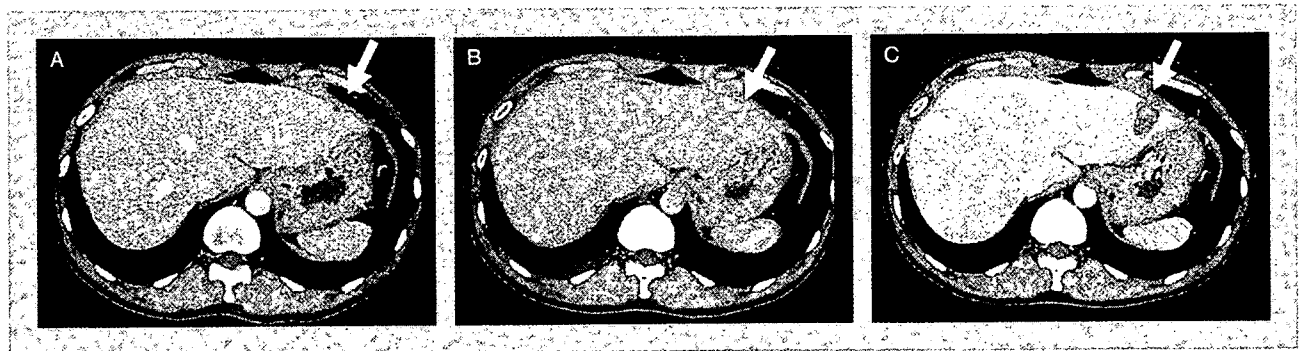**図4 非絶縁部が肝外にある場合**

非絶縁部が肝外へある場合，生理食塩水では十分な焼灼範囲が得られなかった．

図5 転移性肝癌（45歳，男性）

A：RFA前，B：生食水注入下人工腹水法併用RFA後，C：5％ブドウ糖注入下人工腹水法併用RFA後
唯一ブドウ糖液が有用であった症例．心窩部から穿刺．

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各 論：NAFLDの治療

肝庇護剤

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SUMMARY
・NAFLDの治療における肝庇護剤の評価は定まっていない.
・NAFLDの病態は多様であること，肝庇護剤には多彩な作用があることから，今後さらに再検討を要する.
・NAFLDの病態を考えた治療が必要であるので，体重の制御とインリン抵抗性改善薬や抗酸化薬が治療の柱である.
・体重の制御にグラフ化体重日記が有用である.
・生活習慣病を合併していることが多いので，合併症の治療も必要である.

I. 肝庇護剤の位置づけ

　脂肪肝 (NAFLD) は，食事中の中性脂肪や末梢脂肪組織由来の脂肪酸の肝への供給と酸化のバランスの不均衡により生じる．インスリン抵抗性発症にも関与し，酸化ストレスが加わることにより脂肪性肝炎 (NASH) の病態にいたると考えられている[1]．この経過は，いわゆる two hit theory[1] として知られており，first hit として脂肪肝があり，second hit として酸化ストレス，エンドトキシン，インスリン抵抗性などの要因が加わってNASHが発症するとされている (図1).

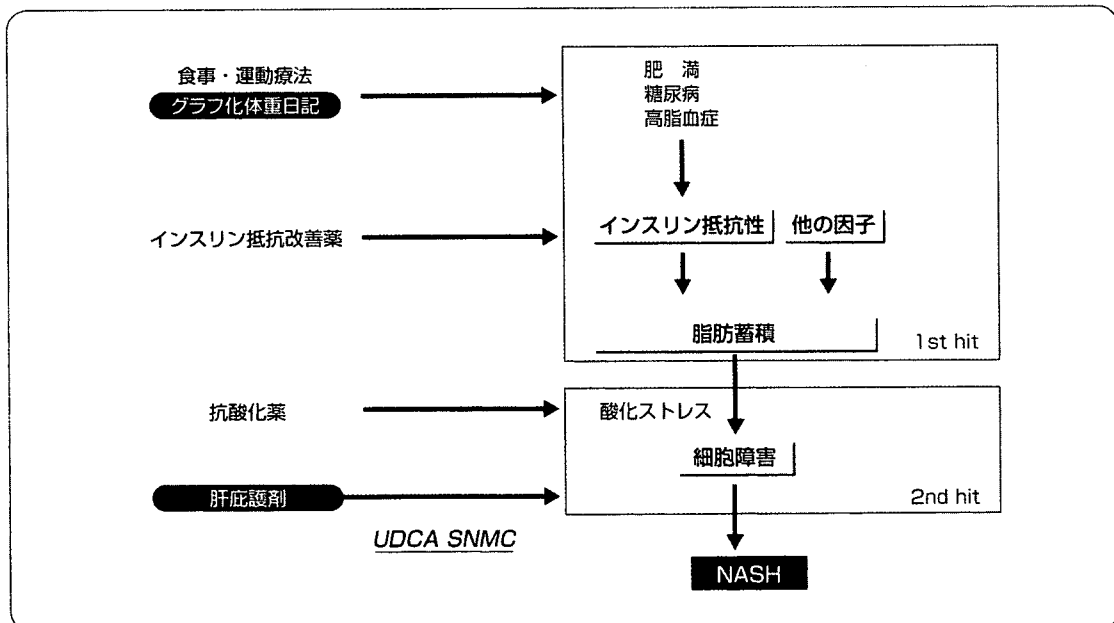図1　一般的なNAFLDの病態と治療

非アルコール性脂肪性肝疾患 (NAFLD) の多く
は，肥満，糖尿病を背景にしていることから，多
くは脂肪酸の肝への供給過剰のため生じている．
そのため，**治療の介入の第一歩は，脂肪酸の肝へ
の流入をまず防ぐことである**．実際，外科的な介
入 (Laparoscopic adjustable gastric banding) を
行い，食事量を減らすことにより体重の減量が得
られ，NAFLDの肝組織所見の改善が得られたと
の報告[2]や栄養指導の短期的な介入も有効であっ
たとの報告[3]がみられ，体重の制御がNAFLDの

治療の中心であることが確認されている．

治療における次の段階はインスリン抵抗性や酸
化ストレスに対する戦略で，現在多くの治療薬で
の治験の報告がなされている．詳細は他稿を参
照．肝庇護剤ではウルソデオキシコール酸
(UDCA) と強力ネオミノファーゲン®C (SNMC)
が知られている．いずれも肝炎や自己免疫性肝疾
患で広く使用されている薬剤でもある．しかし，
その作用は多彩であり，NAFLDに対する効果に
ついても一定の見解は得られていない (図1).

II. グラフ化体重日記

当科では食行動改善プログラムの主要な行動療
法技法として，**グラフ化体重日記**[4, 5]を用いてい
る．本技法はNAFLDやNASHの病態において
も，食行動の改善に伴い，肝への脂肪酸供給を減
少させるうえで，有効な方法である (図2)．本治
療は一日4回体重を測定し，それをグラフ化する

ものである．起床直後，朝食直後，夕食直後，睡
眠直前の4回の測定である．いずれも「直」という
ことを重視している．この体重日記により生活の
状況の把握が容易となる．いわゆる「だらだら食
い」など肥満患者の特有の生活習慣の是正に有用
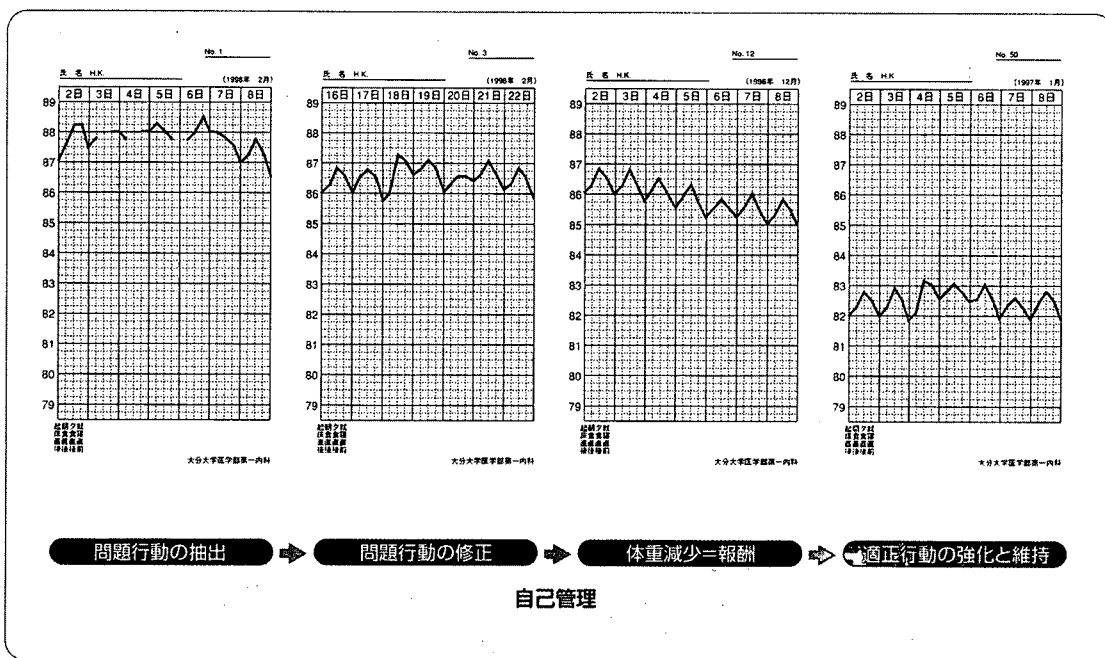である．体重日記をつけ，患者本人がどのような

図2　グラフ化体重日記による行動修正療法

食行動が体重制御によくないかを認識し，自分自身でそれを是正していくという自己管理プログラムである．図に示すように，減量がうまくいくようになると多くはきれいな規則正しいグラフになり，生活リズムが是正されていることがわかる．最近，睡眠前の体重の変動が大きいと体重の制御がうまくいかないという報告[6]がある．間食や夕食後の食事の摂取は，グラフ化体重日記のうえでも体重増加の要因，あるいは減量阻害の要因として描出されてくる．さらに，グラフ化体重日記を用いた生活習慣の是正を行いながら，食事療法や運動療法を導入することも有用である．**グラフ化体重日記は減量体重の維持，リバウンドの防止にも有効である．**

Ⅲ. 肝庇護療法

以上の基本的な指導を行っても改善が得られない場合，薬物療法を考える．本稿に与えられた課題は肝庇護療法である．代表的なものはウルソデオキシコール酸（UDCA）と強力ネオミノファーゲン®C（SNMC）である．

UDCAについては利胆作用，疎水性胆汁酸置換作用による細胞保護作用，免疫調節作用が知られ，ウイルス肝炎，自己免疫性肝疾患ですでに治療薬として用いられている．しかし，NAFLDやNASHについての評価は定まっていない．UDCAについては，有用であるとのpilot studyの報告[7]後，最近，2年間の大規模臨床試験が行われているが，UDCAを13〜15mg/kg/日投与群とプラセボ投与群の両群でトランスアミナーゼと肝組織所見の改善が得られている．しかし，両群で有意な差はなく有効性を否定する報告がなされている[8]．さらに，UDCAにビタミンE，UDCAに食事療法を加えた報告もある[9〜11]が，いずれも決定的なものではない．原発性胆汁性肝硬変症では高用量での効果が知られており，用量や投与期間については，さらに検討を必要とする．

SNMCについてはまとまった報告はないが，症例での報告がある[12]．グリチルリチンやグリチルレチン酸には抗炎症，抗アレルギー，肝炎，皮膚炎などの改善作用に加え，抗酸化，胆汁排泄促進，ステロイド様作用が報告されている．症例報告を詳細に検討すると単独での効果は十分ではなく，結局体重の減量やほかの治療が奏功している．

UDCAやSNMCなどの肝庇護剤は基礎的なデータから，酸化ストレスの改善やインスリン抵抗性改善効果が報告されているので，今後体重の制御を行いながら，肝機能の改善が十分でない症例に長期投与し，治療評価を確実に行う必要があるであろう．

Ⅳ. 症例提示

脂肪性肝炎（NASH）の症例を提示する．SNMCやUDCA600mgの投与で肝機能の改善が十分でないことからNASHの診断に至った症例である（図3）．7年を経て，体重日記で体重を64〜65kgに制御し，組織所見も著しく改善した（図4）．最近5例の治療前後での肝生検を行った結果，GradingとStagingのいずれも改善していた．当科では40例のNASHで急速に進行した例や発癌した例はなく，何らかの治療で介入すれば進展を防ぐことは可能と思われる．
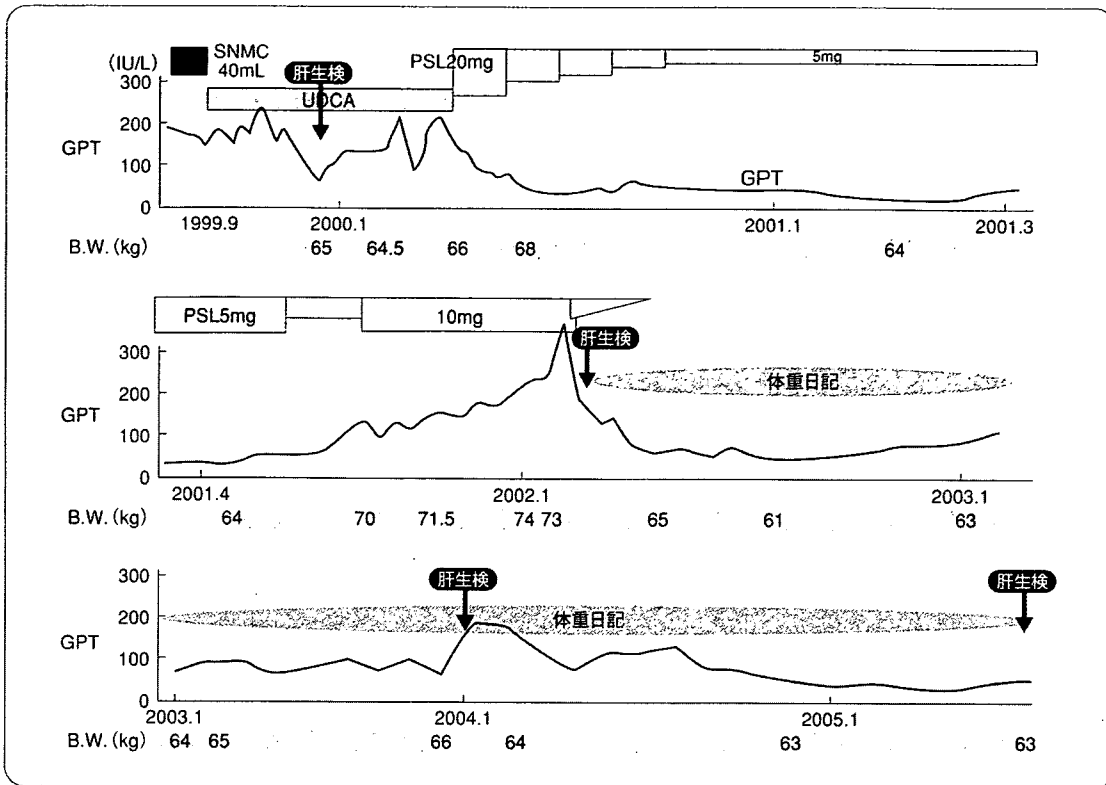
薬物治療の評価は難しい．短期間での評価が多く，長期での評価は少ない．報告の多くは6カ月
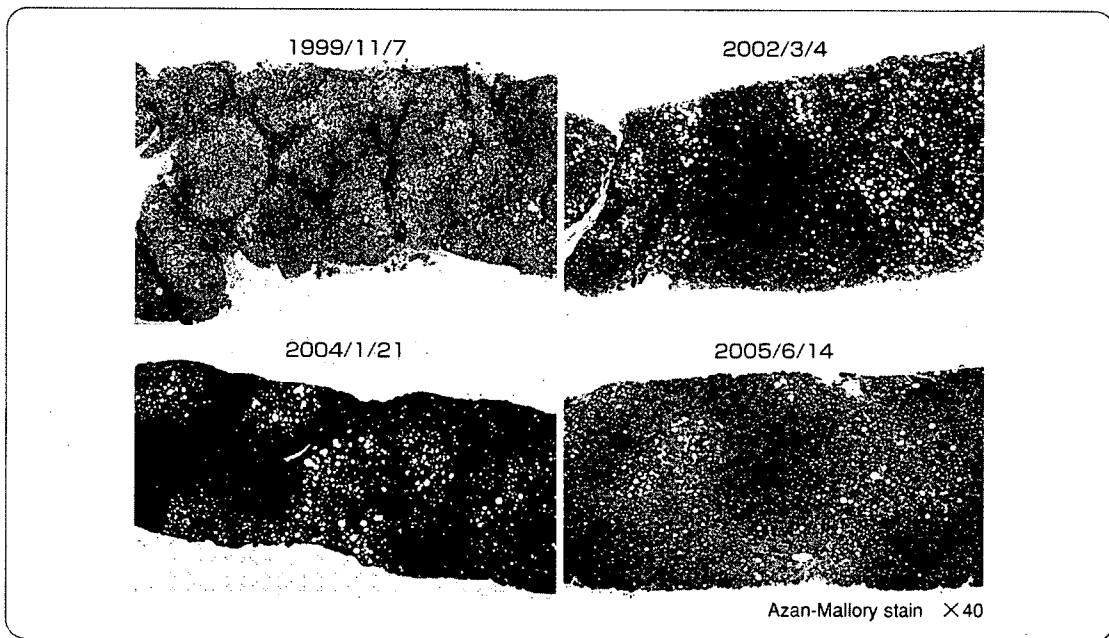
図3 臨床経過
50y.M. 肝庇護剤が効果が不十分で, 体重制御や種々の治療により改善した.

Azan-Mallory stain ×40

図4 肝病理組織像の推移
グラフ化体重日記による体重の制御を中心とした治療で肝の線維化は改善している.

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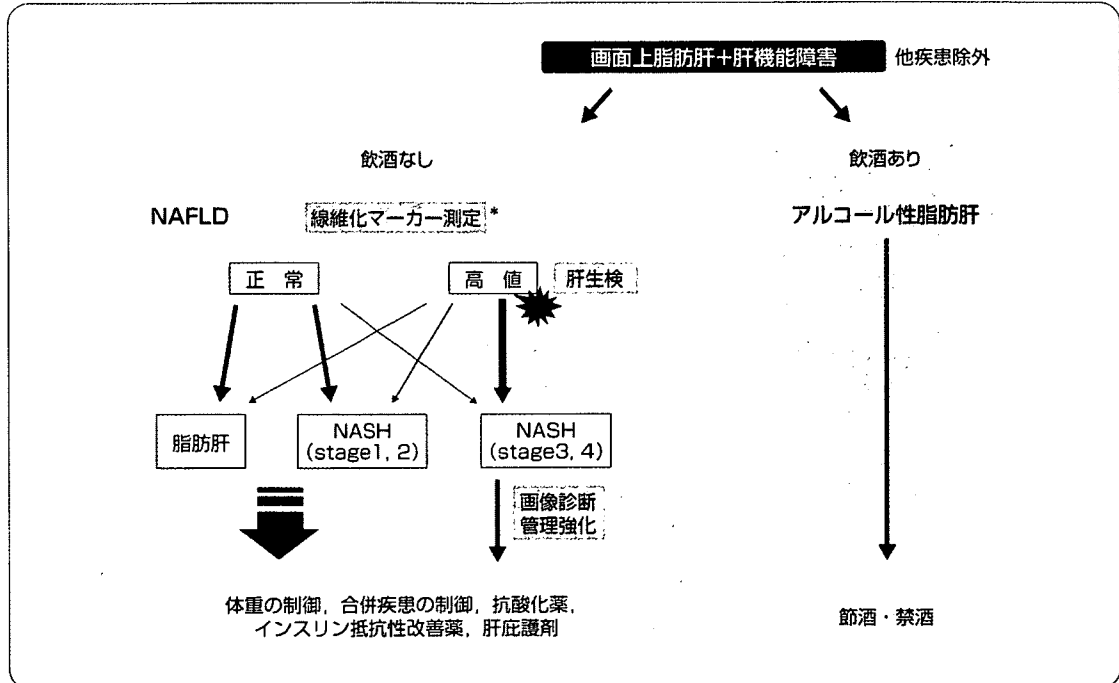
図5　当科におけるNAFLDの診断方針（stageはBluntの分類[14]）　　　　　　　　（文献13）より）
＊：40歳未満ではヒアルロン酸は線維化が進んでいても正常のことが多いのでⅣ型コラーゲンを指標にする．

ないし1年程度である．2年以上の長期の報告例は少ない．短期間であれば偽薬効果や短期での体重制御が薬物治療の効果に影響を及ぼす可能性がある．1～2kg程度の減量でトランスアミナーゼの改善が得られることは臨床でよく経験することである．さらに薬物治療が一時的にうまくいっていても体重の再増加が生じれば肝機能は増悪する．体重の増加中に肝機能安定化へ導くのは困難である．

　当科におけるNAFLDについての診断および治療方針を示す（図5）．すべての症例を肝生検するわけにいかないので，線維化マーカーを測定し，正常値の場合，線維化の程度は軽いと判断し，画像での評価も加え，単純性脂肪肝と同様に扱い，治療を行う．線維化マーカーが異常値であれば，肝生検を行い，高度線維化群では肝硬変症や肝癌への進展を防ぐ目的から，管理を強化し，背景の

生活習慣病への治療や酸化ストレスやインスリン抵抗性改善をさらに進める治療を行うこととしている．NAFLDでは糖尿病，高脂血症，高血圧症，高尿酸血症を伴っていることが多く，これらの疾患についての治療も必要である．

　以上をまとめるとNAFLDではまず体重の制御のうえ，背景にある生活習慣病の治療を行い，肝機能の改善が不十分な場合はインスリン抵抗性改善薬，抗酸化薬を投与し，さらに肝庇護剤の投与を考えるという戦略が選択される．最近の食生活や生活習慣病の欧米化により今後，NAFLDは増加することが予想されることから，比較的容易に把握が可能な肝臓での脂肪蓄積と肝機能検査を注意深く観察することにより，生活習慣病の予防や進展阻止に，貢献できる．